UNIVERSIDAD AUTÓNOMA DE MADRID
FACULTAD DE CIENCIAS

Departamento de Química-Física Aplicada
Sección Departamental de Ciencias de la Alimentación

FORMULACIÓN DE EXTRACTOS VEGETALES BIOACTIVOS PARA EL DESARROLLO DE INGREDIENTES CON APLICACIONES ALIMENTARIAS

BIOACTIVE PLANT EXTRACTS FORMULATIONS FOR THE DEVELOPMENT OF INGREDIENTS FOR FOOD APPLICATIONS

SOMARIS ELENA QUINTANA MARTÍNEZ

TESIS DOCTORAL

Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC)
Departamento de Producción y Caracterización de Nuevos Alimentos

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Memoria presentada por:
SOMARIS ELENA QUINTANA MARTÍNEZ

Para optar al grado de
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Trabajo realizado bajo la dirección de
Dra. Mónica Rodríguez García-Risco
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Instituto de Investigación en Ciencias de la Alimentación (CIAL, UAM-CSIC)
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Madrid, 2020

Dª. Mónica Rodríguez García-Risco, Dra. en Ciencias Químicas y Profesora Contratada Doctor de la Universidad Autónoma de Madrid y **Dª. Tiziana Fornari Reali**, Dra. en Ingeniería Química y Profesora Titular de la Universidad Autónoma de Madrid

INFORMAN,

Que el presente trabajo titulada "**FORMULACIÓN DE EXTRACTOS VEGETALES BIOACTIVOS PARA EL DESARROLLO DE INGREDIENTES CON APLICACIONES ALIMENTARIAS**", que constituye la memoria que presenta Somaris Elena Quintana Martínez, para optar el grado de Doctor en Ciencias de la Alimentación ha sido realizada bajo su dirección en el Departamento de Producción y Caracterización de Nuevos Alimentos del Instituto de Investigación en Ciencias de la Alimentación.

Y para que así conste firman el presente informe el Madrid a 26 de octubre de 2020.

Fdo.: Mónica Rodríguez García-Risco

Fdo. Dª. Tiziana Fornari Reali

A mí família

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Índice

ABREVIATURAS

ANOVA: Análisis de varianza

ATCC: *American Type Culture Collection*

ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ASES: *Aerosol Solvent Extraction Systems*

BIOSAS*: Bioactive Ingredients in Oils by Supercritical Anti-solvent Process*

CG-MS: Cromatografía de Gases - Espectrometría de Masas

Ch: Chitosan / Quitosano

CO_2*:* Dióxido de carbono

CSS: *Crystallization from Supercritical Solutions*

DELOS: *Depressurization of an Expanded Liquid Organic Solutions*

DPPH: Difenil-1-picrilhidrazil

DSC: Calorimetría Diferencial de Barrido

EFSA: Autoridad Europea de Seguridad Alimentaria

E. coli: *Escherichia coli*

FDA: *Food and Drug Administration*

GAE: Equivalente de Ácido Gálico

GAS: *Gas Antisolvent*

GASC: *Gas Antisolvent Cristallization*

GRAS: *Generally Recognized as Safe*

HD: Hidrodestilación / *Hydrodistillation*

HD-UAE: Hidrodestilación asistida con ultrasonido

HPLC: Cromatografía de líquidos de alta eficacia.

HSD: *Honestly-significant-difference*

Hue: *hue angle*

IC_{50}: *Inhibitory Concentration 50*

LE: *Licorice extract* / Extracto de regaliz

LES: *Licorice ethanolic extract*/ Extracto etanólico de regaliz

MCP: *Mixture Critical Point*

PC: Película comestible

RC: Recubrimiento comestible

UAE: extracción sólido-líquido asistida con ultrasonidos

PCA: *Precipitation with a Compressed Fluid Antisolvent*

PEEC: *Plant extract edible coating*

PCM: Punto Crítico de Mezcla

PGSS: *Particle from gas saturated solution*

PSD: *Particle Size Distribution*

QUE: *Quercetin solution*

QWS: *Quillaja saponaria*

RE: *Rosemary ethanolic extract*

RESS: *Rapid expansion of supercritical solutions*

SAE: *Supercritical Antisolvent Extraction*

SAILA: *Supercritical Assisted Injection in a Liquid Antisolvent*

SAS: *Supercritical Antisolvent Precipitation*

SCCO$_2$: Dióxido de carbono supercrítico

SEDS: *Solution Enhanced Dispersions by Supercritical Fluids*

SEM: Scanning Electron Microscopy

SFE: Extracción con Fluidos Supercríticos

SFEE: *Supercritical Fluid Extraction and Expansion*

SFR: Solución Formadora de Recubrimiento

SSC: *Soluble solids content* / contenido de sólidos solubles

SSI: *Supercritical Solvent Impregnation*

S. aureus: *Sstaphylococcus aureus*

TA: titratable acidity / Acidez Titulable

TSI: *Turbiscan Stability Index*

TEAC: Capacidad Antioxidante Equivalente de Trolox

TPC: Compuestos Fenólicos Totales

UFC: Unidades Formadoras de Colonia

SÍMBOLOS

L*: *lightness*

a*: *green-red chromaticity*

b*: *blue-yellow chromaticity*

ΔA: *change of color*

C*: *chromaticity*

$\boldsymbol{\sigma}$**:** *shear stress*

$\dot{\boldsymbol{\gamma}}$**:** *shear rate*

$\boldsymbol{K}$**:** *consistency coefficient*

$\boldsymbol{n}$**:** *flux index*

$\mathbf{G'}$**:** *elastic component*

$\mathbf{G''}$**:** *loss modulus*

Tan δ: *loss tangent*

$\boldsymbol{J}$**:** *compliance*

$\boldsymbol{t}$**:** *time*

$\boldsymbol{J_o}$: *instantaneous compliance*

$\boldsymbol{J_1}$**:** *retarded compliance*

$\boldsymbol{\lambda_{ret}}$: *retardation time of Kelvin-Voight element*

$\boldsymbol{\mu_o}$**:** *Newtonian viscosity*

ω: *frequency*

RESUMEN

Las sustancias presentes en los materiales de origen vegetal poseen diversas propiedades, tecnológicas y funcionales, que han sido utilizadas y siguen siendo aprovechas para el desarrollo e innovación del sector de la alimentación. En este sentido, los extractos que provienen de plantas, verduras, frutas y/o residuos de la industria agraria son objeto de gran interés, debido a la gran variedad de sustancias con demostrada actividad biológica que han sido aisladas de los mismos.

En particular, el uso de los extractos de plantas para la elaboración de películas y recubrimientos comestibles ha alcanzado importantes avances y aplicaciones en las últimas décadas. Los extractos obtenidos a partir de las hojas, tallos, raíces, semillas, flores o frutos, son añadidos a la matriz base del recubrimiento, vehiculizando los componentes del extracto, especialmente los antimicrobianos y antioxidantes, los que contribuyen a la conservación del alimento. Entre las plantas más estudiadas y utilizadas con este fin destacan el tomillo, orégano y romero, por las reconocidas cualidades antioxidantes y antimicrobianas de sus correspondientes aceites esenciales.

Por otro lado, una parte importante de la investigación y el desarrollo de nuevos alimentos está orientada actualmente a los productos que, además de aportar a la dieta los nutrientes necesarios, pueden aportar un beneficio funcional y contribuir a preservar y/o mejorar la salud. Así, es constante la investigación y el desarrollo tecnológico para descubrir y producir nuevos extractos vegetales bioactivos, útiles para formular ingredientes, nutracéuticos y/o suplementos alimenticios. En este sentido, no basta probar la bioactividad del extracto, sino que es fundamental una formulación adecuada para garantizar la biodisponibilidad de los componentes activos y la eficacia del producto.

En esta memoria se presentan los resultados más relevantes obtenidos durante el desarrollo de la Tesis Doctoral “Formulación de Extractos Vegetales Bioactivos para el Desarrollo de Ingredientes con Aplicaciones Alimentarias”, en la que se han utilizado extractos de plantas con dos objetivos fundamentales: la preparación y el análisis de nuevas formulaciones de recubrimientos comestibles, y el desarrollo y validación de un nuevo procedimiento de formulación de ingredientes y nutracéuticos.

Inicialmente, se prepararon extractos y fracciones bioactivas de romero, tomillo, centaura, artemisia y regaliz, empleando las técnicas de hidrodestilación, extracción sólido-líquido asistida por ultrasonidos y extracción supercrítica. Se evaluó la actividad antioxidante y antimicrobiana de los productos, y se seleccionaron los más adecuados para preparar los recubrimientos comestibles. Se analizó la eficacia de los extractos supercríticos de romero y tomillo en comparación con la de sus correspondientes aceites esenciales, aplicados en combinación con quitosano para la conservación de fresas. Si bien, para una misma planta, los recubrimientos preparados con aceites esenciales resultaron superiores a los preparados con extractos supercríticos, éstos mostraron ventajas significativas en comparación con la muestra control (fresas sin recubrimiento).

En particular, se destaca la investigación realizada en relación a las propiedades antimicrobianas de los extractos de raíz de regaliz, siendo evaluados por primera vez los extractos supercríticos de esta planta. Debido a las buenas propiedades determinadas, tanto antioxidantes como antimicrobianas, los extractos de raíz de regaliz también fueron utilizados para elaborar recubrimientos comestibles y se aplicaron a fresas. Se demostró que la adición de estos extractos a la matriz base (quitosano) mejora las propiedades reológicas de las soluciones formadoras de películas y de los recubrimientos obtenidos, influyendo positivamente en la evolución de las propiedades fisicoquímicas, antioxidantes y microbiológicas de las bayas.

Por otro lado, en relación a la formulación de ingredientes de uso para la salud, se estudió el fraccionamiento y la reducción de tamaño de los extractos de romero y raíz de regaliz utilizando la tecnología de precipitación supercrítica antisolvente (SAS), y se evaluaron las propiedades (composición, morfología, distribución de tamaño de partícula, actividad antioxidante) de la fracción precipitada en forma de micro- y nanopartículas. En ambos casos, el fraccionamiento SAS del extracto permitió obtener fracciones mejoradas en comparación con el extracto original. Además, se determinaron las condiciones de operación para obtener partículas secas, con morfología adecuada, y con tamaños de escala micrométrica y distribución regular.

La capacidad de fraccionamiento y precipitación de extractos vegetales mediante el procedimiento denominado SAS en tecnología supercrítica, se utilizó para dispersar micro- y nanopartículas en un medio oleoso, dando lugar al proceso denominado BIOSAS (*Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process*). Este nuevo procedimiento de formulación de preparados oleosos de extractos vegetales bioactivos (solicitud de patente presentada) permite, en un solo paso, el fraccionamiento, micronización y dispersión de los componentes bioactivos del extracto vegetal en un lípido en atmosfera inerte y a bajas temperaturas, ventajas características de los procesos supercríticos, minimizando el riesgo de oxidación y/o degradación térmica de las biomoléculas. BIOSAS también permite la dispersión de microgotas en el medio oleoso, dando lugar a una nueva técnica supercrítica de formación de microemulsiones con buenas expectativas de desarrollo e innovación en la tecnología de alimentos.

En resumen, esta Tesis Doctoral contribuye al avance sobre el aprovechamiento de las sustancias con propiedades bioactivas presentes en las plantas y, en general, en los productos de origen vegetal, útiles para el desarrollo de innovación en el sector alimentario, a través de la investigación y el estudio de los extractos, las alternativas de formulación y las potenciales aplicaciones.

SUMMARY

The substances present in plant materials possess several technological and functional properties, which have been used and are being used for the development and innovation of the food sector. In this respect, the extracts coming from plants, vegetables, fruits and/or residues of the agrifood industry are target of interest, due to the great variety of substances with demonstrated biological activity that have been isolated from them.

In particular, the use of plant extracts for the production of edible films and coatings has achieved important advances and applications in recent decades. The extracts obtained from the leaves, stems, roots, seeds, etc. are added to the coating matrix, incorporating the components of the extract, and especially those with antimicrobial and antioxidant properties, which contribute to the preservation of the food. Among the most studied plants used for this purpose, thyme, oregano and rosemary stand out, due to the recognized antioxidant and antimicrobial qualities of their corresponding essential oils.

On the other hand, an important part of the research and development concerning novel foods is currently focused on products that, in addition to provide the basic diet nutrients, they can also provide a functional benefit and contribute to preserve and/or improve health. Thus, the research and technological development aim to discover and produce new bioactive plant extracts, useful for formulating ingredients, nutraceuticals and/or food supplements. Nevertheless, it is not enough to test the bioactivity of the extract, but an adequate formulation is crucial to guarantee the bioavailability of the active components and the efficacy of the product.

This report presents the most relevant results obtained during the development of the PhD Thesis " *Bioactive Plant Extracts Formulations for the Development of Ingredients for Food Applications*", in which plant extracts have been used with two main objectives: the preparation and the analysis of new edible coating formulations, and the development and validation of a new formulation procedure for producing food ingredients and nutraceuticals.

Initially, extracts and bioactive fractions of rosemary, thyme, centaury, mugwort and licorice were prepared, using hydrodistillation, ultrasound-assisted solid-liquid extraction and supercritical extraction techniques. The antioxidant and antimicrobial activities of the products were evaluated, and the most suitable fractions were selected to prepare the edible coatings. The efficacy of the rosemary and thyme supercritical extracts was analyzed in comparison with that of their corresponding essential oils, applied in combination with chitosan to the conservation of strawberries. Although, for the same plant, the coatings prepared with essential oils were superior to those prepared with supercritical extracts, the last extracts showed significant advantages in comparison with the control sample (uncoated strawberries).

In particular, it is highlighted the research on the antimicrobial properties of licorice root extracts, having been evaluated for the first time in this thesis the antimicrobial activity of

licorice supercritical extracts. Due to the good antioxidant and antimicrobial properties determined, licorice root extracts were also used to elaborate edible coatings and were applied to strawberries. It was shown that the addition of licorice root extracts to the coating matrix (chitosan) improved the rheological properties of the film-forming solutions and the coatings improved the evolution of the physico-chemical, antioxidant and microbiological properties of the berries.

Related to the formulation of ingredients for health use, the fractionation and size reduction of rosemary and licorice root extracts were studied using antisolvent supercritical precipitation (SAS) technology, together with the properties (composition, morphology, particle size distribution, antioxidant activity) of the precipitated fraction in the form of micro and nanoparticles. In both cases, the SAS fractionation of the extract allowed to obtain improved fractions compared to the original extract. In addition, the operating conditions were determined to obtain dry particles, with adequate morphology, and with micrometric sizes and regular distribution.

The capacity of the so-called SAS process in supercritical technology to fractionate and precipitate plant extracts was used to disperse micro and nanoparticles in an oily matrix, giving rise to the process denominated BIOSAS (*Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process*). This new method for formulating oily preparations of bioactive plant extracts (patent application submitted) allows the simultaneous fractionation, micronization and dispersion of bioactive components in a lipid, under an inert atmosphere and at low temperatures, which are the typical advantages of supercritical processes, minimizing the risk of oxidation and/or thermal degradation of the biomolecules. BIOSAS also allows the dispersion of micro-droplets in the lipid, giving rise to a new supercritical technique to produce micro-emulsions, with good opportunities in food technology.

In summary, this PhD Thesis contributes to the progress in the use of substances with bioactive properties present in plants and, in general, in products of plant origin, which are useful for the development of innovation in the food sector, through the research and the study of the extracts, the formulation alternatives and the potential applications.

CAPÍTULO 1

INTRODUCCIÓN

1. INTRODUCCIÓN

Los extractos vegetales son ampliamente utilizados en la industria alimentaria desde hace décadas como complementos o ingredientes en la producción de alimentos. Sin embargo, en la actualidad, se pretende dar a su uso una mayor relevancia, buscando aquellos extractos vegetales concentrados en sustancias biológicamente activas, con el objetivo fundamental de diseñar ingredientes, suplementos alimenticios, nutracéuticos y/o alimentos de uso específico para la salud debido a sus propiedades antioxidantes, antimicrobianas, anti-inflamatorias, antitumorales, entre otras, y la consecuente implicación en una variedad de mecanismos biológicos (Kaur y Kapoor, 2008).

Existen varios ejemplos de extractos de plantas autorizados como aditivos alimentarios. Por ejemplo, está aprobado el uso del extracto de romero (E329) obtenido con diferentes disolventes y/o con dióxido de carbono supercrítico, con dosis máxima permitida de 200 mg/kg o mg/L, como antioxidante en la formulación de alimentos (Reglamento UE No 1129/2011), o el té comercial de hojas de guava (*Psidium guajava* L.), autorizado en la categoría de alimentos relacionados a la prevención de los niveles de azúcar en la sangre y recomendado en patologías como la pre-diabetes por su contenido en polifenoles (Deguchi y Miyazaki, 2010). Adicionalmente, la Comisión Europea, ha autorizado la declaración de propiedad saludable a los polifenoles del aceite de oliva, debido a su contribución a la protección de los lípidos en la sangre contra el estrés oxidativo, y a los flavanoles del extracto de cacao por ayudar a mantener la vasodilatación dependiente del endotelio, contribuyendo al flujo sanguíneo normal (Reglamento UE No 432/ 2012).

Las fuentes de los extractos vegetales son muy diversas, incluyendo plantas, árboles, arbustos, hierbas, así como son muy variadas las partes del material vegetal de donde se pueden obtener (tallos, semillas, frutos, hojas, raíces) dando lugar a extractos con composiciones muy diferentes y por tanto con actividades biológicas que proporciona un amplio campo de aplicaciones.

Los extractos están formados por sustancias bioactivas de diferente naturaleza o estructura química, entre ellas se encuentran los alcaloides, fitoesteroles, carotenoides y los compuestos fenólicos. Estas sustancias atraen la atención de la comunidad científica y de la industria alimentaria, dado que ofrecen la oportunidad de diseñar nuevos productos, con beneficios para la salud más allá de la nutrición básica.

Los compuestos fenólicos incluyen una gran variedad de sustancias, incluidos los ácidos fenólicos, taninos, flavonoides, lignanos y cumarinas (Liu, 2013). Los ácidos fenólicos, también llamados fenol-carboxilos, son aquellos compuestos fenólicos que tienen un grupo ácido carboxílico en su estructura. Por lo general, están presentes en uniones de amidas, ésteres o glucósidos y raramente en forma libre (Pereira y col., 2009) y se encuentran en una gran variedad de especies vegetales, especialmente en las semillas, pieles de las frutas y hojas de algunos de ellas, donde están en concentraciones más altas. Los ácidos fenólicos se dividen principalmente en dos subgrupos: ácidos hidroxibenzoicos y ácidos hidroxicinámicos (Clifford,

1999). Los primeros, poseen una estructura común de C6-C1 y derivan del ácido benzoico. Se encuentran en forma soluble, conjugada con azúcares o ácidos orgánicos, y se unen con fracciones de la pared celular como en la lignina. Se pueden encontrar generalmente en frutos rojos, cebollas, rábanos negros, entre otros (Shahidi y Naczk, 1995). Los ácidos hidroxicinámicos, son derivados del ácido cinámico, y están presentes en los alimentos como ésteres simples con ácido quínico o glucosa. Son especialmente abundantes en bebidas de café (ácidos cafeico, ferúlico, cafeoilquínicos, feruloilquínicos), en arándanos (ácido cafeico), en espinacas (ácido *p*-coumárico) y en brócoli (ácido sinápico) (Clifford, 1999). Los ácidos fenólicos han sido ampliamente investigados, especialmente por sus propiedades antioxidantes (Ranjbar Nedamani y col., 2014) y anti-inflamatorias (Paciello y col., 2020)

En cuanto a los flavonoides, son moléculas de bajo peso molecular, los que estructuralmente contienen dos anillos aromáticos unidos a un anillo de pirano heterocíclico, en una disposición C6-C3-C6. Se clasifican en flavononas, flavonoles, antocianinas, flavonas e isoflavonoides. Esta gran diversidad de compuestos se puede encontrar en muchas especies vegetales como en el té verde (catequinas y sus oligómeros proantocianidinas), en zumos de naranja (naringenina y hesperidina), en cebolla y manzana (quercetina), en bayas (malvidina y delfinidina), en cereales (luteolina), o en soja (genisteína). Los flavonoides han sido destacados como compuestos capaces de prevenir o incluso mejorar enfermedades crónicas como enfermedades cardiovasculares (Perez-Vizcaino y Duarte, 2010) diabetes (Xiao y col., 2015), algunos tipos de cáncer (Fan y col., 2019) y procesos de inflamación (Maleki y col., 2019).

Los taninos son compuestos polifenólicos solubles en agua, reconocidos por sus propiedades antimicrobianas y antivirales (Buzzini y col., 2008). Se clasifican generalmente en taninos hidrolizables y condensados, siendo los primeros mezclas de fenoles simples (ácido elágico o gálico) que pueden hidrolizarse mediante ácidos/bases débiles para producir carbohidratos y ácidos fenólicos (Aroso y col., 2017). Se han extraído fundamentalmente de hojas y pieles de frutas (Shirmohammadli y col., 2018). Los taninos condensados incluyen oligómeros y polímeros compuestos del núcleo flavan -3-ol, como las proantocianidinas y procianidinas (Badal Mccreath y Delgoda, 2017).

Por su parte, los carotenoides son un grupo de terpenoides que contienen una cadena larga de dobles enlaces conjugados (Britton, 1995). Son isoprenoides lipofílicos sintetizados por todos los organismos fotosintéticos, incluidas las plantas, las algas y las cianobacterias, así como varios hongos no fotosintéticos y procariotas (Zheng y col., 2020). Se pueden encontrar en tomate (licopeno) y cáscara de frutas cítricas (d-limoeno, pineno, eucaliptol) (Johnson, 2014; Ma y col., 2012; Shareck y col., 2017) entre muchas otras fuentes. Además de su papel como precursores de la vitamina A, son antioxidantes, y contribuyen a la prevención del cáncer, enfermedades cardiovasculares y trastornos neurodegenerativos.

Los compuestos alcaloides son un grupo de sustancias que generalmente contienen uno o más átomos de nitrógeno, dentro de un anillo heterocíclico. Se encuentran en plantas de la familia *Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae,* y *Loganiaceae* (Shariful y col., 2015). Varios alcaloides, como camptotecina, vincristina, vinblastina,

berberina, sanguinarina, evodiamina, piperina y tetrandrina, son conocidos como potentes agentes quimioterapéuticos (Lu y col., 2012).

1.1. OBTENCIÓN DE EXTRACTOS VEGETALES BIOACTIVOS MEDIANTE FLUIDOS SUPERCRÍTICOS

La composición de los extractos vegetales depende en gran medida de la tecnología empleada para su obtención. Para su producción, se pueden emplear técnicas convencionales como la maceración, infusión, extracción Soxhlet, hidrodestilación, extracción asistida por ultrasonidos o por microondas, en las que se emplean disolventes tanto de carácter hidrofílico como lipofílico en función del tipo de compuestos de interés que se pretenda extraer (Pabón-Baquero y col., 2018). Sin embargo, con el objetivo de reducir los residuos orgánicos contaminantes en los procesos de extracción y, por tanto, el impacto ambiental causado por la eliminación de los mismos, existen actualmente tecnologías de extracción más sostenibles desde el punto de vista medioambiental.

La tecnología de dióxido de carbono supercrítico ($SCCO_2$) es una alternativa muy interesante para la obtención de extractos vegetales bioactivos, gracias a las propiedades del disolvente empleado como fluido supercrítico, así como a la posibilidad de extracción secuencial, fraccionamiento el extracto, uso de diversos co-solventes, permitiendo la obtención de productos con diferente composición y propiedades biológicas. Además, la posibilidad de utilizar temperaturas moderadas y la eliminación del disolvente por descompresión, da lugar a productos de mejor calidad, sin residuos de disolventes y, en muchas ocasiones, con una mejor funcionalidad biológica (Fornari y col., 2012; Ramírez y col., 2005).

La extracción con fluidos supercríticos (SFE) es un proceso de extracción que emplea un fluido supercrítico como disolvente de extracción. Los fluidos supercríticos se definen como fluidos que se encuentra en condiciones de temperatura y presión por encima de su punto crítico (Brunner, 2005), con densidades similares a las de un líquido, lo que le confiere un alto poder de solvatación, pero viscosidades y difusividades similares a las de un gas, lo que le confiere buena capacidad de transporte de materia. El principal fluido supercrítico utilizado industrialmente es el dióxido de carbono (CO_2), debido a su temperatura crítica baja (304,2 K), lo que permite trabajar en condiciones cercanas a la temperatura ambiente, y a una presión crítica moderada (7,38 MPa) y fácilmente alcanzable. Además, es un compuesto no tóxico, no inflamable, económico, de fácil disposición (Attard y col., 2018; Chemat y col., 2017; da Silva y col., 2016) y ha sido asignado por la EFSA y la FDA como un disolvente GRAS (*Generally Recognizad as Safe)*.

El esquema básico de un proceso de extracción con $SCCO_2$ se muestra en la Figura 1.1. El $SCCO_2$ fluye de forma continua y se introduce por la parte inferior de la celda de extracción, donde se encuentra cargado el material vegetal. A la salida de la celda de extracción, el $SCCO_2$ con los solutos extraídos se expanden a través de una válvula de despresurización, los solutos se separan del disolvente gaseoso y se recogen en uno o varios separadores, según se lleve a cabo una despresurización total o en cascada (Fornari y col., 2012). La posibilidad de

obtener extractos con diferente composición y/o características, depende fuertemente de las condiciones de operación, tales como la presión, temperatura, flujo de $SCCO_2$, así como del uso y cantidad de co-solvente. La presión y la temperatura determinan la densidad del disolvente y así, el poder de solvatación de $SCCO_2$. No obstante, en el nivel de temperatura debe considerarse también, la estabilidad térmica, así como la presión del vapor de los solutos, y las propiedades de la matriz, que pueden dificultar la transferencia de masa.

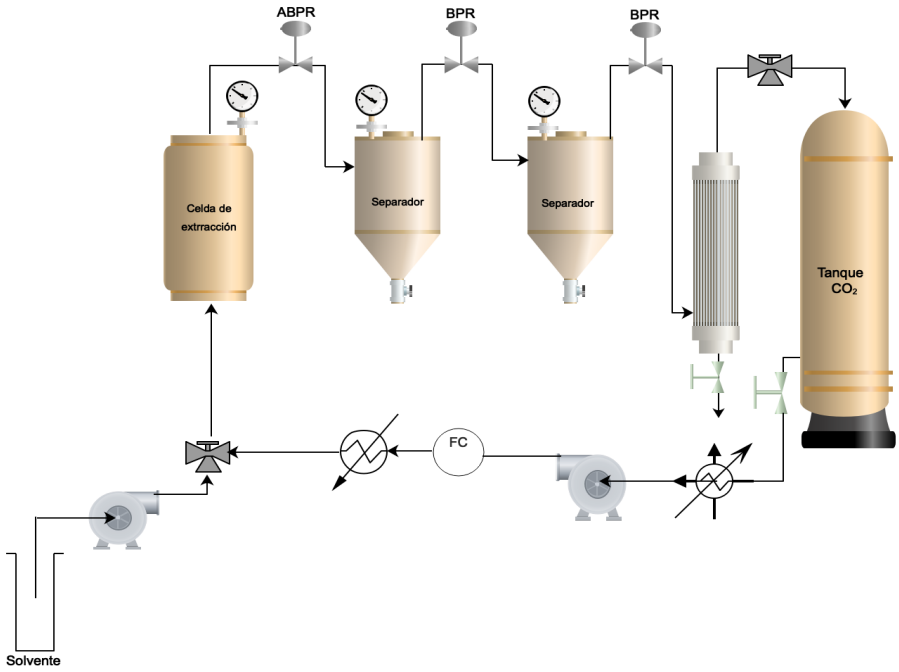

Figura 1.1. Esquema de un equipo de extracción supercrítica

Por otro lado, el $SCCO_2$ es un buen disolvente para la extracción de compuestos apolares, y la solubilidad del soluto disminuye a medida que aumenta su peso molecular. La solubilidad de las sustancias polares se puede mejorar aumentando la presión de operación o agregando un co-solvente polar en pequeñas cantidades. Los co-solventes polares más efectivos suelen ser acetonitrilo, acetona, metanol, éter etílico, etanol y agua (Xu y col., 2017). Sin embargo, debido a su baja toxicidad, el etanol es el más utilizado, y sobre todo en el campo de los alimentos (Khaw y col., 2017; Machado y col., 2013; Xu y col., 2017). El etanol ha sido ampliamente utilizado para mejorar la extracción supercrítica de compuestos fenólicos, (Espinosa-Pardo y col., 2017) flavonoides (Bimakr y col., 2011), terpenoides (Bensebia y col., 2016), incluso carotenoides (de Andrade Lima y col., 2018).

La extracción con $SCCO_2$ (SFE) se emplea para el procesamiento tanto de alimentos sólidos como líquidos (Attard y col., 2018). Entre las aplicaciones, se ha utilizado como una alternativa a los procesos de refinación, como por ejemplo, la SFE del aceite de germen de trigo (Piras y col., 2009), de café verde (Kim y col., 2008), de salvado de arroz (Sparks y col., 2006), de palma (Akanda y col., 2012) o para la recuperación de aceites esenciales (Asbahani y col., 2015). También, se ha utilizado para eliminar compuestos no deseados de matrices vegetales, como por ejemplo la extracción de la cafeína del té verde, evitando la extracción (perdida) de

los apreciados antioxidantes del té (Kim y col., 2008). La SFE ha sido muy investigada para la extracción y fraccionamiento de ácidos grasos (Ben Rahal y col., 2015; Bromberger Soquetta y col., 2018; Canela y col., 2002), como por ejemplo los aceites de pescado (Perretti, 2006), la concentración de fosfolípidos presentes en polvos de crema de suero de leche (Spence y col., 2009), la extracción de pigmentos de microalgas (Yen y col., 2015), de isoflavonas de diversas fuentes vegetales (Lummaetee y col., 2017; Rostagno y col., 2002) y de una gran variedad de compuestos bioactivos (Pereira y Meireles, 2010; Wrona y col., 2017). Por ejemplo, Palsikowski y col. (2019) obtuvieron extractos supercríticos de hojas de *Bauhinia forficata* con un alto contenido de tocoferoles en comparación con el método Soxhlet con etanol, acetato de etilo y hexano, o Vieitez y col. (2017) que obtuvieron extractos de romero (*Rosmarinus officinalis*), boldo (*Peumus boldus*), cidron (*Aloysia citrodora*), cangrosa (*Maytenus ilicifolia*), yerba mate (*Ilex paraguariensis*) y pitanga (*Eugenia uniflora*) con mejor actividad antioxidante y antimicrobiana que los obtenidos por maceración con hexano y/o etanol.

1.2. FUENTES POTENCIALES DE EXTRACTOS VEGETALES

En este apartado se describen los materiales vegetales utilizados durante el desarrollo de la tesis, entre los que se encuentran algunas variedades de plantas muy estudiadas, como el romero y tomillo (García-Risco y col., 2011; Santoyo y col., 2014; Vicente, y col., 2013) y otras especies menos conocidas y/o estudiadas como la raíz de regaliz, centaura y artemisa (Figura 1.2).

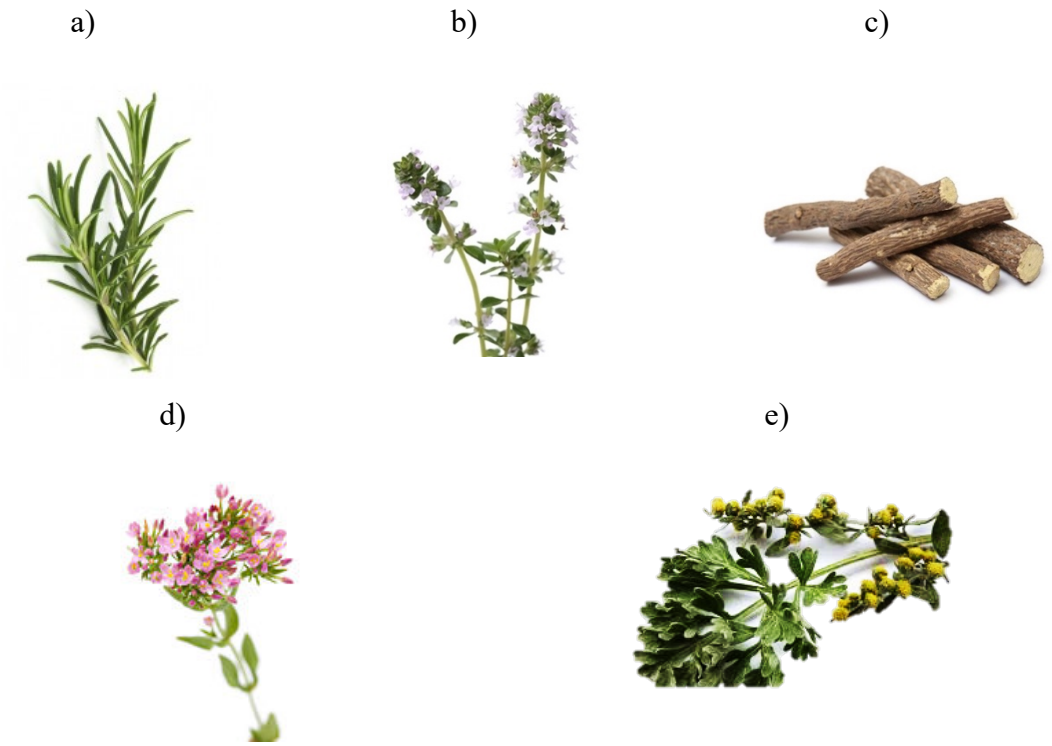

Figura 1.2. Matrices vegetales para la obtención de los extractos: a) romero (*Rosmarinus officinalis* L.), b) tomillo (*Thymus vulgaris*), c) raíz de regaliz (*Glycyrrhiza glabra* L.), d) centaura (*Centaurium erythraea*) y e) artemisia (*Artemisia vulgaris*).

1.2.1. Hojas de romero (*Rosmarinus officinalis* L.)

El romero (Figura 1.2a) es una hierba aromática de la familia *Lamiaceae*, que se caracteriza por ser densa, resistente, de 90-200 cm de altura de hoja perenne, pequeñas, puntiagudas y resinosas. Las hojas, las flores y las ramas producen un aceite esencial y oleorresina valorado en la medicina tradicional y moderna, en aromaterapia, así como en las industrias de perfumes y saborizantes. Algunos extractos obtenidos del romero son considerados productos interesantes como nutracéuticos o como ingredientes para elaborar alimentos funcionales.

Las hojas de romero se caracterizan por poseer cantidades abundantes de diterpenos fenólicos, siendo el ácido carnósico y el carnosol los principales, y otros compuestos fenólicos como el ácido rosmarínico, rosmanol, y en más bajas concentraciones epirosmanol y metoxiepirosmanol (Rezzoug y col., 2005). Por otro lado, el aceite esencial de romero está compuesto principalmente por 1,8-cineol, alcanfor, borneol y α-terpineol. Un gran número de extractos y componentes del romero han evidenciado tener importantes actividades biológicas, tales como actividad antioxidante (Celiktas y col., 2007), antimicrobiana (Wang y col., 2012) y anti-inflamatoria (Villalva y col., 2019) entre otras.

Los extractos de romero obtenidos por SFE en diferentes condiciones han demostrado tener una destacada bioactividad. Por ejemplo, García-Risco y col. (2011) evaluaron los tiempos de extracción para el fraccionamiento del extracto de romero, y demostraron que la actividad antioxidante de las fracciones extraídas está directamente relacionada con el contenido de ácido carnósico, revelando un aumento significativo a medida que aumentaba el tiempo de extracción. Vicente y col. (2013) estudiaron las diferentes condiciones de extracción SFE de romero (presión, cantidad de co-solvente y fraccionamiento en línea), y obtuvieron altos contenidos de compuestos antioxidantes debido al empleo de co-solventes polares (etanol). Ramírez y col. (2006) analizaron la influencia de la presión y de la temperatura para el aislamiento de compuestos bioactivos a partir del romero empleando un sistema preparativo de cromatografía supercrítica, obteniendo una fracción con una mejora del 20 % de la actividad antioxidante y 40 % de la actividad antimicrobiana en comparación con el extracto supercrítico original y sin aroma de romero residual.

1.2.2. Hojas de tomillo (*Thymus vulgaris*)

El tomillo (Figura 1.2b) es una planta aromática ampliamente utilizada como agente saborizante, hierba culinaria y como planta medicinal. Su aceite esencial tiene más de 60 compuestos, siendo los fenoles timol y carvacrol los más abundantes, así como los monoterpenos hidrocarbonados, *p*-cimeno y γ-terpineno (Daferera y col., 2000; Pasqua y col., 2004). Este aceite esencial ha demostrado tener propiedades beneficiosas para la salud, como antivirales (Koch y col., 2008; Nolkemper y col., 2006), antimicrobianas y antioxidantes (Baranauskiene y col., 2003; Chizzola y col., 2008; Rasooli y col., 2006; Rota y col., 2008). Además, el timol está aprobado por la FDA (21 CFR 172.515) como aditivo alimentario y,

junto con el aceite volátil, es reconocido como GRAS (*Generally Recognized as Safe*) (21 CFR 182.20).

La tecnología de fluidos supercríticos para la obtención de extractos de tomillo ha sido estudiada por varios autores. Por ejemplo, Babovic y col. (2010) aislaron un extracto de *Thymus vulgaris* con alta capacidad antioxidante, Fornari y col. (2012) obtuvieron una recuperación del 93,4 % de los compuestos volátiles en un sistema con fraccionamiento en línea, donde el compuesto mayoritario obtenido fue el timol, y Santoyo y col. (2014) obtuvieron extractos supercrítico de tomillo (especies *Thymus vulgaris*, *Thymus hyemalis* y *Thymus zygis*) con capacidad de reducción de infección del virus del herpes simplex tipo 1 (HSV-1) de las especies.

1.2.3. Raíz de regaliz (*Glycyrrhiza glabra* L.)

La raíz de regaliz (Figura 1.2c), también llamada raíz dulce, es un arbusto perenne leñoso, perteneciente al a familia *Fabaceae*, que crece en la región mediterránea, Asia Menor y Medio Oriente, y también se cultiva ampliamente en el sur de Rusia (Ody, 2000). Contiene compuestos activos como saponinas, flavonoides (liquiritin e isoliquiritin), isoflavonoides (isoflavonol, licoricona y glabrol), chalconas, cumarinas (umbeliferona, herniarina), benzofurano, fenoles (Kondo y col., 2007; Simons y col., 2009; Zhang y Ye, 2009), esteroles, lignina, aminoácidos, aminas, gomas y compuestos volátiles; donde su principal compuesto activo es el triterpenoide ácido glicirricínico también conocido como ácido glicirrícico o saponina glicirricina (Murray, 2020).

Existen numerosos trabajos en la bibliografía que han aplicado diferentes metodologías para la obtención de extractos bioactivos a partir de la raíz de regaliz. En la mayoría de los estudios descritos, el objetivo principal fue la obtención de extractos con actividad antioxidante. Visavadiya y col. (2009) realizaron la extracción Soxhlet con etanol y agua, Gupta y col. (2016) y Charpe y Rathod (2012) optimizaron la recuperación de ácido glicirrícico utilizando agua en la extracción asistida por ultrasonidos, Karami y col. (2015) emplearon la extracción sólido-líquido asistida con microondas, con mezclas de agua-etanol y agua-metanol, Cheel y col. (2010) y Tohma y Gulçin (2010) obtuvieron extractos por infusión y Mutaillifu y col. (2020) llevaron a cabo el aislamiento de los polisacáridos de la raíz de regaliz utilizando agua. También, aunque mucho menos estudiada, se ha aplicado la tecnología de extracción supercrítica a la raíz de regaliz. Por ejemplo, Hedayati y Ghoreishi (2015) investigaron la recuperación de ácido glicirrícico por extracción Soxhlet y SFE con metanol y agua como co-solventes del $SCCO_2$, siendo la SFE la que presentó mejores rendimientos.

1.2.4. Parte aérea de centaura (*Centaurium erythraea*)

La centaura es una planta de la familia *Gentinaceae*. Es una hierba anual o bianual de tallo erguido de entre 10 a 50 cm y con numerosas ramas (Figura 1.2d). Tiene hojas de color verde pálido, lisas y con bordes enteros. En esta especie, se han aislado e identificado una gran variedad de compuestos, incluyendo centaurósido, flavonoides, gentiopicrina, gentiopicrosida, isocoumarina, ácidos fenólicos, triterpenos, xantonas (Jovanović y col., 2009; Kaouadjl y col.,

1986; Kumarasamy y col., 2003; Schimmer y Mauthner, 1996; Takagi y col., 1982; Valentão y col., 2002, 2003; Van Der Sluis y Labadie, 1981) varios esteroides (β-sitosterol, estigmasterol, δ-7-estigmasterol y campesterol) y aminoácidos esenciales (leucina, alanina, triptófano y fenilalanina) (Aquino y col., 1985). Algunos de estos compuestos muestran actividades biológicas tales como actividad antimicrobiana (Šiler y col., 2014), antioxidante (Đorđević y col., 2017; Sefi y col., 2011), hepatoprotectora (Mroueh y col., 2004) y gastroprotectora (Tuluce y col., 2011). Se han obtenido también extractos con metanol y cloroformo de las partes aéreas, identificando compuestos como secoiridoides, alcaloides indólicos y compuestos fenólicos (ácidos fenólicos, flavonoides, cumarinas y xantonas) (Šiler y col., 2012, 2014; Stefkov y col., 2014; Valentão y col., 2002, 2003). Recientemente, Bouyahya y col. (2019) obtuvieron aceite esencial por hidrodestilación y los principales compuestos identificados fueron carvacrol, mentol y tricosano, presentando una buena capacidad antioxidante y actividad antibacteriana frente a bacterias Gram positivas.

1.2.5. Hojas de artemisia (*Artemisia vulgaris*)

La artemisa (Figura 1.2e) es un arbusto perteneciente a la familia *Asteraceae*. Es una planta perenne de hoja ancha que se propaga rápidamente con la introducción de un sistema de rizoma bien desarrollado (Barney y Ditommaso, 2003). Se caracteriza por tener hojas pinnatisectas o bipinnatisectas, con segmentos lanceolados u oblongos, suaves, dorsalmente de color blanco plateado. En toda la planta se han identificado compuestos pertenecientes a las clases de flavonas (tricina, jaceosidina, eupafolina, crisoeriol, diosmina, apigenina, luteolina), glucósidos de flavonas (luteolina 7-glucósido y vitexina), flavanonas (homoeriodictyol y eriodictyol), flavonoles (isorhamnetin) y glucosidos de flavonol (kaempferol 3-glucósido, kaempferol 7-glucósido, kaempferol 3-ramnosido, kaempferol 3-rutinósido, quercetina 3-glucósido, quercetina 3-galactósido, quercetina y rutina) (Abiri y col., 2018).

Sobre el aceite esencial de la planta se han realizado diferentes estudios en los que se describe que el aceite está compuesto por α-pineno, alcanfor, canfeno, germacreno D, 1,8-cineol, β-carofileno y α-tujona (Bagci y col., 2010; Blagojević y col., 2006; Bora y Sharma, 2011; Govindaraj y col., 2008, Pandey y col., 2017). Además se han obtenido extractos de la planta con actividad antioxidante mediante maceración con acetona y agua (Bahorun y col., 2004) y extracción por maceración asistida por ultrasonidos con metanol (Melguizo-Melguizo y col., 2014) y con actividad antimicrobiana mediante técnica de extracción asistida por ultrasonidos y por Soxhlet con metanol (Karabegović y col., 2011).

1.3. APLICACIONES DE LOS EXTRACTOS VEGETALES

Existe una creciente preocupación por parte de los consumidores por la seguridad que ofrecen los conservantes sintéticos, marcando así una preferencia hacia los aditivos alimentarios naturales. En la aplicación de extractos de plantas como ingredientes alimentarios es necesario, además de ausencia de toxicidad, que no afecten negativamente en las propiedades sensoriales del alimento, que sean eficaces a bajas concentraciones, compatibles con una amplia gama de alimentos, que sean estables durante el procesado y la vida útil del alimento y, por

último, que sean de fácil aplicación y económicos (Horita y col., 2018). En las últimas décadas, se han estudiado en la bibliografía varias aplicaciones de los extractos naturales de origen vegetal en alimentos, por ejemplo, como colorantes naturales (Aberoumand, 2011), como conservantes para retrasar la oxidación de lípidos (Costa y col., 2015), como aditivos alimentarios para prevenir el crecimiento microbiano y la contaminación (Tohidi y col., 2017), como recubrimientos en películas comestibles (Horita y col., 2018) y para el desarrollo de nutracéuticos (Gonçalves y col., 2018), entre otras. En particular, se describen a continuación las dos aplicaciones de extractos vegetales que se han estudiado en esta tesis.

1.3.1. Extractos vegetales como ingredientes bioactivos en la elaboración de recubrimientos y películas comestibles

El objetivo de los recubrimientos comestibles (RC) y películas comestibles (PC) es prolongar la vida útil del alimento y proporcionan una barrera contra la humedad, el oxígeno o cualquier otro factor causante de su deterioro (Bourtoom, 2008). Además, tienen un alto potencial para transportar ingredientes activos como sabores, especias, agentes anti-pardeamiento, compuestos antimicrobianos y antioxidantes, que pueden contribuir también al aumento de la vida útil del producto o de las propiedades organolépticas o, incluso, del valor nutricional.

Los RC y las PC son envases primarios elaborados con ingredientes comestibles, diferenciándose entre ellos en que el RC es una capa delgada de material comestible formada sobre un producto alimenticio y la PC se define como una capa delgada preformada, hecha de material comestible, que una vez formada se puede colocar sobre o entre componentes alimentarios (McHugh, 2000). Las películas se obtienen como laminados sólidos y posteriormente se aplican sobre los alimentos mientras que los recubrimientos se aplican en forma líquida, y se obtienen generalmente sumergiendo el producto en una mezcla de los ingredientes que forma parte del recubrimiento (Falguera y col., 2011). Para que estos RC y PC puedan utilizarse con estos objetivos deben cumplir algunos requisitos específicos como tener buenos atributos sensoriales, buenas propiedades mecánicas (flexibilidad, tensión), buenas propiedades ópticas (brillo y opacidad), estabilidad bioquímica, fisicoquímica y microbiana, no ser tóxicos ni contaminantes y deben de poder realizarse mediante tecnologías sencillas y de bajo coste tanto de la materia prima como de procesado (Dainelli y col., 2008).

Los RC y las PC se producen principalmente a partir de biopolímeros comestibles que tienen propiedades formadoras de películas, las que se clasifican teniendo en cuenta la solución base. Esta solución base puede formarse a partir de polisacáridos, proteínas, lípidos u otros compuestos. Los *polisacáridos* son polímeros de origen natural que poseen poca capacidad de propiedades de barrera. Son incoloros y tienen una apariencia libre de grasa; los más empleados son el almidón, la celulosa y sus derivados, la pectina, el quitosano, el alginato, el carragenato y la goma gelana (Han, 2013). Las soluciones a base de *proteínas* presentan excelentes propiedades de barrera contra el oxígeno, lípidos y aromas, alta permeabilidad al vapor de agua y propiedades mecánicas moderadas. El colágeno, proteína de suero, gelatina, zeína de maíz, caseínas, gluten de trigo, proteína de soja, proteína de quínoa, proteína de clara de huevo y

queratina son ejemplos de proteínas empleadas para la elaboración de este tipo de películas (Han, 2013). Los *lípidos* son excelentes barreras contra la migración de la humedad (Debeaufort y Voilley, 2009) debido a su baja polaridad. Normalmente, las películas o recubrimientos hechos de lípidos son extra frágiles y más gruesos debido a su hidrofobicidad (Perez-Gago y col., 2002), aportan brillo, minimizan el coste y la complejidad del envase (Akoh y Min, 2008; Shit y Shah, 2014). Ejemplos de lípidos empleados como recubrimiento son la cera de abejas, cera de candelilla, cera de carnauba, triglicéridos, monoglicéridos acetilados, ácidos grasos libres, alcoholes grasos, ésteres de sacarosa y resinas terpénicas comestibles (Brody y col., 2008; Rojas-Graü y col., 2009). Por último, las películas o recubrimientos *compuestos* se pueden crear mezclando dos o más de los componentes relacionados anteriormente (Debeaufort y col., 2000), con el objetivo de minimizar las desventajas de cada uno de ellos por separado (Galus y Lenart, 2013; Kurek y col., 2014) y conseguir por lo tanto, la ventajas de las buenas propiedades de barrera al agua de los recubrimientos lipídicos y la capacidad de formar películas cohesivas con buenas propiedades de permeabilidad a los gases y sin textura grasosa de polisacáridos o proteínas (Kurek y col., 2014).

Adicionalmente, a las PC y los RC se pueden incorporar otros componentes como aditivos de calidad alimentaria que pueden mejorar o modificar la funcionalidad básica del material como los plastificantes (glicerol, sorbitol, propilenglicol, sacarosa, ácidos grasos, polietilenglicol y monoglicéridos) (Sothornvit y Krochta, 2005) que se añaden a los polímeros para reducir la fragilidad y flexibilidad y mejorar la tenacidad y resistencia de las películas. Sin embargo, hay que tener en cuenta que algunos aditivos aumentan la permeabilidad de la película al oxígeno, humedad, aromas y aceites debido a la reducción de las atracciones intermoleculares a lo largo de las cadenas del polímero (Barreto y col., 2003; Rojas-Graü y col., 2009; Sothornvit y Krochta, 2005). También se pueden adicionar agentes de refuerzo como fibras (Pereda y col., 2011), emulsionantes para mejorar su adhesión (Santacruz y col., 2015; Tan y col., 2014), ingredientes para mejorar la calidad, estabilidad y/o seguridad (aromas, colorantes, antioxidantes) y agentes antimicrobianos que actúan como factor de estrés para disminuir el crecimiento de patógenos y proteger los alimentos contra la descomposición.

Las soluciones base para el desarrollo de RC y PC compuestas deben considerar las diferentes polaridades de cada uno de los ingredientes que integran la fórmula, para conseguir una elevada homogeneidad. Por tanto, en la mayoría de los casos se requiere de procesos de emulsificación y homogenización de las fases no miscibles, así como del estudio posterior de su estabilidad y propiedades, puesto que las características de la solución formadora de recubrimientos (SFR) están directamente relacionadas con sus propiedades reológicas y microestructurales. Propiedades como la viscosidad, la densidad y la tensión superficial son determinantes para la formación y un espesor de película apropiados (Skurtys, 2010) y las propiedades reológicas son determinantes para la elección del método de aplicación de los RC.

Las SFR compuestas, debido a las interacciones entre los compuestos hidrófilos e hidrófobos, generalmente se presentan como fluidos no newtonianos, donde la viscosidad varía en función de las velocidades de deformación, y pueden dar problemas para aplicar capas uniformes a velocidades aceptables. Por ejemplo, la adherencia deseada en el producto final a

menudo se asocia con una viscoelasticidad sustancial durante el procesado (Glass y Prud'homme, 1997). Para la elaboración de películas comestibles a partir de las soluciones formadoras se emplean deferentes métodos: 1) formación de moldes por compresión o extrusión, en los que posteriormente se depositará el alimento y 2) por fundición "*casting*" con disolvente o proceso húmedo (Nussinovitch, 2009), en el que las soluciones formadoras se extienden sobre un alimento y se dejan secar. Durante este proceso de secado, la solubilidad del polímero va disminuyendo como resultado de la evaporación del disolvente hasta que las cadenas del polímero se alinean para formar la película sólida (Skurtys, 2010).

El recubrimiento de matrices alimentarias se puede realizar mediante pulverización o inmersión. El método de pulverización consiste en esparcir la solución formadora sobre el producto (González-Forte y col., 2014), mientras que en el proceso de inmersión se sumerge directamente la matriz alimentaria en la SFR y posteriormente se utiliza aire para secar el exceso de solución y para que la solución solidifique sobre el alimento. En ambos procesos, la adhesión del recubrimiento al alimento implica la difusión entre la solución de recubrimiento y el área de la superficie del producto alimenticio (Parreidt y col., 2018; Senturk Parreidt y col., 2018). La efectividad para proteger los alimentos dependerá del control de la humedad (Cerqueira y col., 2009), de una baja solubilidad en agua, para evitar la disolución del recubrimiento (Ozdemir y Floros, 2008), y de la capacidad de la película para mantener la funcionalidad de los compuestos dentro de la matriz, ya que la pérdida de estas moléculas afecta el espesor de la película (Park, 1999).

1.3.1.1. Recubrimientos y películas comestibles con adición de compuestos bioactivos

El objetivo de incluir compuestos bioactivos dentro de las SFR es intensificar aún más las propiedades en propias del RC o de la PC y extender la vida útil de los alimentos por más tiempo. Los ingredientes bioactivos que se incorporan en las soluciones formadoras de películas o recubrimientos pueden ser compuestos puros de diferente naturaleza o bien ingredientes más complejos, como los extractos de plantas, formados por una serie de componentes que pueden actuar solos o de forma sinérgica, potenciando su actividad.

En la actualidad, tanto el estudio como el empleo de recubrimientos comestibles como biopreservantes está progresando a pasos agigantados (Skurtys, 2010). Por ejemplo, Alzate y col. (2017) prepararon películas comestibles usando almidón de tapioca, hidroxipropilmetilcelulosa y glicerol, e incorporaron ingredientes bioactivos puros como sorbato de potasio y carvacrol, obteniendo una película con niveles satisfactorios de resistencia mecánica, incolora, mayor hidrofobicidad y con efectos antimicrobianos frente a *Zygosaccharomyces baili*; Mannozzi y col., (2018) elaboraron recubrimientos a base de quitosano enriquecidos con procianidinas, que fueron capaces de mantener las propiedades antioxidantes de los arándanos frescos durante el almacenamiento a 4 ºC durante 14 días, Zahedi y col. (2019), aplicando quitosano y espermidina aumentaron la vida útil y retrasaron la maduración de los frutos de mango durante 24 días de almacenamiento a 15 ºC con humedad relativa de 85-90 %.

También, los aceites esenciales, extractos de plantas, hierbas y especias, y algunos subproductos agrícolas, se consideran materias primas de interés para el envasado activo de alimentos (Ganiari y col., 2017) y han demostrado, en general, que se obtiene una mejora de las propiedades de los recubrimientos (Maqbool y col., 2011). Por ejemplo, y entre los trabajos más recientes, Utami y col. (2017) adicionaron oleorresina de lima kaffir en recubrimientos comestibles de almidón de mandioca extendiendo la vida útil de carne de res; Correa-Pacheco y col. (2017) estudiaron la incorporación de aceites esenciales en forma de nanopartículas mejorando el control de *Colletotrichum gleosporioides* en aguacate; Shokri y col. (2020) controlaron el aumento de compuestos nitrogenados volátiles y peroxidación de lípidos en filetes de trucha con nanoemulsiones de quitosano y aceite esencial de *Ferulago angulata;* Artiga-Artigas y col. (2017) desarrollaron un recubrimiento comestible a base de nanoemulsiones con aceite esencial de orégano y fibra de mandarina prolongando la vida útil de ciertos quesos; Huang y col. (2020) prepararon un recubrimiento de nanoemulsión a base de gelatina, quitosano y extracto de romero que inhibió el crecimiento de microrganismos en muestras de pollo; y Xiong y col. (2020) prolongaron la vida útil de la carne de cerdo al minimizar el cambio de pH, prevenir la oxidación de lípidos y proteínas e inhibir el crecimiento microbiano con la aplicación de un de recubrimiento de quitosano-gelatina con extracto de semilla de uva y nisina.

1.3.2. Extractos vegetales como ingredientes para el diseño de nutracéuticos y/o suplementos de uso específico para la salud

Nutracéutico es un término híbrido entre nutrición y farmacéutico, propuesto inicialmente por Stephen L. DeFelice en 1989 (DeFelice, 1995). Se define como cualquier sustancia que sea un alimento, o una parte de un alimento, que proporciona beneficios para la salud, incluida la prevención y el tratamiento de enfermedades, alternativo a los fármacos.

Los nutracéuticos son estructural y funcionalmente muy diversos, pueden incluir numerosos compuestos químicos y se pueden clasificar en función de la fuente alimentaria, la naturaleza química y el mecanismo de acción del componente bioactivo (Alamgir, 2017; Kalra, 2003). Una clasificación de los nutracéuticos se puede evidenciar en la Figura 1.3.

Los denominados nutracéuticos tradicionales son aquellos que se obtienen directamente de la naturaleza y se utilizan como tal sin ningún cambio en su forma; y los nutracéuticos no tradicionales (recombinantes o fortificados) son cultivos o alimentos diseñados biotecnológicamente que tienen una cantidad mucho mayor de nutrientes en comparación con los cultivos o alimentos normales.

Teniendo en cuenta su composición química se pueden encontrar (Alamgir, 2017; Kalra, 2003):

⇒ *Fitoquímicos*, como los compuestos fenólicos, incluidos los flavonoides y los no flavonoides, utilizados como antioxidantes; los carotenoides que son potentes anticancerígenos; entre muchos otros.

⇒ *Nutrientes,* como las vitaminas (presentes en cereales, frutas, productos lácteos, cárnicos), los minerales (se pueden encontrar en productos vegetales, animales y lácteos) y los ácidos grasos (por ejemplo ω-3 PUFA, presentes en las semillas de lino y el salmón).

⇒ *Hierbas,* como la corteza de sauce que contiene la salicina, una sustancia con actividad antiinflamatoria y analgésica; la lavanda, que contiene tanino, utilizado para la hipertensión, el estrés, el resfriado.

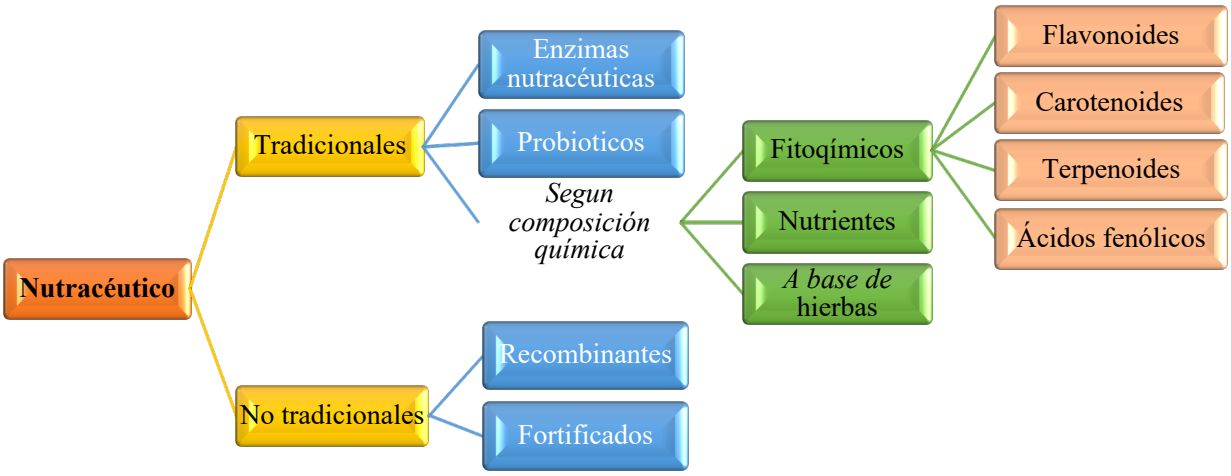

Figura 1.3. Clasificación de los nutracéuticos

La biodisponibilidad de una sustancia bioactiva es la cantidad de la misma que puede llegar al torrente sanguíneo para poder ejercer su función biológica. En este sentido, es esencial la biodisponibilidad de un nutracéutico para que, una vez ingerido, pueda llevar a cabo la función para la que ha sido diseñado. Cuando se ingiere un nutracéutico a través de la dieta, los compuestos deben pasar por boca, estómago e intestino para poder acceder al torrente sanguíneo. Para lograrlo, deben superar con éxito algunas barreras principales, incluida la degradación y la liberación incontrolada en el estómago y el intestino delgado, y también la baja absorción desde el epitelio del intestino al torrente sanguíneo (Jafari y McClements, 2017). Así, la biodisponibilidad del nutraceútico puede verse comprometida por diversos factores como el tiempo de residencia gástrico, su estabilidad, solubilidad dentro del tracto gastrointestinal, sin dejar de lado aquellos factores vinculados al procesado y/o almacenamiento, como sensibilidad a la luz, oxígeno, calor (Bell, 2002). Asimismo, cuando se incorporan nutracéuticos en matrices alimentarias, pueden producirse interacciones indeseables con otros componentes de la matriz, afectando negativamente la apariencia, textura y sensación en boca, y pueden además llegar a presentan menor eficacia *in vivo* en comparación con los ensayos realizados con el nutraceútico *in vitro.*

Por todo esto, es necesario el desarrollo de diferentes estrategias en los sistemas de administración de estos productos para mejorar la biodisponibilidad y la eficacia de los nutracéuticos en el organismo. Entre las estrategias que se han desarrollado se pueden citar la

reducción de tamaño de partícula, la generación de dispersiones sólidas y las formulaciones con base lipídica. También se han definido otras técnicas, como la encapsulación, que permiten proteger a los nutracéuticos frente a factores externos adversos, enmascarar cualquier sabor desagradable y gracias a ella se pueden incorporar más fácilmente a determinados productos alimenticios para posteriormente permitir su liberación controlada y preservar y maximizar sus propiedades bioactivas.

1.4. DISEÑO DE NUTRACÉUTICOS A BASE DE EXTRACTOS VEGETALES BIOACTIVOS

Los compuestos bioactivos que componen a los nutracéuticos pueden ser propensos a la oxidación, acentuada por la exposición a la luz, la descomposición térmica, variaciones de pH y/o contenido de humedad (Hu y col., 2018; Zhang y col., 2017). Su liberación, biodisponibilidad y eficacia dependerán de una formulación que provea estabilidad y preserve la calidad de estos compuestos (Ariyarathna y Karunaratne, 2016; Gómez-Mascaraque y col., 2017).

Estas formulaciones pueden consistir en productos en polvo o en sistemas de administración coloidal (por ejemplo, dispersiones, micelas, emulsiones, partículas de polímero, nanopartículas de lípidos sólidos, liposomas, microgeles) (Aditya y col., 2017; Oh y col., 2008), diseñados para proteger y liberar agentes bioactivos, como los nutracéuticos, y superar los desafíos para su comercialización como son las características de baja solubilidad, susceptibilidad a la degradación química, perfiles de sabor indeseables, bioactividad limitada y/o baja biodisponibilidad (McClements, 2014).

La biodisponibilidad de los compuestos presentes en las formulaciones dependerá en gran medida del tamaño, la distribución y la morfología de las partículas, los que a su vez dependerán de las propiedades de las sustancias y de la técnica de elaboración (Paulo y Santos, 2017; Vinceković y col., 2017). Debido a esto, existe un creciente interés en el desarrollo de tecnologías eficientes de micronización y que logren, además de la reducción de tamaño, la estabilización de los materiales bioactivos, para controlar de manera más fácil y/o mejorada la liberación de los compuestos. Dependiendo de su tamaño podemos encontrar micropartículas (100 nm - 1000 μm) y nanopartículas (<100 nm) (McClements, 2014), las que pueden variar considerablemente en su composición, formas, tamaños y estructuras, lo que impacta en sus propiedades funcionales, ópticas, reológicas, en la estabilidad y las características de liberación de los sistemas de administración. Un tamaño de partícula más pequeño, permite que los compuestos presenten una mayor área de superficie, tienen una solubilidad mejorada, una bioaccesibilidad significativamente mayor y la capacidad de liberación controlada en el sitio de acción específico (Faridi Esfanjani y Jafari, 2016; Katouzian y Jafari, 2016).

Por otro lado, las formulaciones a base de lípidos presentan ventajas desde el punto de vista farmacéutico y nutricional. La co-digestión del principio activo con lípidos, mejora su absorción (Feeney y col., 2016) así como la dispersión en el entorno acuso del tracto gastrointestinal. La ingesta conjunta de compuestos bioactivos y lípidos, incrementa la

solubilización y la captación celular dentro del intestino delgado (Colle y col., 2012; Failla y col., 2014; Huo y col., 2007), por la capacidad de los productos de la digestión de lípidos para formar micelas mixtas que solubilizan y transportan los componentes bioactivos a las células del epitelio (Peng y col., 2018).

Se han empleado diferentes técnicas para la obtención de formulaciones nutracéuticas, como el secado por aspersión, la liofilización, coacervación, emulsificación y extrusión (Shishir y col., 2018). Sin embargo, estas técnicas no permiten un fácil control del tamaño de las partículas producidas. Además, en algunos casos son necesarias temperaturas elevadas que pueden degradar el compuesto bioactivo y/o la eliminación del disolvente residual puede provocar la pérdida de su actividad biológica (Ozkan y col., 2019).

La tecnología de fluidos supercríticos se ha empleado para preparar micro- y nanopartículas de compuestos bioactivos, teniendo en cuenta las ventajas que presenta esta técnica de reducción de tamaño para las sustancias sensibles, puesto que las micro- o nanopartículas se forman utilizando temperaturas bajas, en un ambiente no-oxidante y en ausencia de agua. Los procesos supercríticos utilizados para la formación de partículas empleando la tecnología supercrítica, se pueden clasificar dependiendo del rol que desempeñe el $SCCO_2$, como se muestra en la Tabla 1.1 (Esfandiari, 2015; Jung y Perrut, 2001; Temelli, 2018). En particular se destaca la técnica denominada SAS (*Supercritical Antisolvent Precipitation*) que ha sido utilizada en esta tesis para micronizar extractos vegetales bioactivos.

Tabla 1.1. Procesos de formación de partículas con tecnología supercrítica

Rol del $SCCO_2$	**Procesos con tecnología supercrítica para la formación de partículas**	**Siglas**
Solvente	*Rapid expansion of supercritical solutions*	RESS
	Crystallization from Supercritical Solutions	CSS
	Supercritical Solvent Impregnation	SSI
	Supercritical Fluid Extraction and Expansion	SFEE
Co-solvente	*Depressurization of an Expanded Liquid Organic Solutions*	DELOS
Antisolvente	*Particle from gas saturated solution*	PGSS
	Supercritical Antisolvent Precipitation	SAS
	Gas Antisolvent	GAS
	Gas Antisolvent Cristallization	GASC
	Precipitation with a Compressed Fluid Antisolvent	PCA
	Areosol Solvent Extraction Systems	ASES
	Solution Enhanced Dispersions by Supercritical Fluids	SEDS
	Supercritical Fluid Extraction of Emulsions	SFEE
	Supercritical Antisolvent Extraction	SAE
	Supercritical Assisted Injection in a Liquid Antisolvent	SAILA

En relación a la producción de sistemas basados en formulaciones lipídicas empleando la tecnología supercrítica, Chattopadhyay y col. (2007) emplearon la técnica denominada

Supercritical Fluid Extraction of Emulsions (SFEE) para la producción de formulaciones de nanosuspensiones lipídicas. Para esto, prepararon por homogeneización a alta presión emulsiones de aceite en agua (una mezcla del lípido, el principio activo, cloroformo y la fase acuosa). La emulsión obtenida se introduce en una columna de extracción desde la parte superior y de manera simultánea con el $SCCO_2$ en contracorriente, dando lugar al a formación de suspensiones de nanopartículas de lípidos sólidos.

También, se ha empleado la técnica *Particle from Gas-Saturated Solutions* (PGSS) donde los lípidos sólidos y los compuestos bioactivos a encapsular son cargados en una celda junto con el $SCCO_2$. La mezcla se calienta hasta el punto de fusión de los lípidos, y la solución de gas saturado es rápidamente expandida a través de una boquilla dando lugar a la formación de nanopartículas lipídicas sólidas (Couto y col., 2017, Martín y col., 2017).

Por último, se han desarrollado nanopartículas lipídicas con compuestos bioactivos incorporados empleando la técnica *Supercritical Assisted Injection in a Liquid Antisolvent* (SAILA) (Trucillo y Campardelli, 2019) donde el $SCCO_2$ y un disolvente líquido (en la cual se puede disolver el lípido sólido y un surfactante) son alimentado por líneas independientes a un recipiente (saturador) formándose una mezcla líquida expandida. La disolución a la salida del saturador se inyecta en una fase de agua receptora, utilizando un orificio que tiene un diámetro de 100 μm.

1.4.1. Precipitación supercrítica anti-solvente (SAS)

La Precipitación Supercrítica Antisolvente (SAS) es un proceso de reducción de tamaño especialmente eficaz para precipitar compuestos con baja solubilidad en $SCCO_2$. El fluido supercrítico, utilizado como antisolvente, se disuelve en una disolución que contiene el principio activo y un disolvente orgánico, la expansión de la fase líquida y la saturación del disolvente orgánico con $SCCO_2$ provoca un descenso de la solubilidad del principio activo (efecto antisolvente) y su consecuente precipitación. El proceso se lleva a cabo pulverizando a través de una boquilla la disolución orgánica en un recipiente (celda de precipitación) en la que fluye CO_2 en condiciones supercríticas. El $SCCO_2$ se disuelve rápidamente en la disolución orgánica, y esta rápida transferencia de masa provoca la expansión del disolvente, conduce a la sobresaturación de la disolución y a la precipitación del principio activo, con altas velocidades de nucleación y formándose micro- y/o nanopartículas. Por último, el disolvente y el antisolvente se separan mediante despresurización en un separador ubicado después de la celda de precipitación (Reverchon y De Marco, 2011).

El proceso SAS se ha empleado para la micronización de compuestos puros como ácido gálico (Montes y col. 2016), curcumina (Matos y col., 2019), pigmentos (Santos y Meireles, 2013), así como una variedad de compuestos antioxidantes (Liu y col., 2020; Montes y col., 2016).

En el campo de los extractos vegetales, el proceso SAS se ha utilizado como técnica de precipitación y fraccionamiento del extracto, también conocido como SAF (*Supercritical*

Antisolvent Fractionation) que consiste en la pulverización continua, a través de una boquilla, de una disolución polar de un extracto vegetal en presencia de $SCCO_2$. El carácter lipofílico de $SCCO_2$ permite la precipitación de uno o más componentes polares de la disolución, mientras que los componentes menos polares permanecen disueltos y pueden recuperarse por reducción de presión en un separador posterior (Martín y col., 2011). Catchpole y col. (2004) emplearon esta técnica para fraccionar el extracto un hidroalcohólico de propóleo, obteniendo una fracción enriquecida en flavonoides y el aceite esencial en el separador. También se ha empleado para la producción de micro- y nanopartículas de compuestos fenólicos antioxidantes a partir de residuos de mango (Meneses y col., 2015), hojas de mango (Guamán-Balcázar y col., 2017, 2018) y hojas de *Achillea millefolium* (Villalva y col., 2019). También, Visentín y col. (2011) realizaron el fraccionamiento de los principales compuestos bioactivos de oleorresinas viscosas de hojas romero con una boquilla casera, obteniendo una purificación del extracto con un alto contenido de ácido carnósico y Sánchez-Camargo y col. (2016) realizaron el SAF de un extracto de romero obtenido por extracción con líquidos presurizados, con el fin de separar los diterpenos fenólicos (ácido carnósico, carnosol) de los ácidos fenólicos (ácido rosmarínico).

La micronización SAS permite superar los principales inconvenientes de las técnicas de micronización convencionales, como el secado por aspersión, la liofilización, el molido por chorro, la evaporación de solventes y/o la trituración (Ha y col., 2020; Park y col., 2010) y aporta beneficios muy interesantes en el producto precipitado, tanto en términos de morfología como de dimensiones de las partículas que lo constituyen. Un esquema general del proceso SAS se muestra en la Figura 1.4. El equipo está compuesto por la celda de precipitación y dos separadores con control independiente de presión y temperatura, dos bombas, una para la alimentación del $SCCO_2$ y otra para la disolución.

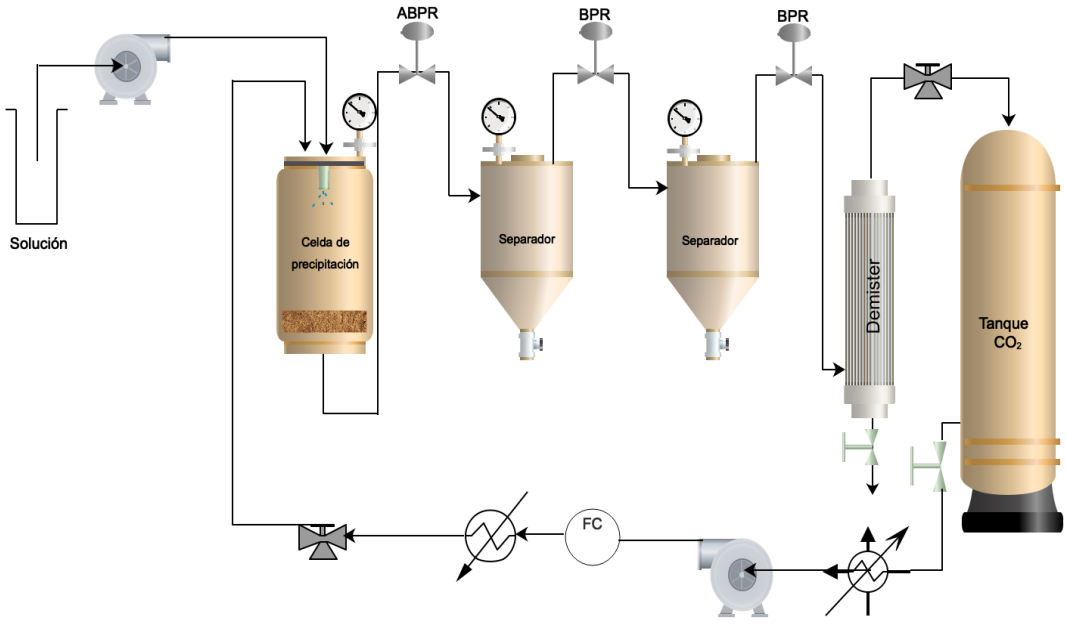

Figura 1.4. Esquema general del proceso de precipitación supercrítica antisolvente (SAS).

En la parte superior la celda de precipitación (Figura 1.5) está equipada con una boquilla de diámetro interno micrométrico para la inyección de la disolución líquida y un fritado en la parte inferior para la recolección del material sólido (Villanueva-Bermejo y col., 2017).

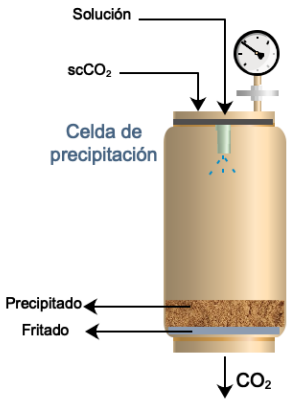

Figura 1.5. Diagrama de la celda de precipitación supercrítica antisolvente (SAS).

El proceso SAS se desarrolla en tres etapas (Mattea y col., 2009): inicialmente se introduce el fluido supercrítico a la celda de precipitación por la parte superior, hasta alcanzar las condiciones de temperatura y presión deseadas; una vez alcanzadas estas condiciones, y simultáneamente con el flujo de $SCCO_2$, se bombea la disolución (compuestos bioactivos + disolvente) a través de la boquilla durante el tiempo necesario, hasta agotar la disolución; finalmente se sigue bombeando el fluido supercrítico a flujo constante durante un tiempo adicional, hasta eliminar el disolvente orgánico que pueda haber quedado retenido en las partículas sólidas precipitadas. El disolvente y el antisolvente, formando una fase supercrítica homogénea, salen por la parte inferior de la celda de precipitación, donde son separados mediante despresurización en el separador situado después de la celda de precipitación.

Para llevar a cabo el proceso SAS es necesario que el disolvente orgánico empleado sea miscible con el antisolvente ($SCCO_2$) y los solutos a precipitar deben ser insolubles en la fase fluida $SCCO_2$ + disolvente que abandona la celda de precipitación. La solubilidad de los solutos en esta fase dependerá de sus propiedades físicas y químicas, como la estructura molecular, la polaridad y la volatilidad, y también de las condiciones de operación (temperatura, presión, concentración de solutos en la disolución, velocidad de flujo del fluido supercrítico y de la disolución). Por otro lado, es importante mantener una buena transferencia de masa entre $SCCO_2$ y la disolución, que depende de las diferencias de densidad entre el disolvente y el antisolvente, la viscosidad, la difusividad, el diámetro de las gotas o partículas y la velocidad de flujo del disolvente (Montes y col., 2011).

No obstante, la viabilidad de producir partículas secas y obtener una morfología homogénea y una distribución de tamaños de partícula regular, está fuertemente condicionada por la ubicación del punto de operación de la precipitación SAS en relación al punto crítico de la mezcla (PCM). Para explicar este concepto en forma sencilla, se muestra en la Figura 1.6 un esquema del diagrama de fases de la mezcla CO_2 + disolvente, donde se muestran las diferentes

regiones de fases del sistema binario: región de equilibrio líquido + vapor, región de fase subcrítica homogénea, y región de fase supercrítica (fase homogénea).

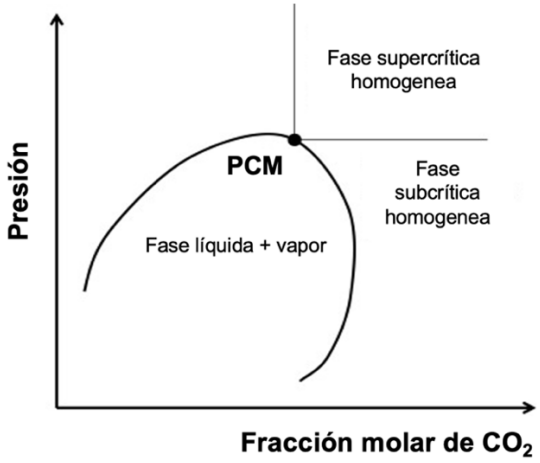

Figura 1.6. Esquema del diagrama de fases de una mezcla CO_2 + disolvente PCM: punto crítico de la mezcla. Fuente: (Quintana y col., 2020)

Si las condiciones de proceso SAS están por debajo del PCM, pero en la región subcrítica homogénea, la formación de partículas es inducida por el efecto antisolvente de $SCCO_2$ y por el agotamiento del solvente orgánico en las gotas formadas por la boquilla, obteniendo micropartículas expandidas (partículas de núcleo hueco) con formas irregulares. En el caso de que las condiciones SAS se encuentran dentro de la región de equilibrio líquido + vapor, se producen partículas irregulares y aglomerados debido a la presencia de disolvente residual en el precipitado. Por último, cuando las condiciones de operación SAS están por encima del PCM, en la región supercrítica homogénea, la mezcla de CO_2 con el disolvente es instantánea, no se produce una interfase líquido-gas, lo que resulta en partículas pequeñas y regulares debido a la condensación de los solutos desde una fase gaseosa (Reverchon y De Marco, 2011).

Por tanto, desde un punto de vista termodinámico, para garantizar la precipitación exitosa de partículas, la presión y la temperatura deben situarse por encima del punto crítico de mezcla (PCM), evitando las condiciones subcríticas, donde existe la posibilidad de producción de una interfase líquido-vapor. Pero la presencia de sustancias en la fase CO_2 + disolvente (las sustancias que no precipitan en la celda de precipitación) puede inducir cambios importantes del PCM en comparación con el punto crítico correspondiente al sistema binario CO_2 + disolvente. Cuando se trata de un único principio activo, con baja solubilidad e pequeñas interacciones con la fase CO_2 + disolvente, su influencia en los diagramas de fase suele ser despreciable y, por lo tanto, es posible establecer condiciones de operación adecuadas sobre la base del conocimiento del equilibrio de fases del sistema binarios CO2 + disolvente (Kikic y col., 2006). No obstante, en sistemas con muchas sustancias no precipitadas, como es el caso de los extractos naturales, es posible un cambio significativo del PCM de la mezcla CO_2 + disolvente + solutos, en comparación con el PCM de la mezcla binaria CO_2 + disolvente.

CAPÍTULO 2

JUSTIFICACIÓN Y OBJETIVOS

2. JUSTIFICACIÓN Y OBJETIVOS

Los extractos de muchas plantas y hierbas contienen una gran cantidad y diversidad de compuestos bioactivos; algunos de estos extractos han sido tradicionalmente utilizados como conservantes alimentarios naturales, aprovechando sus propiedades antioxidantes y antimicrobianas. No obstante, más recientemente, se ha demostrado que presentan una gran variedad de actividades biológicas y funcionales, beneficiosas para la salud, más allá de sus propiedades como conservantes, por lo que han recibido una particular atención para el desarrollo de ingredientes, complementos alimenticios y/o nutracéuticos de uso específico para la salud.

En el diseño de ingredientes de uso para la salud, la reducción de tamaño de partícula a escala micro- y nanométrica contribuye significativamente a la biodisponibilidad del principio activo y potencian el efecto beneficioso a nivel biológico. Técnicas tradicionales de reducción de tamaño, como el secado por pulverización, la recristalización, liofilización, entre otras, presentan algunos inconvenientes tales como la degradación térmica y oxidativa del principio activo, cambios estructurales, alta concentración de solvente residual, así como la dificultad para controlar el tamaño y obtener una distribución homogénea del tamaño de las partículas. Una alternativa que minimiza estas desventajas, y resulta más eficaz en la formación de miro- o nanopartículas, es la tecnología de fluidos supercríticos.

Por otro lado, en el desarrollo de ingredientes saludables, se ha demostrado la efectividad de las estrategias de vehiculización con lípidos para favorecer la biodisponibilidad y la absorción de ciertos compuestos bioactivos de origen vegetal, combinándolos en su formulación con lípidos portadores (Martin y col., 2016; Mouhid y col., 2017; Vargas y col., 2015).

Hipótesis

- Los extractos vegetales concentran sustancias con propiedades bioactivas específicas y naturalmente disponibles en la matriz vegetal, por lo que pueden ser aprovechados en el diseño de ingredientes alimentarios de alto valor añadido.

- Para utilizar estos extractos de forma eficaz, la tecnología de fluidos supercríticos presenta ventajas respecto de otras técnicas tradicionales para la obtención y formulación de ingredientes, conservando las propiedades del extracto original.

- La combinación de extractos vegetales con sustancias lipídicas portadoras permite mejorar la biodisponibilidad y absorción de las sustancias bioactivas presentes en el extracto.

OBJETIVO GENERAL

El objetivo general de este trabajo ha sido preparar nuevas fórmulas y desarrollar nuevos procedimientos de formulación utilizando extractos vegetales bioactivos, útiles para aplicaciones alimentarias como es el caso de (i) los recubrimientos comestibles, con propiedades antioxidantes y antimicrobianas, o (ii) los nutracéuticos antioxidantes de uso para la salud.

Los objetivos parciales planteados para la consecución del objetivo general son los siguientes:

(i) Para la preparación de recubrimientos comestibles, con propiedades antioxidantes y antimicrobianas:

- Obtener una batería de extractos a partir de diversas plantas y hierbas, aplicando diferentes técnicas de extracción, incluida la tecnología de fluidos supercríticos, y evaluar su actividad antioxidante y antimicrobiana con el fin de seleccionar los mejores extractos para ser utilizados en la formulación de recubrimientos comestibles.

- Elaborar distintos recubrimientos comestibles, evaluar sus propiedades reológicas y aplicarlos como conservantes en alimentos listos para el consumo.

(ii) Para la preparación de nutracéuticos antioxidantes de uso para la salud:

- Estudiar la reducción de tamaño mediante tecnología supercrítica antisolvente de extractos bioactivos seleccionados, evaluar la morfología, distribución de tamaño de partícula y actividad antioxidante de la fracción micronizada.

- Elaborar preparados oleosos de los extractos antioxidantes en forma de micro- o nanopartículas utilizando la precipitación supercrítica antisolvente. Estudiar la morfología, distribución de tamaño de partícula y actividad antioxidante de los preparados.

CAPÍTULO 3

PLAN DE TRABAJO

3. PLAN DE TRABAJO

Según los antecedentes expuestos y los objetivos planteados, el plan de trabajo desarrollado se divide en tres actividades principales, como se muestra en la Figura 3.1.:

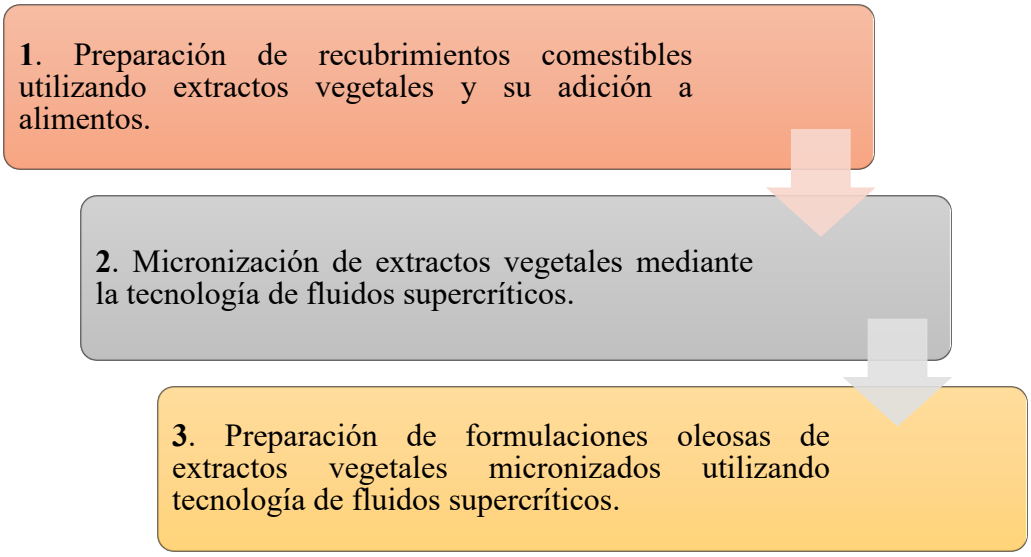

Figura 3.1. Esquema general del plan de trabajo.

Las tareas desarrolladas en cada una de estas actividades fueron las siguientes:

3.1. Preparación de recubrimientos comestibles utilizando extractos vegetales y su adición a alimentos.

Tarea 1.1: Obtención de extractos de plantas y hierbas por hidrodestilación, extracción asistida por ultrasonidos y extracción con fluidos supercríticos.

Tarea 1.2: Caracterización química: compuestos fenólicos totales (método de Folin-Ciocalteu), identificación de compuestos volátiles (CG-MS) y de compuestos fenólicos (HPLC).

Tarea 1.3: Determinación de la actividad antioxidante utilizando los métodos de captación de radicales libres del $ABTS^{\cdot+}$ y del DPPH. Determinación de la actividad antimicrobiana frente a bacterias gram positivas (*S. aureus*) y gram negativas (*E. coli*) por el método de micro-dilución.

Tarea 1.4: Elaboración de recubrimientos comestibles con extractos los vegetales seleccionados y combinados con una matriz de tipo polisacárido (quitosano).

Tarea 1.5: Estudio de las propiedades reológicas (propiedades de flujo en estado estacionario y viscoelásticas oscilatorias) de las formulaciones y de las películas formadas.

Tarea 1.6: Aplicación de los recubrimientos comestibles en fresas, y análisis de las propiedades fisicoquímicas (pH, sólidos solubles, color) y microbiológicas de las fresas tratadas utilizando los diferentes recubrimientos.

Un esquema del plan de trabajo de las tareas 1.1 a 1.3 se muestra en la Figura 3.2. y de las tareas 1.4 a 1.6 en la Figura 3.3.

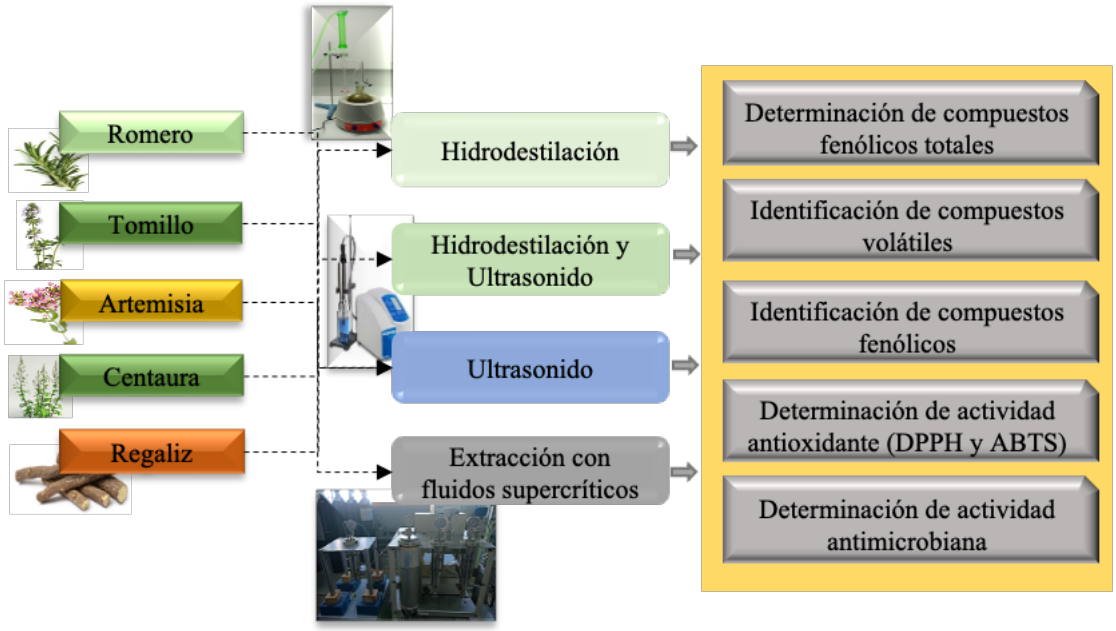

Figura 3.2. Esquema de la obtención y caracterización de extractos naturales de plantas comestibles.

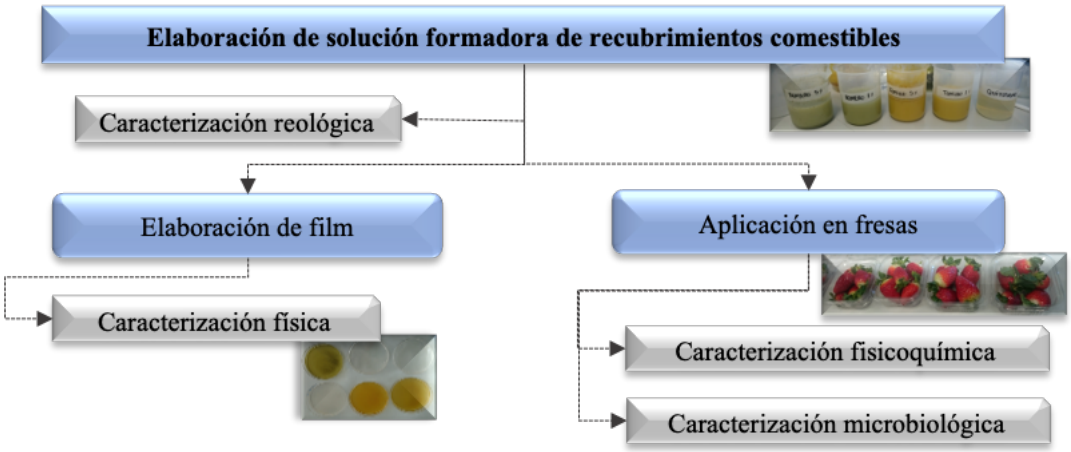

Figura 3.3. Esquema de la elaboración y estudio de recubrimientos comestibles.

3.2. Micronización de extractos vegetales mediante tecnología de fluidos supercríticos.

Tarea 2.1: Precipitación de micro- y nanopartículas de extractos vegetales seleccionados mediante tecnología supercrítica antisolvente (SAS).

Tarea 2.2: Cuantificación de compuestos antioxidantes por HPLC, compuestos fenólicos totales (método de Folin-Ciocalteu) y actividad antioxidante (DPPH) de los micronizados precipitados.

Tarea 2.3: Estudio de la morfología (microscopía electrónica de barrido) y distribución de tamaño de partícula (difracción láser) de los precipitados.

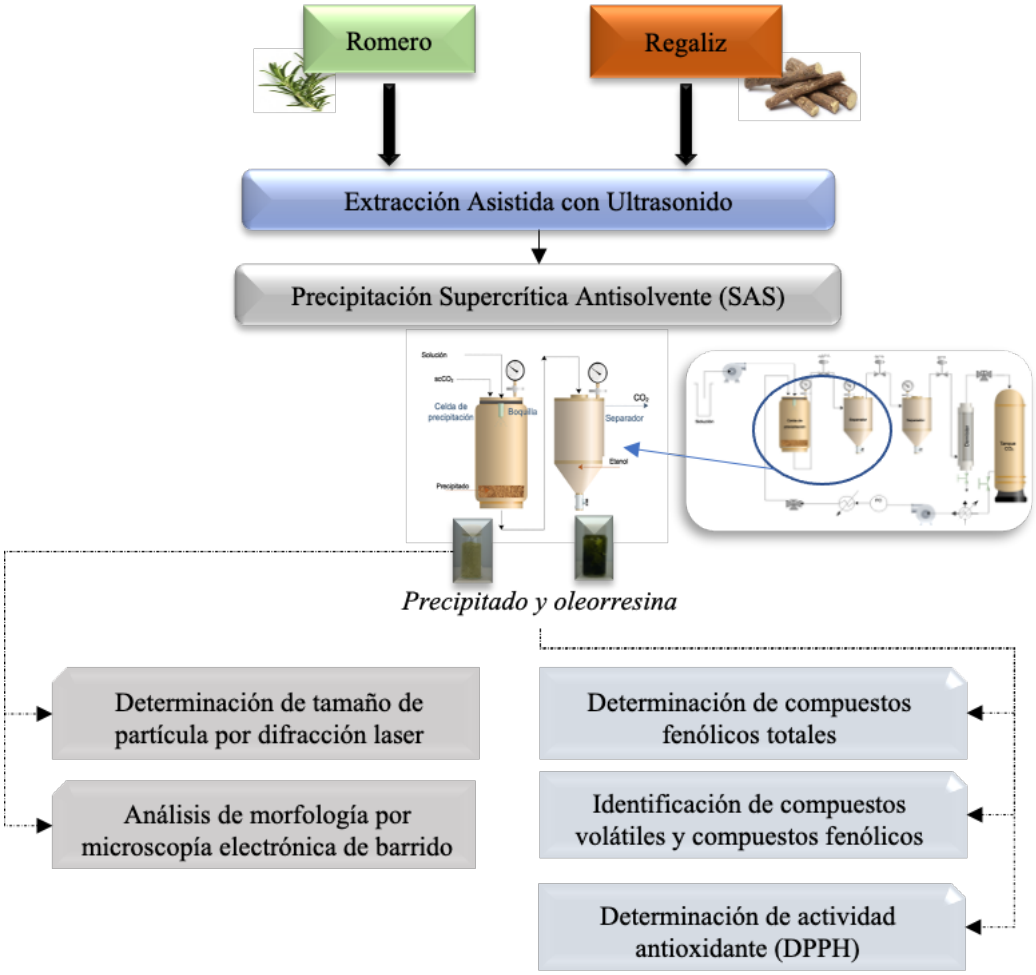

Figura 3.4. Esquema de proceso de micronización de extractos vegetales.

3.3. Preparación de formulaciones oleosas de extractos vegetales micronizados utilizando tecnología de fluidos supercríticos.

Tarea 3.1: Incorporación de las micro- y nanopartículas de extractos vegetales en sustancias lipídicas utilizando la tecnología SAS.

Tarea 3.2: Evaluación de la estabilidad (dispersión de luz múltiple), morfología (microscopía de escaneo láser confocal), distribución de tamaño de partícula (difracción láser) de las formulaciones oleosas obtenidas, y determinación de sus propiedades térmicas (calorimetría diferencial de barrido).

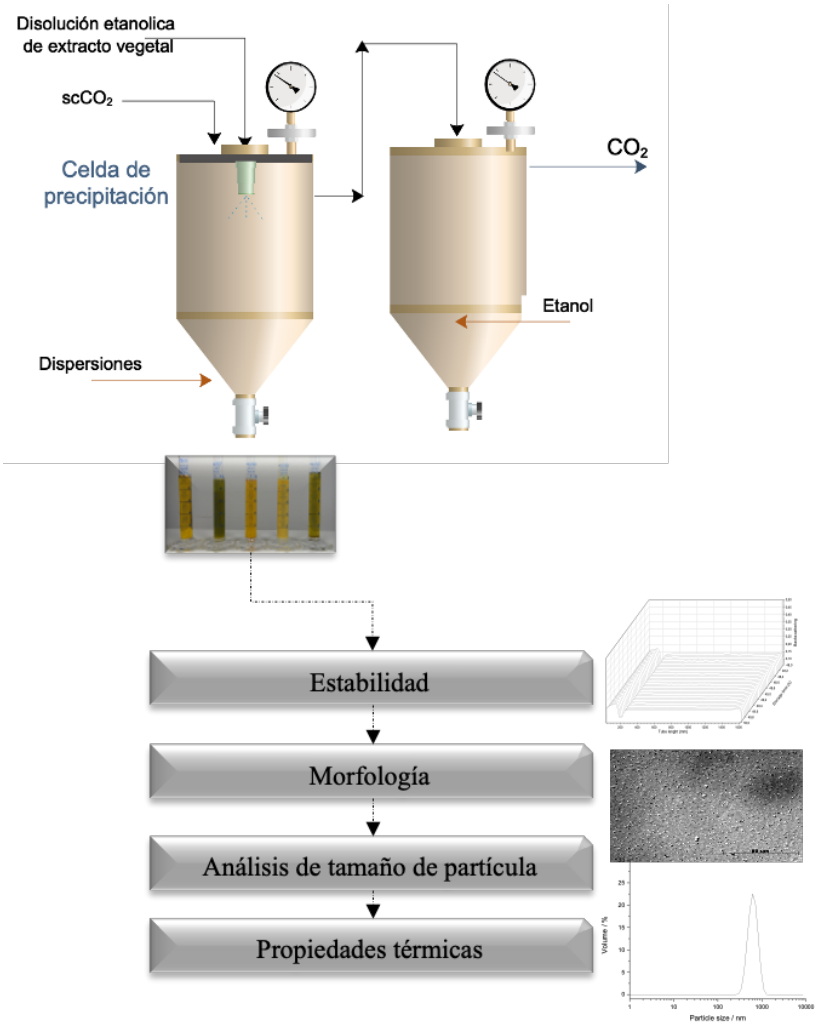

Figura 3.5. Esquema de la preparación de dispersiones oleosas de extractos vegetales micronizados.

CAPÍTULO 4

RESULTADOS

4. RESULTADOS

4.1. PREPARACIÓN DE RECUBRIMIENTOS COMESTIBLES CON EXTRACTOS VEGETALES

Este capítulo contiene los resultados obtenidos en relación a la preparación de recubrimientos comestibles utilizando extractos vegetales con propiedades antioxidantes y antimicrobianas, los que se presentan a través de tres artículos científicos.

Para producir los extractos vegetales se utilizaron diversas plantas, algunas muy tradicionales y bien conocidas por sus propiedades antioxidantes y antimicrobianas, como son las hojas de romero (*Rosmarinus officinalis* L.) y de tomillo (*Thymus vulgaris*), y otras más recientemente y/o escasamente estudiadas en la bibliografía, como por ejemplo la raíz de regaliz (*Glycyrrhiza glabra* L.), la centaura (*Centaurium erythraea*) y la artemisia (*Artemisia vulgaris*).

En cuanto a las técnicas se utilizó la hidrodestilación y la hidrodestilación asistida por ultrasonidos, con el objetivo de recuperar el aceite esencial de las plantas con un alto grado de pureza, puesto que, en la fracción más volátil de los metabolitos secundarios de origen terpénico de las plantas, se suelen concentrar sustancias con buenas cualidades antimicrobianas. También, con el objetivo de comparar con la hidrodestilación, se utilizó la extracción con $SCCO_2$ para producir extractos con sustancias de baja y moderada polaridad, mientras que la extracción asistida con ultrasonidos se combinó con disolventes más polares (etanol, agua, y mezclas etanol/agua), con el fin de extraer de la matriz vegetal las sustancias de tipo fenólico, ácidos orgánicos, saponinas, entre otras.

En el primer trabajo, titulado "*Comparison between essential oils and supercritical extracts into chitosan-based edible coatings on strawberry quality during cold storage*", en revisión para su publicación en *The Journal of Superccritial Fluids*, se muestran los resultados de la batería de extractos vegetales obtenida, su caracterización química, así como los resultados del análisis de su actividad antioxidante y antimicrobiana. Las buenas propiedades obtenidas para los extractos supercríticos de romero y tomillo motivaron su aplicación en la formulación de recubrimientos conservantes de un alimento fresco (fresas), mantenido en almacenamiento durante 10 días a 4 °C, analizando el cambio de ciertos parámetros (pH, sólidos solubles, color) en el alimento. Además, se compararon estos recubrimientos con los elaborados utilizando el correspondiente aceite esencial de la planta (obtenidos por hidrodestilación). Mientras que los aceites esenciales de romero y tomillo ya fueron presentados en la bibliografía para la formulación de recubrimientos comestibles, el empleo de extractos supercríticos de estas plantas ha sido menos investigado, y la comparación de ambos productos (obtenidos por hidrodestilación y por tecnología supercrítica) se presenta por primera vez en este trabajo.

Asimismo, una de las plantas que presentó buena actividad antimicrobiana y antioxidante fue la raíz de regaliz. Siendo muy escasa la información de la bibliografía sobre la actividad antimicrobiana de extractos supercríticos de raíz de regaliz, se llevó a cabo un estudio

que dio lugar al artículo titulado: "*Antioxidant and Antimicrobial Assessment of Licorice Supercritical Extracts*", publicado en el *Journal of Industrial Crops & Products* (139 (2019) 111496). En este trabajo se evaluó la composición y la actividad antioxidante y antimicrobiana de los extractos supercríticos de raíz de regaliz, obtenidos en distintas condiciones de operación, utilizando como disolvente tanto $SCCO_2$ puro como $SCCO_2$ y etanol como co-solvente. Simultáneamente, se estudiaron extractos de raíz de regaliz obtenidos por extracción asistida con ultrasonidos, utilizando diferentes disolventes, temperaturas y potencia, y se seleccionaron aquellos extractos que presentaron un mayor rendimiento y actividad antimicrobiana, para ser utilizados en la formulación de nuevos recubrimientos comestibles, combinados con quitosano, y aplicados a fresas. Los resultados dieron lugar al trabajo titulado "*Preparation and characterization of licorice-chitosan coatings for postharvest treatment of fresh strawberries*" enviado para publicación en el *Journal Applied Science* (10 (2020) 8431).

En resumen, en este primer capítulo se presenta la producción de una batería de extractos de diferentes plantas, utilizando diferentes tecnologías verdes de extracción, y ensayando diferentes condiciones, el análisis de su actividad antioxidante y antimicrobiana, así como de componentes claves vinculados a estas actividades. Se seleccionaron los mejores extractos, para la preparación de recubrimientos comestibles combinados con quitosano, y los mismos se aplicaron a fresas, evaluando sus propiedades reológicas y su efecto como conservantes.

4.1.1. Comparison between essential oils and supercritical extracts into chitosan-based edible coatings on strawberry quality during cold storage

The Journal of Supercritical Fluids (En revisión)

Comparison between essential oils and supercritical extracts into chitosan-based edible coatings on strawberry quality during cold storage

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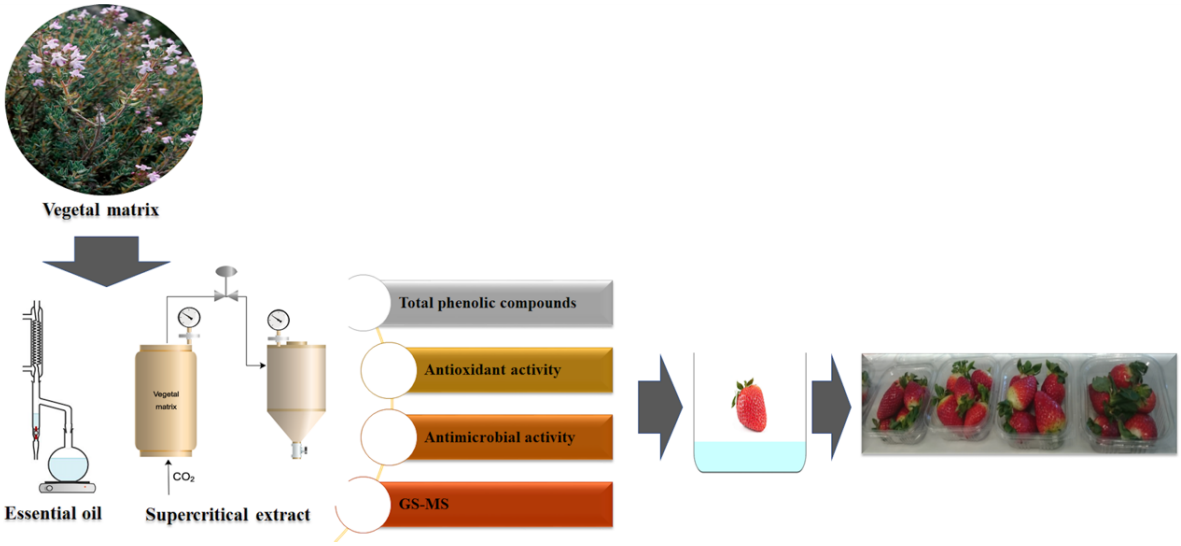
Abstract

Plant extracts are being studied in the development of novel edible food coatings. The antioxidant and antimicrobial compounds that naturally occur in some plants are the key substances that contribute to preserving food quality. Besides the plant material, the method utilized to produce the extract influences its chemical and preservative characteristics. In general, hydrodistillation produce plant extracts (essential oils) that are well recognized for containing high concentration of antioxidant or antimicrobial volatile compounds. Supercritical fluid technology produces high quality bioactive extracts with higher yield but lower concentration of volatile compounds, in comparison with hydrodistillation. In this work, six different natural extracts of five different plants were produced by hydrodistillation and supercritical fluid extraction, and their antioxidant and antimicrobial activities were compared. The most active extracts were used to produce chitosan-based edible coatings with the aim of assessing the effect of essential oils vs. supercritical extracts on the preservation of strawberries.

Keywords

Edible coatings, supercritical extracts, essential oils, chitosan, strawberries.

Graphical abstract



Highlights

- Plant extracts were produced by hydrodistillation and supercritical extraction.
- Selected extracts were used in chitosan-based edible coatings applied to berries.
- Coatings with supercritical extracts showed less loss weight and fungal decay.
- Spoilage preservation was better with essential oil coatings.
- All coating treatments did not change significantly the color appearance of berries.

1. Introduction

Plant extracts have been used in the food industry for a wide variety of purposes for thousands of years [1]. During last decades, the research on the biological activity of plant extracts endorsed several innovative applications, such as the development of natural coatings for food preservation. Some plant extracts contain natural antioxidant and antimicrobial substances and therefore, can be used for the protection of food quality [2]. Because of their natural origin, plant extract edible coatings (PEEC) are environment friendly coatings. As other food coatings, PEEC are thin layers of edible substances which are applied on food surface, and they should be not harm for human health when consumed at the concentrations required for maintaining the food quality. PEEC can act as a barrier to restrain the exchange of moisture, oxygen and solid solutes transformation, and thus reducing water loss, respiration and oxidation reaction rates, and extend shelf life [3].

Some applications of PEEC as preservatives are limited due to the flavor associated to the plant volatile compounds (plant essential oil) and thus, both the plant extract and the food to be coated should be smartly selected, in order to minimize alterations of food taste and flavor.

In comparison with conventional packaging, edible coating films are directly applied on the surface of food and the film materials are mainly derived from natural food-grade substances, with non-toxic, bioactive and biodegradable properties, and this may not be the case in synthetic packaging materials [4]. Main substances that have been used to develop edible films are polysaccharides (e.g. alginate, pectin and chitosan), proteins (e.g. whey, collagen, gelatin) and lipids (e.g. wax), alone or in combination [4].

Chitosan is a cationic polysaccharide which can be obtained by the deacetylation of chitin, a polymer found in the exoskeleton of crustaceans and insects [5]. Due to its biodegradability, biocompatibility, antimicrobial activity and non-toxicity, chitosan is considered a very promising and eco-friendly material for different purposes [5–7]. Chitosan has been extensively studied and applied in the food industry owing to its unique film-forming properties, low gas permeability, antioxidant activity against lipid oxidation, and more importantly, antimicrobial activity against bacteria and fungi [8,9] and can trap essential oils and bioactive compounds in its chemical structure [10]. However, the most important disadvantage is its relatively high moisture permeability [8]. Following this

concept, the combination of chitosan with essential oils and plant extracts represents an interesting alternative to the development of novel PEEC [5]. Furthermore, the possibility of incorporating the distinctive bioactive compounds of the plant into the edible coating can provide functional properties, with a specific health target, besides a novel way to maintain food quality during storage.

In general, the addition of essential oils in the development of PEEC has demonstrated to improve food quality and extend shelf life. For example, Chiabrando & Gialcalone [11] add rosemary essential oil to a alginate based coating and demonstrated a significant improvement on quality attributes and enzyme activities of apple slices during storage. Cano-Embuena et al [12] and Yanguilar [13] develop a mixture of rosemary and oregano essential oil for the coating of cheese preventing the loss weight and inhibiting fungal growth during ripening. Alotaibi & Tahergorabi [14] used a sweet potato starch coating combined with thyme essential oil spread on shrimps, reducing the microbial growth, melanosis and loss of firmness during eight days of storage. Also thyme plant was used in the study of Sapper et al [15], in which the effect of applying a starch-gellan coating with thyme essential oil on the surface of apples, tomatoes and persimmons was reported. Tabassum & Khan [16] evaluate the potential of alginate edible coatings with a mixture of thyme and oregano essential oils on fresh cut papaya, concluding that the PEEC delayed the degradation rate of the fruit physicochemical properties and improved its microbiological safety. Naeem et al [17] studied the preservation of unripe green mangoes using guar gum edible coating combined with different essential oils (*Nigella sativa*, *Coriandrum sativum*, *Foeniculum vulgare* and *Laurus* nobilis) and Etemadipoor et al [18] analyzed for 28 days the preservation of color, pH, firmness, flavor index, chlorophylls, carotenoids and soluble solids contents of guava fruit coated with cinnamon essential oil.

In this work, different extracts of five plants were produced using hydrodistillation and supercritical carbon dioxide extraction techniques, with the aim of comparing their antioxidant and antimicrobial properties, and the effect of the essential oil vs. the supercritical extract on the preservation of strawberries, a fruit which is very sensitive to quality changes after harvest. The plants used include some varieties well studied in the literature, such as rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris*) and licorice (*Glycyrrhiza glabra*), and another two less studied, such as centaury (*Centaurium erythraea*) and mugwort (*Artemisia vulgaris*). The hydrodistillation and supercritical

extracts of the plants with the most suitable antioxidant and antimicrobial properties were combined with chitosan in different ratios and applied on fresh strawberries to analyze the effect of the different PEEC on some crucial quality properties of the fruit during 10 days of cold storage.

2. Materials and methods

2.1.Raw material

CO_2 (N38) was supplied from Carburos Metálicos (Madrid, Spain). Ethanol (99.5 % purity) and sodium carbonate anhydrous (99.5 % purity) were purchased from Panreac (Barcelona, Spain). Gallic acid standard (> 98 % purity), 2.2-Diphenyl-1-pycrilhydrazyl (DPPH, 95 % purity), 2.2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥ 95 % purity), (±) 6-Hydroxy-2.5.7.8-tetramethyllchromane-2-carboxylic acid (Trolox, 97 % purity), Folin-Ciocalteu´s reagent, Chloramphenicol (≥ 98 % purity), Chitosan low molecular weight, Deacetylated chitin, Ply (D-glucosamine), tween 20, acetic acid (≥ 99.5 % FCC, FG) and glycerol (>99%, FCC,FG) were supplied from Sigma–Aldrich (St. Louis, MO, USA). Difco Wilkins-Chalgren Agar and BBL Mueller Hinton II Broth was obtained from Becton, Dickinson and Company (France), and sodium hydroxide (ACS, Reag. Ph Eur, ISO) from EMSURE.

2.2.Sample preparation

Rosemary (*Rosmarinus officinalis*), thyme (*Thymus vulgaris*), centaury (*Centaurium erythraea*) and mugwort (*Artemisia vulgaris*) leaves and root of licorice (*Glycyrrhiza glabra*) harvested in Spain were obtained from Murciana herbalist's (Murcia, Spain). The samples were ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain). All samples were stored in polyethylene bags under vacuum and kept at 4 °C until extraction.

2.3.Extraction procedures

2.3.1. Hydrodistillation

The plant samples were subject to hydrodistillation using a Clevenger-type apparatus for 3 h, using a 1:10 plant: water ratio. The oil phase was separated from the water phase, dried and stored at -20ºC. The essential oil yields were calculated with respect to the dry mass of plant subject to hydrodistillation (yield = mass essential oil / mass plant x 100).

2.3.2. Hydrodistillation combined with ultrasound pretreatment

The plant samples in a 1:10 plant: water ratio was subject to ultrasound (Branson Digital Sonifier 550 model, Danbury, USA) frequency of 20 kHz, 550 W and 60 % of sonication output amplitude pretreatment for 15 min and 25 ºC, before the 3 h hydrodistillation process. The essential oil was recovered, dried and stored at -20ºC, and yields were calculated as described previously.

2.3.3. Supercritical fluid extraction

Supercritical fluid extraction (SFE) was carried out using a pilot-plant extractor (SF2000 Thar Technology, Pittsburgh, USA) including a 0.273 L cylinder extraction vessel and two-cylinder separators (S1 and S2), each of 0.5 L capacity, with independent control of temperature and pressure. Also, the pilot-plan device includes a CO_2 recirculation system. A detailed description of the equipment can be found elsewhere [19]. Different extractions were accomplished, with pure CO_2 and using 10 % mass of ethanol cosolvent, at 313.15 K, 70 g/min of CO_2 flow rate, and pressures in the range 15-30 MPa. All the experimental conditions of the extraction procedures are listed in Table 1.

The plant extracts were obtained by depressurization in the separators of the supercritical stream flowing out of the extractor vessel. In the case of SFE15 and SFE15-EtOH extractions both separators were maintained at the system recirculation pressure (5 MPa) and the extracted mass was collected from both separators and mixed in a single fraction. Moreover, runs SFE30 was carried out at higher pressures (30 MPa) and the extract was fractionated in the decompression cascade system comprising the two separators. For this purpose, the first separator cell (S1) was kept at 15 MPa and the second one was maintained at the recirculation pressure (5 MPa). Thus, two fractions (S1 and S2) were collected in each run.

2.4.Characterization of extracts

2.4.1. Total phenolic compounds determination

The total phenolic compounds (TPC) content in essential oils and supercritical extracts was determined using the Folin-Ciocalteu method [20]. Briefly, 50 µl of extract were mixed with 3 mL of milliQ water and 250 µl of Folin Ciocalteu reagent, thoroughly mixed for 3 min, 750 µl of sodium carbonate solution (20 % mass) and 950 µl of milliQ water were added to

149 the mixture. Then, samples were remained 2 h in darkness at room temperature. The
150 absorbance was measured at 760 nm using a Genesys 10S UV-Vis spectrophotometer
151 (Thermo Fischer Scientific Inc., USA). The results were expressed as GAE (mg of gallic
152 acid equivalents/g of extract). All analyses were done in triplicate.

153 **2.4.2. Antioxidant activity**

154 The antioxidant activity of samples was determined by the ABTS and DPPH assays. The
155 ABTS assay was carried out following the method described by Re et al [21]. $ABTS^{\cdot+}$
156 radical cation was generated by mixing ABTS stock solution (7 mM) with 2.45 mM
157 potassium persulfate after incubation of the mixture at room temperature for 16 h under
158 darkness. Once the $ABTS^{\cdot+}$ radical was formed, the solution absorbance was adjusted to
159 0.700 ± 0.02 at 734 nm using ethanol in a Genesys 10S UV-Vis spectrophotometer (Thermo
160 Fischer Scientific Inc., MA, USA). Afterwards, 990 µl of $ABTS^{\cdot+}$ solution was added to 10
161 µl of sample and the reaction mixture was allowed to stand at room temperature and under
162 darkness, until the absorbance reached a plateau. The absorbance was recorded at 734 nm
163 and the results were expressed as Trolox equivalents (TEAC) (µmol Trolox/g extract),
164 which were calculated taking into account the Trolox standard and sample concentrations
165 that produce the scavenging of 50 % of $ABTS^{\cdot+}$ radical.

166 The ability of extracts to scavenge DPPH free radicals was determined according to the
167 method described by Brand-Williams et al., [22]. Samples were added to 975 µl of DPPH
168 radical in ethanol, which was daily prepared. The reaction took place at room temperature
169 in the dark until it reached a plateau. Then, the absorbance was measured at 515 nm in a
170 Genesys 10S UV-Vis spectrophotometer (Thermo Fischer scientific, USA). The DPPH
171 concentration in the reaction medium was calculated from a calibration curve (linear
172 regression). A control sample, containing the same volume of solvent instead of extract, was
173 used to measure the maximum DPPH absorbance. Trolox was used as reference standard,
174 and then results were expressed as TEAC values (µmol trolox/g extract). All the analyses
175 were carried out in triplicate.

176 **2.4.3. Antibacterial activity assay**

177 The extracts and fractions collected were individually tested against a Gram-positive
178 bacterium (*Staphylococcus aureus* ATCC 25923) and a Gram-negative bacterium

(*Escherichia coli* ATCC 25922). A broth microdilution method was used, as recommended by the National Committee for Clinical Laboratory Standards [23], for the determination of the minimum inhibitory concentration (MIC). All tests were performed in Mueller–Hinton broth supplemented with 0.5 % tween 20. The inoculum of bacterial strains were prepared from overnight Mueller–Hinton broth cultures at 37 ºC. Test strains were suspended in Muller–Hinton broth to give a final density 10^7 CFU/mL. Then, the samples were diluted in ethanol ranging from 1 to 50 mg/mL.

96-microwell plates were prepared by dispensing into each well 185 µl of culture broth, 10 µl of the different sample's dilutions, antibiotic solution (chloramphenicol as positive control) or solvent (ethanol as negative control), and 5 µl of the inoculums. In addition, blanks were prepared adding 190 µl of broth medium to the solvent or extracts wells. The final volume of each well was 200 µl. After dispensing the inoculum, the plates were read in an Infinite 200 PRO plate reader (TECAN, Trading AG, Switzerland) spectrophotometer at 620 nm for T_0 (Zero Time). Then, the plates were incubated at 37 °C for 24 h and the absorbance was read for T_F (Final Time). Each test was performed in triplicate and repeated twice and was used to calculate IC_{50} values.

2.4.4. CG-MS

Identification and quantification of volatile compounds of samples was carried out in a GS-MS-FID 7890A system (Agilent Technologies, USA) comprising a split/splitless injector, FID detector and a mass spectrometer detector 5975C triple-axis. An HP-5MS capillary column (30 m x 0.25 mm i.d. and 0.25 µm phase thickness) was used. The chromatographic method starts with an initial temperature of 40 ºC, then increased to 150 ºC, at 3 ºC/min and was held at 150 ºC for 10 min, then from 150 to 300 ºC, at 6 ºC/min and finally held at 300 ºC for 1 min. Volume of 1 µl of samples was injected in splitless mode. Helium (99.99 %) was employed as carrier gas (1 mL/min flow rate). The temperatures were: 250 ºC for injector, 230 ºC for the mass spectrometer ion source, 280 ºC for interface and 150 ºC for quadrupole. The mass spectrometer operated under electron impact mode (70 eV) and it was used in total ion current (TIC) mode and scanned the mass range from 40 to 500 m/z.

2.5. Preparation and application of edible composite coatings with chitosan, essential oils or supercritical extracts

PEEC were prepared with chitosan and the addition of different amounts (1 and 5 % w/w) of thyme and rosemary essential oils obtained by HD, and thyme and rosemary supercritical extracts obtained, respectively, by SFE15 and SFE15-ETOH.

Chitosan solution was prepared following the procedures described by Ali et al. (36) with some modification. Briefly, 2.0 g of chitosan was dissolved in 100 mL of distilled water containing 0.5 mL of glacial acetic acid. The solution was heated with constant stirring for 12 h, the pH of the solution was adjusted to 5.5 with 1 N NaOH, and 0.1 mL of tween-80 was added as emulsifier. Then, 1 or 5 % w/w of the sample (essential oil or supercritical extract) was added and homogenized using an Ultra Turrax homogenizer (T18 basic IKA, Germany) at 7500 rpm for 3 min.

The strawberry coating was carried out by dipping the fruit into the different edible coatings for 1 min. Then, the fruits were air dried, packed in commercial corrugated boxes and stored at 4.0 ± 1 °C.

Nine different PEEC were evaluated: fruit coated with pure chitosan (Ch), with chitosan and 1% (EOT-1) or 5% (EOT-5) thyme HD extract, chitosan and 1% (EOR-1) or 5% (EOR-5) rosemary HD extract, chitosan and 1% (SCT-1) or 5% (SCT-5) thyme supercritical extract, chitosan and 1 % (SCR-1) or 5 % (SCR-5) of rosemary supercritical extract. Uncoated fruit was used as control (C). Twenty-five berries for each coating treatment were used and the experiments were performed in duplicate. Quality characteristics of control and coated fruits were determined during storing at 4.0 ± 1 °C.

2.6.Evaluation of fruit quality

2.6.1. Fungal decay percentage

Strawberries were visually evaluated for the presence of mold growth during the storage time (10 days). Any strawberry with visible spoilage was considered to be decayed. Fungal decay percentage was calculated by using the equation (1):

$$Fungal\ decay\ (\%) = \frac{number\ of\ decayed\ fruits}{total\ number\ of\ fruits} \times 100 \qquad (1)$$

2.6.2. Weight loss

Strawberries were weighed just after coating and air drying. Twenty-five berries for each coating treatment were utilized and the experiments were performed in duplicate. The berry weights were determined until 10 days after coating. The weight loss was calculated as the percentage of weight loss related to the initial weight value.

$$Weight\ loss\ \% = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100 \quad (2)$$

2.6.3. Soluble solid content (SSC) and pH

SSC and pH of strawberries were measured at different time intervals after the coating treatment following the procedures described by AOAC method. The SSC of the pulp sample was measured at 20 °C using a Brix refractometer. Similarly, pH of the pulp was measured with a pH-meter. Sampling and measurements were done in triplicate.

2.6.4. Color

The color of the strawberry surface was measured with a colorimeter. Values of L* (lightness), a* (green-red chromaticity) and b* (blue-yellow chromaticity) were recorded to calculate the change of color (ΔE), chromaticity (C*) and hue angle (Hue), based on the following formulas:

$$\Delta E = \sqrt{(\Delta L^{*2}) + (\Delta a^{*2}) + (\Delta b^{*2})} \quad (3)$$

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2} \quad (4)$$

$$Hue = tan^{-1}\left({}^{b^*}/_{a^*}\right) \quad (5)$$

2.6.5. Analysis of total phenolic compounds (TPC)

5 g of pulp finely chopped were extracted with 10 mL of methanol and were homogenized. After a cleaning-up step via centrifugation (5 min at 4500 rpm and 25 ºC) and filtration, the supernatants were recovered and allowed to stand at room temperature for evaporation of the solvent. The experiment was done in triplicate. Then, the total phenolic compounds content of the extracted pulp was determined using Folin-Ciocalteu method [20].

2.7. Statistical analysis

Two replicates were studied for each coating treatment and each day of analysis, and each measurement was done in triplicate. All data collected were expressed as mean ± standard error, and the statistical analysis of data was performed using R software version 3.6.2. The significant differences of mean values were determined using one-way ANOVA with Tukey HSD (honestly-significant-difference) grouping at 95% confidence level.

3. Results and Discussion

3.1.Extraction yield and content of volatile compounds

The extraction yields (mass of extract/mass of plant) obtained in the different extraction procedures are given in Table 2. In general, rosemary and thyme yields are the highest while licorice root yields are the lowest.

Considering the plant hydrodistillation processes, rosemary, thyme, mugwort and centaury HD resulted in yields of, respectively, 1.33, 0.97, 0.02 and 0.02 % w/w. In the case of licorice root, HD yield was very low and thus, it was not possible to recover the essential oil accurately. Furthermore, according to Table 2, no important differences were observed between HD and UAE-HD yields, and these yields are in reasonably agreement with the values reported by other authors. Rasooli et al., [24] informed rosemary hydrodistillation yield c.a. 1 %, and. Boutekedjret et al., [25] values of 1.2 and 0.44 % using, respectively, steam distillation and hydrodistillation. Morsy [26] reported 1 % yield in the thyme leaves hydrodistillation, and Janáckovic et al [27] yields in the range 0.027 to 0.295 % for different varieties of *Artemisia sp*. Furthermore, low hydrodistillation yields were obtained by Jerkovic et al [28] in the case of centaury leaves (c.a. 0.02 %).

As expected, SFE yields are considerably higher than HD yields, with values in the range 0.66 to 4.75 % w/w at low pressures (SFE15). The effect on yield of increasing pressure from 15 to 30 MPa varied with the plant variety, producing and increase in the case of rosemary, thyme and licorice (see yields of the two fractions recovered at 30 MPa, SFE30-1 and SFE30-2) but almost no change in the case of mugwort or centaury. Several authors reported a positive influence of pressure on supercritical extraction yield, which is attributed to the increase of solvent density and its enhanced solvation power [29]. Additionally,

284 considering the fractionation accomplished at 30 MPa, yields obtained were in general
285 higher in the second separator (SFE30-2) than in the first separator (SFE30-1).

286 Also, as expected, the addition of 10 % ethanol cosolvent (SFE15-EtOH) resulted in a 2 to
287 3.3 fold increase of yield, in comparison with the extraction without ethanol (SFE15) for all
288 plant varieties. The increase of the supercritical phase polarity when ethanol is added to CO_2
289 as cosolvent, absolutely enhanced the extraction of the more polar compounds present in
290 the plant matrix and thus, extraction yield increased.

291 The results of GC-MS analysis of plant extracts are given in Table 3. Volatile compounds
292 were identified for rosemary, thyme, mugwort and some centaury fractions, while no
293 volatile compounds were identified in the case of licorice extracts. Eucalyptol, borneol, α-
294 terpineol and camphor were the major compounds identify in rosemary extracts. In general,
295 the rosemary essential oils produced by HD and UAE-HD presented higher concentrations
296 of these compounds in comparison with supercritical rosemary extracts. Eucalyptol and
297 borneol are the principal compounds present rosemary essential oil as reported elsewhere
298 [25]. In the case of thyme, as it is well-known [30], the major and characteristic compounds
299 of this plant are thymol and carvacrol, which were both observed in the products obtained
300 by hydrodistillation and supercritical extraction. Significant lower amounts of volatile
301 compounds were identified and quantified in mugwort and centaury. While c.a. 45-65 %
302 w/w of the extracts produced by hydrodistillation was quantified in the case of rosemary
303 and thyme, less than 10 % w/w was determined in the case of mugwort and centaury

304 Regarding the results obtained in the GC-MS analysis (Table 3) it can be observed that the
305 volatile compounds identified in each plant variety are present in higher concentrations in
306 the essential oils (HD and UAE-HD extracts) in comparison with the supercritical extracts.

307 **3.2.Antioxidant and antimicrobial activity**

308 Table 4 present the content of total phenolic compounds (TPC), together with the
309 antioxidant and antimicrobial capacity of the different plant extracts obtained.

310 The highest TPC content was observed in thyme essential oil, with values in the order of
311 300 mg GAE/g. Also, rosemary, thyme and licorice supercritical extracts presented high
312 TPC values (56-174 mg GAE/g), while values lower than 27 mg GAE/g were obtained for
313 all mugwort and centaury extracts.

As reported in the literature, the content of phenolic compounds present in a vegetal sample is very close related with its antioxidant capacity [31,32]. Figure 1 shows the antioxidant capacity (ABTS and DPPH tests) of all extracts obtained as a function of the TPC content of the sample. Only thyme essential oil presented high ABTS and DPPH values. For the rest of hydrodistillation extracts these values were very low and, in general, could not be accurately measured. As can be observed in Figure 1, in general, the higher the TPC value the higher the ABTS (Figure 1a) or DPPH (Figure 1b) values, denoting the high influence of phenolic compounds in the antioxidant activity of the plant extracts. Particularly, good linear regression coefficient ($R^2 = 0.8373$) was obtaining in the correlation of ABTS values with TPC, which may be attributed to the ability of this method to assess the antioxidant effect of both hydrophilic and lipophilic phenolic compounds.

In supercritical extraction technology, the use of ethanol as CO_2 cosolvent is usually related with an increase of the supercritical solvent polarity and thus, it is expected to obtain extracts with higher content of phenolic compounds and in turn better antioxidant activity. Figure 2 shows an analysis of this general statement for the five plant varieties studied in this work, by comparison of the results of the SFE assays carried out at the same temperature, pressure and CO_2 flow, but with and without ethanol cosolvent (SFE15-EtOH and SFE15). As can be observed in Figure 2(a), except in the case of thyme, the general tendency observed is that the TPC values are higher in the supercritical extractions carried out with ethanol (SFE15-EtOH). Consequently, in the case of rosemary, centaury and licorice, the ABTS values are higher for the corresponding SFE15-EtOH extracts (Figure 2(b)) and thus presenting better antioxidant capacity than SFE15 extracts. In this respect, results obtained with mugwort plant matrix did not show statistical differences ($p > 0.05$). Furthermore, although in the case of thyme the use of ethanol cosolvent did not produce an increase in TPC content (as expected according to SFE premise), the higher TPC value observed in thyme SFE15 extract is in accordance with a higher ABTS value.

Regarding the antimicrobial activity, it is generally recognized that plant extracts obtained by hydrodistillation contain large amounts of the volatile compounds (essential oil) of the vegetal matrix, comprising high antimicrobial substances [33]. Accordingly, HD and UAE-HD plant extracts presented good antimicrobial activity (IC_{50} values in the range 0.05-2.12 mg/mL) except in the case of centaury (see Table 4). Furthermore, thyme HD showed the best antimicrobial activity (lower IC_{50} values), followed by mugwort HD and rosemary

346 UAE-HD extracts. Only in the case of rosemary, the ultrasound pretreatment applied to the
347 hydrodistillation process produced a positive effect in the extraction of antimicrobial
348 rosemary volatile compounds.

349 Supercritical extracts also presented antimicrobial activity, with IC_{50} values in a higher
350 range (0.76-2.93 mg/mL) in comparison with hydrodistillation extracts, although the
351 content of identified volatile compounds is significantly lower in these extracts in
352 comparison with hydrodistillation extracts. A random trend was observed in the *E. coli* and
353 *S. aureus* IC_{50} values when comparing SFE extracts obtained with or without ethanol
354 cosolvent. Nevertheless, in the case of rosemary, mugwort and licorice, a general trend can
355 be stated concerning the effect of the fractionation carried out at 30 MPa. As can be observed
356 in Figure 3, SFE30-2 fractions presented better antioxidant activity than SFE30-1 fractions
357 (Figure 3(a)), and the opposite tendency was observed for the antimicrobial activity,
358 particularly concerning *E. coli* IC_{50} values (Figure 3(b)).

359 Finally, considering the extraction yields, which are significantly higher for SFE in
360 comparison with hydrodistillation, it can be conclude that supercritical technology maybe
361 an adequate alternative for the production of plant extracts with good antimicrobial and/or
362 antioxidant activity to be used in the formulation and development of PEEC. According to
363 the plant varieties analyzed in this work, rosemary and thyme were selected to prepare the
364 edible coatings to make a comparison between supercritical and hydrodistillation extracts
365 on strawberries treatment.

366 **3.3. Effect of edible coatings on strawberries physicochemical properties**

367 As described previously, different coatings were prepared using mixtures of chitosan and
368 1% or 5% w/w of rosemary (HD and SFE15) or thyme (HD and SFE15-EtOH) extracts.
369 These samples were selected taking into account their extraction yield, so as the antioxidant
370 and antibacterial activity determined in this work. A total of eight different coatings
371 combining chitosan and plant extracts were evaluated. Furthermore, uncoated berries and
372 berries coated with pure chitosan were used as controls.

373 The effectiveness of chitosan-based plant extracts edible coatings to inhibit fungal
374 development on strawberries was visually inspected. All coated samples present a lower
375 percentage of fungal decay in comparison with control samples, as can be observed in Figure

4. Nevertheless, the incidence of fungal decay was not evidence during the first six days of storage, and the percentages calculated using Eq. (1) are related just to the surface deterioration observed in the berries. After seven days of storage, visual decay due to fungal infection was observed, with significant differences between uncoated strawberries and strawberries treated with the different edible coatings. On day 10, the highest decay percentage occurred in control sample (31.7 %) and the lowest values were found in the case of EOR-5 treatment (6.7 %), probably due to its high content of a renowned antifungal compound (eucalyptol). The worse fungal decays with significant differences were observed for SCT-5 (18.3 %) and SCR-1 (21.7 %), and it can be highlighted that rosemary essential oil contains c.a. 13 times higher content of eucalyptol than rosemary supercritical extracts. For the rest of samples similar fungal decay values were obtained (around 14 %).

As reported elsewhere [34], low loss weight of fruits and vegetables during storage is an indicator of freshness. Figure 5 show the loss weight observed in this study for the coated and uncoated samples. An increase of the weight loss during storage time was observed for coated and uncoated samples, with statistically significant differences ($p < 0.05$). After 10 days of treatment, pure chitosan resulted in a lower loss of weight than all chitosan + plant extract coatings, but edible coatings obtained with supercritical extracts presented minor loss weight than those coatings produced with hydrodistillation extracts. Furthermore, berries coated with rosemary supercritical extracts exhibit the lowest loss weight in comparison with all chitosan + plant extract coating treatments.

The effect of the different edible coatings on pH and content of soluble solids (SS) is depicted in Figure 6. The increase of pH and SS is due to metabolic processes and reactions during post-harvest storage, which continue to convert starch and acids into sugar [35]. Significant differences ($p < 0.05$) were obtained for the pH values concerning the storage time, observing an increase of pH for all samples during the entire storage period (Figure 6a). In general, the application of supercritical extract coatings increased significantly the pH of samples on day 1 in comparison with essential oil coatings, which better delayed the pH increment, and thus diminished the effect of the maturation of the fruit through the consumption of organic acids.

Fruit ripening with storage is directly related to an increase in soluble solids (SS) content [36] owing to the solubilization of complex carbohydrates into simpler structures [37]. In this study, an increase in the content of soluble solids (SS) in fruits coated with the different

408 treatments was observed in comparison with the untreated fruit (Figure 6b). These changes
409 could be due to a reduction in fruit respiration rate and slower hydrolysis of carbohydrates
410 into sugars [38] as a result of the coating treatment. Significant differences ($p < 0.05$)
411 between coated and uncoated strawberries were observed, but in general minor differences
412 were observed concerning storage time.

413 Table 5 show the TPC values of uncoated strawberries and strawberries treated with
414 chitosan and the different plant extracts, measured on day 1 and day 10 of storage. Total
415 phenolic compounds content of strawberry was significantly affected by the different
416 treatments ($p > 0.05$). On day 1, the highest content of total phenolic compounds was
417 observed in the EOT-1 (1.541 ± 0.087 mg GAE/g fruit), showing a significant difference in
418 comparison with the control sample. The TPC values of coated and uncoated fruit decreased
419 during storage, as reported in Table 5. Nevertheless, the uncoated samples present the
420 highest TPC loss (Figure 7). Furthermore, the treatments combining chitosan with rosemary
421 (hydrodistillation or supercritical extracts) presented lower TPC loss than coatings produced
422 with chitosan and thyme extracts.

423 Color is an important quality attribute, which plays a key role in determining the consumer
424 preference and acceptability. Artés-Hernández et al [39] reported that the delay in the
425 development of color can be explained by a slower synthesis of pigments, such as
426 carotenoids and/or anthocyanins in fruits. The senescence delay attained by chitosan-oleic
427 acid edible coatings [40] and alginate coatings [41], was evidenced by the decrease in color
428 changes of strawberries during storage. Color attributes of coated and uncoated strawberries
429 were analyzed to evaluated color change, hue angle and chroma values during storage
430 period, and the results obtained are given in Figure 8.

431 The C* values for samples coated with rosemary or thyme extracts are showed, respectively,
432 in Figure 8a and Figure 8b. Chroma (C*) is a measurement of color saturation: higher C*
433 values mean than samples are brighter. Lower C* values were observed on day 1 for treated
434 berries in comparison with control samples or samples treated with pure chitosan.
435 Furthermore, C* values were in general higher for samples treated with rosemary extracts
436 in comparison with thyme extracts, but differences were not significant concerning the type
437 (supercritical or hydrodistillation) or the concentration of the plant extract. Regarding
438 storage time, it was observed a sharply decrease of C* values in control (C) and chitosan
439 (Ch) on day 3 of treatment, which could be attributed to the drying of the surface, while the

samples coated with chitosan + plant extract exhibit a moderately decrease (particularly, thyme extracts). Figure 8c and 8d present the variation of hue angle (Hue) for the different samples along with storage time. A decrease of Hue values indicates a decline of red color and increase of yellow color. Only in the case of thyme supercritical extracts, a significant decrease of Hue values was observed on day 1. Furthermore, the most important Hue values decrease with storage time occurred in control samples, while for treatments differences were in general not significant. Finally, ΔE values (change of color) are represented in Figure 8d and 8e. On day 1 significant higher ΔE values with respect to control were observed for chitosan and thyme hydrodistillation extracts. Then, ΔE values significantly increased with the storage time for all samples.

4. Conclusions

SFE yields of all studied plants were considerably higher than HD yields, and yields were significantly higher when ethanol was used as CO_2 cosolvent. The ABTS values of all extracts produced (SFE and HD) presented a high linear regression coefficient with the TPC content of the extract, confirming the influence of phenolic compounds in the antioxidant activity of plant extracts. Rosemary and thyme extracts exhibited the best combination of antioxidant and antimicrobial properties.

After 10 days of cold treatment, it can be concluded that berries coated with chitosan and 1% or 5% of supercritical rosemary SFE15 or thyme SFE15-ETOH extracts presented lower fungal decay in comparison with control, but spoilage preservation of the essential oils was better than that of supercritical extracts. No important effects of coatings with SFE or HD extracts on the color appearance of strawberries were established. Furthermore, in general, edible coatings produced with HD extracts exhibited somewhat higher loss weight, but lower pH and content of soluble solids increase, and better preserved the content of phenolic compounds in strawberries, in comparison with the berries coated with SFE extracts.

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616

Table 1. Experimental conditions of the extraction procedures used to obtain natural extracts. HD: hydrodistillation; UAE: ultrasound assisted extraction; SFE: supercritical extraction; S1 and S2: first and second decompression separators in SFE process.

	Extraction conditions
HD	Hydrodistillation at 100 °C, 1:10 plant: water ratio for 3 h
UAE-HD	Ultrasound Assisted Extraction pretreatment (1:10 plant: water, 550 W, 25 ºC, 15 min) followed by HD
SFE15	SFE at 15 MPa, 313.15 K and 70 g CO_2/min (S1 and S2 decompression pressure = 5 MPa)
SFE30	SFE at 30 MPa, 313.15 K and 70 g CO_2/min (S1 and S2 decompression pressure, respectively, 15 and 5 MPa)
SFE15-EtOH	SFE at 15 MPa, 313.15 K, 70 g CO_2/min and 10 % ethanol cosolvent) (S1 and S2 decompression pressure = 5 MPa)

Table 2. Extraction yields (mass of extract/mass of plant). SFE30-1 and SFE30-2 refer to the samples collected, respectively, in S1 and S2 cells.

Extraction	rosemary	thyme	mugwort	centaury	licorice root
HD	1.33	0.97	0.02	0.02	--
UAE-HD	1.23	0.94	0.01	0.02	--
SFE15	4.75	3.93	1.81	3.40	0.66
SFE30-1	1.92	1.85	0.29	2.02	0.24
SFE30-2	5.49	3.45	0.93	1.52	1.52
SFE15-EtOH	9.75	13.48	4.65	9.27	2.19

Table 3. Quantification of volatile compounds (mg/g extract) identified in plant extracts by GC-MS.

		Eucalyptol	γ-Terpinene	cis β-Terpineol	Linalool	Camphor	Borneol	4-Terpineol	α-Terpineol	Thymol	Carvacrol	Caryophyllene	Caryophyllene oxide
	Retention time	**13.088**	**14.38**	**14.72**	**16.33**	**18.35**	**19.40**	**19.91**	**20.62**	**25.53**	**26.13**	**31.11**	**38.05**
Rosemary	**HD**	313.48			4.04	23.95	56.43	10.65	39.03			29.23	
	UAE-HD	369.19			4.03	24.68	57.42	12.51	37.41			26.60	
	SFE15	24.62			0.07	44.15	14.71	2.75	10.77	6.86		8.34	5.18
	SFE30-1	58.20			1.45	88.71	19.87	4.48	14.66			14.14	5.40
	SFE30-2	14.97				19.85	6.96	1.37	5.04			3.69	5.12
	SFE15-EtOH	24.00				35.96	9.35	1.44	6.40			5.27	
Thyme	**HD**	5.60		4.21	12.24		8.93			560.20	67.30		
	UAE-HD	4.91		5.79	13.52					537.85	65.01		
	SFE15		1.46	0.10			0.40	0.08		41.54	10.98		
	SFE30-1			1.08	1.16		1.47	1.90		121.85	18.78		
	SFE30-2			0.61	0.13		0.99	0.87		70.41	13.50		
	SFE15-EtOH			1.46	2.66		1.83	2.20		122.94	18.69		
Mugwort	**HD**						4.52	10.66	4.46				65.48
	UAE-HD						11.51	22.25	7.56				
	SFE15												4.35
	SFE30-1						0.19						3.96
Centaury	**HD**					10.22	3.53	2.22	2.59	6.13	26.22		
	UAE-HD					15.25	5.76	3.51	4.62	8.06	23.54		
	SFE15					2.69							
	SFE30-1					2.61							4.19
	SFE15-EtOH					2.59							

Table 4. Total phenolic compounds (TPC, mg GAE/g extract), antioxidant activity (TEAC, mMol Trolox/g extract) and antimicrobial activity (IC_{50}, mg/mL) of the plant extracts obtained in this work.

		TPC	TEAC		IC_{50}	
			(ABTS)	(DPPH)	*E. coli*	*S. aureus*
Rosemary	**HD**	2.61 ± 0.04	n.d.	0.007±0.01	1.34	2.12
	UAE-HD	3.94 ± 0.06	n.d.	0.006±0.01	0.93	1.12
	SFE15	104.92 ± 0.68	0.21±0.01	0.61±0.01	1.25	1.08
	SFE30-1	82.71±0.66	0.16±0.01	0.63±0.01	0.84	1.02
	SFE30-2	167.39±1.00	0.24±0.01	0.75±0.02	1.32	0.96
	SFE15-EtOH	174.11±1.74	0.43±0.01	1.38±0.01	1.03	1.31
Thyme	**HD**	331.24±0.57	1.10±0.01	n.d.	0.05	0.70
	UAE-HD	271.94±1.17	0.90±0.01	n.d.	0.71	0.71
	SFE15	123.98±0.06	0.15±0.01	0.16±0.01	0.94	2.13
	SFE30-1	127.24±0.91	0.18±0.02	0.13±0.01	1.07	2.93
	SFE30-2	75.47±0.96	0.10±0.07	0.17±0.01	1.22	1.33
	SFE15-EtOH	56.42±0.26	0.08±0.01	0.11±0.01	1.06	1.08
Centaury	**HD**	0.04±0.50	n.d.	n.d.	>2.50	>2.50
	UAE-HD	20.50±1.10	n.d.	n.d.	>2.50	>2.50
	SFE15	11.14±0.96	0.012±0.01	0.017±0.01	>2.50	>2.50
	SFE30-1	15.37±0.34	0.015±0.01	0.008±0.01	>2.50	>2.50
	SFE30-2	27.24±0.60	0.018±0.01	0.009±0.01	>2.50	>2.50
	SFE15-EtOH	26.99±0.24	0.039±0.01	0.019±0.01	>2.50	>2.50
Mugwort	**HD**	21.38±0.22	n.d.	n.d.	0.99	1.17
	UAE-HD	12.13±0.01	n.d.	n.d.	1.32	1.23
	SFE15	7.78±0.69	0.028±0.01	0.005±0.01	1.39	2.05
	SFE30-1	22.807±0.43	0.028±0.01	0.005±0.01	1.23	1.55
	SFE30-2	23.11±0.41	0.041±0.01	0.003±0.01	1.46	1.55
	SFE15-EtOH	11.60±0.08	0.035±0.01	0.007±0.01	2.00	1.41
Licorice	**SFE15**	76.19 ± 1.45	0.10±0.01	0.112±0.01	1.17	1.41
	SFE30-1	69.05 ± 0.94	0.04±0.01	0.057±0.01	0.76	1.49
	SFE30-2	159.23 ± 1.18	0.13±0.01	0.331±0.02	2.17	2.20
	SFE15-EtOH	141.18 ± 1.09	0.15±0.20	0.442±0.04	1.50	2.26

n.d.: non determined

Table 5. Total phenolic compounds (TPC) of uncoated strawberries (C), strawberries treated with chitosan (Ch) and different plant extracts, stored 4±1 ºC for 10 days.

No.	Sample	% w/w plant extract	TPC (mg GAE/g of fruit)	
			Day 1	Day 10
1	C		1.414 ± 0.116	0.977 ± 0.045
2	Ch		1.281 ± 0.057	0.997 ± 0.079
3	EOR-1	1% rosemary-HD	1.283 ± 0.057	1.218 ± 0.060
4	EOR-5	5% rosemary-HD	1.246 ± 0.088	1.239 ± 0.146
5	EOT-1	1% thyme-HD	1.541 ± 0.087	1.380 ± 0.014
6	EOT-5	5% thyme-HD	1.381 ± 0.102	1.223 ± 0.016
7	SCR-1	1% rosemary-SFE15	1.205 ± 0.059	n.d.
8	SCR-5	5% rosemary-SFE15	1.195 ± 0.012	1.123 ± 0.057
9	SCT-1	1% thyme-SFE15-EtOH	1.230 ± 0.065	1.098 ± 0.013
10	SCT-5	5% thyme-SFE15-EtOH	1.247 ± 0.069	1.064 ± 0.048

n.d.: non determined.

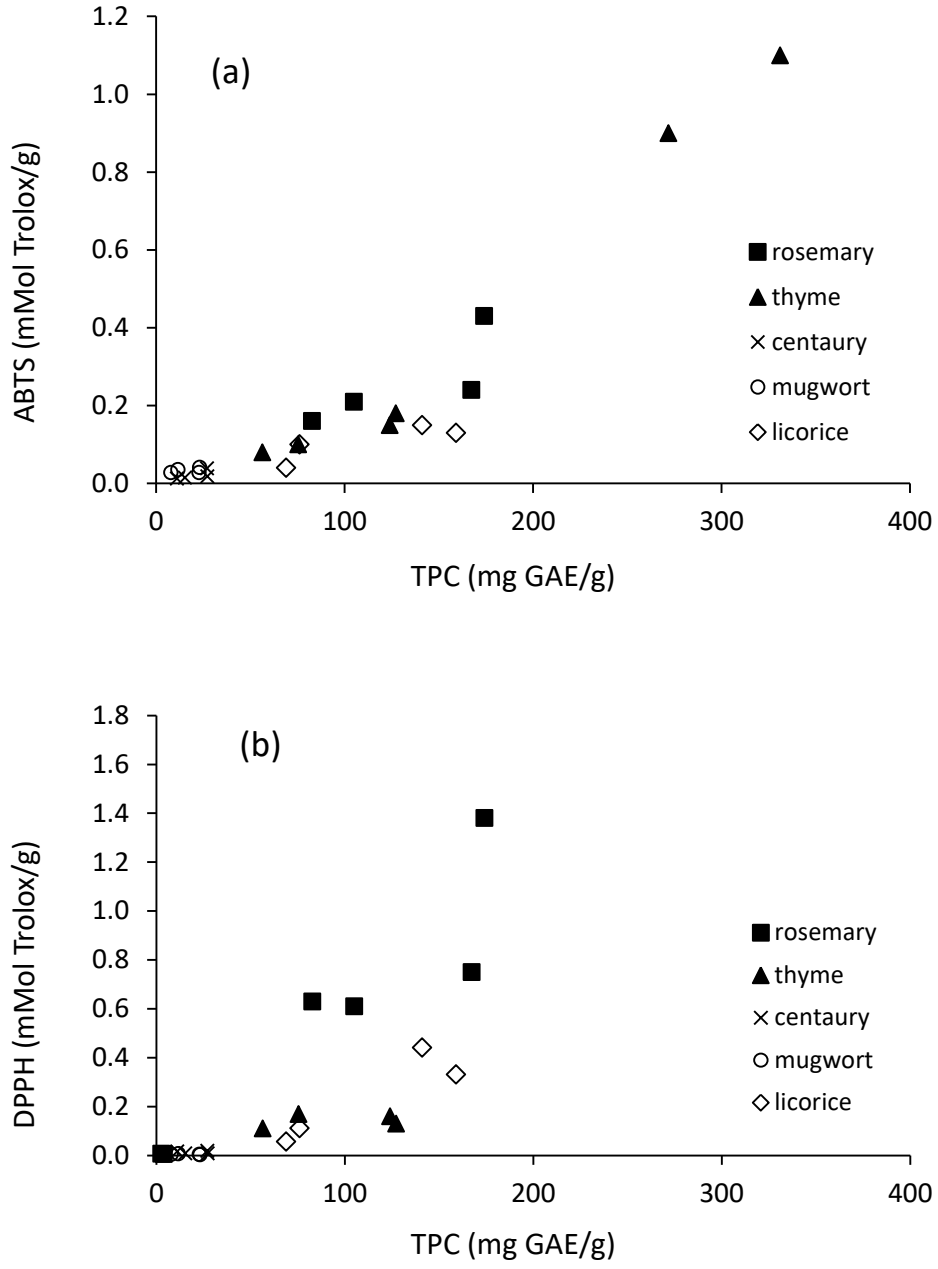

Figure 1. (a) ABTS and (b) DPPH values of plant extracts obtained by hydrodistillation and supercritical extraction as a function of the content of total phenolic compounds (TPC).

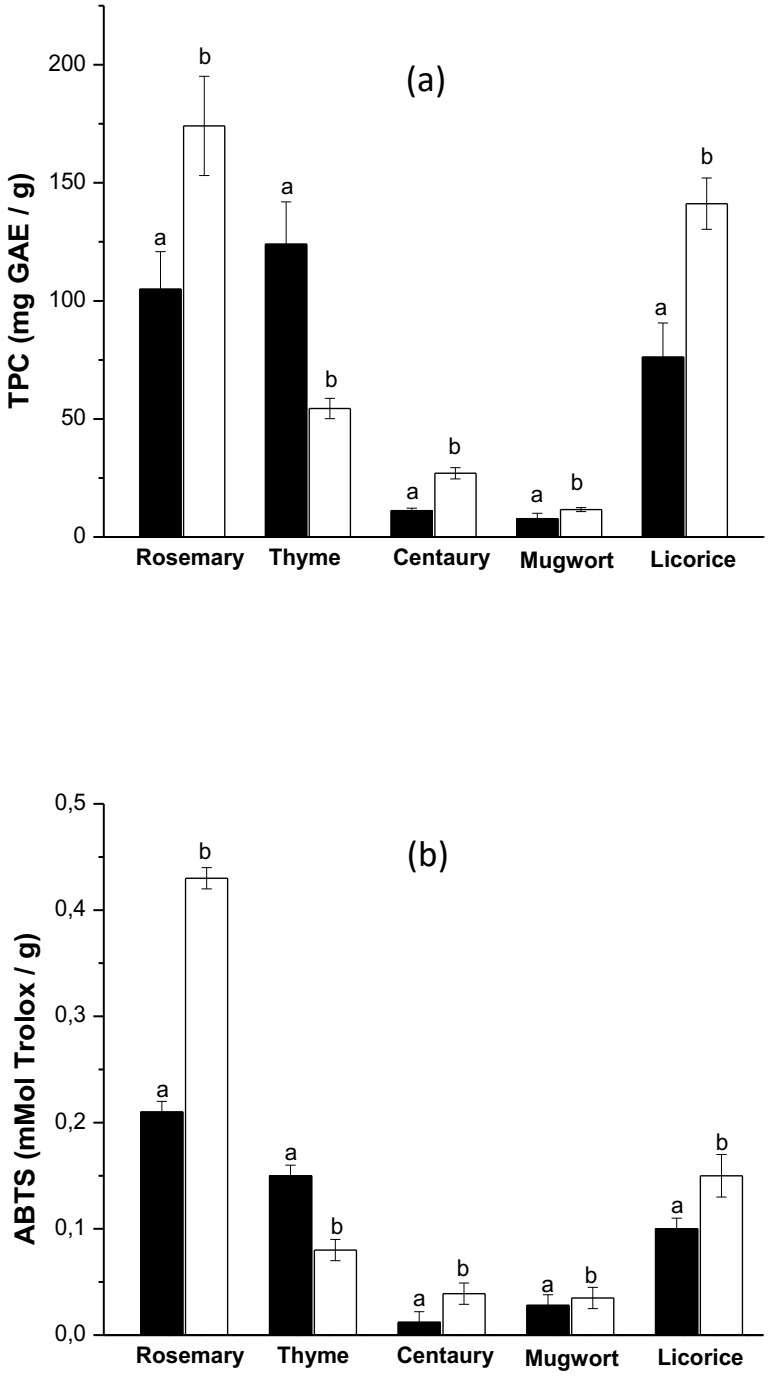

Figure 2. (a) Content of total phenolic compounds and (b) ABTS values of the plant extracts obtained by supercritical extractions SFE15 (■) and SFE15-EtOH (□). Bars with different letters are significantly different ($p < 0.05$).

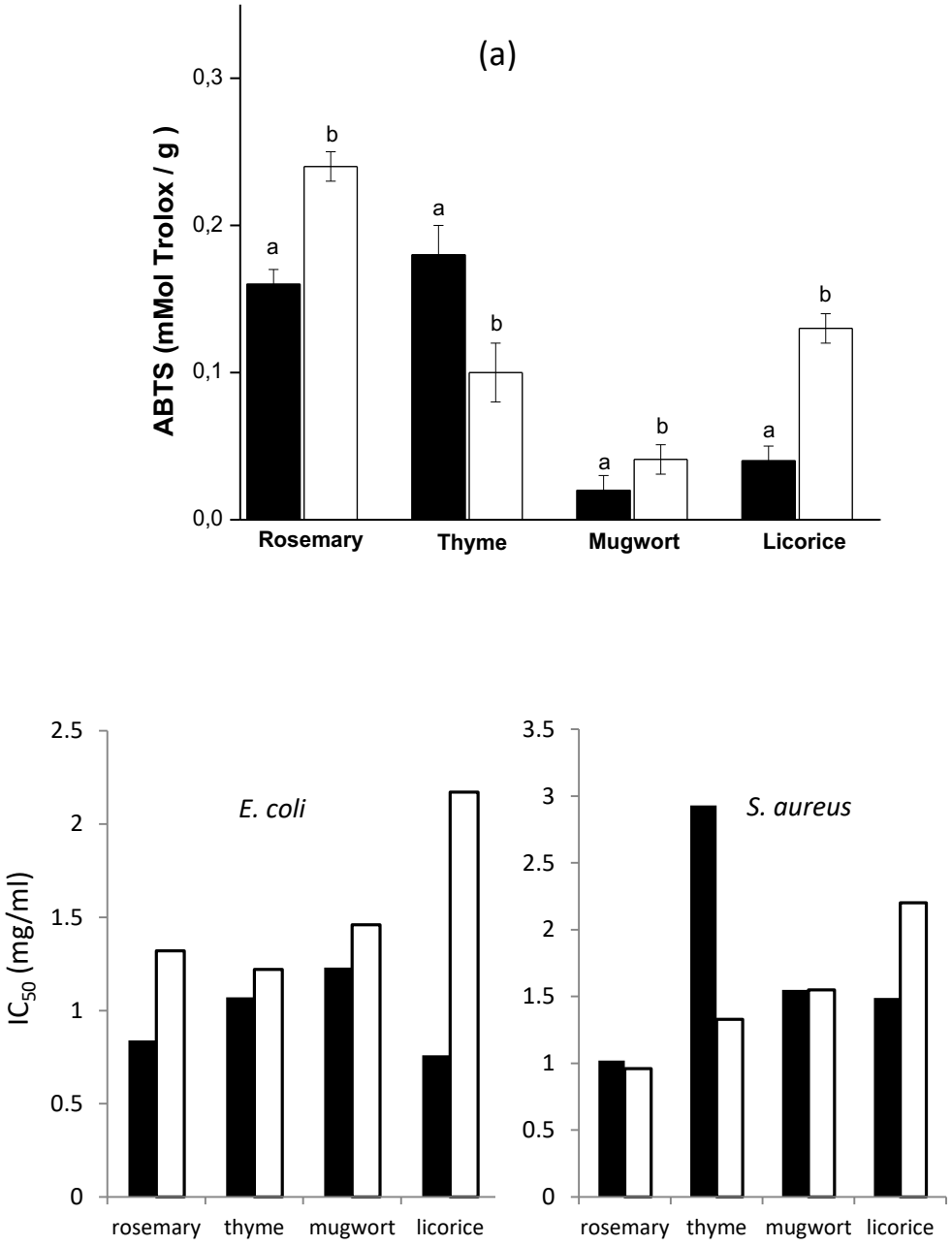

Figure 3. (a) ABTS values and (b) IC_{50} values of SFE30-1 (■) and SFE30-2 (□) plant extract fractions obtained by a cascade decompression of the supercritical extract (30 MPa, 313.15 K and 70 g/min of CO_2). Bars with different letters are significantly different ($p < 0.05$).

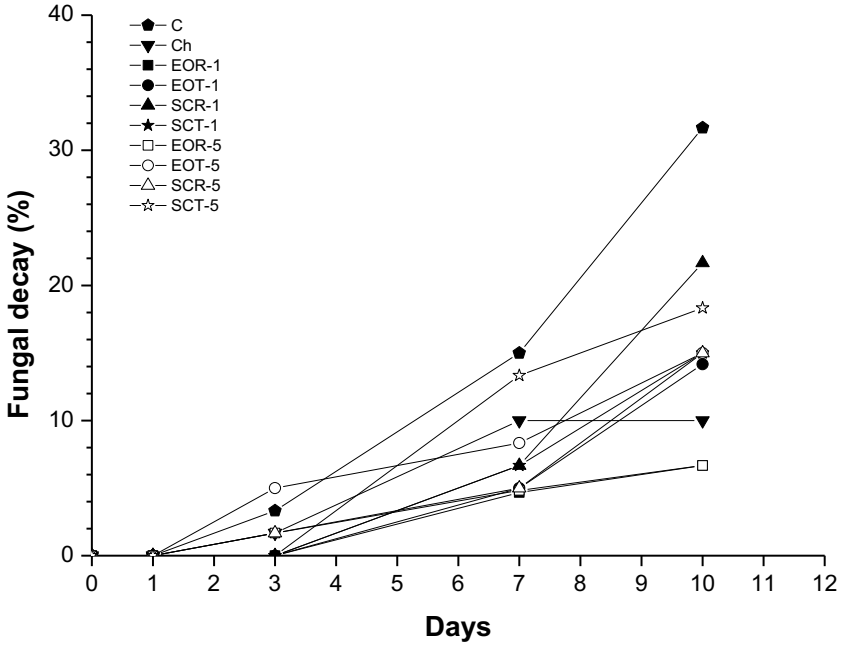

Figure 4. Fungal decay of coated and uncoated strawberries stored at 4±1 °C for 10 days.

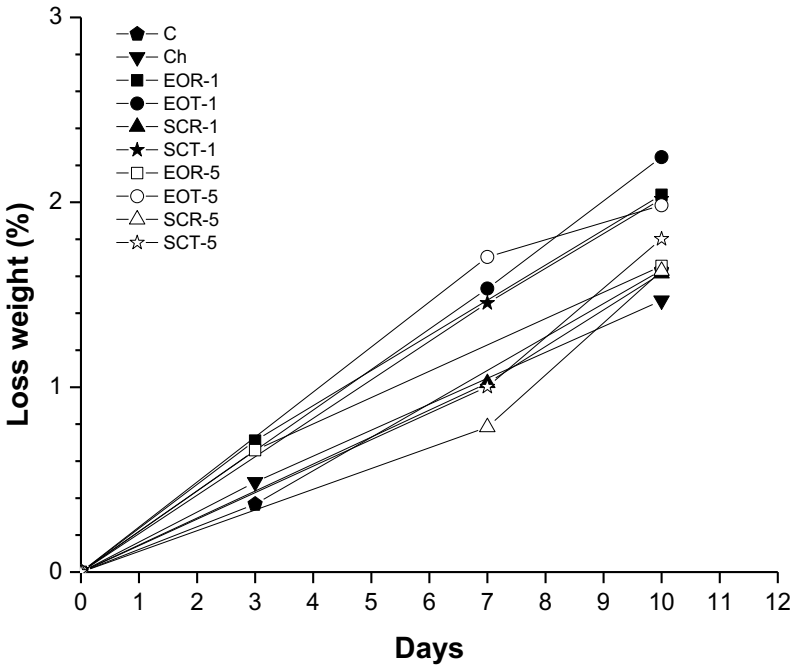

Figure 5. Loss of weight of coated and uncoated strawberries, stored at 4±1 °C for 10 days.

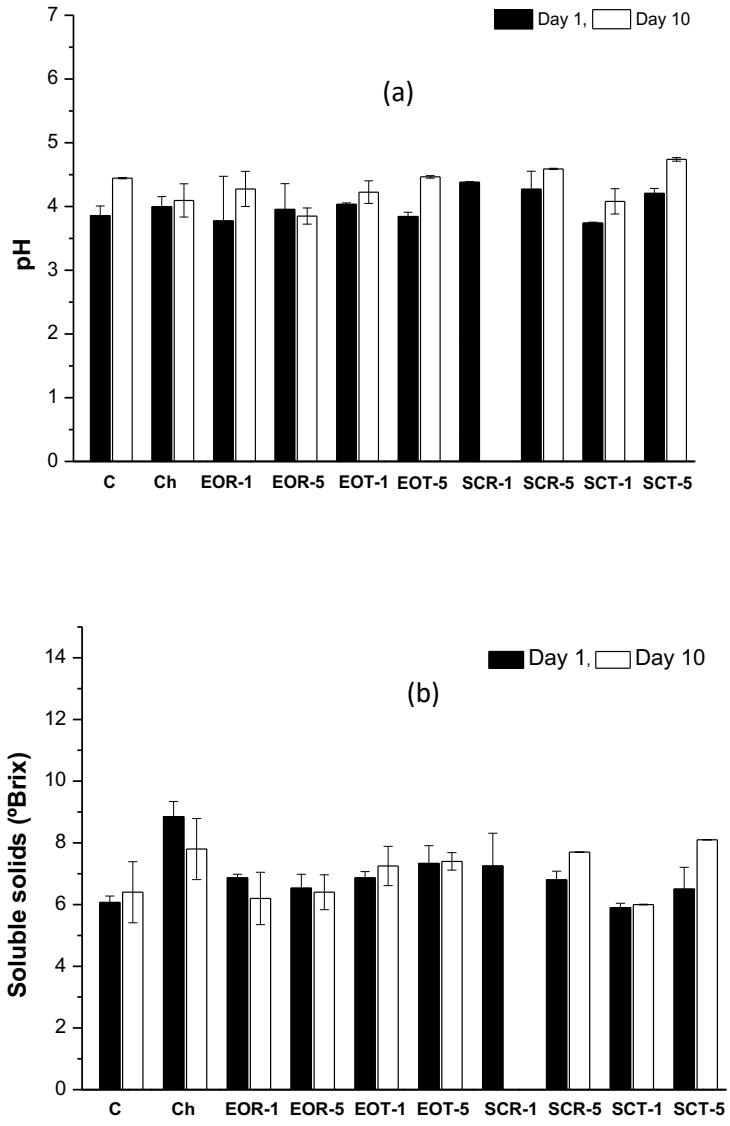

Figure 6. (a) pH and (b) content of soluble solids of coated and uncoated strawberries during storage at 4±1 °C for 10 days.

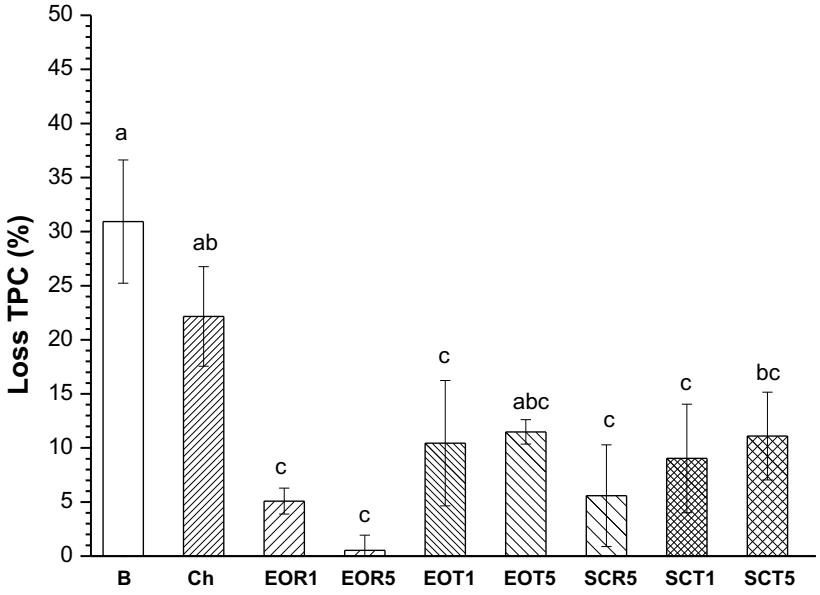

Figure 7. Loss of total phenolic compounds (TPC) of coated and uncoated strawberries during storage at 4±1 °C for 10 days. Data non available for berries coated with SCR-1. Bars with different letters are significantly different ($p < 0.05$).

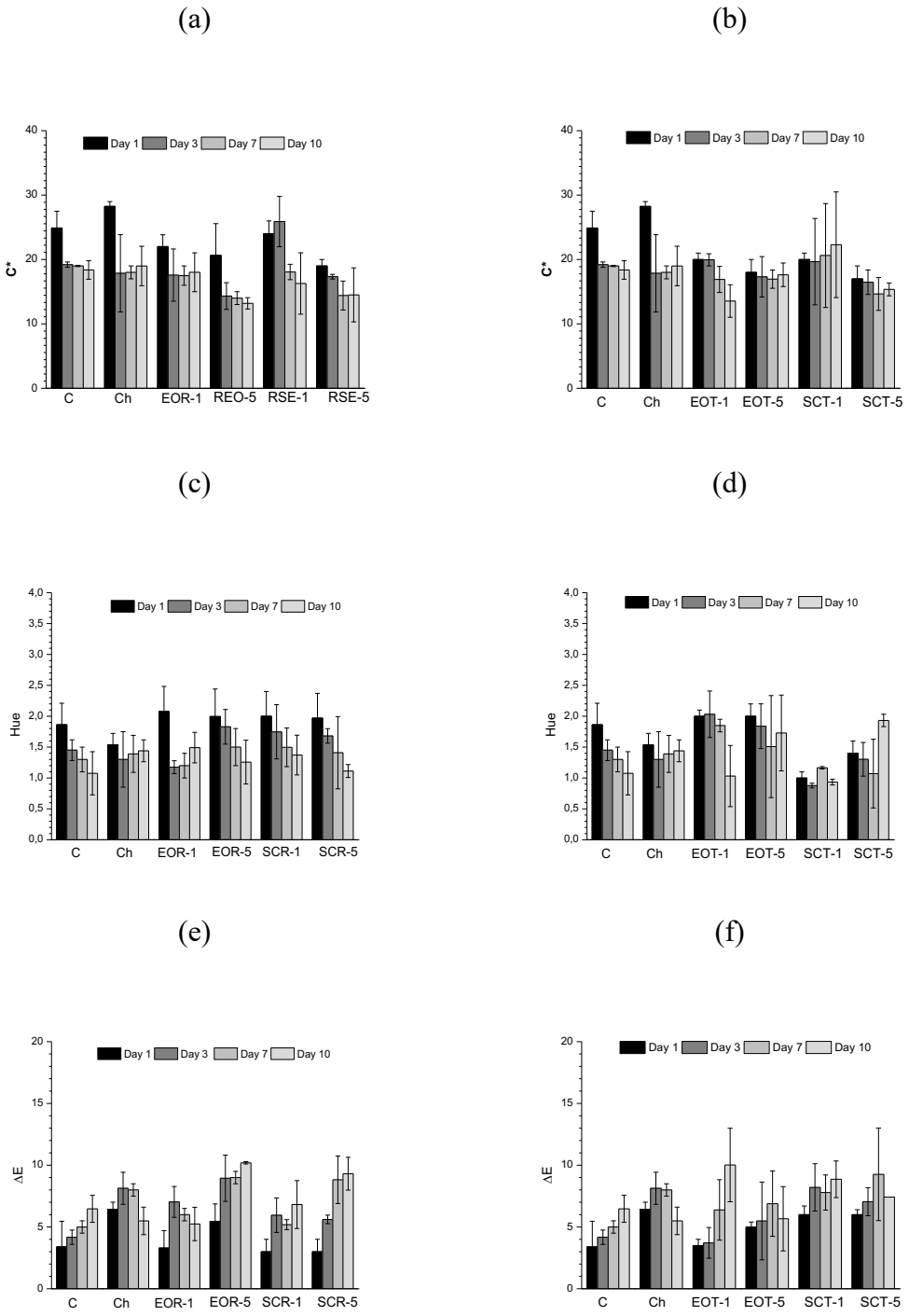

Figure 8. Color characteristics of strawberries coated with chitosan and plant extracts during storage at 4 ± 1 °C for 10 day.

The Journal of Supercritical Fluids

Comparison between essential oils and supercritical extracts into chitosan-based edible coatings on strawberry quality during cold storage

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Abstract:	Plant extracts are being studied in the development of novel edible food coatings. The antioxidant and antimicrobial compounds that naturally occur in some plants are the key substances that contribute to preserving food quality. Besides the plant material, the method utilized to produce the extract influences its chemical and preservative characteristics. In general, hydrodistillation produce plant extracts (essential oils) that are well recognized for containing high concentration of antioxidant or antimicrobial volatile compounds. Supercritical fluid technology produce high quality bioactive extracts with higher yield but lower concentration of volatile compounds, in comparison with hydrodistillation. In this work, six different natural extracts of five different plants were produced by hydrodistillation and supercritical fluid extraction, and their antioxidant and antimicrobial activities were compared. The most active extracts were used to produce chitosan-based edible coatings with the aim of assessing the effect of essential oils vs. supercritical extracts on the preservation of strawberries.
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Antioxidant and antimicrobial assessment of licorice supercritical extracts

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ABSTRACT

Licorice (*Glycyrrhiza glabra* L.) is a plant used widely in herbal medicines due to their several biological potentials. The supercritical extraction of licorice roots was investigated to assess the antioxidant and antimicrobial activity of the extracts. Extraction conditions were pressures from 15 to 40 MPa, 313.15 and 333.15 K, and ethanol cosolvent in the range of 0 to 20% mass. In the case of high-pressure extractions using pure carbon dioxide (CO_2) fractionation of the supercritical extract was accomplished in a two-cell decompression system. Fractionation was carried out with the aim to examine the potential separation of the antioxidant and antimicrobial licorice compounds and thus increase the bioactive properties of the fractions obtained in each separation cell. Main licorice bioactive compounds, liquiritin, liquiritigenin, glycyrrhizin, isoliquiritigenin and glabridin, were identified by HPLC and quantified using standards. Extracts obtained with supercritical CO_2 and ethanol cosolvent contain the higher amounts of phenolic compounds and also the higher antioxidant activity but exhibit low or even no antimicrobial activity. Using pure CO_2 at high pressure coupled with the on-line fractionation of the extract, two samples were obtained which showed, respectively, lower phenolic compounds content and good antimicrobial capacity (first fraction) and higher phenolic compounds content and antioxidant capacity (second fraction). Thus, the advantages of supercritical on-line fractionation are demonstrated in the extraction of Licorice roots.

1. Introduction

Licorice is a ligneous perennial shrub typical of the Mediterranean region, Asia Minor and Middle East and also widely cultivated in southern Russia and Iran (Ody, 2000). The roots and rhizomes of licorice are used extensively in herbal medicines due to their, among others, emollient, detoxification, anti-ulcer, anti-inflammatory, gastroprotective and anti-allergenic properties (Mukhopadhyay and Panja, 2008). Licorice contains a variety of bioactive compounds, different sugars, phenolic compounds, isoflavones, coumarins, stilbenoids and saponins such as glycyrrhizin (Qiao et al., 2015), with positive pharmaceutical functions, such as anti-inflammatory, anti-viral, anti-carcinogenic (Fukai et al., 2004; Hatano et al., 2000), antifungal properties (Fatima et al., 2009), as well as antioxidant and antimicrobial activities (Thakur et al., 2016). Different extraction procedures have been investigated to obtain bioactive extracts from licorice roots, being water, ethanol and methanol, the main solvents studied.

Visavadiya et al. (2009) and Gupta et al. (2016) reported the antioxidant activity of extracts obtained by soxhlet extraction with ethanol and water. Hejazi et al. (2017) used the same extraction technique, but with methanol solvent. Methanolic extracts were subsequently fractionated with water and several organic solvents covering different polarities, in order to evaluate the fractions for the *in vitro* antioxidant capacity and apoptotic effects in cell systems. Cheel et al. (2010) and Tohma and Gulçin (2010) studied the radical scavenging activity of extracts obtained by infusion using ethanol and water. Karami et al. (2015) evaluated the antioxidant capacity of extracts obtained by microwave assisted extraction (MAE) with water and 80% ethanol and methanol. From these works, polar solvents were generally more effective to obtain extracts containing high concentrations of phenolic compounds and better antioxidant capacities, specially water, ethanol and their mixtures. Furthermore, the antioxidant activity of licorice extracts was strong related with the presence of glycyrrhizin, a compound with an antioxidant mechanism studied and reported in the literature (Beskina et al., 2006).

Recently, several studies aiming to expand the knowledge about the biological properties of licorice extracts in regard to antimicrobial activity have been reported. Several studies have shown the ability of

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licorice aqueous, ethanol and methanol extracts, obtained by different extraction processes, to inhibit the growth of Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Salmonella spp*. (Chandra and Gunasekaran, 2017; García-Ruiz et al., 2015; Astaf eva and Sukhenko, 2014; Ercisli et al., 2008). Based on the above inhibitory activities against bacteria, it has been suggested that licorice may have a therapeutic and/or preventive capacity for oral infections (Gafner et al., 2011).

Supercritical fluid extraction (SFE) is an extraction technology also applied to obtain antioxidant and antimicrobial extracts from natural sources (Fornari et al., 2012). Supercritical fluids allow high extraction rates due to their high solvation power (liquid-like density), low viscosities and high diffusion coefficients. Supercritical CO_2 ($SCCO_2$) is the preferred supercritical fluid, due to its low critical temperature (304 K) and moderate critical pressure (7.4 MPa), which prevents or minimizes the degradation of bioactive compounds. Moreover, CO_2 is inexpensive, inert, non-toxic, non-flammable and allows obtaining solvent-solvent free products. Despite these interesting properties, only a few studies have been reported for the SFE of licorice roots and their objective was the extraction of glycyrrhizic acid. Kim et al. (2004) examined the extraction behavior of this compound at different pressures (11–50 MPa), temperatures (313–393 K) and using water and 70% aqueous methanol as cosolvent. The best result (98% glycyrrhizic acid recovery) was obtained at 30 MPa, 343 K and 15% of aqueous methanol cosolvent. Likewise, Hedayati and Ghoreishi (2015) studied glycyrrhizic acid extraction by a combined static-dynamic procedure at several pressures (10–34 MPa), temperatures (318–358 K), dynamic extraction times (40–120 min), CO_2 flow rates (0.8–2 mL/min) and using different methanol/water mixtures as cosolvent. The highest extraction (54% compound recovery) was reached at 30 MPa, 341 K, extraction time of 108 min and 46.5% methanol (v/v). In a further contribution, Hedayati and Ghoreishi (2016) studied the same extraction parameters but using water as cosolvent, and determined the optimal conditions simulating the experimental results by an artificial neural network model.

Based on this background, this work presents a study of the SFE of licorice roots using CO_2 with and without the addition of ethanol as a cosolvent and, for the first time, the *in vitro* evaluation of the antioxidant and antimicrobial activity of the supercritical fractions obtained.

2. Materials and methods

2.1. Chemicals

Ethanol (99.5% purity) and Sodium Carbonate anhydrous (99.5% purity) were purchased from Panreac (Barcelona, Spain). CO_2 (N38) was supplied from Carburos Metálicos (Madrid, Spain). Gallic acid standard (> 98% purity), 2,2-Diphenyl-1-pycrilhydrazyl (DPPH, 95% purity), 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥ 95% purity), (±)-6-Hydroxy-2,5,7,8-tetramethyllchromane-2-carboxylic acid (Trolox, 97% purity), Folin-Ciocalteu´s reagent, chloramphenicol (≥ 98% purity), liquiritin, glabridin, liquiritigenin, glycyrrhizic acid ammonium salt and isoliquiritigenin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Difco Wilkins-Chalgren Agar and BBL Mueller Hinton II Broth was purchased from Becton, Dickinson and Company (France).

2.2. Sample preparation

Root of licorice harvested in Spain was obtained from Murciana herbalist's (Murcia, Spain) and the water content was 9.90% wt. The sample was ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain) and the mean particle size was 100 µm (all particles were lower than 500 µm). All samples were stored in polyethylene bags under vacuum and kept at 4 °C until extraction.

Table 1
Experimental conditions and extraction yield (mass of extract/mass of raw material) obtained in the supercritical extraction of licorice roots. Extraction time: 3 h. (PS1) pressure in first separator, pressure in second separator was maintained at $SCCO_2$ recirculation pressure. Run 1–8 at 313.15 K and 9–10 at 333.15 K.

Run	Pressure (MPa)	CO_2 flow (g/min)	Ethanol (% mass)	P S1 (MPa)	Extraction yield (%)
1	15	70	0	5	0.66
2	15	70	10	5	2.19
3	15	50	5	5	1.64
4	15	50	10	5	2.01
5	15	50	15	5	2.48
6	15	50	20	5	3.07
7	30	70	0	15	1.76*
8	40	70	0	15	0.56*
9	30	70	0	15	0.74*
10	40	70	0	10	0.89*

* Extraction yield expressed as the sum of the mass collected from both separator vessels (S1and S2).

2.3. Supercritical fluid extraction

A pilot-extractor (model SF2000; Thar Technology, Pittsburgh, PA, USA) was used for the SFE assays. The extractor comprises a cylinder cell (0.273 L) and two separator-cells (S1 and S2) (0.5 L capacity each), with independent control of temperature and pressure. A detailed description of the equipment used can be found elsewhere (Villanueva-Bermejo et al., 2017). The experimental conditions are listed in Table 1. The mass of licorice root was 0.160 kg of licorice root in all the experiments, being the apparent density of the packed bed 586 kg/m^3 (estimated porosity of 0.7). Different extractions were accomplished at 15–40 MPa, 313.15 and 333.15 K, and two CO_2 flow rates, 50 and 70 g/min, during 3 h in dynamic extraction (no static extraction was applied), representing a CO_2/plant ratio of 56 and 79 kg/kg, respectively. Ethanol was used as a cosolvent, being the concentration of ethanol in the supercritical extractive solvent in the range 0–20% wt (CO_2/ethanol ratio of 4 kg/kg). CO_2 and the cosolvent were mixed in the desire ratio previous to be pumped into the extraction cell.

The extracts were obtained by reducing pressure in the separator-cells. In the case of runs 1 to 6 (Table1) both separators were maintained at the system recirculation pressure (5 MPa) and the extracted material was recovered from S1 and S2 and mixed in a single fraction. Moreover, runs 7 to 10 were carried out at higher pressures and the extract was fractionated in the decompression cascade system comprising two separators. For this purpose, the first separator vessel (S1) was kept at 15 MPa and the second one was maintained at the recirculation pressure (5 MPa). Thus, two fractions (S1 and S2) were collected in each of the runs 7 to 10.

2.4. HPLC analysis

HPLC analysis was performed as previously described by the authors Wei et al. (2015). A Prominence-i LC-2030C 3D Plus (Shimadzu) equipped with a quaternary solvent delivery system, an autosampler and DAD detector and RP-C18 (250 × 4.6 mm; 3 µm) was used. The column temperature was set at 25 °C. The mobile phase consists of acetonitrile (A) and 0.026% aqueous H_3PO_4 (v/v), applying the following gradient elution: at 0–20 min, 20%–25% A, 20–30 min, 25%–34% A, 30–50 min, 34%–50% A, 50–60 min, 50%–60% A and 60–80 min, 60% A. After 5 min, the initial conditions were achieved. The flow rate was 0.7 ml/min and was kept constant during analysis. Injection volume was 20 µl and detection was accomplished at 254, 280 and 370 nm. Calibration curves with the standards were used to determine the content of these bioactive compounds in the different extracts.

2.5. Total phenolic compounds (TPC) determination

The total phenolic content in licorice extracts was determined using the Folin-Ciocalteu method (Singleton et al., 1999). Briefly, 50 µl of extract were mixed with 3 mL of milliQ water and 250 µl of Folin Ciocalteu reagent. The content was thoroughly mixed and after 3 min, 750 µl of sodium carbonate solution (20% mass) and 950 µl of milliQ water were added to the mixture. After 2 h at room temperature and remained in darkness, the absorbance was measured at 760 nm using a Genesys 10S UV–vis spectrophotometer (Thermo Fischer Scientific Inc., MA, USA). The results were expressed as GAE (mg of gallic acid equivalents/g of extract). All analyses were done in triplicate.

2.6. Antioxidant capacity

2.6.1. ABTS assay

The antioxidant capacity of the extracts was determined by the $ABTS^{\cdot+}$ radical scavenging assay following the method described by Re et al. (1999). $ABTS^{\cdot+}$ radical cation was generated by mixing $ABTS^{\cdot+}$ stock solution (7 mM) with 2.45 mM potassium persulfate after incubation of the mixture at room temperature for 16 h under darkness. Once the $ABTS^{\cdot+}$ radical was formed, the solution absorbance was adjusted to 0.700 ± 0.02 at 734 nm by ethanol in a Genesys 10S UV–vis spectrophotometer (Thermo Fischer Scientific Inc., MA, USA). Afterwards, 990 µL of $ABTS^{\cdot+}$ solution was added to 10 µl of sample and the reaction mixture was allowed to stand at room temperature and under darkness, until the absorbance reached a plateau. The absorbance was recorded at 734 nm and the results were expressed as IC_{50} value (Inhibitory concentration: concentration of extract necessary to inhibit the initial concentration of radical by 50%), as well as Trolox equivalents (TEAC) (µmol Trolox/g extract), which were calculated taking into account the Trolox standard and sample concentrations that produce the scavenging of 50% of $ABTS^{\cdot+}$ radical. All the analyses were carried out in triplicate.

2.6.2. DPPH assay

The ability of extracts to scavenge DPPH free radicals was determined according to the method described by Brand-Williams et al. (1995). Licorice extract were added to 975 µL of DPPH radical in ethanol ($6.1 \cdot 10^{-5}$), which was daily prepared. The reaction took place at room temperature in the dark until it reached a plateau. Then, the absorbance was measured at 515 nm in a Genesys 10S UV–vis spectrophotometer (Thermo Fischer scientific, MA, USA). A calibration curve (linear regression) was used to determine the DPPH concentration in the reaction medium. A control sample (i.e. a sample comprising the same volume of solvent instead of extract) was used to measure the maximum DPPH absorbance. Trolox was used as reference standard, so results were expressed as TEAC values (µmol Trolox/g extract), as well as IC_{50} value (µg/mL). All analyses were done in triplicate.

2.7. Antibacterial activity assay

The extracts and fractions collected were individually tested against a Gram-positive bacteria, *Staphylococcus aureus* American Type Culture Collection-ATCC 25923 and a Gram-negative bacteria, *Escherichia coli* ATCC 25922. A broth microdilution method was used, as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999), for determination of the minimum inhibitory concentration (MIC). All tests were performed in Mueller–Hinton broth supplemented with 0.5% tween 20. The inocula of bacterial strains were prepared from overnight Mueller–Hinton broth cultures at 37 °C. Test strains were suspended in Muller–Hinton (bacteria) broth to give a final density 10^7 cfu/mL. The extract and fractions were diluted in ethanol ranging from 50 to 1 mg/mL.

The 96-microwell plates were prepared by dispensing into each well 185 µl of culture broth, 10 µl of the different extracts dilutions, antibiotic solution (chloramphenicol as positive control) or solvent (ethanol as negative control), and 5 µl of the inoculums. In addition, blanks were prepared adding 190 µL of broth medium to the solvent or extracts wells. The final volume of each well was 200 µl. After dispensing the inoculum, the plates were read in an Infinite 200 PRO plate reader (TECAN, Trading AG, Switzerland) spectrophotometer at 620 nm for T0 (Zero Time). Then, the plates were incubated at 37 °C for 24 h and the absorbance was read for TF (Final Time). Each test was performed in triplicate and repeated twice.

The inhibition percentage was calculated following the method described by Cueva et al. (2010) as (Eq. 1):

$$\%\,\text{Inhibition} = \left(1 - \frac{(TF_{Sample} - T0_{Sample}) - (TF_{Blank\ of\ sample} - T0_{Blank\ of\ sample})}{(TF_{Growth} - TO_{Growth}) - (TF_{Blank} - T0_{Blank})}\right) \times 100 \quad (1)$$

where TF_{Sample} and $T0_{Sample}$ corresponded to the absorbance at 620 nm of the strain growth in the presence of the licorice extracts after and before incubation, respectively; $TF_{Blank\ of\ sample}$ and $T0_{Blank\ of\ sample}$ corresponded to the broth medium with extracts after and before incubation, respectively; TF_{Growth} and $T0_{Growth}$ correspond to the strain growth in the presence of the solvents after and before incubation; and TF_{Blank} and $T0_{Blank}$ corresponded to the broth medium with solvent after and before incubation.

For active extracts, the survival parameter IC_{50} value was defined as the concentration required to obtain 50% inhibition of growth after 24 h of incubation at 37 °C and was estimated by nonlinear regression using the sigmoidal dose-response (with variable slope) Eq.(2):

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{(1 + 10^{(\log IC_{50} - X) \times \text{Slope})})} \quad (2)$$

where, X represents the logarithm of concentration, Y is the % Inhibition which starts at the Bottom and goes to the Top with a sigmoid shape, Log IC_{50} is the logarithmic of IC_{50}, and Slope represents the slope parameter. The PRISM program (GraphPad Software, Inc.) was used for the approximation of the four parameters. For each data set, comparison of the fit to the previous sigmoidal dose response model (4 parameters) was carried out using PRISM, and also the fit to the same model with the Bottom and Top parameters constrained, respectively, to 0 and 100% was possible.

3. Results and discussion

3.1. Supercritical fluid extraction

The experimental conditions applied for the SFE of licorice roots, as well as extraction yield obtained (mass of extract/mass of licorice) are reported in Table 1. As can be observed, the extraction yields ranged from 0.66% (run 1) to 3.07% (run 6).

With regard to the effect of pressure (Table 1, runs 1 and 7), a rise in this parameter considerably increased the extraction yield. In this sense, when no cosolvent is used, an extraction yields 2.7-fold higher was reached at 30 MPa (1.76%) in comparison with yield at 15 MPa (0.66%). Temperature effect seem to exhibit crossover behavior, since at 40 MPa a temperature rise produced a yield increase (runs 8 and 10), but at 30 MPa the same temperature rise resulted in a yield decrease (runs 7 and 9).

Indeed, the use of ethanol had a considerably influence over the amount of extract obtained. In this respect, the addition of 10% cosolvent at 15 MPa and 70 g/min (run 2) implied an extraction yield 3.3-fold higher than the obtained without ethanol (run 1). This effect is clearly represented in the Fig. 1. As it is showed, the extraction yields linearly increased ($r^2 = 0.960$) with the addition of cosolvent in the range studied. Maximum yield is obtained in run 6 (3.07%) when the maximum amount of ethanol (20%) is used. These results are in agreement with the analysis reported by Hedayati and Ghoreishi (2015) concerning the yield and recovery of glycyrrhizin (no total yields were

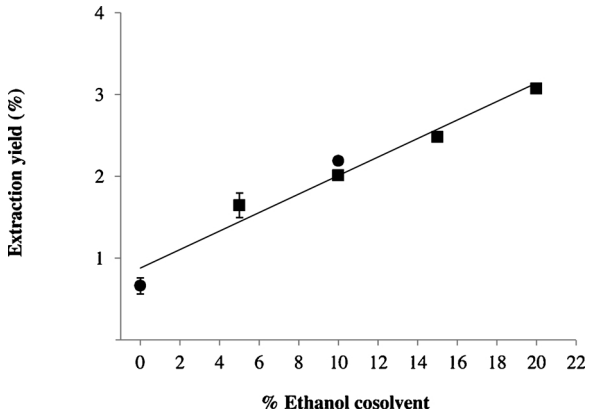

Fig. 1. Extraction yield obtained in the supercritical fluid extractions of licorice root as a function of the amount of cosolvent (%mass). (□, ■) 15 MPa. Squares and circles correspond, respectively, to 50 and 70 g/min CO_2. The standard deviations obtained in duplicate experiments carried out in run 1 (pure CO_2) and run 3 (5% ethanol) are depicted in the figure.

reported) in the supercritical extractions carried out using methanol: water mixtures as modifier, in percentages up 5% (v/v). Furthermore, Kim et al. (2004) also highlighted the relevant effect of methanol: water as CO_2 modifier to extract the same compound, glycyrrhizin, from licorice root.

The influence of the amount of cosolvent over yield is especially relevant if it is compared to the yields obtained at higher pressure. Despite the increase in the solvation power as a consequence of the higher density of CO_2 when pressure rises from 15 to 30 MPa at a constant temperature, the extraction yield obtained at 15 MPa, 70 g/min and 10% cosolvent (2.19%) was -1.25-fold higher than the obtained at 30 MPa at the same flow rate but without cosolvent (1.76%).

The yields obtained in the fractions S1 and S2 collected in runs 7–10 are given in Table 2. In general, most of extract was recovered in the second separator, with yields up to 8 times higher than the yield obtained in the first separator.

3.2. Total phenolic compounds (TPC) and antioxidant capacity

Table 3 shows the amount of TPC (mg GAE/g extract) in the extracts obtained at the different experimental conditions and their antioxidant capacity expressed as IC_{50} (μg extract/mL) and TEAC value (μmol trolox/g extract) as determined by the ABTS and DPPH assays.

The TPC content varied from 48.47 (run 8-S1) to 180.06 mg GAE/g extract (run 6). The lower concentrations were obtained by using pure CO_2 (run 1) and particularly in the first fraction (S1) of runs 7 to 10. The higher concentrations of TPC were obtained when ethanol was used and, at constant pressure of 30 MPa, 313 K of temperature and 50 g/min of CO_2 flow rate, TPC increased linearly (r^2 = 0.979) as the amount of ethanol did (Fig. 2). The concentrations of TPC obtained in the SFE licorice extracts were higher than those reported in other studies. The TPC extracted in run 6 (180.06 mg GAE/g extract) was almost 4-fold higher than the obtained with 80% ethanol by MAE (Karami et al., 2015, 2013), and 7.7, 3.8 and 2.3-fold higher than the achieved using, respectively, water (Gupta et al., 2016), ethanol (Visavadiya et al., 2009) and methanol (Hejazi et al., 2017) by Soxhlet extraction.

The effect of supercritical solvent flow on TPC can be analysed by comparison of the values obtained for runs 2 and 4, which were carried out at 15 MPa, 313 K, 10% ethanol cosolvent and, respectively, 70 and 50 g/min CO_2 (total supercritical solvent flow of 77.8 and 55.6 g/min). The increase of flow resulted in a decrease of TPC from 163.03 to 141.18 mg GAE /g extract probably due to the co-extraction of substances other than phenolics, as can be inferred from the higher yield obtained in run 2 (almost 10% higher).

Regarding the effect of pressure and temperature on TPC, Fig. 3 show the total amount of TPC extracted (S1 + S2 samples) in runs 7–10 as a function of pressure, together with the total yield obtained in these runs. As can be observed in the figure, the TPC values show the same crossover behaviour described previously concerning extraction yield. Then, it can be concluded that at 30 MPa the increase of temperature decrease total yield and TPC content, while at 40 MPa an increase of temperature increase total yield and TPC content.

Regarding the antioxidant capacity, the ABTS and DPPH assays show the same tendency, as it can be deduced from the relation between the IC_{50} values depicted in Fig. 4. As in the case of TPC, the antioxidant capacity increased with the amount of ethanol, so the highest values were reached with 20% cosolvent (556 and 760 μmol/g for the ABTS and DPPH assay). Nevertheless, despite the linear tendency TPC-%ethanol (Fig. 2), no linear correlation between the amount of ethanol and the antioxidant capacity was observed, with a significant increase in the antioxidant capacity of the extract obtained with 20% ethanol (run 6) assessed by both the ABTS and DPPH assay. Moreover, the TEAC values exhibit a general trend to increase with increasing TPC (Fig. 5), but TEAC and TPC values could not be linearly correlated for both the ABTS and DPPH assay. Several studies related a strong and positive correlation between TPC and antioxidant activity (Casagrande et al., 2018; Skotti et al., 2014). Nevertheless, this trend is not always satisfied and maybe related to the presence of antioxidants other than phenolic compounds, such as carotenoids, ascorbic acid, among others (Millao and Uquiche, 2016; Rufino et al., 2010). In the case of supercritical licorice root extracts, other no-phenolic compounds may be present (although were not analyzed in this work), in particularly those comprising licorice essential oil, which may also contribute to the antioxidant activity (e.g. estragole, eugenol and anethol (Fenwick et al., 1990) and thus, no linear relation between TPC and TEAC values was obtained.

Taking into account that lower IC_{50} values indicates a stronger radical scavenging activity, the extract obtained in run 6, at 15 MPa, 50 g/min CO_2 and 20% cosolvent (IC_{50} 7.74 and 18.59 μg/mL, respectively, in ABTS and DPPH assays) showed a considerably higher antioxidant capacity than extracts which were obtained from licorice using water or organic solvents and other extraction techniques. In this regard, the IC_{50} of run 6 extract was around 75-fold lower than the obtained by Gupta et al. (2008) with ethanolic extracts (575 μg/mL and 1424 μg/mL in ABTS and DPPH assay, respectively), as well as 7.4 (ABTS assay) and 1.5 (DPPH assay) fold lower than the obtained by Visavadiya et al. (2009) with the same solvent. Hejazi et al. (2017) obtained DPPH IC_{50} values of 71.93 and 77.86 μg/mL in methanol and chloroform fractions, respectively, so the IC_{50} of run 6 extract was 3.9 and 4.2 times lower than the obtained with these two organic solvents. In the case of the water extract obtained by Thakur et al. (2016) by means of sonication (IC_{50} value of 189.9 and 334.7 μg/mL for DPPH and ABTS assay), our run 6 supercritical extract showed an efficacy to scavenge these free radicals between 10.2 (DPPH assay) and 18.0 (ABTS assay) fold higher. Furthermore, extracts with similar antioxidant capacity were obtained using pure CO_2 at high pressure (30 and 40 MPa) and 333.15 K by fractionation of the extract at 15 MPa (S2 fractions of runs 9 and 10).

Table 2
Extraction yield (mass of extract/mass of raw material) obtained in the S1 and S2 fractions of licorice roots extracts. Extraction time: 3 h. Pressure in first separator was 15 MPa and pressure in second separator was maintained at $SCCO_2$ recirculation pressure.

Run	Extraction yield in separators (%mass)	
	S1	S2
7	0.24	1.52
8	0.06	0.50
9	0.09	0.65
10	0.27	0.62

Table 3
Mean values and standard deviations of total phenolic compounds (TPC) and antioxidant capacity (ABTS and DPPH assay) of licorice root supercritical extracts. S1: first separator fraction, S2: second separator fraction.

Run	TPC	ABTS		DPPH	
	(mg GAE/g extract)	IC_{50} (μg/mL)	TEAC (μmol Trolox/g extract)	IC_{50} (μg/mL)	TEAC (μmol Trolox/g extract)
1	76.2 ± 1.5	42.1 ± 3.2	102.2 ± 7.8	126.0 ± 0.5	112.5 ± 0.4
2	141.2 ± 1.1	28.4 ± 3.6	147.4 ± 13.7	32.4 ± 2.5	442.1 ± 34.2
3	153.0 ± 1.1	13.7 ± 0.1	312.5 ± 0.7	42.6 ± 0.9	331.4 ± 8.6
4	163.0 ± 1.3	13.0 ± 0.8	329.6 ± 21.1	37.8 ± 04	418.7 ± 4.4
5	174.9 ± 1.9	12.0 ± 0.1	358.6 ± 3.9	32.3 ± 0.4	440.8 ± 5.2
6	180.1 ± 0.9	7.7 ± 0.6	555.7 ± 45.7	18.6 ± 0.4	759.6 ± 16.2
7-S1	69.05 ± 0.9	105.3 ± 1.7	40.7 ± 0.7	248.1 ± 4.1	57.1 ± 0.9
7-S2	159.3 ± 1.2	33.3 ± 1.0	128.6 ± 4.0	42.8 ± 2.5	330.6 ± 9.0
8-S1	48.5 ± 3.6	44.7 ± 0.1	95.8 ± 0.3	185.8 ± 0.9	76.0 ± 0.4
8-S2	128.3 ± 3.4	11.84 ± 0.1	361.7 ± 3.0	21.9 ± 0.1	647.9 ± 3.1
9-S1	62.5 ± 2.5	38.8 ± 2.8	110.6 ± 7.9	149.5 ± 0.8	94.4 ± 0.5
9-S2	140.5 ± 7.7	10.3 ± 0.2	416.0 ± 8.9	54.4 ± 3.6	259.9 ± 17.3
10-S1	95.2 ± 8.7	15.7 ± 0.1	272.5 ± 0.2	74.0 ± 0.4	190.9 ± 0.6
10-S2	138.0 ± 8.9	7.7 ± 0.1	554.0 ± 6.6	59.2 ± 0.2	238.7 ± 0.7

DPPH: 2,2-Diphenyl-1-pycrilhydrazyl.
ABTS: 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt.
GAE: Gallic acid equivalent.
IC_{50}: Concentration of extract necessary to inhibit the initial concentration of radical by 50%.
TEAC: Trolox equivalent antioxidant capacity.

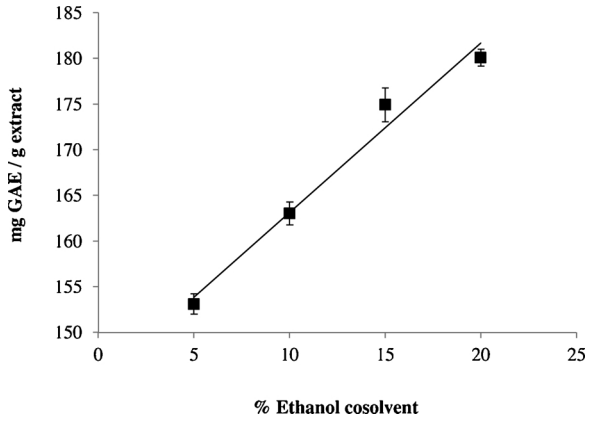

Fig. 2. Supercritical fluids extractions of licorice roots at 15 MPa, 313.15 K and 50 g/min CO_2 flow: Effect of ethanol cosolvent addition in the extraction of phenolic compounds expressed as mg of gallic acid equivalent (GAE)/g of extract.

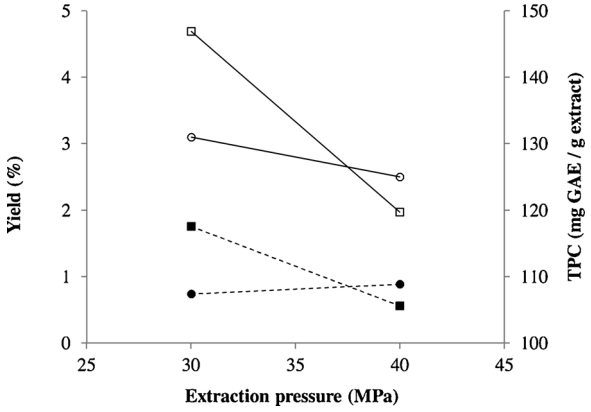

Fig. 3. Total yield and total phenolic compounds (TPC) extracted (S1 + S2 samples) obtained in runs 7–10. (■, □) 313.15 K; (●, ○) 333.15 K. Full symbols represent extraction yield and empty symbols represent TPC content.

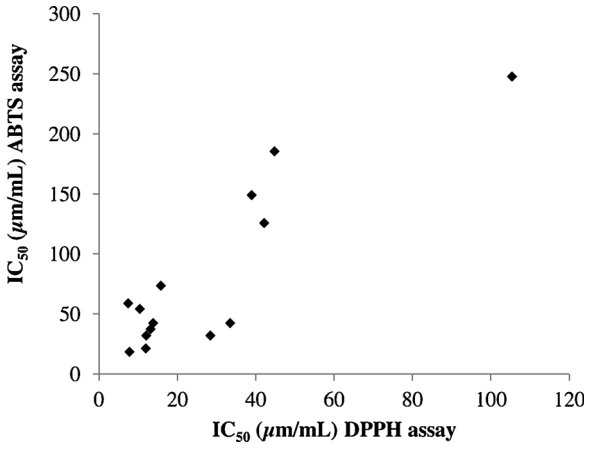

Fig. 4. Analysis of the relation between the IC_{50} (Concentration of extract necessary to inhibit the initial concentration of radical by 50%) values of licorice supercritical extracts calculated with the ABTS and DPPH assay. (ABTS: 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH: 2,2-Diphenyl-1-pycrilhydrazyl).

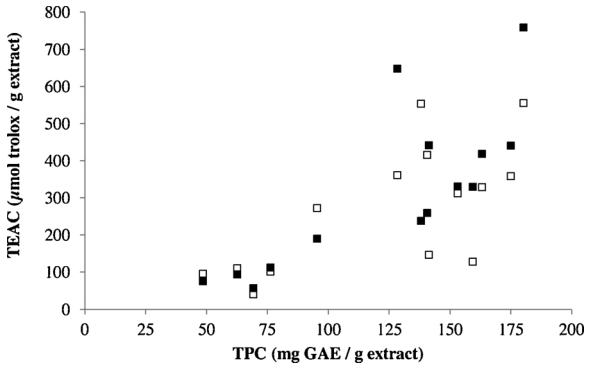

Fig. 5. Trolox equivalent antioxidant capacity (TEAC) vs. total phenolic compounds (TPC) values of licorice root supercritical extracts: (□) ABTS and (■) DPPH assays. (ABTS: 2.2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH: 2,2-Diphenyl-1-pycrilhydrazyl).

Table 4
Antimicrobial activity of licorice root supercritical extracts.

Sample	*E. coli* IC_{50} (mg/ml)	*S. aureus* IC_{50} (mg/ml)
1	1.17	1.41
2	1.50	2.26
3	1.60	1.60
4	2.19	–
5	–	–
6	–	–
7-S1	0.76	1.49
7-S2	2.16	2.20
8-S1	1.86	2.00
8-S2	1.62	2.21
9-S1	1.30	1.49
9-S2	1.61	1.77
10-S1	1.02	1.36
10-S2	1.21	1.50
Chloramphenicol	0.08	0.09

IC_{50}: Concentration of extract required to obtain 50% inhibition of bacterial growth.

3.3. Antibacterial activity

Antimicrobial activity of licorice extracts against *E. coli* and *S. aureus*, as representative of Gram-negative and Gram-positive bacteria, was evaluated as the inhibition of the bacterial growth, and expressed as the IC_{50} parameter. The antimicrobial assays showed that licorice extracts can inhibit the growth of the evaluated bacteria. The IC_{50} values (mg/ml) corresponding to the inhibition of *E. coli* (ATCC 25299) and *S. aureus* (ATCC 25923) are given in Table 4.

In general, results of antimicrobial activity showed that *S. aureus* ATCC 25923 (Gram-positive) was more resistant (higher IC_{50} values) than *E. coli* ATCC 25299 (Gram-negative) to the antimicrobial effect of all extracts tested. These results are in agreement with the data presented by Chandra and Gunasekaran (2017) in their study of licorice extracts obtained by solid-liquid extraction with chloroform solvent. A greater susceptibility of *E. coli* than *S. aureus* to other antimicrobial materials was also reported by other authors (García-Ruiz et al., 2015). The structures of cell envelope (cytoplasmic membrane and cell wall component), which differ between Gram-positive and Gram-negative bacteria, may influence these observed results. Antimicrobial agents commonly contact the cell envelope first and thus, the structural differences produce a key role in the antimicrobial susceptibility. In particular, the extracts obtained using ethanol (higher contents of phenolic compounds, runs 2–6), exhibit lower and even no antimicrobial activity in comparison with the extract produced with pure CO_2 (run 1). On the other hand, fractions obtained in the first separator (lower content of phenolic compounds) exhibited the best antimicrobial activity for both bacteria. These findings indicate that licorice antimicrobial compounds are soluble in CO_2 at pressures higher than 15 MPa and can be concentrated by cascade decompression of high pressure SFE. It suggests that antimicrobial activity of licorice extracts seems to be dependent on the type of phenolic structure rather than the total content of phenolic compounds.

3.4. HPLC analysis

The major bioactive constituents of licorice are glycyrrhizin and several flavonoids, such as liquiritin, isoliquiritin and their aglycones. These compounds are supposed as the active principles responsible for its pharmacological efficacy (Zhang and Ye, 2009). The bioactive compounds identified in this work by HPLC analysis were five compounds: liquiritin (RT: 12.80 min), liquiritigenin (RT: 35.06 min), glycyrrhizic acid ammonium salt (glycyrrhizin) (RT: 48.21), isoliquiritigenin (RT: 49.73 min) and glabridin (RT: 69.15 min). The results of the HPLC analysis are presented in Table 5. In the case of experiments accomplished with on-line fractionation (runs 7–10), the compound concentrations obtained in the total extract (S1 + S2 fractions) are also reported in Table 5.

The most abundant compound found in all the extracts obtained in this work is glabridin, a compounds well-characterized in the literature due to several important biological activities such as antioxidant, anti-inflammatory and anti-atherogenic (Asl and Hosseinzadeh, 2008; Simmler et al., 2013). Extract obtained using pure CO_2 in run 1 contains 49.50 ± 0.14 mg glabridin per g extract. Without cosolvent, the concentration of glabridin in the extract (mg glabridin / g extract) increases with pressure, as is represented in Fig. 6 for runs 1, 7 and 8 (313.15 K, 70 g/min of CO_2 without cosolvent). Nevertheless, glabridin recovery (mg glabridin / g root) seem to attain a maximum around 30 MPa. Significantly higher glabridin concentration and recovery were obtained when ethanol is used as cosolvent. Moreover, an increase of ethanol cosolvent produces a decrease in glabridin concentration (runs 3–6 in Tables 5) probably due to the simultaneous extraction of other compounds (and according with the increase of extraction yield observed). In relation with the samples obtained by supercritical fractionation, temperature seems to affect significantly glabridin fractionation since fractions obtained in S1 at 313.15 K present lowest concentration of glabridin than the corresponding S2 fraction (runs 7 and 8) while the opposite behavior was found at 333.15 K (runs 9 and 10).

Another metabolite identified in all samples was liquiritin, which is a flavone observed in the sweetening agent licorice and has been associate with anti-inflammatory effects (Gao et al., 2017). Liquiritin content slightly increase with the percentage of ethanol and considerably lower amounts of this compound were quantified in all samples (0.2–1.2 mg/g) in comparison with glabridin (26–198 mg/g).

Among phenolic compounds in licorice are typically isoliquiritigenin and liquiritigenin (Kondo et al., 2007). These were identified and quantified in the extracts of runs 2, 3, 4, 5 and 6, observing an increase of their concentration with the increase of the percentage of ethanol cosolvent. Thus, these compounds could not be quantified in the supercritical fractionation assays (runs 7–10) since no ethanol was used as cosolvent and despite the higher extraction pressures applied. On the contrary, the content of triterpenoid glycyrrhizin was found to decrease with increasing ethanol cosolvent and thus, it was not possible to quantify this compound in runs 4, 5 and 6 while it was identified and quantified in all samples obtained in runs 7–10. Glycyrrhizin is an important ingredient in various medicines, such as antimicrobial, anti-ulcer, anti-hepatotoxic and antivirus formulas (Cinatl et al., 2003; Dehpour et al., 1995). Yet, plant extracts use to be much more effective than isolated compounds, as highlighted Cheel et al. (2010) for the case of licorice aqueous extracts.

Fig. 6 shows the effect of pressure on glycyrrhizin concentration in the extract and recovery per gram of root extracted. The figure refers to runs 1, 7 and 8 (313.15 K, 70 g/min of CO_2 without cosolvent). Glycyrrhizin concentration show a slight decrease with increasing pressure, but the recovery attains a maximum close to 30 MPa, similarly to glabridin. Yet, this maximum recovery is lower than 1.5 mg /g root, considering the content of glycyrrhizin in licorice root reported by Kim et al. (2004) (138 mg glycyrrhizin / g root). Nevertheless, Kim et al. (2004) could not extract glycyrrhizin with pure supercritical CO_2 even applying pressures of 50 MPa. Hedayati and Ghoreishi (2015) reported a maximum glycyrrhizin recovery of 54% (74 mg glycyrrhizin / g root) when using methanol: water as CO_2 cosolvent, which is a value significantly higher than the maximum recovery obtained in this work using ethanol cosolvent (4.6 mg glycyrrhizin/g root).

Concerning the relation between the compounds detected and the biological activities observed (antioxidant and antibacterial) in supercritical extracts, it can be stated that the content of liquiritigenin and isoliquiritigenin do not affect the antibacterial activity, since these compounds were not detected in the most active extracts (S1 fractions and extract of run 1). Furthermore, the content of glabridin seems not to affect the antibacterial activity of these most active extracts (Fig. 7). On the contrary, regarding the antioxidant activity a dependence of the

Table 5
Concentration of bioactive compounds of supercritical extracts of licorice under different conditions.

Run	Liquiritin (mg/g)	Liquiritigenin (mg/g)	Glycyrrhizin (mg/g)	Isoliquiritigenin (mg/g)	Glabridin (mg/g)
1	0.40 ± 0.01	ND	0.80 ± 0.01	ND	49.50 ± 0.14
2	0.60 ± 0.01	0.80 ± 0.01	2.10 ± 1.27	1.40 ± 0.85	168.60 ± 1.70
3	0.80 ± 0.01	LLQ	1.20 ± 0.01	0.40 ± 0.01	198.10 ± 0.71
4	0.80 ± 0.01	1.60 ± 0.01	UDL	2.40 ± 0.01	192.10 ± 0.99
5	0.80 ± 0.01	1.80 ± 0.01	UDL	0.90 ± 0.14	151.50 ± 0.71
6	1.20 ± 0.01	2.60 ± 0.01	UDL	2.30 ± 1.27	147.40 ± 0.28
Concentration in samples S1 and S2					
7-S1	UDL	ND	0.20 ± 0.01	ND	26.60 ± 1.41
7-S2	UDL	ND	0.70 ± 0.14	ND	140.80 ± 7.35
8-S1	UDL	ND	0.40 ± 0.01	ND	47.40 ± 7.07
8-S2	UDL	UDL	0.60 ± 0.01	UDL	143.50 ± 6.65
9-S1	0.20 ± 0.01	ND	0.70 ± 0.14	UDL	140.50 ± 0.99
9-S2	0.20 ± 0.01	UDL	0.60 ± 0.01	ND	74.40 ± 1.98
10-S1	UDL	UDL	0.40 ± 0.01	ND	118.30 ± 0.14
10-S2	0.80 ± 0.01	ND	0.80 ± 0.01	UDL	113.80 ± 0.01
Concentration in total extract (S1 + S2 samples)					
7	UDL	ND	0.732 ± 0.14	ND	125.23 ± 6.54
8	UDL	UDL	0.579 ± 0.01	UDL	133.20 ± 6.70
9	0.20 ± 0.01	UDL	0.612 ± 0.03	UDL	82.44 ± 1.86
10	0.56 ± 0.01	UDL	0.679 ± 0.01	UDL	115.17 ± 0.04

ND: no detected.
UDL: under detection limit.

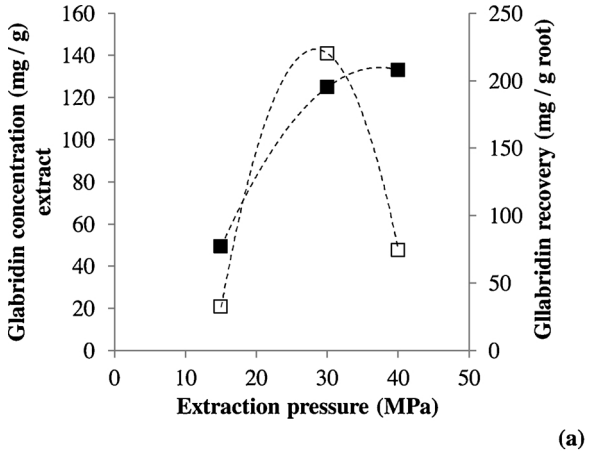

Fig. 6. Effect of pressure ((313.15 K, 70 g/min of CO_2 without cosolvent)) on the concentration and recovery of (a) glabridin and (b) glycyrrhizin: (■) concentration; (□) recovery.

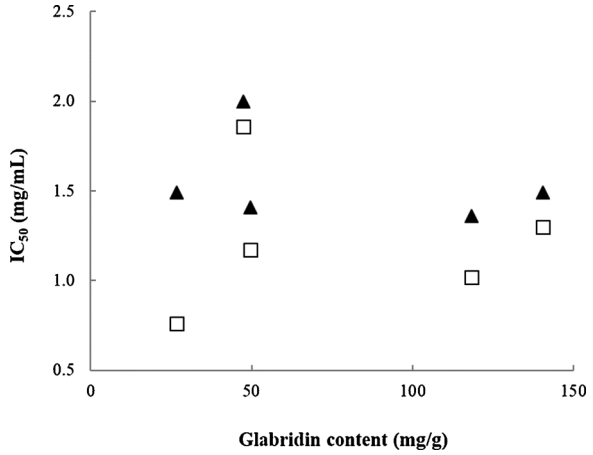

Fig. 7. Antibacterial activity of the most active samples (S1 fractions and extract of run 1): IC_{50} (Concentration of extract required to obtain 50% inhibition of bacterial growth) values obtained for (□) *E. coli* and (▲) *S. aureus* as a function of the glabridin content.

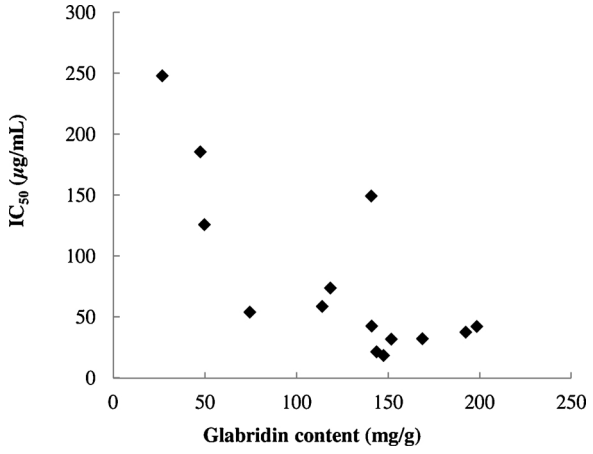

Fig. 8. Antioxidant activity: IC_{50} (Concentration of extract necessary to inhibit the initial concentration of radical by 50%) value obtained with the DPPH assay as a function of the glabridin content.

antioxidant capacity on glabridin content is observed, as depicted in Fig. 8 for all licorice supercritical extracts obtained in this work.

4. Conclusions

Supercritical licorice roots extracts were obtained using different extraction conditions to investigate the viability of producing antioxidant and/or antimicrobial fractions. Two strategic approaches were investigated: the effect of using ethanol as a polar cosolvent and the effect of supercritical fractionation using a two-cell cascade decompression system.

In general, licorice supercritical extracts obtained in this work present considerably higher antioxidant activity in comparison with extracts produced using liquid solvents (methanol, chloroform, ethanol, water) as reported in the literature. As expected, the higher contents of phenolic compounds were obtained in the extracts produced with supercritical CO_2 and ethanol cosolvent, which in turn exhibit the higher antioxidant activity. The extraction yield and total phenolic compounds content varies linearly with the amount of ethanol used. Nevertheless, this linear correlation was not observed in regards the antioxidant activity. Furthermore, the increase of the content of glabridin favors the antioxidant activity of the extract.

On the other hand, the extracts produced with supercritical CO_2 and ethanol cosolvent show low or even no antimicrobial activity against *E. coli* ATCC 25299 and *S. aureus* ATCC 25923. In this respect, high pressure extraction (30–40 MPa) using pure CO_2 and on-line fractionation resulted in a first fraction (at 15 MPa) with a better antimicrobial effect although with lower content of phenolic compounds.

The study concludes and highlights the capability of supercritical fractionation applied to licorice roots to selective separate substances and to obtain antimicrobial and antioxidant fractions.

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4.1.3. Preparation and characterization of licorice-chitosan coatings for postharvest treatment of fresh strawberries.

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Preparation and Characterization of Licorice-Chitosan Coatings for Postharvest Treatment of Fresh Strawberries

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Abstract: Several plant extracts are being investigated to produce edible coatings, mainly due to their antioxidant and antimicrobial activities. In this study, licorice root extracts were produced by ultrasound-assisted extraction and were combined with chitosan to elaborate edible coatings. Different solvents and temperatures were used in the extraction process, and the antioxidant and antimicrobial activity of the extracts were assessed. The most bioactive extracts were selected for the development of the edible coatings. The rheological properties of the coatings were studied, and they were applied on strawberry to evaluate their physicochemical and microbiological properties. The addition of licorice extract to chitosan resulted in positive effects on the rheological properties of the coatings: the incorporation of phytochemicals to chitosan decreased the shear stress and improved the restructuring ability of the coating solutions. The films presented a reduction of the Burger model parameter, indicating a reduction of rigidity. Furthermore, the strawberry coated with chitosan and licorice extract maintained good quality parameters during storage and showed the best microbiological preservation in comparison with controls. Hence, the use of chitosan with licorice extract is a potential strategy to produce edible coating for improving the postharvest quality of fruits.

Keywords: licorice; chitosan; edible coating; strawberry shelf life; rheological properties; ultrasound-assisted extraction

1. Introduction

An increase in consumer requirements for safe food has led to the development of new improved packaging systems, including active, intelligent and edible materials. The use of edible biopolymers in food-packaging applications has become an alternative due to their film-forming properties and environmentally friendly behavior [1]. Edible coatings and films are different: edible films are used as wrapping packaging materials, while edible coatings can be directly applied on the surface of food products [2]. Edible coatings are natural, safe and ecologically friendly substitutes, suitable to be applied to reduce water transfer, gaseous exchange and oxidation of fresh products [3]. The edible coatings should preserve the quality, nutritional value and texture of food products by reducing moisture loss and oxygen effects, while maintaining adherence, without altering the original taste and odor [4]. Furthermore, the combination of natural food grade substances in the coating should

improve the physical properties of the formed films [5]. In fact, the rheological properties of the film-forming solution, such as thickness, spreadability and uniformity of the liquid coating layer, and the film performance, can be significantly affected by the type and composition of the coating constituents, such as polysaccharides, protein, hydrocolloid or composite materials. In general, a reduction in the solution's viscosity provides a processing advantage during high-shear processing operations, whereas high apparent viscosity at low-shear rates provides a better application by dipping [4].

Chitosan is a cationic biopolymer that can be mainly obtained from the deacetylation process of chitin, a renewable natural resource [2] which is widely used in the coating of fresh products. Biological properties have been attributed to chitosan, such as antibacterial and antifungal properties [6]. Then, the application of this substance has been applied in several areas such as agriculture, cosmetic, pharmacy and also food industry [7].

Chitosan has been studied and used for coating fish, meat, fruits and vegetables, to improve the quality and extend the food shelf life [8]. In addition, chitosan can be combined with other functional compounds with antioxidant, antimicrobial or other activities, to enhance the quality and efficacy of the coating [9]. Antioxidants, antimicrobial agents, coloring agents, flavors, nutrients, prebiotics and probiotics are examples of functional compounds that can be incorporated into edible chitosan-based coatings [10]. Particularly, for the case of strawberry (*Fragaria* × *ananassa*), some works studied the application of chitosan-based coatings [11,12] but a relatively high moisture permeability was reported [2]. Then, the incorporation of natural bioactive compounds such as nisin, natamycin, pomegranate, grape seed extracts [13], aloe vera gel [14], *Thymus capitatus* [15] and *Mentha spicata* essential oils [16] and green tea extract [17], were studied, with the aim of enhancing the performance of the edible coating and the preservation of strawberry. This fruit has been intensively studied, because it is highly consumed [18] and is a relevant source of bioactive compounds such as vitamin C, vitamin E, β-carotene and anthocyanins [19]. Nevertheless, several factors can reduce the fruit quality and limit its commercialization, such as intrinsic physiology, physiological and mechanical lesion, fungal infections and postharvest decay [20–22].

On the other hand, licorice is a traditional therapeutic herb, which grows in various parts of the world, and is well recognized due to its bioactive properties, including antioxidant, anti-fungal, anti-ulcer, anti-inflammatory, as well as anticancer and antiviral. Licorice was commonly used in traditional medicine recipes for the treatment of respiratory complaints, inflammatory disorder and liver problems [23,24]. Accordingly, licorice extracts, particular those obtained from the root, were studied as natural food preservatives. Jiang et al. [25] investigated the efficacy of the addition of licorice extract in the preparation of meat patty to inhibit lipid oxidation during refrigerated and frozen storage. Qui et al. [26] combined chitosan citric acid and aqueous licorice extract to preserve the quality of sea bass (*Lateolabrax japonicas*) fillets by preventing lipid oxidation and microbial growth and thus extending the fish shelf life. Mandanipour et al. [27] evaluated the influence of an ethanolic licorice extract combined with chitosan for controlling blue mold and extending the shelf life in the postharvest storage of apples.

Different methods have been employed for the production of extracts. Among these, the ultrasound-assisted extraction (UAE) is a versatile, flexible and efficient technique employed for the extraction of bioactive compounds [28] due to its high reproducibility, and it is very appreciated for the reduction of solvent consumption [29]. Application of ultrasounds causes the implosion of the solvent cavitation bubbles, leading to the disruption of the vegetal cell membranes. This action facilitates the penetration of solvent into the cells, thereby improving mass transfer and increasing the releasing bioactive compounds.

In this study, ultrasound-assisted extraction of licorice root was studied, using solvents of different polarities, to examine the bioactive composition and the antioxidant and antimicrobial activity of the extracts. Selected extracts were tested for the elaboration of chitosan-based edible coatings. The rheological properties of the coating solutions were studied to assess their ability to form films. Furthermore, the rheological properties of the films were analyzed in order to determine the influence of the incorporation of licorice extract. Finally, a study to evaluate the effect of the

application of the edible coatings on strawberry was accomplished, considering the physicochemical and microbiological characteristics of the berries during storage.

2. Materials and Methods

2.1. Chemicals and Fruit

Sodium carbonate anhydrous (99.5% purity) and ethanol (99.5% purity) were purchased from Panreac (Barcelona, Spain). Folin–Ciocalteu reagent, Gallic acid standard (>98% purity), (±)-6-hydroxy-2.5.7.8-tetramethyll-chromane-2-carboxylic acid (Trolox, 97% purity), 2.2-diphenyl-1-pycrilhydrazyl (DPPH, 95% purity), chloramphenicol (≥98% purity), low molecular weight chitosan deacetylated chitin, tween-20, tween-80, acetic acid (≥99.5% FCC, Food Grade), glycerol (>99%, FCC, FG), isoliquiritigenin, liquiritin, glycyrrhizic acid ammonium salt, liquiritigenin and glabridin, were purchased from Sigma–Aldrich (St. Louis, MO, USA). BBL Mueller Hinton II Broth and Difco Wilkins-Chalgren Agar were purchased from Becton, Dickinson and Company (France). Sodium hydroxide (ACS, Reag. Ph Eur, ISO) was purchased from EMSURE®, Merck (Germany).

Strawberries were purchased from a local market. They were brought to the lab for the experimental studies and used immediately. Strawberries were selected for uniformity of color, shape and size and with the absence of physical defects or decay.

2.2. Preparation of Licorice Extracts

Roots of licorice were obtained from Murciana herbalist's (Murcia, Spain) and ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain). Then, UAE using an ultrasonic probe (Branson Digital Sonifier 550 model, Danbury, CT, USA) with an electric power of 550 W and frequency of 60 kHz was accomplished. The extractions were carried out for 15 min with a plant/solvent ratio of 1:10, at temperatures of 25 and 50 °C. Four different solvents were used: ethanol, methanol, ethanol:water (50:50) and methanol:water (50:50) (Table 1). The extracts obtained were stored at −20 °C until their use.

Table 1. Extraction yields (% mass extracted related to the mass of licorice root) of licorice root ultrasound-assisted extraction (UAE).

Sample	Solvent	Temperature °C	Yield %
ELe25	Ethanol	25	3.85 ± 0.46 [d]
MLe25	Methanol	25	9.08 ± 2.77 [c]
EWLe25	Ethanol:water (50:50 *v*/*v*)	25	25.14 ± 1.07 [a]
MWLe25	Methanol:water (50:50 *v*/*v*)	25	15.69 ± 2.93 [b]
ELe50	Ethanol	50	3.69 ± 0.14 [d]
MLe50	Methanol	50	9.94 ± 2.08 [c]
EWLe50	Ethanol:water (50:50 *v*/*v*)	50	24.75 ± 0.76 [a]
MWLe50	Methanol:water (50:50 *v*/*v*)	50	23.36 ± 0.18 [a]

Values with different letters (a–d) differ significantly ($p < 0.05$).

2.3. Content of Total Phenolic Compounds (TPC) and Antioxidant Activity

The content of TPC of licorice extract was determined following the method of Folin–Ciocalteu [30]. Initially, 10 μL of extract was mixed with 600 μL of milliQ water and 50 μL of Folin–Ciocalteu reagent. The content was thoroughly mixed and after 3 min, 150 μL of sodium carbonate solution (20% mass) and 190 μL of milliQ water were added to the mixture. After 2 h at room temperature in darkness, the absorbance was measured at 760 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific Inc., MA, Waltham, USA). The results were expressed as GAE (mg of gallic acid equivalents/g of extract).

The ability of licorice extracts to scavenge DPPH free radicals was determined according to the method described by Brand-Williams et al. [31]. For the reaction, 25 μL of samples were added to 975 μL of DPPH radical in ethanol (6.1×10^{-5}), which was prepared daily. The reaction took place at room temperature, in the dark, until it reached a plateau. Then, the absorbance was measured at 515 nm in a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer scientific, Waltham, MA, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve (linear regression). A control sample, containing the same volume of solvent instead of the extract, was used to measure the maximum DPPH absorbance. Trolox was used as a reference standard, so results were expressed as TEAC values (μmol Trolox/g extract). All analyses were done in triplicate.

2.4. Determination of Antimicrobial Activity of Licorice Extracts

The licorice extracts were tested against a Gram-positive bacterium, *Staphylococcus aureus* ATCC 25923, and a Gram-negative bacteria, *Escherichia coli* ATCC 25922, following the methods described by Quintana et al. [32]. These tests were carried out to assess a general comparison of the antimicrobial capacity of the extracts and decide which of them could be the most effective for the edible coating. A broth microdilution method was used for the determination of IC_{50} values (i.e., the concentration required to obtain 50% inhibition of growth after 24 h of incubation at 37 °C). All tests were performed in Mueller–Hinton broth supplemented with 0.5% tween-20. The inoculum of bacterial strains was prepared from overnight Mueller–Hinton broth cultures at 37 °C. Test strains were suspended in Muller–Hinton (bacteria) broth to give a final density 10^7 CFU(Colony forming units)/mL. The extract and fractions were diluted in ethanol ranging from 1 to 50 mg/mL. The 96-microwell plates were prepared by dispensing into each well 185 μL of culture broth, 10 μl of the different extract's dilutions, antibiotic solution (chloramphenicol as positive control) or solvent (ethanol as negative control), and 5 μL of the inoculums. In addition, blanks were prepared by adding 190 μL of broth medium to the solvent or licorice extracts wells. The final volume of each well was 200 μl. After dispensing the inoculum, the plates were read in an Infinite 200 PRO plate reader (TECAN, Trading AG, Männedorf, Switzerland) spectrophotometer at 620 nm for T0 (Zero Time). Then, the plates were incubated at 37 °C for 24 h and the absorbance was read for TF (Final Time). Each test was performed in triplicate and repeated twice.

2.5. HPLC Analysis

High Performance Liquid Chromatography (HPLC) analysis was performed as previously described by the authors [33] to identify and quantify main bioactive compounds of licorice, i.e., isoliquiritigenin, liquiritin, glycyrrhizic acid, liquiritigenin and glabridin. A Prominence-i LC-2030C 3D Plus (Shimadzu) unit equipped with a quaternary solvent delivery system, an autosampler, diode-array detection (DAD) detector and a RP-C18 (250 × 4.6 mm; 3 μm) column was used. The column temperature was set at 25 °C. The mobile phase was 0.026% aqueous H_3PO_4 (*v*/*v*) (A) and acetonitrile, applying the following gradient elution: 80–75% (0–20 min), 75–66% (20–30 min), 66–50% (30–50 min), 50–40 (50–60min) and 40% (60–80min) of A. After 5 min, the initial conditions were achieved. The flow rate was 0.7 mL/min and was kept constant during analysis. Injection volume was 20 μL and detection was accomplished at 254, 280 and 370 nm. Calibration curves with the standards were used to determine the content of these bioactive compounds in the different extracts.

2.6. Preparation of Coating Forming Solutions

Edible coatings were prepared with chitosan and the addition of different amounts (1% and 5%) of the licorice extract (LE). Chitosan solution was prepared following the procedures described by Ali et al. [34] with some modifications. Briefly, 4.0 g of chitosan was dissolved in 200 mL of distilled water containing 1.0 mL of glacial acetic acid. The solution was heated with constant stirring for 12 h. The pH of the solution was adjusted to 5.5 with 1 N NaOH, and 0.2 mL of tween-80 was added as emulsifier. Then, 1% or 5% of licorice extract was added and homogenized using an Ultra Turrax homogenizer (T18 basic IKA, Staufen, Germany) at 7500 rpm for 3 min.

Rheological Properties of Coating Solutions and Films

Rheological assay was done in order to evaluate the steady and viscoelastic properties of edible coating solutions employing a modular advanced rheometer system Mars 60, Haake (Thermo-Scientific, Karlsruhe Germany), equipped with a coaxial cylinder (inner radius 12.54 mm, outer radius 11.60 mm, cylinder length 37.6 mm).

Steady-State Shear Test

The continuous shear test was performed at a temperature of 25 °C, over a shear rate in the range of 10^{-3} to 10^{3} s^{-1}, to study the influence of the natural extract on the rheological behavior. Experimental data were fitted to the rheological Power Law model, according to Equation (1):

$$\sigma = K\dot{\gamma}^{n} \quad (1)$$

where σ is the shear stress (Pa), $\dot{\gamma}$ is the shear rate (s^{-1}), K is the consistency coefficient ($Pa \cdot s^{n}$) and n is the flux index (dimensionless).

Oscillatory Test

The stress amplitude sweep test was carried out within the range of 0.01 to 1000 Pa and with angular frequency of 0.1 Hz on all samples, in order to determine the linear viscoelastic regime (LVR). Based on the results of these experiments, the frequency sweep was done at 0.1 Pa, to keep the stress in the LVR, within the range of 0.01 to 100 rad·s^{-1}. Thermo-viscoelasticity properties were investigated in a ramp temperature from 20 to 80 °C, under constant frequency (0.1 Hz) in the LVR and at a heating rate of 5 °C/min. Dynamic data were obtained in oscillatory shear experiments. The data recorded include the storage modulus (G′) which provides the elastic component, the loss modulus (G′′) which is related to the viscous component of the material and the loss tangent (Tan δ) which is the ratio G′′/G′ and provides the ratio of elastic to viscous response of the material under consideration.

Preparation of the Film

In order to evaluate the influence of the addition of licorice extracts to chitosan on the ability of the coating solutions to form films, the casting method [35] was used to prepare the films. The coating solutions were casted in sample holder followed by drying at 47 °C for 12 h. The dried films were peeled and stored in a desiccator containing silica gel to prevent moisture absorption. Films of 0.5 mm of thickness were obtained.

Creep and Recovery

The rheological measurement of films was conducted using a parallel plate geometry (diameter 35 min, gap 0.5 mm). The storage modulus (G') and loss modulus (G'') were measured from 0.1 to 20 rad·s^{-1} at 25 °C in the LVR. Creep and recovery tests were performed at 25 °C for each sample (with the same size). The tests were recorded at constant stress amplitude at 25 °C for 180 s, followed by release of the stress and recovery for 180 s. Creep curves were analyzed according to the Burgers model with one Kelvin-Voigt element [36] (Equation (2)):

$$J = J_o + J_1\left(1 - exp\left(\frac{-t}{\lambda_{ret}}\right)\right) + \frac{t}{\mu_o} \quad (2)$$

where J is compliance, t is the time, J_o is the instantaneous compliance (Pa^{-1}), J_1 is the retarded compliance (Pa^{-1}), λ_{ret} is the retardation time of Kelvin-Voight element (s) and μ_o is Newtonian viscosity (Pa·s).

2.7. Application of Coatings on Strawberry and Quality Parameters

The strawberries were dipped into three different edible coating samples (chitosan, chitosan + 1% LE, and chitosan + 5% LE) for 1 min. Then, the fruits were air-dried, packed in commercial

corrugated boxes and stored at 4.0 ± 1 °C. Uncoated strawberries were used as control. Twenty-five berries for each coating treatment were used and the experiments were performed in duplicate. Quality characteristics of control and coated fruits were determined during storing at 4.0 °C.

2.7.1. Fungal Decay Percentage

Strawberries were visually evaluated for the presence of mold growth during the storage time (10 days). Any strawberry with visible spoilage was considered to be decayed. Fungal decay percentage was calculated by using the following equation:

$$Fungal\ decay\ (\%)\ = \frac{number\ of\ decayed\ fruits}{total\ number\ of\ fruits} \times 100 \quad (3)$$

2.7.2. Determination of Weight Loss

Strawberries were weighed just after coating and air drying. Then, berries' weight was monitored during 10 days after coating. Weight loss was calculated as the percentage of loss related to initial weight (Equation (4)):

$$Weight\ loss\ \% = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100 \quad (4)$$

2.7.3. Determination of pH and Titratable Acidity (TA)

Following the procedures describe by the Association of Official Agricultural Chemist -AOAC method [37], the pH was measured using a pH-meter. TA was determined by titrating the diluted juice (5 g fruit diluted in 95 mL distilled water) up to pH 8.2 using 0.1N NaOH. The results were expressed as the percent of citric acid (Equation (5)):

$$\mathrm{TA} = \frac{\mathrm{V(NaOH)} \cdot 0.1 \cdot 0.064}{\mathrm{m}} \times 100\ (\%) \quad (5)$$

where V(NaOH) is the volume (mL) of NaOH consumed for titration, 0.1 is the molarity of the NaOH solution, 0.064 is a conversion factor for citric acid and m is the mass of the aliquot sample taken for analysis. Measurements were done in triplicate.

2.7.4. Content of Total Phenolic Compounds (TPC)

Strawberries' pulp was finely chopped and 5 g of it was extracted and homogenized with 10 mL of methanol. After a cleaning-up step via centrifugation (5 min at 4500 rpm and 25 °C) and filtration, the supernatants were recovered and allowed to stand at room temperature for evaporation of solvent. The experiments were done in triplicate. Total phenolic content of the extracted pulp was determined using the Folin–Ciocalteu method [30].

2.7.5. Microbiological Analysis of Strawberries

The microbiological analysis of strawberries during storage was measured according to Haiji et al. [38] with slight modifications. The mesophilic and psychrophilic bacteria and yeast count were considered the most comprehensive method to evaluate the microbiological quality of the strawberries. On day 1 and until day 10 of storage, 10 g of fruit was aseptically transferred into stomacher bags, and 100 mL of sterile saline solution was added to each sample. Serial decimal dilutions of the homogenized sample were prepared. Determination of total aerobic bacteria was carried out on Plate Count Agar (37 °C for 48 h). The enumeration results were signified as log CFU (colony forming units)/g.

2.8. Statistical Analysis

Two replicates were prepared for each coating treatment and for each day. Each sample was measured in triplicate. All data were expressed as mean ± standard error, and the statistical analysis of data was performed using R software version 3.6.2. The significant difference between the treatments (three coating treatments) and days were determined using one-way analysis of variance (ANOVA) with Tukey's HSD (honestly-significant difference) test, grouping at the 95% confidence level.

3. Results and Discussion

3.1. Extraction Yield of Licorice Extracts and Quantification of Bioactive Compounds

Extraction yields are reported in Table 1, together with the deviations obtained in duplicate experiments. Taking into account the solvents used, extraction yield increased in the following order: ethanol, methanol, methanol:water (50:50) and ethanol:water (50:50). Based on the dielectric constants of the solvents (ethanol 24.3, methanol 32.7 and water 78.4, at 25 °C), it can be established that the addition of 50% water to the organic solvents and thus, the increase of solvent polarity, resulted in a significant increase of extraction yield ($p < 0.05$). Nevertheless, extraction temperature did not affect yield significantly ($p > 0.05$), at least in the range of temperatures explored (25 and 50 °C).

While the addition of 50% water to methanol produced nearly a 2-fold increase of extraction yield, the addition of 50% water to ethanol resulted in a 6.5-fold yield increase. Then, the combination of a medium polar solvent (ethanol) with a high polar solvent (water) was the most suitable alternative to obtain high yields in the extraction of licorice root. These results are in accordance to other reported extraction studies, such as the work of Nieto et al. [39] concerning the extraction of grape stems, Arranz et al. [40] in the extraction of marjoram with different ethanol:water mixtures, or Kaderides et al. [41] in the UAE extraction of pomegranate peels, reporting an increase in the extraction yield with an increase in solvent polarity. Moreover, it was found that the combination of solvents is more efficient for extraction of phenolic compounds than a single solvent [42].

The main well-known bioactive compounds of licorice (glycyrrhizin, glabridin, liquiritigenin, isoliquiritigenin and liquiritin) were identified and quantified by HPLC analysis and the results obtained are reported in Table 2. Values in the range of 79.0 to 157.9 mg of these bioactive compounds were determined per gram of the different extracts obtained. Except in the case of ethanolic extracts, the higher concentrations were determined for glycyrrhizin, followed by glabridin, and in considerably lower concentrations, liquiritigenin, isoliquiritigenin and liquiritin.

Table 2. Concentration (mg/g extract) of bioactive compounds identified in licorice UAE extracts (High Performance Liquid Chromatography - HPLC analysis).

Sample	Liquiritin	Glabridin	Glycyrrhizin	Isoliquiritigenin	Liquiritigenin
ELe25	0.60 ± 0.01 ^{a}	55.80 ± 5.03 ^{a}	17.30 ± 1.56 ^{c}	0.87 ±0.03 ^{a}	0.95 ± 0.10 ^{c}
MLe25	0.60 ± 0.01 ^{a}	21.35 ± 0.72 ^{b}	53.45 ± 0.85 ^{b,c}	0.60 ± 0.01 ^{a}	2.00 ± 0.01 ^{b}
EWLe25	0.70 ± 0.07 ^{a}	2.73 ± 0.12 ^{c}	159.50 ± 12.51 ^{a}	0.40 ± 0.01 ^{a}	3.95 ± 0.30 ^{a}
MWLe25	0.80 ± 0.01 ^{a}	1.00 ± 0.02 ^{c}	123.85 ± 10.11 ^{a,b}	0.40 ± 0.01 ^{a}	3.30 ± 0.35 ^{a,b}
ELe50	1.10 ± 0.12 ^{a}	55.13 ± 2.77 ^{a}	13.60 ± 0.23 ^{c}	0.60 ± 0.01 ^{a}	1.00 ± 0.01 ^{c}
MLe50	1.00 ± 0.06 ^{a}	24.40 ± 0.90 ^{b}	96.80 ± 4.27 ^{b,c}	1.60 ± 0.09 ^{a}	2.60 ± 0.22 ^{b}
EWLe50	0.60 ± 0.01 ^{a}	2.45 ± 0.03 ^{c}	145.10 ± 10.23 ^{a}	0.40 ± 0.01 ^{a}	3.45 ± 0.30 ^{a}
MWLe50	0.80 ± 0.01 ^{a}	0.80 ± 0.07 ^{c}	133.40 ± 15.24 ^{a,b}	0.40 ± 0.00 ^{a}	3.60 ± 0.46 ^{a,b}

Values with different letters (a–d) in the column and row differ significantly ($p < 0.05$).

In the case of ethanolic extracts, the concentration of glabridin was higher than glycyrrhizin, while significantly lower concentrations were observed for the rest of the components. Glabridin, an isoflavan, and glycyrrhizin, a triterpene glycoside, are the most abundant and distinctive compounds of licorice roots with several recognized pharmacological properties [43,44]. While glabridin is nearly

insoluble in water, the glycosylated sugars present in glycyrrhizin provides some polarity to this compound and thus is better extracted using water. Figure 1 shows the concentration of these compounds in the extracts (mg/g) obtained at 25 °C as a function of the dielectric constant of the solvent used. In the case of water:organic solvent mixtures, the dielectric constant was estimated as the weighted average of the pure solvent dielectric constants. As it can be observed in Figure 1, the extraction of glycyrrhizin increased considerably as the solvent dielectric constant increased, while the opposite effect is observed for the non-polar compound glabridin. Similar results were observed in the case of licorice root UAE extracts obtained at 50 °C.

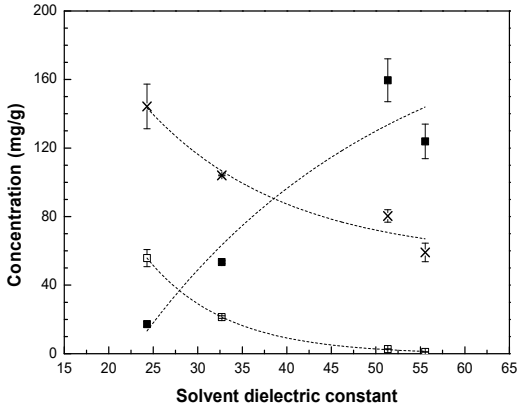


Figure 1. Concentration of glabridin (□), glycyrrhizin (■) and (×) total phenolic compounds (TPC) in UAE licorice extracts as a function of the estimated dielectric constant of the solvent (extraction temperature 25 °C); (-----) trend line.

3.2. TPC, Antioxidant and Antimicrobial Activity of Licorice Extracts

3.2.1. TPC and Antioxidant Activity

The TPC of licorice extracts (mg GAE/g), together with their antioxidant (DPPH test) and antimicrobial activities (IC_{50} values), are reported in Table 3. The extracts with the higher content of TPC were obtained with ethanol (ELe25 and ELe50), followed by methanol (MLe25 and MLe50), ethanol:water (EWLe25 and EWLe50) and methanol:water (MWLe25 and MWLe50), with values in the range 60–160 mg GAE/g extract. Figure 1 shows that the TPC values of the extracts obtained at 25 °C decreased when the dielectric constant of the solvent increased. Similar results were obtained at the extraction temperature of 50 °C. Then, it can be established that the addition of water to the organic solvents (methanol or ethanol) enhanced the extraction yields, while the TPC concentration decreased, maybe due to the extraction of other polar compounds, as suggested by Spingo et al. [45]. For example, the glycyrrhizic acid (GA) molecule has several hydroxyl groups and thus, it is easily soluble when extracted with polar solvents [46]. Then, higher concentrations of GA were obtained in the extracts produced using ethanol:water (EWLe25 and EWLe50) and methanol:water (MWLe25 and MWLe50).

Concerning the effect of temperature, an increase of the extraction temperature (25 to 50 °C) increases the TPC content, showing significant differences ($p < 0.05$). A similar positive effect of temperature on total polyphenols recovery has been previously reported, for example in the extraction of olive leaves [47]. However, it should be noted that extremely high extraction temperature may promote possible degradation of phenolic compounds, or may enhance solvent loss through vaporization [48].

As reported elsewhere, phenolic compounds significantly contribute to the antioxidant activity of plant extracts [49,50]. It is generally stated that the higher the TPC value, the higher the antioxidant activity. In this work, a positive relationship between the antioxidant activity (evaluated by the DPPH

assay) and the TPC content was obtained, considering all licorice UAE extracts produced. Figure 2 shows the linear correlation (R^2 = 0.974) obtained between TPC (mg GAE/g) and TEAC (μmol Trolox/g) values. Regarding the effect of temperature and following the observed tendency in the case of TPC content, for all solvents used, the increase of extraction temperature increased the antioxidant activity of the extract. As mentioned before, the extracts obtained using 50% water were those with the lower TPC values and thus, these extracts are those with the lower antioxidant activity (Figure 2). This result strengthens the hypothesis that the addition of water to the organic solvent enhanced the extraction of polar but non-antioxidant compounds.

Table 3. Total phenolic compounds (TPC), antioxidant activity (DPPH free radicals scavenging assay expressed as TEAC value) and antimicrobial activity (IC_{50} mg/mL) of licorice UAE extracts.

Sample	TPC (mg GAE/g)	DPPH (μmol Trolox/g)	*E. coli* IC_{50} (mg/mL)	*S. aureus* IC_{50} (mg/mL)
ELe25	144.25 ± 3.03 ^{a}	447.37 ± 1.83 ^{a}	>2.5	>2.5
MLe25	104.05 ± 0.22 ^{a,b}	210.77 ± 0.17 ^{a,b}	>2.5	>2.5
EWLe25	80.36 ± 3.67 ^{b}	86.92 ± 0.99 ^{b}	1.21 ± 0.03^{b}	1.38 ± 0.11 ^{a}
MWLe25	59.09 ± 5.40 ^{b}	68.01 ± 1.26 ^{b}	1.09 ± 0.10^{ab}	1.56 ± 0.06 ^{c}
ELe50	162.20 ± 1.30 ^{a}	546. 52 ± 2.16 ^{a}	>2.5	>2.5
MLe50	126.20 ± 2.88 ^{a,b}	277.50 ± 0.24 ^{a,b}	> 2.5	>2.5
EWLe50	85.71 ± 3.07 ^{b}	102.52 ± 0.49 ^{b}	0.84 ± 0.04^{b}	1.43 ± 0.06 ^{d}
MWLe50	79.14 ± 2.16 ^{a,b}	62.18 ± 1.28 ^{b}	0.97 ± 0.03^{b}	1.93 ± 0.01 ^{b}

Values with different letters (a–d) in the column and row differ significantly ($p < 0.05$).

Among the identified and quantified licorice bioactive compounds, only in the case of glabridin, the less polar identified polyphenol, did an increase of its concentration show a positive effect on the antioxidant activity of the extract.

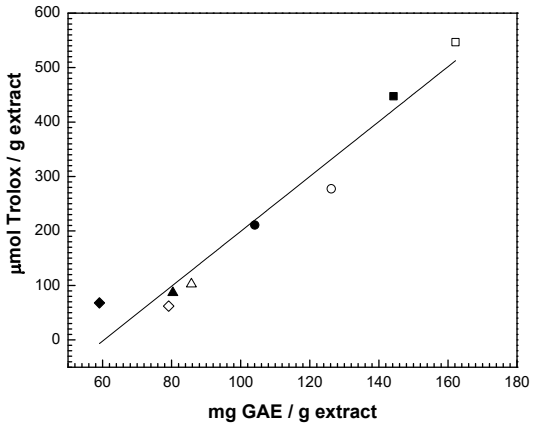


Figure 2. Correlation between antioxidant activity (μmol Trolox/g) and total phenolic compounds (mg GAE/g) of licorice UAE extracts (Table 3). T = 25 °C: (■) ethanol, (●) methanol, (▲) ethanol:water and (◆) methanol:water. T = 50 °C: (□) ethanol, (○) methanol, (Δ) ethanol:water and (◊) methanol:water.

3.2.2. Antimicrobial Activity

Table 3 reports the IC_{50} values (concentration of the extract necessary to attain 50% inhibition) of the licorice UAE extracts tested against a Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, and a Gram-negative bacteria, *Escherichia coli* ATCC 25922. The IC_{50} values corresponding to Chloramphenicol, an antibiotic useful for the treatment of a number of bacterial infections, are 0.09

mg/mL for *S. aureus* bacteria and 0.08 mg/mL for *E. coli*. Only extracts obtained using mixtures of water and organic solvent (methanol or ethanol) exhibit antimicrobial activity. In comparison with Chloramphenicol, the IC_{50} values of these extracts are around one order of magnitude larger for both types of bacteria. The observed antimicrobial activity may be related to the high glycyrrhizin concentration observed in these extracts (>130 mg/g) due to the recognized antimicrobial activity of this compound [51] and licorice extracts [32].

3.2.3. Selection of Licorice Extract for Preparing Edible Coatings

Considering the Food and Drug Administration recommendation for the use of non-toxic and environmentally safe solvents, such as ethanol [52], and the antimicrobial and antioxidant activity of samples, the extract produced with ethanol:water (50:50) at 50 °C (EWLe50) was selected and produced in sufficient amounts in order to prepare the edible coatings and carry out the study of strawberries' preservation. This licorice extract (LE) can be produced with high yield (c.a. 25%), and contains 85.71 ± 3.07 mg GAE/g, moderate antioxidant activity (102.52 ± 0.49 μmol Trolox/g) and good antimicrobial activity (IC_{50} values of 0.84 ± 0.04 and 1.43 ± 0.06 mg/mL for *E. coli* and *S. aureus*, respectively).

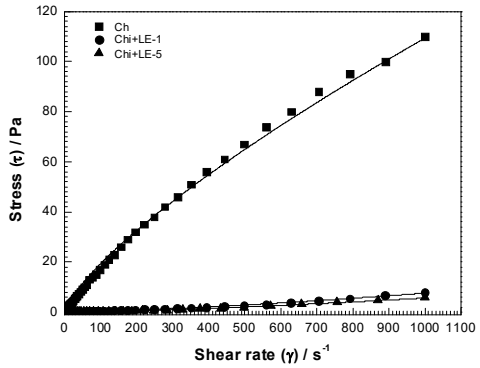
3.3. Rheology of the Edible Coating Solution

The rheological properties of coating solutions are of great importance because they affect the structure and apparent viscosity of the film matrix. The uniformity, the spreadability and the thickness of the coating could be greatly influenced by the flow characteristics of the coating solution [53]. Different chitosan-licorice coating solutions were prepared employing 1% (Ch + LE-1) and 5% (Ch + LE-5) of the licorice extract EWLe50 and pure chitosan (Ch) was used as control. In order to obtain a good knowledge of fluid behavior, the viscosity and viscoelastic properties of coating solutions were measured to assess the processability of the coating.

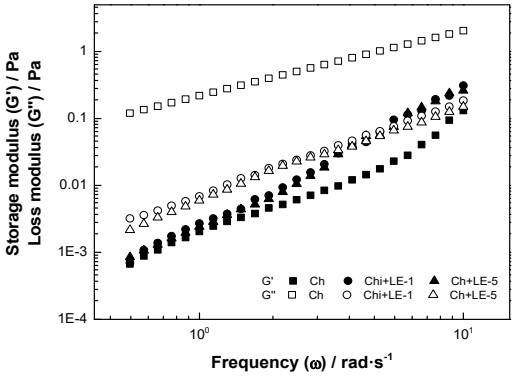
3.3.1. Steady Rheological Properties

Figure 3a shows the flow curve of different samples: the changes observed in shear stress are a consequence of the addition of licorice root extract to chitosan control, due to the interaction between chitosan and licorice phytochemicals and complexation. All coating solutions exhibited the behavior of non-Newtonian fluid and can be adjusted to the Power Law model (Equation (1)). The model parameters (K and n) are given in Table 4, and high fitting degree ($R^2 > 0.994$) was achieved. The K (consistency coefficient) values decrease significantly and n (flux index) values increase with the addition of LE. Chitosan solution presents a consistency coefficient of 0.469 ± 0.015 and flux index of 0.795 ± 0.005. Moreover, the samples Ch + LE-1 and Ch + LE-5 present K values of $3.72 \cdot 10^{-4}$ ± 0.001 and $1.76 \cdot 10^{-4}$ ± 0.001 and n values of 1.436 ± 0.018 and 1.503 ± 0.012, respectively. n values equal to 1 indicate the Newtonian fluids, lower than 1 indicate shear thinning fluids, while n values higher than 1 indicate a shear thickening behavior [54]. Then, the chitosan coating solution presents a shear thinning behavior due to alignment of molecules in the direction of flow, and a decrease of viscosity with the increase of shear rate. Similar results were obtain by Zhang [55] in a coating solution of chitosan/zein with the addition of alpha-tocopherol.

On the other hand, the addition of LE caused a decrease in the shear stresses and a non-Newtonian behavior. The shear thickening behavior ($n > 1$) observed in samples Ch + LE-1 and Ch + LE-5 are probably a consequence of particle hydro-clustering and an order-to-disorder transition. At large shear rates and stresses, convective and hydrodynamic force dominate over inter-particle force, and cause hydro-clustering of particles [56,57]. The rheological properties of coating solutions are consistently used among the literature to provide data on films' microstructure, thickness, coating spreadability and uniformity on substrate. In general, highly viscous solutions retained air bubbles in the casting process, and low viscous solutions facilitated its spreading on the plate where the films were formed [58].



(**a**)



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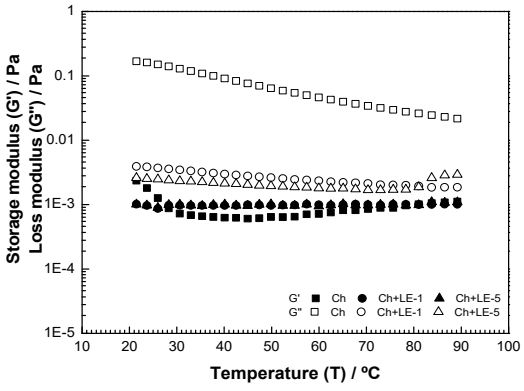


(**c**)

Figure 3. Rheological properties of edible coating solutions produced with chitosan and chitosan-licorice: (**a**) flow curve, (**b**) frequency sweep and (**c**) temperature sweep.

Table 4. Power Law parameters obtained for the edible coating solutions. Ch = Chitosan; Ch + LE-1 = Chitosan + 1% of licorice extract; Ch + LE-5 = Chitosan + 5% of licorice extract.

Treatment	K (Pa·s^n)	n	R^2	σ ($\dot{\gamma} = 10\ s^{-1}$) (Pa)
Ch	0.469 ± 0.015	0.795 ± 0.005	0.998	0.2
Ch + LE-1	$3.72 \cdot 10^{-4}$ ± 0.001	1.436 ± 0.018	0.994	0.0047
Ch + LE-5	$1.76 \cdot 10^{-4}$ ± 0.001	1.503 ± 0.012	0.998	0.0021

3.3.2. Dynamic Rheological Properties

The study of dynamic viscoelastic properties of coating solutions led to obtain information about the molecular entanglement and molecular network formation during drying [59]. The LVR used in this study was the maximum stress value in the flat region of storage modulus (G′) and stress curve. Stress value applied for Ch, Ch + LE-1 and Ch + LE-5 was of 0.1 Pa.

Figure 3b shows the frequency dependence of the storage (G′) and loss modules (G″) of the different edible coating solutions studied, from 0.01 to 100 rad·s^{-1}. According to mechanical spectra (Figure 3b), increased G′ and G″ values were observed with the increase of angular frequency (ω), with G″ exhibiting a larger increase for all the samples in comparison with G′. Higher G″ values were obtained for chitosan-licorice solutions, indicating that these solutions will behave as liquids during the mixer process for lower processing times. Then, to deform the material, the supplied energy would be lower in the case of the chitosan-licorice coatings [60].

Furthermore, G″ > G′ for the pure chitosan coating solution in the entire frequency range applied, without any crossover point observed, exhibited the typical behavior of liquid-like solutions [61]. But, in the case of chitosan-licorice solutions, crossover points were reached, indicating a longest time required for chain disengagement of the polymer structures in the solution [62,63]. This observed frequency dependence of G′ and G″ indicates that chitosan-licorice solutions are a useful class of materials to be applied as films in food coating, since they can enhance adhesiveness and the hardness of the coating solution.

Figure 3c shows the effect of temperature on G′ and G″ values of the edible chitosan and chitosan-licorice coating solutions for a heating rate of 5 °C/min in the LVR and 0.1 Hz frequency. The effect of temperature on the variation of G′ and G″ of the coating solutions exposes the phase transitions and elasticity and allows the selection of appropriate temperature ranges for the formulation and the application of the coating solution on the product.

For all coatings, G″ was higher than G′ in the whole range of temperatures studied, denoting the liquid-like behavior. Chitosan coating solution presents higher G′ values than samples with 1% and 5% of LE, the ones which present similar values. Gelatinization does not occur because the high temperatures accelerate the mobility of chitosan molecules in the solution and reduce the intermolecular hydrogen bonding interactions, removing energized water molecules surrounding the chitosan chains [64].

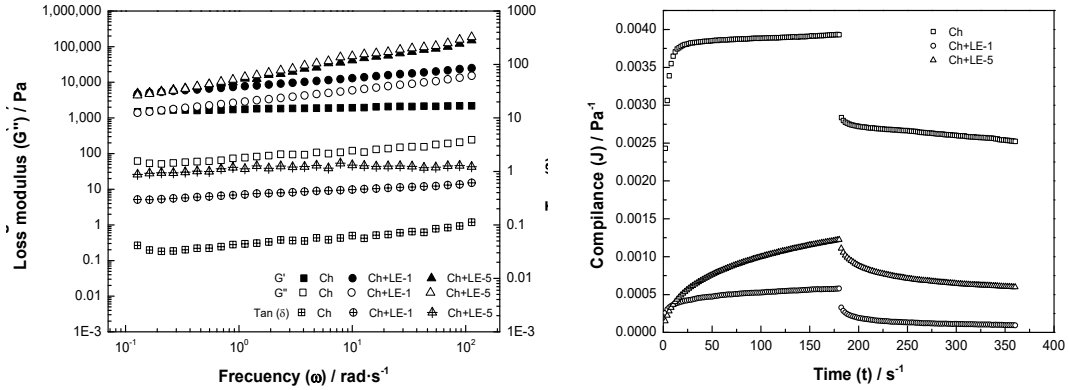
3.3.3. Rheological Properties of Edible Film

The physicochemical and functional properties of edible films, such as mechanical, rheological and optical properties, are directly related to their microstructure and the interaction between film components and the drying conditions [65]. The rheological properties of edible films were evaluated in order to analyze the influence of combining chitosan with licorice extracts in the coating solutions, and to assess the particular characteristics and modifications in comparison with using only chitosan.

All the films were flexible and easily detachable from the sample holder, without evident defects in the form of cracks or pores. Figure 4a shows the frequency dependence of storage modulus (G′) and loss modulus (G″) of films of Ch, Ch + LE-1 and Ch + LE-5. For Ch films, G′ >> G″ over the entire frequency range studied, indicating that chitosan film exhibits dominant elastic behavior. G′ > G″ in the case of Ch + LE-1, and the film presents a more robust network and exhibits a gel-like behavior. Finally, for Ch + LE-5 film, G′ > G′, exhibiting a viscous behavior. Then, the addition of licorice phytochemicals to chitosan increases the viscous properties of film, displaying a variation in G′. Tan

δ (= G″/G′) increase with the addition of licorice extract to chitosan and values become Tan δ ≈ 1, corroborating that the viscous quality of the film is greatly affected by the addition of LE and suggesting that the films containing LE have higher flexibility compared to the chitosan film and thus, they are more suitable than pure chitosan for application as edible food coatings.

The creep compliance data are shown in Figure 4b. The extensional creep curves of all films showed a typical behavior of viscoelastic material, with varying degrees of viscoelasticity. The compliance of films decreases with the addition of licorice extracts and with the percentage amount of extract added. It can be inferred that films became more flexible with the addition of LE to chitosan, indicating an interaction between LE phytochemicals with possible hydrogen bonds formation.



(**a**) (**b**)

Figure 4. Rheological properties of chitosan and chitosan-licorice edible films: (**a**) frequency sweep and (**b**) creep and recovery.

The creep results were simulated by applying the Burger model (Equation (2)) and a satisfactory description of the essential features of the film viscoelasticity was attained, with high correlation coefficients ($R^2 > 0.999$) (Table 5). The parameters of the Burger model reflect the structure of any system, and the decreasing/increasing of these parameters shows the weakening/strengthening of the structure. The instantaneous compliance (J_o) of chitosan was 3.793, and decreased to 0.482 and 0.801 for Ch + LE-1 and Ch + LE-5 respectively, indicating a reduction of rigidity or strength [66,67]. The retarded compliance (J_1) of chitosan was 1.408 mPa^{-1}, decreasing to 0.481 and 0.626 respectively, with the addition of 1% and 5% of LE, indicating a reduction of the gel cohesive force and also a reduction of the resistance to deformation caused by the three-dimensional network structure [66,67]. Newtonian viscosity (μ_o) increased significantly with the addition of LE to chitosan, revealing that mechanical strength and mobility of polymer chains increased with the addition of LE to chitosan in the coating formulation.

Table 5. Burger model parameters obtained for the films. Ch = Chitosan; Ch + LE-1 = Chitosan + 1% of licorice extract; Ch + LE-5 = Chitosan + 5% of licorice extract.

Table 1	J_o (mPa^{-1})	J_1 (mPa^{-1})	λ_{ret} (s)	μ_o (Pa·s)	R^2	Recovery (%)
Ch	3.793	1.408	4864.0	1.282×10^6	0.998	35.82
Ch + LE-1	0.482	0.481	906.3	1.878×10^6	0.999	83.78
Ch + LE-5	0.801	0.6258	340.8	4.252×10^5	0.997	51.09

Recovery percentages are an indirect measure of gel elasticity and the calculated values for the different coating films are given in Table 5. The recovery percentages of the films with LE extracts

(83.78% and 51.09% for Ch + LE-1 and Ch + LE-5, respectively) were considerably higher than that of chitosan (35.82%). Then, the addition of licorice extract changed the viscous behavior and improved the elasticity of the films, with the Ch+LE-1 coating being the one which presents the highest recovery percentage (best gel elasticity).

3.4. Effect of Chitosan-Licorice Edible Coatings on Strawberries' Quality

Four sets of strawberries were prepared to carry out the study of the effect of chitosan-licorice edible coatings on the fruit preservation: uncoated (control) fruit (C), coated with chitosan (Ch) and coated with Ch + LE-1 and Ch + LE-5. The samples were stored at 4 °C for 10 days.

Physicochemical Effects

In order to determine the effect of licorice extracts on the coated fruits, photographs were picked up during storage, which are shown in Figure 5. Pictures corresponding to 14 days of storage were also included in the figure, because the effect of coatings became very obvious. The coated strawberries presented a brilliant appearance, which was better than the uncoated sample.

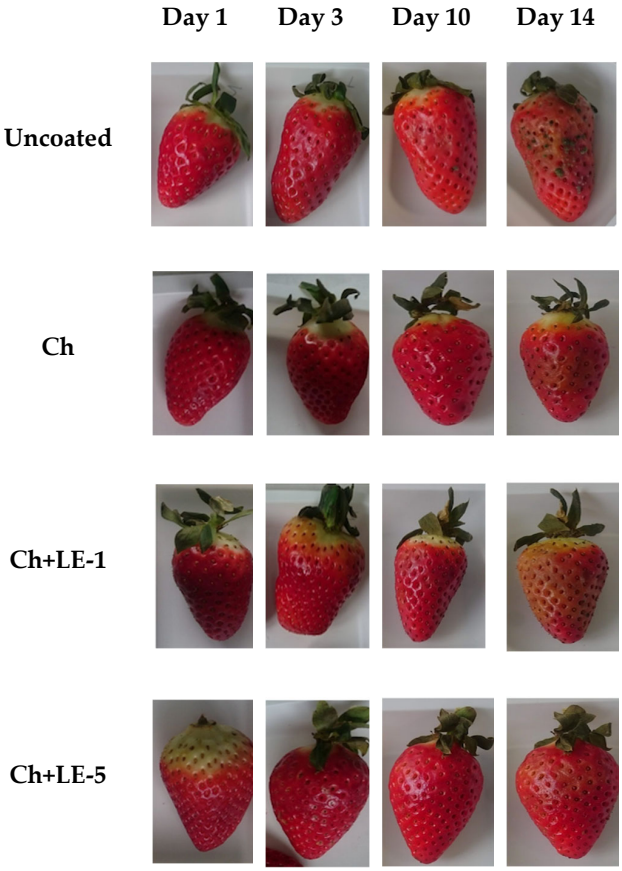


Figure 5. Visual appearance of uncoated, chitosan- and chitosan-licorice-coated strawberries corresponding to 1, 3, 10 and 14 days of storage at 4 °C.

The coated forming on berries depends on the rheological parameters of the coating solutions, which present different shear stress and viscoelastic properties. The amount of coating solution adhered to strawberry during application strongly depends on the viscosity and the surface tension of the coating solutions. The roughness on the surface of strawberries require formulations with ingredients that can reduce the coating solution surface tension, in order to ensure coating uniformity and absence of void holes [4]. The total sugars present in fruit can act as a plasticizer, interacting with the polymer chains and generating "free" volumes within the chains, weakening the intermolecular

forces and, consequently, reducing the resistance of the films [68]. Some authors determined that solutions with high viscosity and low surface tension promote a better film-forming surface [69]. In this study, the chitosan coating solution presented a viscosity of 170 mPa·s, while lower values were observed in the case of chitosan-licorice solutions (4.40 and 2.10 mPa·s for Ch + LE-1 and Ch + LE-5, respectively). Nevertheless, despite the observed viscosity reduction, the addition of LE improved the viscoelastic properties and allowed a better surface extensibility on berries, forming thinning coatings with higher uniformity and adherence, which can be related with the better physical appearance observed (Figure 5). The application of coatings did not affect the color of berries, maintaining good visual quality and no water migration on the surface was observed.

The presence of mold was visually observed in the control sample on day 10 (Figure 6), while no mold was observed in the coated berries. Ch + LE-5 coating exhibits the best preservation of strawberry appearance. The fungal decay of samples is depicted in Figure 6. The percentage of fungal decay was higher in control samples, followed by chitosan samples, while chitosan-licorice coatings did not present fungal decay during 10 days of storage. The licorice extract increased the antimicrobial properties of chitosan, according to the antibacterial activity assessed in this study and in other studies available in the literature [32].

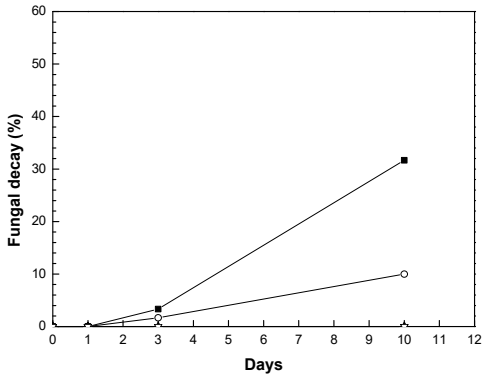


Figure 6. Fungal decay of uncoated and licorice-chitosan-coated strawberries stored for 10 days at 4 °C: (○) Ch (Chitosan); (■) C (uncoated).

Figure 7 shows the weight loss of uncoated and coated samples with the different films throughout the cold storage time. Postharvest mass loss of fruits and vegetables is mostly due to moisture loss through transpiration, and is recognized to accelerate the susceptibility of fruit and vegetables to physiological disorders [70,71]. After only three days of storing, the uncoated sample presented less weight loss than the samples treated with any of the coatings. Nevertheless, on day 10, the barrier effect exerted by the coatings could be observed, since all coatings reduced the loss of fruit weight in comparison with uncoated samples, with the coatings containing Ch + LE-1 and Ch + LE-5 being more efficient than the one containing solely chitosan. For these coatings, weight losses of samples are lower than 2% after 10 days of storing. Similar weight loss values were obtained by Martínez et al. [15] using chitosan coatings combined with *Thymus capitatus* essential oil. Furthermore, these loss weight values were lower than those observed by Petriccione et al. [72] when studying different species of strawberry coated with 2% chitosan (weight losses of 7–9%) or Ventura-Aguilar [73] using an edible film of chitosan combined with a *Roselle calyces* extract (weight loss of 6%).

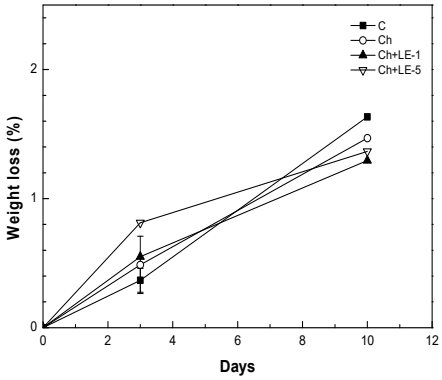
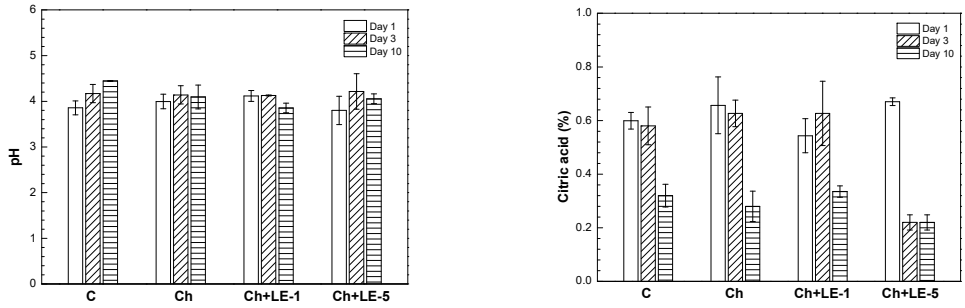


Figure 7. Effect of different licorice-chitosan coatings on the weight loss of strawberries stored for 10 days at 4 °C: (○) Ch (chitosan) (▲) Ch + LE-1 (Chitosan + 1% of licorice extract); (▽) Ch + LE- 5 (Chitosan + 5% of licorice extract; (■) C = uncoated.

The effect of the different coatings in comparison with uncoated samples on the pH and titratable acidity (TA) of strawberries were also examined, since these properties are a measure of ripening effect. Fruit maturation produces the enzymatic reaction of sugars and the amounts of acids tend to decrease and the pH increases. The initial pH values of coated strawberries were slightly higher than uncoated samples (Figure 8a). Although there were some differences on day 1, pH values of coated samples did not change significantly during storage and no significant differences were observed between coated samples on day 10 (mean pH value is 4.05 ± 0.10), while a significantly higher pH value was obtained for the uncoated fruit (4.5 ± 0.20).



(**a**) (**b**)

Figure 8. Effect of different coatings based on chitosan and licorice extracts on (**a**) pH and (**b**) titratable acidity (TA) expressed as citric acid percentage of strawberries stored for 10 days at 4 °C. C = uncoated; Ch = chitosan; Ch + LE-1 = Chitosan + 1% of licorice extract; Ch + LE-5 = Chitosan + 5% of licorice extract. Means values and intervals of Tukey's test at 95% according to the analysis of variance (ANOVA) test.

Furthermore, the TA values were significantly reduced ($p < 0.05$) both in coated and uncoated strawberries, but due to the nature of fruit organic acids, these usual decreases in fruit acidity during maturity did not induce a notable change in the pH value. As can be observed in Figure 8b, a significant decrease of TA values ($p < 0.05$) with storing time was observed in all cases, but differences between control and the different coatings were not significant. These results are in agreement with similar research conducted by Bhimrao et al. [74], who studied the extension of shelf life of strawberries using a coating formed with chitosan and whey protein isolate.

3.4.2. Total Phenolic Compounds Content and Microbiology Analysis

As stated before, the phenol content of a vegetal sample can be related with its antioxidant activity. Thus, the TPC content of the uncoated and coated strawberries was evaluated after 10 storing days, as an indicator of the coating antioxidant functional capacity (Table 6). The TPC values of uncoated strawberry on day 1 are in accordance with the values reported in the literature [75], which indicated that TPC values of 27 strawberry cultivars varied from 0.57 to 1.33 GAE mg/g of fresh weight.

Table 6. Total phenolic compounds (TPC) of strawberries treated with different coatings. Ch = Chitosan; Ch + LE-1 = Chitosan + 1% of licorice extract; Ch + LE-5 = Chitosan + 5% of licorice extract.

Treatment	TPC (mg GAE/g)	
	Day 1	Day 10
Uncoated	1.41 ± 0.12	0.97 ± 0.04
Ch	1.28 ± 0.06	0.99 ± 0.08
Ch + LE-1	1.52 ± 0.10	1.32 ± 0.05
Ch + LE-5	1.44 ± 0.02	1.38 ± 0.35

The results given in Table 6 indicate that, on day 10 and for all cases (uncoated and coated fruit), the TPC values were lower after the cold storage. But, the type of coating had a significant effect on the observed TPC decrease, as is depicted in Figure 9, and the control samples (uncoated fruit) presented the highest TPC loss percentage (30.93 mg GAE/g), followed by chitosan coating (22.65 mg GAE/g), chitosan + 1% LE (13.1 mg GAE/g) and chitosan + 5% LE (13.3 mg GAE/g). Then, LE extract contributed significantly to preserve the phenolic compounds of samples.

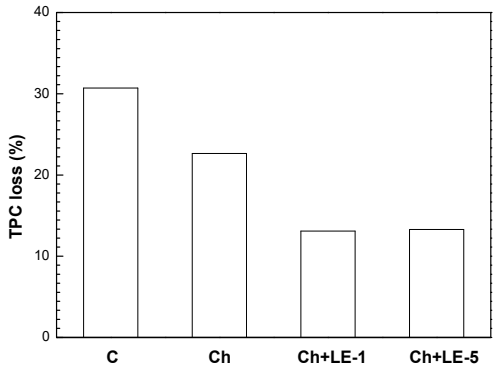


Figure 9. Effect of chitosan-licorice coatings on total phenolic compounds (TPC) of strawberries stored for 10 days at 4 °C: C = uncoated; Ch = chitosan; LE1 = Chitosan + 1% of licorice extract; LE5 = Chitosan + 5% of licorice extract.

Concerning the microbiological analysis, Table 7 shows the aerobic bacteria and yeast determined in coated and uncoated strawberries after 6 days of cold storage. No initial growth of bacteria or yeasts was observed in the uncoated and coated strawberries. But after 6 days of storage, viable count of bacteria and yeasts were determined in uncoated samples and in berries coated with chitosan, while in the samples coated with chitosan-licorice mixtures, no growth was observed. Then, the addition of phytochemicals of licorice in order to prepared edible coating improved the antimicrobial properties of chitosan.

Table 7. Effects of edible coatings prepared with chitosan and chitosan + licorice extract on the growth of mesophilic and psychrophilic bacteria (total aerobic psychrophilic bacteria) and yeasts during storage at 4 °C. Ch = Chitosan; Ch + LE-1 = Chitosan + 1% of licorice extract; Ch + LE-5 = Chitosan + 5% of licorice extract.

Bacteria and Yeasts	Treatment	Total Viable Count (Log CFU/g) on Day 6 of Cold Storage
Mesophilic	Uncoated	7.0 ± 2.0
	Ch	4.5 ± 0.7
	Ch + LE-1	n.g.
	Ch + LE-5	n.g.
Psychrophilic	Uncoated	4.0 ± 0.01
	Ch	4.0 ± 0.01
	Ch + LE-1	n.g.
	Ch + LE-5	n.g.
Yeasts	Uncoated	4.0 ± 0.01
	Ch	4.0 ± 0.01
	Ch + LE-1	n.g.
	Ch + LE-5	n.g.

n.g.: no growth observed. CFU: Colony forming units

4. Conclusions

In this study, the ultrasound-assisted extraction using solvents with different polarities and two temperatures (25 and 50 °C) was carried out, in order to obtain bioactive licorice extracts (LE) for the elaboration of edible coatings. The polarity of the solvent contributed to the extraction of specific compounds, and while temperature did not significantly affect the extraction yield, it influenced the TPC content of samples. A higher concentration of glabridin was obtained with ethanol solvent, showing a positive effect on the antioxidant activity of the extract. Nevertheless, ethanolic licorice root extracts did not present antimicrobial activity. On the other hand, the extraction with mixtures of ethanol or methanol with water (50:50 *v*/*v*) enhanced the extraction of polar biomolecules (such as glycyrrhizin), and the extracts presented good antimicrobial activity but less antioxidant capacity in comparison with the ethanolic extract.

The edible coating solutions elaborated with pure chitosan and with chitosan and licorice ethanol:water extract, presented a non-Newtonian flow behavior. Solutions of pure chitosan presented shear thinning behavior, and the addition of LE decreased the shear stress value and presented a shear thickening behavior, probably due to particle hydro-clustering and an order-to-disorder transition. The viscoelastic properties of the coating solution show that the restructuring ability of Ch + LE physical gels improve the properties of film forming. Then, the addition of licorice phytochemical to chitosan increased the viscous properties of film, displayed a variation in the dissipation energy (G″) and films became more flexible with the addition of LE. Furthermore, the addition of LE to chitosan decreased the shear stress values of samples, allowing better physical properties of the films applied on strawberries.

Concerning the preservation capacity, the addition of LE increased the antimicrobial properties of chitosan, according to the antibacterial activities studied in vitro. The values of weight loss of these coatings were significantly lower than those corresponding to pure chitosan. Additionally, the chitosan + LE edible coatings better preserved the content of phenolic compounds in the berries.

Then, the ultrasound-assisted extraction of bioactive compounds of licorice root using ethanol:water (50:50 *v*/*v*) is an alternative for producing bioactive licorice extracts with potential application in the elaboration of edible coatings with good rheological properties and preservative capacity.

Author Contributions: Conceptualization, M.R.G.-R.; L.A.G.-Z., and T.F.; methodology, S.E.Q.; M.R.G.-R.; L.A.G.-Z., and T.F.; software, O.L. and S.E.Q.; validation, T.F., formal analysis, S.E.Q., M.R.G.-R., L.A.G.-Z., and T.F.; investigation, S.E.Q., O.L., resources, S.E.Q., O.L., M.R.G.-R.; L.A.G.-Z. and T.F., writing—original draft preparation, S.E.Q. and T.F.; writing—review and editing, S.E.Q., O.L., L.A.G.-Z., M.R.G.-R. and T.F., supervision, T.F., M.R.G.-R. and L.A.G.-Z. and project administration, M.R.G.-R. and T.F.; funding acquisition, S.E.Q.; O.L., M.R.G.-R. and T.F. All authors have read and agreed to the published version of the manuscript.

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

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4.2. MICRONIZACIÓN DE EXTRACTOS VEGETALES MEDIANTE TECNOLOGÍA DE FLUIDOS SUPERCRÍTICOS

En este capítulo se presentan los resultados obtenidos en relación a la reducción de tamaño de las partículas de extractos vegetales bioactivos seleccionados, empleando la tecnología de precipitación supercrítica antisolvente (SAS), los que componen dos artículos científicos publicados.

La micronización se desarrolló con el objetivo de obtener un producto en polvo, homogéneo, con tamaño de partícula del orden micro- y/o nanométrico, considerando que el tamaño y la forma de las partículas desempeñan un papel fundamental en las propiedades fisicoquímicas y funcionales de los preparados nutracéuticos de uso para la salud. Asimismo, la micronización facilita la manipulación del extracto vegetal para su posible adición en matrices alimentarias.

Se utilizaron los extractos etanólicos de hojas de romero (*Rosmarinus officinalis* L.) y de raíz de regaliz (*Glycyrrhiza glabra* L.), obtenidos mediante la técnica de extracción asistida con ultrasonidos. Estos extractos habían presentado una buena recuperación y concentración de compuestos fenólicos y actividad antioxidante en los estudios preliminares de esta tesis. La técnica de micronización empleada fue la precipitación supercrítica antisolvente (SAS), la que permite asegurar un buen rendimiento de precipitación de aquellos compuestos del extracto vegetal con características más polares, puesto que son los de baja solubilidad en la fase supercrítica (CO_2 + etanol) que abandona la celda de precipitación. En cambio, los componentes del extracto vegetal más apolares (más solubles en la fase supercrítica) tienden a no precipitar y abandonar la celda de precipitación disueltos en la fase supercrítica. Es decir, a la vez que se lleva a cabo la precipitación de partículas en forma de polvo se produce un fraccionamiento de los compuestos bioactivos del extracto vegetal.

En un primer trabajo, titulado "*Supercritical antisolvent particle precipitation and fractionation of rosemary (Rosmarinus officinalis* L.*) extracts*", publicado en el *Journal of CO_2 Utilization* (34 (2019) 479-489), se muestran los resultados de la evaluación de las condiciones de precipitación SAS sobre la micronización y el fraccionamiento de un extracto de romero. Además, se evaluó la composición química y la actividad antioxidante de las fracciones obtenidas (precipitado y oleorresina), en las distintas condiciones de operación estudiadas, así como la morfología, tamaño y distribución de tamaño de partícula de los precipitados.

En el segundo trabajo se llevó a cabo un estudio similar, correspondiente a la precipitación SAS del extracto etanólico de raíz de regaliz, artículo titulado "*Fractionation and precipitation of licorice (Glycyrrhiza glabra* L.*) Phytochemicals by supercritical antisolvent (SAS) technique*" y publicado en el *Journal LWT – Food Science and Technology* (126 (2020) 109315). En este trabajo se evaluó el efecto de la concentración del extracto vegetal, la temperatura y presión del proceso de precipitación sobre el fraccionamiento y recuperación de los compuestos bioactivos de interés, del contenido de compuestos fenólicos totales y de la actividad antioxidante de las fracciones obtenidas. También, de forma similar al primer trabajo

se estudió la morfología, tamaño y distribución de tamaño de las partículas de los precipitados en polvo obtenidos.

En resumen, en este segundo capítulo se presenta la producción de precipitados micronizados de extractos de dos plantas diferentes, utilizando la tecnología de $SCCO_2$, ensayando diferentes condiciones de proceso, tales como la concentración de la disolución etanólica de extracto vegetal, la temperatura y presión en la cámara de precipitación, y la relación CO_2/disolución utilizada. En ambos casos, estos parámetros del proceso SAS se estudiaron en relación a las condiciones críticas, demostrándose la importancia de mantener los parámetros de operación alejados del punto crítico de la mezcla para conseguir partículas de tamaño micro- y/o nanométrico, homogéneas y regulares. La precipitación SAS, en las condiciones de operación adecuadas, permitió obtener extractos de romero y raíz de regaliz en forma de polvo, con tamaños de partículas micro- y nanométricos, y con una distribución y morfología adecuadas. Además, el análisis de la composición química y actividad antioxidante de los precipitados permitió determinar los cambios más significativos de los productos obtenidos en comparación con el extracto vegetal original.

4.2.1. Supercritical antisolvent particle precipitation and fractionation of rosemary (*Rosmarinus officinalis* L.) extracts

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Supercritical antisolvent particle precipitation and fractionation of rosemary (*Rosmarinus officinalis* L.) extracts

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ABSTRACT

The simultaneous fractionation and precipitation of an ethanolic extract of rosemary (*Rosmarinus officinalis* L.) using supercritical carbon dioxide anti-solvent technique was studied, with the target of separate in two different fractions the key antioxidants of rosemary (i.e. rosmarinic acid, carnosic acid and carnosol). The effect of pressure and temperature on the fractionation process was investigated, together with the morphology and particle size distribution of the precipitates. Additionally, the chemical composition of the oleoresins were analyzed and reported.

In the range of pressures (9–20 MPa) and temperatures (313–333 K) used in this work, the precipitates presented a 2–3 fold enrichment of rosmarinic acid, while carnosic acid and carnosol were concentrated (2–3 fold enrichment) in the oleoresin fractions. Furthermore, in general, oleoresins presented higher antioxidant activity than precipitates. Particles produced with a nozzle of diameter 101.6 μm were smaller and more spherical with increasing pressure (mean value 4–10 μm at 20 MPa) and decreasing temperature.

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) is a Lamiaceae perennial shrub which grows in many parts of the world [1,2]. It has been cultivated since ancient times and recognized as a natural preservative due to its high antioxidant and antimicrobial activities. These activities are related to the presence of phenolic compound, mainly carnosol, rosmanol, isorosmanol, rosmadiol, carnosic acid, rosmarinic acid, and methyl carnosate [3,4]. Extraction techniques like ultrasound or microwave assisted [5], maceration [6], pressurized liquid extraction [7] or using supercritical carbon dioxide [8,9], are mainly employed to improve the extraction of rosemary antioxidants. Generally, natural extracts are marketed in liquid form, as oily preparations, or in solid form as powders. In general, dried powdered extracts have some advantages over liquid extracts, including lower storage costs and a higher concentration and stability of active substances [10].

Different techniques have been studied and developed to obtain powdered extracts and produce particles such as spray drying, spray chilling, jet milling, spray cooling, lyophilization, liquid antisolvent precipitation, or dry/milling processes [11–14]. However, these methods have several disadvantages, such as a wide particle size distribution (PSD), the possible degradation of the product due to mechanical or thermal stresses, and difficulties in the complete elimination of the organic solvents used in the process [15]. Supercritical Antisolvent (SAS) precipitation is an alternative to these conventional processes and has arisen extensive attention as an environmental-friendly and appropriate way for the production of micro- or nanoparticles of pharmaceutical/bioactive compounds [16–18].

In SAS method, supercritical carbon dioxide ($SCCO_2$) is utilized as an antisolvent. The solute is precipitated from a solution which is expanded through a nozzle while mixed with $SCCO_2$, followed by the nucleation and crystal growth [19]. The morphology and size of the particles are influenced greatly by many factors and their combined effects, such as SAS operating conditions [20–22], solvent type [20], surface tension of the solution [23], fluid dynamics [24–27] and mass transfer [28–32].

Abbreviations: MCP, mixture critical point; PSD, particle size distribution; RE, rosemary ethanolic extract; SAS, supercritical antisolvent precipitation process; $SCCO_2$, supercritical carbon dioxide; SEM, scanning electron microscopy; TEAC, trolox equivalent; TPC, total phenolic compounds

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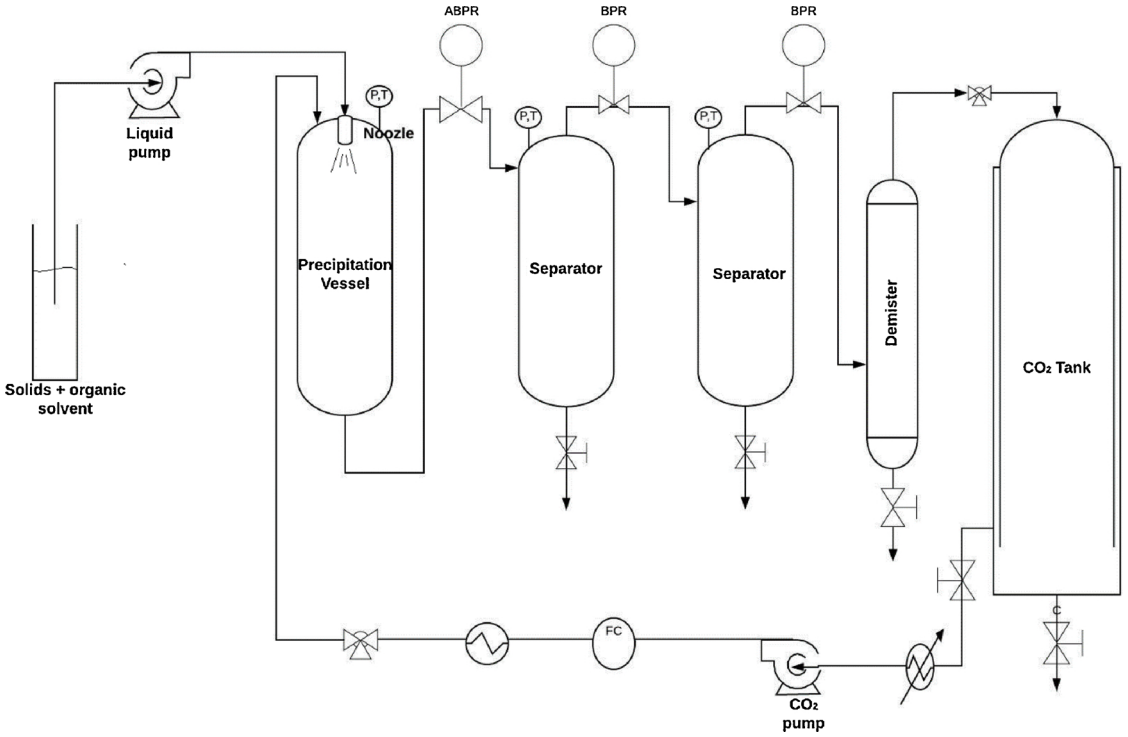

Fig. 1. Schematic diagram of the SAS process. ABPR: Automatic back pressure regulator, BPR: manual back pressure regulator, P: manometer, T: temperature probe, FC: flowmeter.

In general, the precipitation temperature and pressure, the $SCCO_2$ flow, and the concentration of the extract in the solution have significant effect on the size and structure of the particles precipitated during the process [23,33].

Many natural compounds such as carotenoids, quercetin, caffeine, ellagic acid [34], β-carotene [35] or herbal extracts have been precipitated using SAS approach. Likewise, the technique is being used in pharmaceutical products such as ibuprofen or mandelic acid, as well as inorganic materials such as hydrides or polymers (polyethylene glycol or polylactic acid). Also, Matos et al. [36] evaluated the effect of SAS parameters on the co-precipitation of curcumin and poly-vinylpyrrolidone from mixtures of ethanol and acetone, obtaining particle sizes between 96 nm and 135 nm. Franco et al. [37] proposed SAS technique to co-precipitate zein with diclofenac sodium at different operational conditions, gaining a prolonged drug release. SAS precipitation of mango leaf extracts was reported by Guamán-Balcázar et al. [38] obtaining spherical nano- and submicron-particles, with diameters in the range 0.04 – 0.38 μm, and stronger antioxidant activity than the original extracts. Moreover, Widjojokusumo et al. [39] applied SAS to micronizate *Manilkara kauki* L. Dubard's leaf extracts, and Villanueva et al. [40] carried out the fractionation of green tea extracts to obtain decaffeinated fractions with high content of catechins.

Regarding the application of SAS technique to rosemary, Visentin et al. [41] studied the fractionation of an ethanolic oleoresin using a home-made nozzle. They obtained a solid fraction in the precipitation vessel (precipitate) with low yield and concentration of carnosic acid, and a liquid fraction in the separator with high content of this phenolic diterpene (dissolved in ethanol). In addition, Visentin et al. [10] performed the encapsulation of rosemary antioxidants (carnosic and rosmarinic acids) with polymers using SAS precipitation technique at different operating conditions. More recently, Sánchez-Camargo et al. [42] used a pressurized liquid extraction device coupled with an anti-solvent precipitation vessel, with the purpose of determining the bioactivity of the fractions obtained concerning their antiproliferative effect in the HT-29 colon cancer cells. In all these referred works, the SAS fractionation of rosemary antioxidants has been carefully evaluated, but the effect of SAS process conditions on the morphology and particle size distributions of the precipitates has not been considered. In this work, the SAS fractionation of a rosemary ethanolic extract (obtained by ultrasound assisted extraction) to produce a powdered precipitate was studied, considering the effect of process parameters on the recovery of rosemary antioxidants, along with the antioxidant activity and the morphology and particle size distribution of the precipitates.

2. Materials and methods

2.1. Chemicals

CO_2 (99.98% purity) was supplied from Carburos Metálicos (Madrid, Spain). Ethanol (99.5% purity) and Sodium Carbonate anhydrous (99.5% purity) were purchased from Panreac (Barcelona, Spain). Gallic acid standard (> 98% purity), 2,2-Diphenyl-1-pycrilhydrazyl (DPPH, 95% purity), 2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, ≥ 95% purity), (±)-6-Hydroxy-2,5,7,8-tetramethyllchromane-2-carboxylic acid (Trolox, 97% purity), Folin-Ciocalteu´s reagent and rosmarinic acid (≥ 98% purity), eucalyptol (≥ 99% purity), linalool (≥ 97% purity), camphor (≥ 95% purity), borneol (≥ 99% purity), 4-terpineol, (+)–terpineol (≥ 99% purity), (1S) - (-) verbenone (≥ 94% purity), -carophyllene Kosher (≥ 80% purity) and carophyllene oxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Carnosic acid (> 97% purity), purchased from Cymit (Cymit Química S.L., Barcelona, Spain).

2.2. Preparation of rosemary extract

Rosemary (*Romarinus officinalis* L.) leaves harvested in Spain obtained from Murciana herbalist's Murcia Spain) was ground using a Premill 250 hammer mill Lleal S.A. Granollers, Spain). Then, ultrasound assisted extraction (UAE) using an ultrasonic device (Branson Digital Sonifier 550 model Danbury, USA) with an electric power of 200 W and frequency of 60 kHz was accomplished. The extraction was:

carried out with ethanol at 1: 10 (w/v) plant/solvent ratio for 15 min and keeping constant the extraction temperature at 25 °C. The rosemary ethanolic extract (RE) (ethanol + solutes extracted) contained 16.04 mg of solutes in 1 ml of dissolution (1.98% wt). The dissolution was stored at -293.15 K for its use in the SAS process.

2.3. Supercritical antisolvent precipitation

The supercritical antisolvent precipitation was performed using the supercritical technology equipment Thar SF2000 (Thar Technology, PA, USA) (Fig. 1). The equipment comprises two pumps for feeding the supercritical $SCCO_2$ and the liquid solution, respectively, the precipitation vessel and two separators (S1 and S2) each of 500 ml capacity, with independent control of temperature and pressure, coupled with a demister unit. The demister unit is specially designed to separate liquid or solid particles from the outgoing stream, before driving CO_2 to the recycling system and the storage tank. The precipitation vessel consists on a stainless-steel precipitation cell of 273 ml volume where the $SCCO_2$ and liquid solution streams are fed from the top in a co-current manner (coaxial nozzle). The precipitation cell is equipped with a 101.6 μm inner diameter nozzle for the injection of the liquid solution and a porous metallic frit (5 μm in diameter) that is located at the bottom of the precipitator to collect the precipitate.

$SCCO_2$ was pumped into the precipitation vessel until precipitation pressure and temperature conditions were attained. Then, the ethanolic RE was pumped into the precipitator, while maintaining the $SCCO_2$ flow. After 60 min, once the extract fed ends, additional $SCCO_2$ was pumped during 15 min to wash out the residual solvent from the precipitator. During the process, both separators were kept at ambient pressure. The depressurization of the supercritical stream in the separators produced the ethanol precipitation together with those components which did not precipitate into the precipitation vessel (i.e. the components soluble in the $SCCO_2$/ethanol supercritical phase). Finally, the precipitation vessel was depressurized, and the precipitate was collected.

The simultaneous precipitation and fractionation of RE were studied at pressures in the range 8 to 20 MPa, temperatures of 313.15 to 353.15 K, 50 and 60 g/min of $SCCO_2$ flow, and 1.6 g/min of ethanolic RE. The mole fraction of CO_2 in the pseudo-binary supercritical ethanol + CO_2 mixtures ranged from 97.03 to 97.51%. These mole fractions intent to ensure a homogenous phase according to the ethanol + CO_2 binary phase equilibria data reported in the literature [43–45] although at 333.15 K and 8, 9 and 10 MPa these mole fractions are very close to the two-phase gas-liquid boundary. All experiments were carried out feeding the liquid extract for 60 min. The precipitate (solid fraction) was recovered in the frit of the precipitator vessel. The liquid fractions (non-precipitated compounds dissolved in ethanol) were obtained in the two separators. Both liquids were combined, and ethanol was removed by rotary evaporation under vacuum obtaining an oleoresin. Samples were kept at −297.15 K under darkness until analysis.

2.4. Total phenolic compounds and antioxidant activity

The content of total phenolic compounds present in the samples was determined using the Folin-Ciocalteu method [46]. Briefly, 50 μl of extract were mixed with 3 ml of milliQ water and 250 μl of Folin Ciocalteu reagent. The content was thoroughly mixed and after 3 min, 750 μl of sodium carbonate solution (20% mass) and 950 μl of milliQ water were added to the mixture. After 2 h at room temperature in darkness, the absorbance was measured at 760 nm using a Genesys 10S UV–vis spectrophotometer (Thermo Fischer Scientific Inc., MA, USA). The results were expressed as GAE (mg of gallic acid equivalents / g of extract).

The ability of extracts to scavenge DPPH free radicals was determined according to the method described by Brand-Williams et al, [47]. For the reaction, 25 μl of samples were added to 975 μl of DPPH radical in ethanol ($6.1{\cdot}10^{-5}$), which was daily prepared. The reaction took place at room temperature, in the dark, until it reached a plateau. Then, the absorbance was measured at 515 nm in a Genesys 10S UV–vis spectrophotometer (Thermo Fischer scientific, MA, USA). The DPPH concentration in the reaction medium was calculated from a calibration curve determined by linear regression. A control sample, containing the same volume of solvent instead of extract, was used to measure the maximum DPPH absorbance. Trolox was used as reference standard, so results were expressed as TEAC values (μmol Trolox equivalent/g extract). All analyses were done in triplicate.

2.5. HPLC analysis

Carnosol, carnosic and rosmarinic acid were identified and quantified in the samples following the procedures describes by Vicente et al. [48] using a HPLC Prominence-i LC-2030C 3D Plus (Shimadzu) equipped with a RP-C18 (250 × 4.6 mm; 3 μm) chromatography column. The mobile phase consisted of 0.1% of phosphoric acid in water (solvent A) and acetonitrile (solvent B) applying the following gradient: 0–8 min, 77% A, 8–25 min, 25% A, 25–40 min 25% A and the 40–45 min 77% A. Initial conditions were gained in 5 min. The flow rate was constant at 0.7 ml/min. Injection volume was 20 μl and the detection was accomplished using a diode array detection system, storing the signal at a wavelength of 230, 280 and 350 nm.

2.6. GC–MS analysis

Identification and quantification of volatile compounds of samples was carried out by a GS-MS-FID using 7890A system Agilent Technologies Santa Clara, CA, USA) comprising a split/splitess injector FID detector and a mass spectrometer detector 5975C triple-axis. An HP-5MS capillary column (30 m x 0.25 mm i.d. 0.25 μm phase thickness) was used. The chromatographic method starts with an initial temperature of 313.15 K then increased to 423.15 K at 276.15 K/min and was held at 423.15 K for 10 min then from 423.15 to 573.15 K at 279.15 K/min and finally held at 573.15 K for 1 min. Volume of 1 μl of samples was injected in spitless mode. Helium (99.99%) was employed as carrier gas (1 ml/min flow rate). The temperatures were: 523.15 K for injector 503.15 K for the mass spectrometer ion source 553.15 K interface and 423.15 K for quadrupole. The mass spectrometer operated under electron impact mode (70 eV) and it was used in total ion current (TIC) mode and scanned the mass range from 40 to 500 *m/z*.

2.7. Morphology and particle size analysis

Morphologies of particles collected from the precipitation vessel were visually studied by scanning electron microscopy (SEM) with an energy-dispersive X-ray spectrometer (SEM-EDS) XL-30S FEG, Philips (Japan). Samples were placed on carbon tapes and then were coated with a thin chrome layer by a sputter coater. Particle size distributions were measured by light scattering with a laser diffraction system Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK), equipped with a wet dispersion unit.

3. Results and discussion

3.1. The supercritical antisolvent process

The flow rates of $SCCO_2$ and RE were set in order to reach a percentage of ethanol in CO_2 around 3.2% weight ethanol. It was expected to attain a homogenous CO_2 + ethanol phase at all the different precipitation conditions (pressure and temperature) and thus, ensure the complete elimination of ethanol and produce the precipitation of solid particles [49].

The phase equilibria of the CO_2 + solvent + solute multicomponent

system strongly affect the phenomena taking place in the SAS precipitation process. Different behavior concerning jet mixing and mass transfer were described in the literature, depending on the SAS operational conditions, which can be located below the mixture critical point (MCP), near above the MCP or far above the MCP [25]. Furthermore, fluid dynamics and mass transfer determine the production of precipitates, and the complexities of these mechanisms are responsible for the great variety of particle size and morphology generally obtained.

When the operational conditions of SAS process are below the MCP (subcritical conditions) small droplets of the solvent (containing the solute) are produced as a result of the jet break-up. The surface tension persists and a multiphase gas-liquid-solid mixing system results. The mass transfer of CO_2 into the droplets (solvent-rich phase) together with the solvent evaporation into the CO_2-rich phase lead to the supersaturation of the solute and produce its precipitation. That is, the formation of particles is induced by the $SCCO_2$ antisolvent effect and by the organic solvent depletion in the droplets formed by the nozzle. Consequently, in the case of SAS subcritical gaseous conditions, microparticles and expanded microparticles (hollow core particles) with regular forms are obtained. Nevertheless, if the SAS subcritical conditions are located within the gas-liquid region, irregular particles and agglomeration due to residual solvent generally occur.

On the other hand, for SAS operational conditions far above the MCP, the mixing of CO_2 with the solvent is produced instantaneously since the surface tension vanishes before an appreciated jet break-up is obtained and thus, no liquid-gas interphase occurs. It was generally stated [49] that smaller particles (nanoparticles) are obtained at pressures relatively larger than those corresponding to the MCP, produced by their condensation from a gaseous phase. Yet, for conditions near above the MCP the behavior size and morphology depend on the time that it takes to vanish the surface tension and the time of jet break-up. In general, spherical microparticles were observed when SAS process was performed in the proximity of the MCP.

In the present studyas stated before,not all the solutes of RE precipitate in the precipitation chamber since some of them are soluble in the supercritical CO_2 + ethanol phase. In this way two different fractions were obtained from the RE a powder in the precipitation vessel (: P: precipitate) and a viscous fraction (: O: oleoresin) which was recovered from the separators after evaporation of ethanol.

The SAS experimental assays carried out in this work, together with the corresponding yield (Y), total phenolic compound (TPC) and antioxidant capacity (TEAC values) of the fractions obtained (P and O) are shown in Table 1.

First, supercritical antisolvent precipitation was carried out by duplicate at the selected conditions 20 MPa, 313.15 K and 50 g/min of $SCCO_2$ flow rate (Exp. 10 and 11 in Table 1) to assess the reproducibility of the method. Regarding precipitation yield (mass of extract precipitated / mass of extract feed) the values obtained were 44.74 and 45.07%, respectively, and the oleoresin yields (mass of extract recovered in the separators / mass of extract feed) were 43.03 and 43.64%, respectively, for Exp. 10 and 11. These results indicate a mean deviation of 0.23 and 0.43, respectively, for P and O yields, and a total recovery of the mass feed larger than 87%. Further analysis of the precipitates obtained in Exp. 10 and 11 confirmed low mean deviation in the rosmarinic acid, carnosol and carnosic acid content (Table 2), so as total phenolic content and TEAC value (Table 1) and mean size of particle (Table 4).

Table 2
Mass fraction of main rosemary antioxidants (rosmarinic acid, carnosol and carnosic acid) determined in the precipitates and oleoresins by HPLC analysis. C: mass fraction, E: enrichment factor.

Exp.	antioxidant compound	Precipitate (P)		Oleoresin (O)	
		C / mg/g	E	C /mg/g	E
1	Rosmarinic acid	45.40	2.79	12.20	0.74
	Carnosol	4.80	0.33	19.40	1.33
	Carnosic acid	24.50	0.24	123.20	1.20
2	Rosmarinic acid	21.60	1.33	7.00	0.43
	Carnosol	13.90	0.95	19.40	1.33
	Carnosic acid	79.00	0.77	172.50	1.68
3	Rosmarinic acid	21.60	1.65	3.60	0.22
	Carnosol	13.40	0.92	24.00	1.64
	Carnosic acid	77.80	0.76	224.60	2.18
4	Rosmarinic acid	19.80	1.51	1.40	0.09
	Carnosol	13.40	0.92	35.70	2.45
	Carnosic acid	76.20	0.74	172.50	1.68
5	Rosmarinic acid	24.80	1.89	1.80	0.11
	Carnosol	2.00	0.14	40.40	2.77
	Carnosic acid	13.40	0.13	299.20	2.91
6	Rosmarinic acid	24.60	1.88	1.20	0.07
	Carnosol	2.00	0.14	21.20	1.45
	Carnosic acid	13.20	0.13	132.20	1.29
7	Rosmarinic acid	20.00	1.23	2.80	0.17
	Carnosol	13.80	0.95	15.50	1.06
	Carnosic acid	81.40	0.79	138.80	1.35
8	Rosmarinic acid	25.50	1.56	1.40	0.09
	Carnosol	3.00	0.21	39.60	2.71
	Carnosic acid	16.20	0.16	299.20	2.91
9	Rosmarinic acid	32.20	1.98	0.00	0.00
	Carnosol	0.80	0.05	30.20	2.07
	Carnosic acid	5.80	0.06	233.80	2.27
10	Rosmarinic acid	39.40	2.42	2.80	0.17
	Carnosol	1.00	0.07	29.90	2.05
	Carnosic acid	9.60	0.09	228.20	2.22
11	Rosmarinic acid	38.00	2.33	2.80	0.17
	Carnosol	0.80	0.05	29.90	2.05
	Carnosic acid	7.40	0.07	226.20	2.20

Table 1
Yield (Y) expressed as mass recovered of precipitate or oleoresin/mass of extract feed, Total Phenolic Content (TPC) expressed as GAE (mg of gallic acid equivalents/g) and antioxidant activity expressed as TEAC value (μMol Trolox equivalent/g).

Precipitation conditions				Precipitate (P)			Oleoresin (O)		
Exp.	P / MPa	T / K	CO_2 flow / g/min	Y %	TPC mg GAE/g	TEAC μMol Trolox equivalent/g	Y %	TPC mg GAE/g	TEAC μMol Trolox equivalent/g
1	8	333.15	60	8.43	222.98 ± 0.32	975.42 ± 3.06	56.28	137.42 ± 0.01	760.89 ± 1.34
2	9	333.15	60	39.36	148.91 ± 0.32	756.23 ± 0.35	11.49	145.12 ± 0.01	850.63 ± 7.69
3	10	333.15	60	46.84	138.54 ± 2.05	523.47 ± 3.06	16.60	137.64 ± 0.47	895.93 ± 6.88
4	10	333.15	50	68.10	136.53 ± 1.10	454.19 ± 0.60	18.82	155.60 ± 4.73	1066.85 ± 2.41
5	10	313.15	60	53.82	103.84 ± 0.01	448.44 ± 2.88	38.10	162.52 ± 0.01	1160.23 ± 1.29
6	10	313.15	50	57.63	107.41 ± 0.95	449.06 ± 2.95	36.48	159.62 ± 0.95	1187.27 ± 7.96
7	10	353.15	60	41.16	138.76 ± 1.10	749.57 ± 1.67	7.29	119.21 ± 0.21	663.91 ± 2.06
8	15	333.15	60	59.17	115.11 ± 0.16	456.97 ± 1.33	32.32	121.47 ± 0.32	1344.52 ± 0.69
9	20	333.15	60	42.84	123.37 ± 0.47	500.62 ± 0.39	38.16	147.79 ± 2.52	1291.76 ± 5.95
10	20	313.15	50	44.74	177.35 ± 2.91	557.00 ± 0.01	43.03	218.08 ± 9.72	1356.65 ± 0.01
11	20	313.15	50	45.07	175.44 ± 6.70	561.43 ± 0.03	43.64	221.68 ± 6.70	1376.87 ± 0.06

3.2. Effect of pressure, temperature and $SCCO_2$ flow on precipitation yield

The effect of temperature on the fractionation and precipitation of RE was studied at 10 MPa, 50–60 g/min of $SCCO_2$ and varying temperature in the range of 313.15 to 353.15 K (Exp. 3, 4, 5, 6, and 7 in Table 1). Additionally, the effect of pressure was investigated keeping temperature constant at 333.15 K, $SCCO_2$ flow at 60 g/min, and varying pressure from 8 to 20 MPa (Exp. 1, 2, 8 and 9 in Table 1). The flow rate of the RE was set to 1.6 g/min in all cases.

Considering both the mass of precipitate (P) and the mass of oleoresin (O) recovered in the separators48 to 94% of the total mass of solids fed to the SAS process was recovered. The major losses of solids pumped were produced at the higher temperature 353 K Exp. 7) and at lower precipitation pressures (Exp. 1 and 2). In general yields in the precipitation chamber were higher than oleoresin yields. This result is in accordance with previous results obtained by Sánchez-Camargo et al. [42] in the SAS precipitation of an : ethanol: water rosemary extract obtained by pressurized liquid extraction. Nevertheless a distinct behavior at 8 MPa and 313 K (Exp. 1) was observed with extremely low precipitation yield in comparison with the rest of experiments. At these pressure and temperature conditions the CO_2 + ethanol mixture is very close to the vapor-liquid equilibrium state [4344] and thus it is presumed that some small drops formed by the nozzle were dragged out of the precipitation chamber and precipitation was drastically reduced. The increase of pressure from 9 to 15 MPa at constant temperature (333 K) and $SCCO_2$ flow (60 g/min) produced a linear increase of both P and O yields (R^2 values of 0.95 and 0.99 respectively improving the total mass of RE recovered from 50.85% at 9 MPa to 91.49% at 15 MPa. Yet further increase of pressure to 20 MPa seem not to improve precipitation yield and/or total extract recovery.

The opposite behavior was observed concerning the effect of temperature on P and O yields. That is, increasing temperature at constant pressure (10 MPa) and $SCCCO_2$ flow (60 g/min) a linear decrease of both P and O yields (R^2 values of 0.99 and 0.95, respectively) was observed, decreasing the total mass of RE recovered from 53.82% at 313 K to 41.16% at 353 K. Regarding the effect of the $SCCO_2$ flow on process yields, despite the narrow range of values studied in this work, a decrease of precipitation yield was observed when $SCCO_2$ flow increased form 50 and 60 g/min (Exp. 3 and 4 at 333 K, and 5 and 6 at 313 K). Both the increase of temperature and the increase of CO_2 flow suppose a higher dragging of volatile compounds with the CO_2 stream and thus, lower amounts of solutes were recovered with increasing temperature and CO_2 flow.

3.3. Phenolic compounds and antioxidant activity of precipitates and oleoresins

Table 1 shows the amount of Total Phenolic Compounds (TPC) expressed as mg GAE/g in the samples obtained (P and O) at the different experimental conditions, along with their antioxidant capacity expressed as TEAC value (μmol Trolox equivalent/g extract).

In general, samples with high TPC values present also high antioxidant activity (high TEAC values). This result is in general accordance with the literature [50]. For the precipitates (P), the TPC are between 103.84 and 177.35 mg GAE/g and the TEAC values ranged from 448.44 to 756.23 μMol Trolox equivalent/g. The TPC values of oleoresins (O) ranged from 119.91 to 218.08 mg GAE/g and TEAC values were in the range 663.91–1356 μMol Trolox equivalent/g. Although differences in the TPC content of samples P and O obtained in a particular experiment were not very large, in general, the antioxidant activity of the oleoresin is considerably higher than that of the precipitate (Fig. 2). Taking into account the TPC content and TEAC value of the RE (121.58 mg GAE/g and 661.84 ± 0.25 μmol Trolox equivalent/g, respectively) is mainly highlighted the potential enrichment of TPC and improvement of antioxidant activity achieved by means of the supercritical fractionation.

The effect of pressure on the content of phenolic compounds and the antioxidant activity is depicted in Fig. 3. Phenolic compounds are substances with recognized antioxidant activity [51,52] and thus, the higher the TPC the higher the antioxidant activity (higher TEAC values). TPC values and TEAC values decrease with increasing pressure in the precipitation chamber, denoting a relation among polyphenols content and antioxidant activity. Nevertheless, in the case of the oleoresins, TEAC values increase with pressure although TPC slightly varied. The opposite is observed concerning the effect of temperature (Fig. 4) since the increase of temperature produce increasing values of both TPC and TEAC in precipitates while decreasing values in the oleoresins. Minor influence of the $SCCO_2$ flow on the TPC and TEAC values were observed, probably due to the narrow range of values explored (Exp. 3 and 4; Exp. 5 and 6).

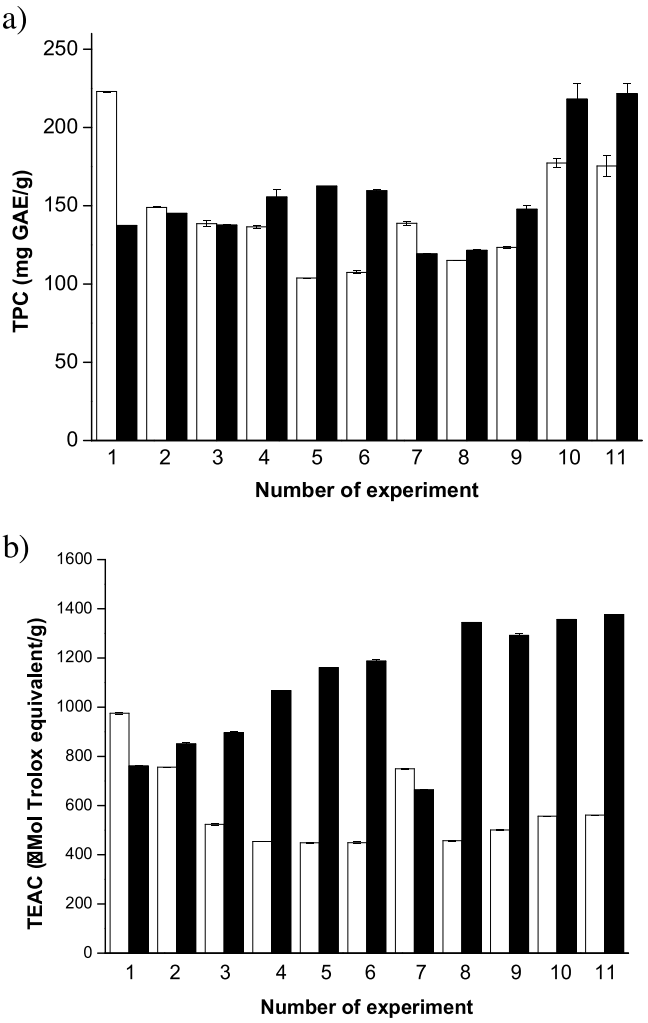

Fig. 2. (a) Content of total phenolic compounds (TPC values) and (b) antioxidant activity (TEAC values) obtained in (□) precipitates and (■) oleoresins.

Then, as a general trend, it can be concluded that the precipitates with the higher antioxidant activity were obtained at the lower pressures and higher temperatures, while the opposite resulted in the case of oleoresins. This general trend can be explained in terms of the TPC of precipitates and oleoresins, since higher amounts of TPC were determined for the precipitates at lower pressures and higher temperatures and the opposite resulted in the case of oleoresins (Figs. 3 and 4).

3.4. Fractionation of rosmarinic acid, carnosic acid and carnosol

Table 2 shows the results of the quantification of the main and well-known rosemary antioxidants, e.g. rosmarinic acid, carnosic acid and carnosol, in precipitates and oleoresin. The varied conditions applied in

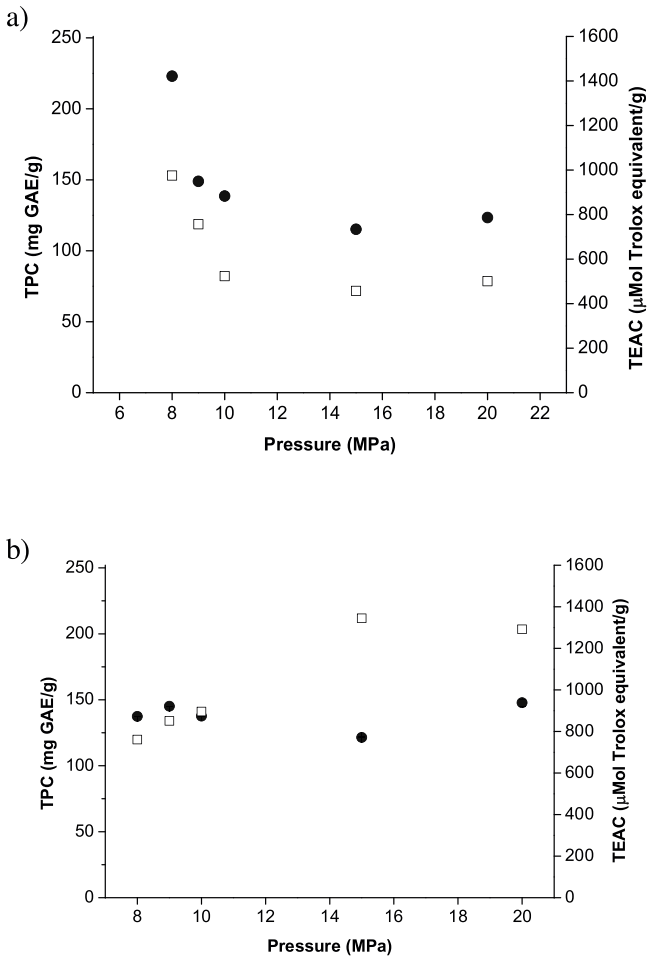

Fig. 3. Effect of pressure on (●) TPC and (□) TEAC values in (a) precipitates and (b) oleoresins. T = 333.15 K; CO_2 flow = 60 g/min.

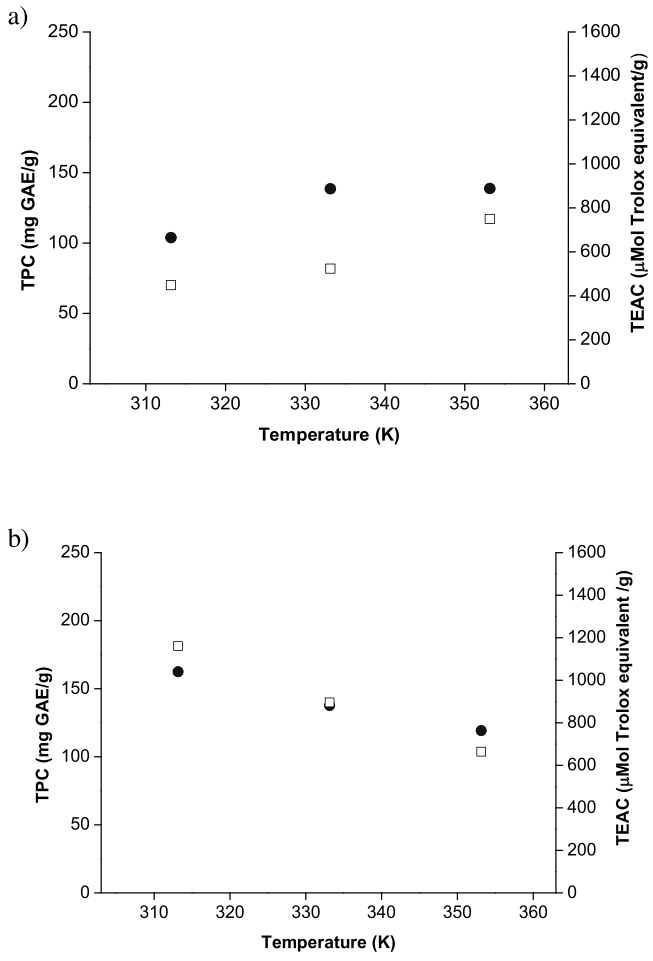

Fig. 4. Effect of temperature on (●) TPC and (□) TEAC values in (a) precipitates and (b) oleoresins. P = 10 MPa; CO_2 flow = 60 g/min.

the SAS precipitation process resulted in a different fractionation of these rosemary antioxidant compounds.

In general, the precipitate presented higher concentrations of rosmarinic acid in comparison with the oleoresin. On the contrary, higher concentrations of carnosic acid and carnosol were obtained in the oleoresins. This behavior was expected taking into account the solubility of these compounds in a supercritical phase comprising $SCCO_2$ and ethanol acting as a cosolvent. According with the literature, while rosmarinic acid is almost no soluble in $SCCO_2$, carnosic acid exhibits solubilities up to 0.4 mg/g [53,54]. Thus, rosmarinic acid is mainly recovered in the precipitation vessel while carnosic acid (dissolved in the supercritical stream which flows out of the precipitation vessel) is recovered in the separators.

Fig. 5 shows the recovery of rosmarinic acid, carnosic acid and carnosol (mg compound/100 g of RE) at 333.15 K and different pressures (9–20 MPa) obtained in precipitates and oleoresins. In the range of pressures explored, rosmarinic acid recovery (Fig. 5(a)) was significantly higher in the precipitates, increased with pressure and attained a maximum at 15 MPa. Regarding carnosic acid and carnosol (Fig. 5(b) and (c)) the higher recoveries were obtained in the separators (oleoresin product) and as in the case of rosmarinic acid a maximum recovery was observed at 15 MPa.

Moreover, in comparison with the RE (14.6 mg/g of rosmarinic acid, 102.8 mg/g of carnosic acid and 16.4 of carnosol) a rosmarinic acid enrichment of 1.33–2.79 was produced in the precipitates while 1.20–2.91 carnosic acid enrichment and 1.06–2.77 carnosol enrichment were obtained in the oleoresins. Thus, using SAS technique, a selective fractionation of these compounds was attained.

3.5. Oleoresin composition

Table 3 shows the components of rosemary essential oil identified and quantified in the oleoresins by GC–MS analysis. These compounds were not identified or identified in very small amounts in the precipitates (data not shown). Table 3 also includes the mass fraction of main rosemary antioxidants (rosmarinic acid, carnosic acid and carnosol) determined in the oleoresins by HPLC.

According with the literature, the main compounds of rosemary essential oil are eucalyptol, camphor and borneol. The rosemary oleoresins produced in this work contain large amounts of camphor (30–198 mg/g), eucalyptol (25–65 mg/g) and borneol (10–70 mg/g), being the most abundant compounds identified by GC–MS. Considering the compounds identified by GC–MS together with those determined by HPLC, the total amount of oleoresin quantified varies from 23.4 to 60.9%.

3.6. Analysis of morphology and particle size of precipitates

3.6.1. Particle morphology

Scanning electron micrograph (SEM) of the RE and the precipitates (P) are shown in Fig. 6. The image corresponding to RE (Fig. 6(a)) showed that the powder is mostly non-equiaxed and angular in morphology.

The shape of the precipitated particles showed micronized size with different characteristics depending on SAS pressure. At 313.15 K, 9 and 10 MPa (Fig. 6(b) and (c)) the particles are thin, elongated, with the form of fibers and agglomerates of particles are observed showing

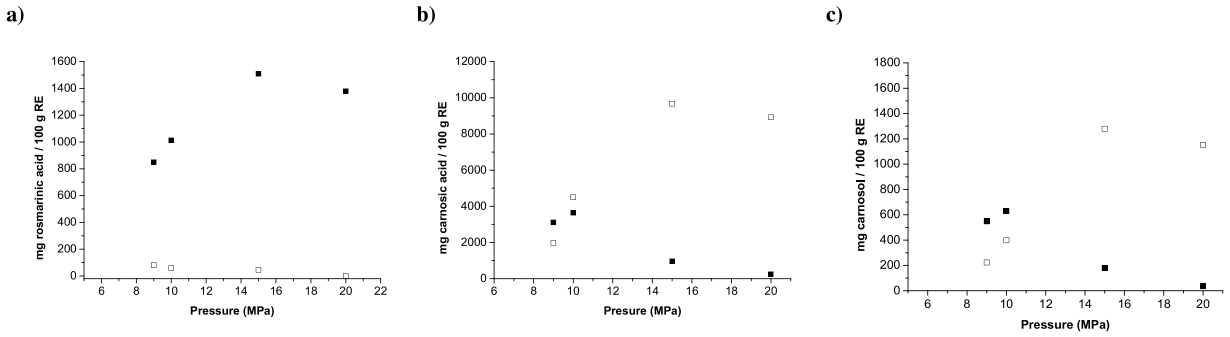

Fig. 5. Recovery of (a) rosmarinic acid, (b) carnosic acid and (c) carnosol (mg/100 g of RE) at 333.15 K and different pressures (9–20 MPa) obtained in precipitates (■) and oleoresins (□).

Table 3
Composition of the oleoresins obtained in the supercritical anti-solvent fractionation of rosemary extract. The values are expressed as mg/g.

Exp	1	2	3	4	5	6	7	8	9	10	11
P (MPa)	8	9	10	10	10	10	10	15	20	20	20
T (K)	333.15	333.15	333.15	333.15	313.15	313.15	353.15	333.15	333.15	313.15	313.15
CO_2 flow (g/min)	60	60	60	50	60	50	60	60	60	50	50
GC-MS											
(-)-4-terpineol	1.97	6.68	6.04	4.52	2.14	2.52	9.38	4.65	0.98	1.43	1.67
camphor	29.44	133.38	153.28	111.02	51.80	54.63	198.99	54.05	64.69	34.21	34.04
alpha terpineol	4.06	28.88	22.82	19.16	10.24	11.07	40.12	11.28	3.29	7.55	7.26
borneol	9.84	50.44	47.13	35.57	17.66	19.03	71.70	15.46	9.38	9.48	9.75
caropyllene	3.00	19.81	20.38	7.63	4.58	5.06	10.33	5.94	2.43	4.12	4.30
eucalyptol	25.31	31.90	65.60	48.39	25.83	26.78	58.34	31.56	28.68	24.23	25.04
verbenone	5.78	22.50	22.57	14.83	6.18	6.58	30.16	13.68	8.35	3.86	3.86
alpha carophyllene	n.i.	8.31	4.54	n.i.	6.10	n.i.	n.i.	1.86	n.i.	n.i.	n.i.
linalool	n.i.	3.61	6.81	3.18	0.87	1.21	7.05	1.78	1.97	u.q.l	u.q.l
carophyllene oxyde	n.i.	13.52	7.60	8.79	n.i.	6.28	16.23	4.15	n.i.	n.i.	n.i.
HPLC											
rosmarinic acid	12.20	7.00	3.60	1.40	1.80	1.20	2.80	1.40	0.00	2.80	2.60
carnosol	19.40	19.40	24.00	35.70	40.40	21.20	15.50	39.60	30.20	31.20	30.40
carnosic acid	123.20	172.50	224.60	172.50	299.20	132.20	138.80	299.20	233.80	231.20	223.20
% mass quantified	23.4	51.8	60.9	46.3	46.7	28. 8	60.0	48.5	38.4	35.0	34.2

n.i.: non identified.
u.q.l.: under quantification limit (< 0.02 mg/ml).

Table 4
Mean particle size of SAS particles in the precipitates.

Exp.	P /MPa	T / K	CO_2 flow / g/ min	d (0.1) μm	d (0.5) μm	d (0.9) μm
1	8	333.15	60	3.12	21.08	45.98
2	9	333.15	60	5.37	20.32	40.60
3	10	333.15	60	4.22	18.15	42.14
4	10	333.15	50	6.39	15.49	30.21
5	10	313.15	60	3.40	10.17	22.58
6	10	313.15	50	3.77	9.81	20.64
7	10	353.15	60	6.96	31.89	66.29
8	15	333.15	60	0.27	3.43	8.97
9	20	333.15	60	0.16	0.62	9.77
10	20	313.15	50	0.28	1.16	5.28
11	20	313.15	50	0.24	1.52	4.15

varying sizes. When the pressure increased to 15 and 20 MPa (Fig. 6(d) and (e)) the particles tend to be smaller (< 1 μm) and more spherical. Similar results were reported by Villanueva-Bermejo et al., [55] concerning the precipitation of *Achilea millefolium* L. ethanolic extract, where more irregular morphologies were found in the precipitates obtained at 10 MPa in comparison to those obtained at 20 MPa. Also, Guamán-Balcázar et al. [38] found spherical and small particles in the precipitation of a mango leaf extracts using pressures over 15 MPa.

Certainly, the size and morphology of the particles depends on the type of solutes, but as mentioned before also depend on the operating conditions, particularly concerning the location of SAS operational conditions with respect to the MCP. The phase equilibria data of the multicomponent CO_2 + ethanol + RE system is not available in the literature (and difficult to attain), but some analysis and conclusions can be established considering the MCP of the CO_2 + ethanol binary mixture. Nevertheless, it has to be highlighted that in the case of solutes with high solubility in the CO_2 + ethanol phase, as is the case of some RE solutes, the MCP of the multicomponent mixture can be rather different from that of the binary CO_2 + ethanol MCP [25].

According to the phase equilibria data reported in the literature [43–45] the MCP of the binary CO_2 + ethanol at 313.15, 333.15 and 353.15 K are, respectively, 8.2, 10.6 and 15.0 MPa. Thus, considering these data, the operating conditions of experiments 5, 6 and 8–11 are clearly above the MCP, while the rest of experiments are very close or below of the MCP. Consequently, at 333.15 K and the lower pressures (Exp. 2 and 3) irregular forms and agglomerates of different size were observed, probably due to operating subcritical conditions located within the gas-liquid region. When pressure is increased (Exp. 8 and 9) and thus the SAS conditions are above the MCP, spherical and regular microparticles were obtained (Fig. 6).

The effect of temperature at constant pressure (10 MPa) on the particle's morphology showed more regular spherical structures in the precipitates at the lower temperature (313.15 K) since at this temperature the experimental SAS conditions are far above the MPC (Exp. 5 and 6). Increasing temperature at 10 MPa, particles tend to present more irregular forms including filament pieces, conglomerates and spheres (imagens not shown) due to subcritical and most probably two-phase operational conditions.

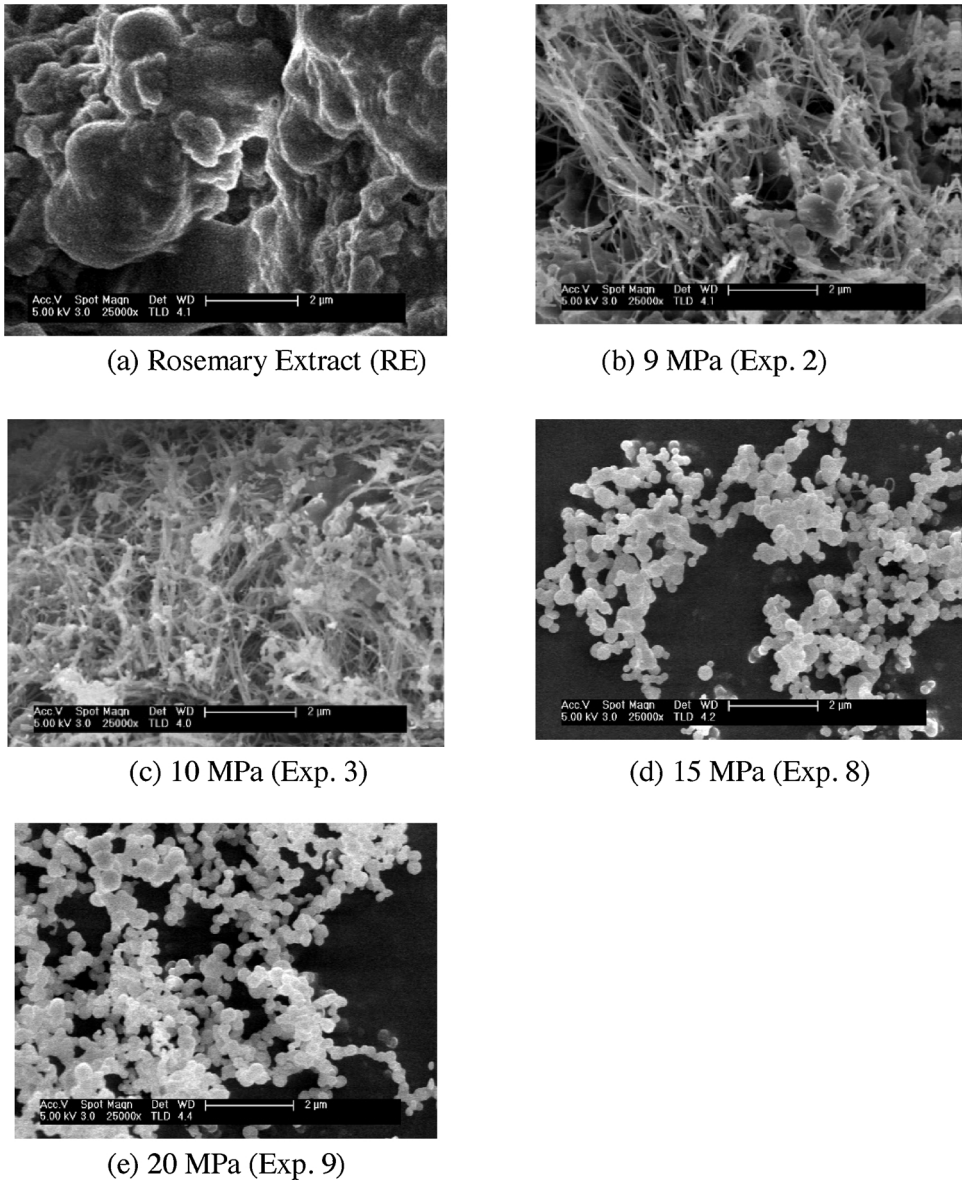

Fig. 6. SEM images (25000x) of rosemary extract (RE) and SAS precipitates (P) at 333.15 K and 60 g/min and different pressures.

3.6.2. Particle size

As mentioned before, it is generally recognized that smaller particles (nanoparticles) are obtained at pressures relatively larger than those corresponding to the MCP [22]. Nevertheless, some authors have reported the opposite effect [55].

The particle size distribution and the most significant statistical variables obtained in this work are shown in Fig. 7 and Table 4. The size of the precipitates is in the range of 0.62–21.06 µm. As expected, all samples presented particle sizes lower than the RE. Smaller particles were successfully achieved by increasing the precipitation pressure, i.e. the mean diameter of particles obtained at 9 MPa (Exp. 2) was 20.31 µm while at 15 MPa (Exp. 8) was 5.39 µm.

With respect to the effect of pressure on particle size, Fig. 7 shows the particle size distribution of the RE and the SAS precipitates at 333.15 K, 60 g/min of CO_2 flow and different precipitation pressures. The distributions are moderately narrow, normal and in appearance bimodal for RE and for the precipitates obtained at 9 and 10 MPa (Exp. 2 and 3). When the precipitation pressure increases to 15 and 20 MPa (Exp 8 and 9) the behavior appears as a multi-modal distribution, with significant smaller sizes. Furthermore, the CO_2 flow did not influence particle size in the range of flow rates explored, and regarding temperature (Exp. 3, 5 and 7) it was observed a decrease of particle size with a decrease of temperature (Table 4).

The particle size distribution obtained can be explained in terms of the SAS operational conditions in relation to the MCP [25]. Conditions far above the MCP resulted in smaller particles as can be observed in Table 4 for Exp. 5, 6 and 8–11, being the smaller particles at the higher pressures.

4. Conclusion

Supercritical anti-solvent SAS precipitation of an ethanolic RE permits the simultaneous fractionation and precipitation of two rosemary fractions: a dry powder with small aggregate particles of irregular shape and an oleoresin resulted after ethanol removal.

A selective separation of the main rosemary antioxidants was observed in the range of conditions studied. In comparison with the RE, a 2-fold increase of the rosmarinic acid mass fraction was obtained in the precipitates at 15–20 MPa and 313 K, while a 2-fold increase of the mass fraction of carnosic acid and carnosol in the corresponding oleoresin. Furthermore, particles with smaller size and more spherical shape were obtained with increasing pressure and decreasing

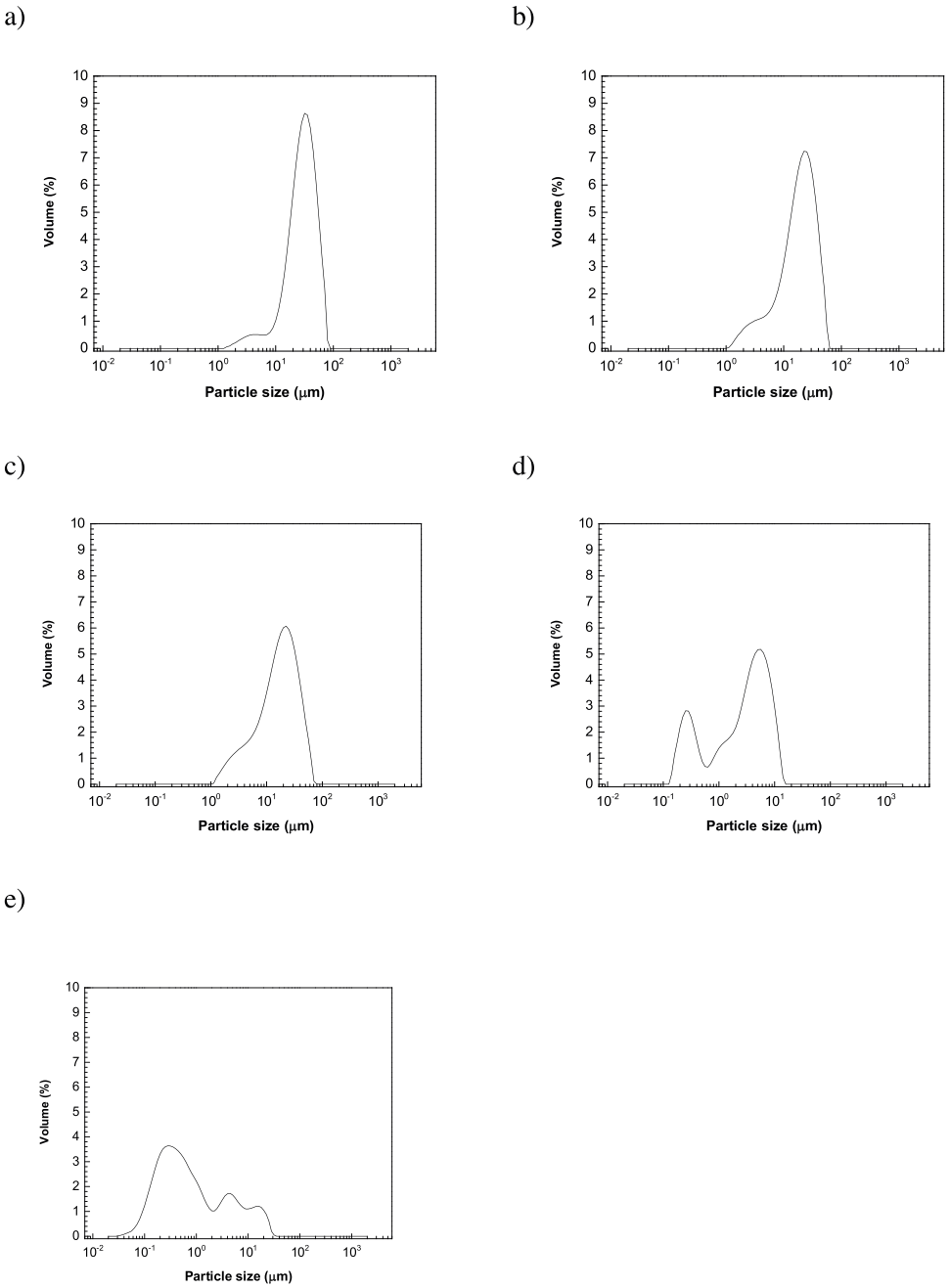

Fig. 7. Particle size distribution of (a) RE and precipitates at 333.15 K, 60 g/min of CO_2 flow and different pressures: (b) 9 MPa, (c) 10 MPa, (d) 15 MPa and (e) 20 MPa.

temperature. Thus, the smaller particle size and more homogeneous particle size distribution were obtained at 313 K and 15–20 MPa, which are SAS operational conditions far above the mixture critical point.

Considering the selective effect of SAS precipitation to separate rosemary antioxidants, it can be concluded the utility of this technique to produce high valued bioactive ingredients with potential application in food products or nutraceuticals. Further studies are under development to determine the anti-inflammatory activity of precipitates and oleoresins in comparison with the initial rosemary extract.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4.2.2. Fractionation and precipitation of licorice (*Glycyrrhiza glabra* L.) phytochemicals by supercritical antisolvent (SAS) technique

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Fractionation and precipitation of licorice (*Glycyrrhiza glabra* L.) phytochemicals by supercritical antisolvent (SAS) technique

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ABSTRACT

Supercritical anti-solvent precipitation (SAS) using carbon dioxide is a novel technique that can be used to produce powdered ingredients in small size particles, facilitating their incorporation into food matrices. In this work, the SAS precipitation of a licorice root ethanolic extract was studied. SAS assays were carried out at 15–20 MPa, 308.15 and 313.15 K, and two different concentrations (9.6 and 14.2 mg/ml) of the ethanolic licorice extract. In the range of conditions investigated, SAS pressure and temperature did not affect significantly the precipitation yield, but phytochemicals recovery was higher with the lower licorice extract concentration. Moreover, the fractionation of licorice bioactives (liquiritin, liquiritigenin, isoliquiritigenin, glabridin and glycyrrhizic acid) was assessed, together with the content of total phenolic compounds and antioxidant activity of the powders and oleoresin by-products obtained. In this respect, precipitates and oleoresins presented significant differences in the concentration of some licorice bioactives, and higher antioxidant activity was observed in precipitates. Additionally, significant effect of pressure, temperature and licorice extract concentration on the morphology and particle size of precipitates was observed, recovering smaller and more regular particles at 15–20 MPa, 313.15 K and 9.6 mg/ml licorice extract concentration, attaining satisfactory yield and antioxidant activity.

1. Introduction

Licorice (*Glycyrrhiza glabra* L.) is an important industrial crop that grows in Mediterranean countries, Asia and Southeast Europe (Saxena, 2005). Licorice has been recognized for its biological properties as antitussive, antiulcer, antimicrobial and antiviral, thrombin inhibitor, anti-inflammatory, antidiabetic, hepato-protective and anticancer. These activities were related to the presence of triterpenoid-type and phenolic-type compounds, mainly liquiritin, liquiritigenin, glycyrrhizic acid, isoliquiritigenin and glabridin (Chin et al., 2007; Kaur, Kaur, & Dhindsa, 2013).

The recovery of these bioactive licorice compounds has been investigated and reported in the literature, using different extraction techniques such as ultrasound assisted extraction (Pan, Liu, Jia, & Shu, 2000), maceration (Sankeshwari, Ankola, Bhat, & Hullatti, 2018), pressurized liquid extraction (Baek, Lee, & Lee, 2008) or supercritical carbon dioxide (CO_2) extraction (Hedayati & Ghoreishi, 2015; S.E.; Quintana, Cueva, et al., 2019). The extracts obtained were oleoresin-type products, presented in the form of oily preparations. In this respect, it is recognized that dried powdered formulas have some advantages over liquid-type preparations, since higher concentration and stability of the bioactive substances together with lower storage costs can be attained (Visentin, Rodríguez-Rojo, Navarrete, Maestri, & Cocero, 2012). Furthermore, powders containing micro- and/or nanoparticles allow a better incorporation of bioactive substances in complex food matrices, and smaller sizes may improve the bioavailability of the bioactive compounds, improving absorption and effectiveness (Martín & Cocero, 2008).

Some conventional micronization techniques, such as spray-drying, centrifugal extrusion, emulsification/solvent evaporation, freeze-drying and coacervation have been used to obtain precipitated small particles (Gonnet, Lethuaut, & Boury, 2010). However, using these techniques, it is not easy to control the size of the particles produced. Also, in some cases, high temperatures are necessary and may degrade the bioactive compound and/or the elimination of the residual solvent may cause the loss of its biological activity (Ozkan, Franco, De Marco, Xiao, & Capanoglu, 2019). Micronized particles can be produced by supercritical CO_2 antisolvent (SAS) method at moderate temperatures,

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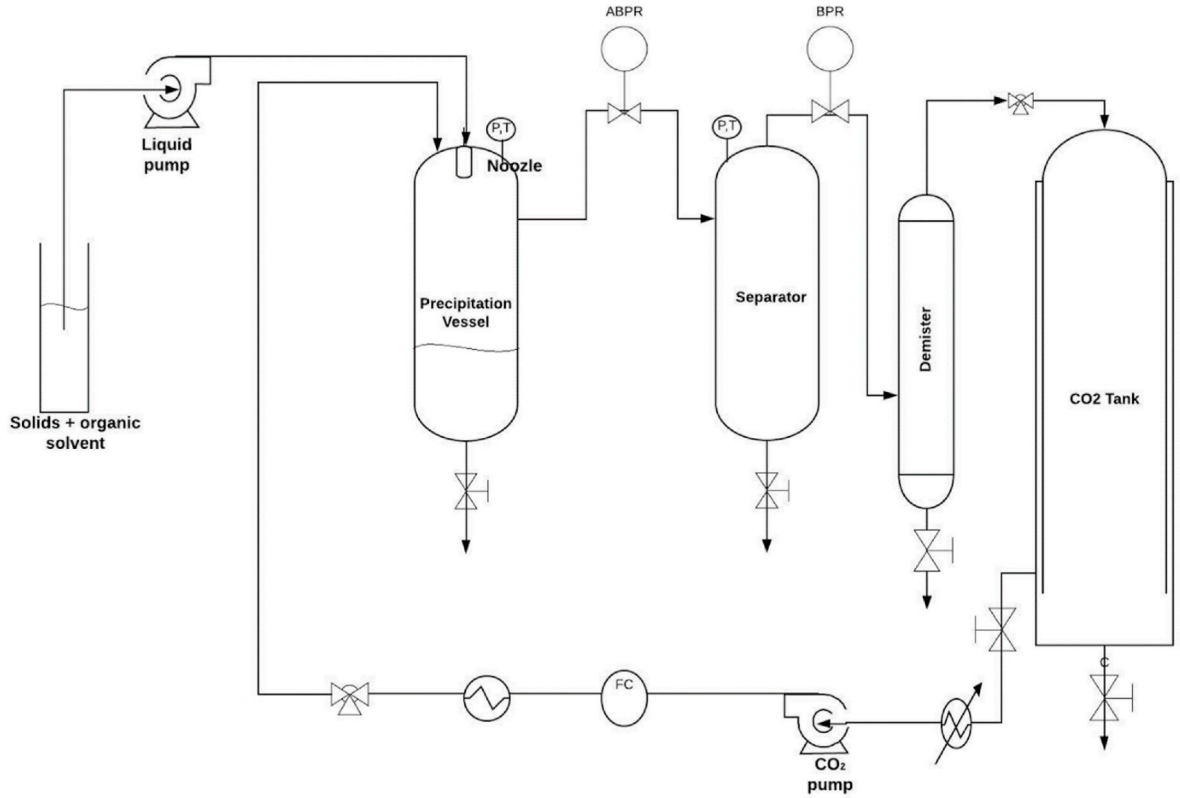

Fig. 1. Schematic diagram of the SAS process. ABPR: Automatic back pressure regulator, BPR: manual back pressure regulator, P: manometer, T: temperature probe, FC: flowmeter.

resulting in products with better particle size distribution, morphology and smaller particle sizes, in comparison with conventional techniques (Mezzomo & Ferreira, 2013).

SAS precipitation is based on the continuous contact between supercritical carbon dioxide ($SCCO_2$) and a solution containing an organic solvent and the targeted bioactive compounds. The solution is introduced in the precipitation vessel through a nozzle, forming small drops. The $SCCO_2$ penetrate in the droplets, inducing the solution supersaturation, followed by the bioactive substance precipitation (anti-solvent effect) into small solid and dry particles (Langa et al., 2019; Martín & Cocero, 2008). To obtain dry particles, the complete removal of the organic solvent from the precipitation vessel is required (Reverchon, Torino, Dowy, Braeuer, & Leipertz, 2010). Therefore, the operating conditions depend largely on the solvent used and the CO_2 + solvent phase equilibria behavior, what in turn influence the morphology and size of the particles produced (De Marco, Knauer, Cice, Braeuer, & Reverchon, 2012).

SAS technique was extensively used in the last years in pharmaceutical applications (Deshpande et al., 2011; Girotra, Singh, & Nagpal, 2013; Sarkari, Darrat, & Knutson, 2000). Many bioactive pure substances, such as quercetin, caffeine, β-carotene, ellagic acid ibuprofen, mandelic acid, curcumin, among others, were micronized using SAS technique. Furthermore, this method was used to simultaneously fractionate and precipitate complex mixtures of phytochemicals (e.g. vegetal extracts), such as the case of rosemary (Quintana et al., 2019b), mango (Guamán-Balcázar, Montes, Pereyra, & Martínez de la Ossa, 2019), orange (Montes et al., 2019) and yarrow leaves (Villanueva-Bermejo et al., 2017). Nevertheless, even though the extraction of licorice bioactives has been studied and described in the literature, the licorice SAS precipitation has not been reported yet. In this work, the SAS fractionation and precipitation of a licorice ethanolic extract to produce micro- and nano-particles was studied for the first time. The effect of process parameters on the recovery of licorice bioactive compounds was analyzed, along with the content of total phenolic compounds and antioxidant activity of the products obtained, and the morphology and particle size distribution of the precipitates.

2. Materials and methods

2.1. Chemicals

CO_2 (99.98% purity) was supplied from Carburos Metálicos (Madrid, Spain). Ethanol (99.8% purity), Sodium Carbonate anhydrous (99.5% purity) and Folin-Ciocalteu's reagent were purchased from Panreac (Barcelona, Spain). Gallic acid standard (> 98% purity), 2,2-Diphenyl-1-pycrilhydrazyl (DPPH, 95% purity), (±)-6-Hydroxy-2,5,7,8-tetramethyllchromane-2-carboxylic acid (Trolox, 97% purity), liquiritin, liquiritigenin, isoliquiritigenin, glabridin and glycyrrhizic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Orthophosphoric acid (85% purity) was purchased from Scharlab S.L. (Sentmenat, Spain). Acetonitrile (99,8% purity) was purchased from Macron (Poland).

2.2. Preparation of licorice ethanolic solutions

Roots of licorice harvested in Spain were obtained from Murciana herbalist's (Murcia, Spain) with water content of 9.90% wt. The sample was ground using a Grindomix GM 200 knife mill (Verder International B.V., Vleuten, Netherlands) in particles with size lower than 500 μm. Then, ultrasound assisted extraction (UAE) using an ultrasonic device (Branson Digital Sonifier 550 model, Danbury, USA) with an electric power of 550 W and frequency of 20 kHz was accomplished. The extraction was carried out at 323 K for 15 min using ethanol at 1:10 (w/v) plant/solvent ratio. Ethanol was selected as solvent because is a food grade solvent, and due to its high solubility in $SCCO_2$ which allows the completely elimination of the organic solvent during SAS process and

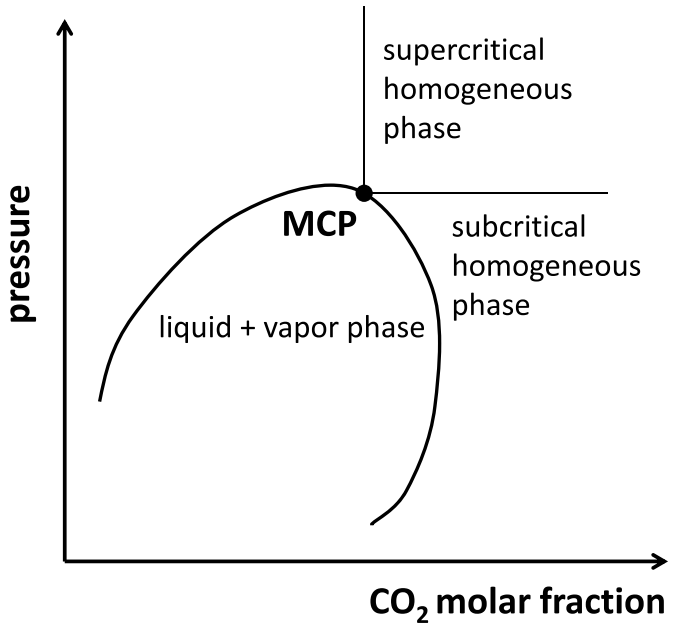

Fig. 2. Scheme of the pressure vs. composition phase diagram of the binary mixture of $SCCO_2$ + ethanol. MCP: mixture critical point.

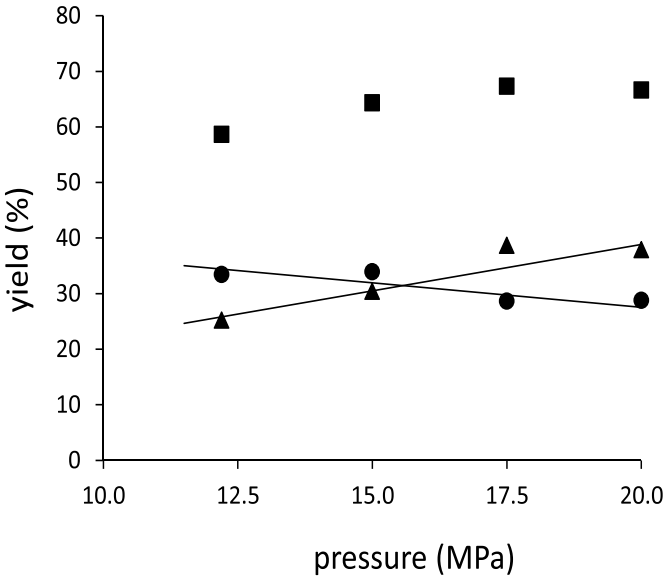

Fig. 3. Precipitate (●), oleoresin, (▲) and total (precipitate + oleoresin) (■) yields as a function of SAS precipitation pressure, corresponding to experiments 3 to 6 of Table 1 (308.15 K and LES concentration of 14.2 mg/ml). (——) Trend line.

thus, the production of dry powders of licorice phytochemicals. Extraction yield was 3.18% (mass of phytochemicals extracted/mass of plant material utilized) and the concentration of licorice phytochemicals in the ethanolic solution was 14.2 mg/ml (LES1). This ethanolic solution (704.2 ml) was further diluted with ethanol to a final volume of 1000 ml to obtain another ethanolic solution containing 9.6 mg/ml (LES2) of licorice phytochemicals. Both ethanolic solutions (14.2 mg/mL and 9.6 mg/mL) were stored at 253.15 K for its use in the SAS process.

2.3. Supercritical antisolvent (SAS) precipitation

Fig. 1 shows the supercritical antisolvent precipitation device used for this study (Model Thar SF2000, Thar Technology, PA, USA). A detailed description of the equipment can be found elsewhere (Villanueva-Bermejo et al., 2017). The equipment comprises a precipitation vessel and a separator with independent control of temperature and pressure. The precipitation vessel (273 ml) is equipped with a 101.6 μm inner diameter nozzle to spray the ethanolic solution. $SCCO_2$ and the ethanolic solution are fed from the top in a co-current manner (coaxial nozzle).

$SCCO_2$ was pumped at 50 g/min flow rate until pressure and temperature conditions were attained into the precipitation vessel. Then, the licorice ethanolic solution (LES) was pumped through the nozzle at 2 g/min for 45 min, while maintaining the $SCCO_2$ flow rate. Additional $SCCO_2$ was pumped during 15 min to wash out the residual solvent from the precipitator. During the process, the separator was kept at 313.15 K and ambient pressure. In the separator, ethanol and the phytochemicals which did not precipitate into the precipitation vessel (i.e. the licorice phytochemicals which are soluble in the $SCCO_2$ + ethanol supercritical phase) were recovered. Finally, the precipitation vessel was depressurized, and the precipitate was collected from a frit placed at the bottom of the precipitator vessel. The ethanolic fraction was further rotary evaporated until an oleoresin-type product was obtained. Samples (oleoresins and precipitates) were kept at 253.15 K under darkness until analysis.

2.4. Total phenolic compounds and antioxidant activity

Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999) was used to determine the total phenolic compounds (TPC) content in the samples. In order to determine the antioxidant capacity of the samples RPPH assay was done following the procedure describe by Brand-Williams, Cuvelier, and Berset (1995). All analyses were done in triplicate.

Table 1
SAS conditions in the fractionation and precipitation of licorice ethanolic solution (LES). Yield (Y) expressed as mass recovered/mass of licorice phytochemicals feed, total phenolic compounds content (TPC) expressed as GAE (mg of gallic acid equivalents/g), antioxidant activity expressed as TEAC (mmol Trolox equivalent/ml) and IC_{50} values (μg/ml). LES concentration = 14.2 mg/ml. $SCCO_2$ and LES flows were, respectively, 50 and 2 g/min. Precipitation time = 45 min.

SAS conditions			Precipitate (P)				Oleoresin (O)			
Exp.	P/MPa	T/K	Yield %	TPC/mg/g	TEAC/mmol/ml	IC_{50}/μg/ml	Yield %	TPC/mg/g	TEAC/mmol/ml	IC_{50}/μg/ml
1	15.0	313.15	13.28 ± 9.21[a]	145.2 ± 3.4[b]	0.690 ± 0.015[a]	18.37 ± 0.73[a]	41.35 ± 17.02[a]	166.9 ± 3.2[a]	0.484 ± 0.01[a]	26.67 ± 0.16[a]
2	20.0	313.15	23.48 ± 8.11[a]	133.1 ± 1.8[b]	0.709 ± 0.021[a]	18.19 ± 0.40[a]	34.75 ± 16.50[a]	161.0 ± 5.1[a]	0.524 ± 0.01[a]	24.70 ± 0.55[a]
3	12.5	308.15	33.42 ± 3.34[a]	167.1 ± 9.4[a]	0.760 ± 0.023[a]	16.88 ± 0.25[a]	25.23 ± 3.14[a]	160.0 ± 10.6[a]	0.483 ± 0.03[a]	26.94 ± 2.06[a]
4	15.0	308.15	33.93 ± 1.41[a]	154.4 ± 11.4[a]	0.764 ± 0.015[a]	16.98 ± 0.09[a]	30.40 ± 1.29[a]	167.8 ± 5.1[a]	0.511 ± 0.02[a]	25.56 ± 1.33[a]
5	17.5	308.15	28.64 ± 1.22[a]	152.3 ± 11.1[a]	0.711 ± 0.028[a]	18.37 ± 1.13[a]	38.66 ± 4.03[a]	164.9 ± 3.9[a]	0.487 ± 0.02[a]	26.48 ± 1.32[a]
6	20.0	308.15	28.77 ± 0.45[a]	157.7 ± 3.1[a]	0.697 ± 0.035[a]	18.75 ± 1.15[a]	37.89 ± 9.40[a]	160.3 ± 23.9[a]	0.481 ± 0.02[a]	27.15 ± 1.09[a]

[a, b] Different letters within each column are significantly different at $p < 0.05$ when compared using Tukey's test.

Table 2
SAS conditions in the fractionation and precipitation of licorice ethanolic solution (LES). Yield (Y) expressed as mass recovered/mass of licorice phytochemicals feed, total phenolic compounds content (TPC) expressed as GAE (mg of gallic acid equivalents/g), antioxidant activity expressed as TEAC (mmol Trolox equivalent/ml) and IC_{50} values (μg/ml). LES concentration = 9.6 mg/ml. $SCCO_2$ and LES flows were, respectively, 50 and 2 g/min. Precipitation time = 45 min.

SAS conditions			Precipitate (P)				Oleoresin (O)			
Exp.	P/MPa	T/K	Yield %	TPC/mg/g	TEAC/mmol/ml	IC_{50}/μg/ml	Yield %	TPC/mg/g	TEAC/mmol/ml	IC_{50}/μg/ml
7	15	308.15	52.70 [a]	160.8 ± 3.2[a]	0.734 ± 0.008 [a]	17.29 ± 0.59 [a]	38.25 [a]	160.3 ± 23.9 [a]	0.498 ± 0.001 [a]	26.00 ± 0.11 [a]
8	20	308.15	30.35 [a]	163.8 ± 4.8[a]	0.776 ± 0.005 [a]	16.67 ± 0.08 [a]	37.62 [a]	168.9 ± 5.6 [a]	0.456 ± 0.006 [a]	27.89 ± 0.84 [a]
9	15	313.15	44.20 [a]	161.4 ± 3.5[b]	0.721 ± 0.003 [a]	18.11 ± 0.31 [a]	36.63 [a]	150.2 ± 4.4 [a]	0.445 ± 0.002 [a]	28.81 ± 0.49 [a]
10	20	313.15	33.95 [a]	148.8 ± 7.3[b]	0.733 ± 0.008 [a]	17.36 ± 0.54 [a]	56.63 [a]	177.8 ± 6.2 [a]	0.511 ± 0.016 [a]	25.28 ± 0.55 [a]

[a, b] Different letters within each column are significantly different at $p < 0.05$ when compared using Tukey's test.

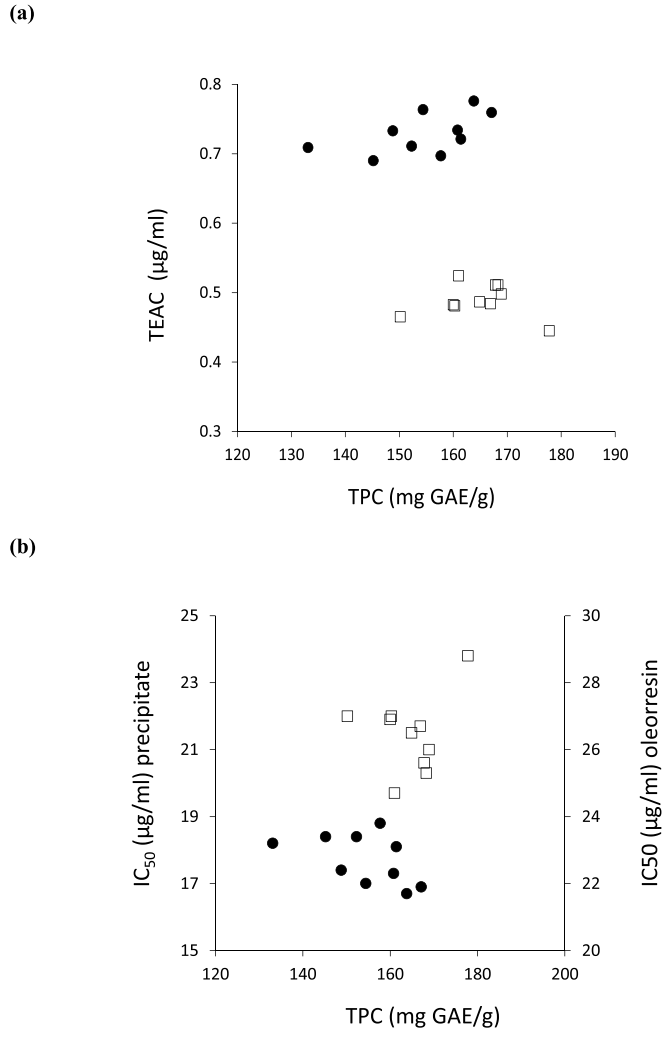

Fig. 4. (a) TEAC values and (b) IC_{50} values of (●) precipitates and (□) oleoresins as a function of total phenolic compounds (TPC, mg GAE/g).

2.5. HPLC-DAD analysis

HPLC analysis was carried out by duplicate using the method described by Wei, Yang, Chen, Wang, and Cui (2015). A LC-2030C 3D Plus (Shimadzu) device equipped with a quaternary pump, auto-injector and DAD detector was used. The column was ACE Kromasil 100 C18 (250 × 4.6 mm; 5 μm) and analyses were accomplished at 298 K. The mobile phase comprised acetonitrile (A) and 0.026% aqueous H_3PO_4 (v/v), and the following elution gradient was applied: 20–25% A for 0–20 min, 25–34% A for 20–30 min, 34–50% A for 30–50 min, 50–60% A at 50–60 min and 60% A for 60–80 min. The initial conditions were attained in 5 min. The flow rate was 0.7 ml/min and was kept constant throughout the analysis. The injection volume was 20 μl and the detections were carried out at 230, 254, 280 and 370 nm. Calibration curves with standards were used to determine the content of the bioactive licorice phytochemicals (liquiritin, liquiritigenin, isoliquiritigenin, glabridin and glycyrrhizic acid) in the different samples.

2.6. Morphology and particle size analysis

The morphology of precipitates was studied by scanning electron microscopy (SEM) with an energy-dispersive X-ray spectrometer (SEM-EDS) XL-30S FEG, Philips (Japan). Samples were placed on carbon tapes and then were coated with a thin chrome layer by a sputter coater. Particle size and size distributions were carried out by duplicate using light scattering with a laser diffraction system Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK) equipped with a wet dispersion unit.

2.7. Statistical analysis

The statistical analysis of the data was performed using Minitab 19 ((Minitab, LLC, State College, PA). Results were expressed as mean ± standard error. The significant difference between mean values was determined using one-way ANOVA with Turkey HSD (Honestly-significant-difference) grouping at 95% confidence level.

3. Results and discussion

3.1. The supercritical antisolvent process

The CO_2 + ethanol + licorice phytochemicals is a complex multicomponent system and the phase equilibria of this mixture strongly affect the performance and the result of SAS process. The temperature and pressure of the mixture critical point (MCP) in comparison with SAS temperature and pressure conditions may determine the success of the precipitation process, since affect jet mixing, fluid dynamics and mass transfer (Reverchon et al., 2010). These complex mechanisms are responsible for the great variety of particle sizes and morphologies that can be obtained in SAS precipitation process; it was described in the literature (Reverchon et al., 2010), these mechanisms strongly depend on the SAS temperature and pressure conditions, which can be located below the MCP, near above the MCP or far above the MCP (Fig. 2).

In general, it was stated (Reverchon & De Marco, 2011) that when SAS conditions are below the MCP but in the homogenous subcritical region, the formation of particles is induced by the $SCCO_2$ antisolvent effect and by the organic solvent depletion in the droplets formed by the nozzle. Consequently, microparticles and expanded microparticles (hollow core particles) with irregular forms are obtained. Nevertheless, if the SAS subcritical conditions are located within the liquid-vapor region, irregular particles and agglomerates are produced due to the presence of residual solvent in the precipitate. On the other hand, when

Table 3
Licorice bioactive compounds identified and quantified (mg/g) in SAS precipitates and oleoresins (HPLC-DAD analysis).

	Liquiritin	Liquiritigenin	Glycyrrhizic acid	Isoliquiritigenin	Glabridin
UAE extract	5.23 ± 0.01[a]	0.73 ± 0.05[a]	0.52 ± 0.17[a]	0.63 ± 0.00 [a]	28.99 ± 2.13[a]
(a) Precipitates					
1	8.34 ± 0.06[b]	0.58 ± 0.02[a]	0.32 ± 0.01[a]	0.52 ± 0.00 [ab]	12.75 ± 0.38[b]
2	8.11 ± 0.07[b]	0.32 ± 0.01[a]	0.28 ± 0.00[a]	0.46 ± 0.00 [b]	10.89 ± 0.03[b]
3	9.12 ± 0.77[b]	0.26*	0.40 ± 0.03[a]	0.60 ± 0.01[ab]	23.29 ± 6.63[ab]
4	8.59 ± 0.34[b]	0.57*	0.37 ± 0.04[a]	0.56 ± 0.01[ab]	16.31 ± 0.74[b]
5	8.92 ± 0.24[b]	0.57 ± 0.18[a]	0.43 ± 0.10[a]	0.54 ± 0.01[ab]	16.26 ± 1.44[b]
6	8.72 ± 0.25[b]	0.56 ± 0.04[a]	0.44 ± 0.05[a]	0.52 ± 0.01[b]	14.82 ± 1.76[b]
7	8.84 ± 0.00[b]	0.38 ± 0.00[a]	0.42 ± 0.00[a]	0.48 ± 0.00[ab]	12.66 ± 0.08[b]
8	9.37 ± 0.01[b]	0.44 ± 0.07[a]	0.56 ± 0.16[a]	0.47 ± 0.00[b]	10.94 ± 1.77[b]
9	8.05 ± 0.02[b]	0.58 ± 0.00[a]	0.39 ± 0.01[a]	0.52 ± 0.00[ab]	12.53 ± 3.90[b]
10	9.61 ± 0.11[b]	0.56 ± 0.05[a]	0.42 ± 0.00[a]	0.50 ± 0.01[b]	15.23 ± 5.40[b]
(b) Oleoresins					
1	0.89 ± 0.00[c]	0.89 ± 0.00[a]	0.22 ± 0.00[b]	0.81 ± 0.00 [b]	56.26 ± 1.59[c]
2	0.71 ± 0.01[c]	0.95 ± 0.01[a]	0.25 ± 0.03[b]	0.83 ± 0.01 [ab]	51.90 ± 0.96[c]
3	n. d.	0.87 ± 0.03[a]	0.19 ± 0.01[b]	0.79 ± 0.02 [ab]	57.07 ± 0.99[c]
4	0.74 ± 0.01[c]	0.84 ± 0.02[a]	0.18 ± 0.01[b]	0.78 ± 0.01 [ab]	54.70 ± 1.17[c]
5	n. d.	1.02 ± 0.15[a]	0.22 ± 0.03[b]	0.87 ± 0.08 [a]	61.66 ± 8.00[c]
6	n. d.	0.89 ± 0.09[a]	0.22 ± 0.00[b]	0.88 ± 0.06 [a]	54.562 ± 2.38[c]
7	n. d.	1.00 ± 0.00[a]	0.19 ± 0.00[b]	0.84 ± 0.00 [ab]	53.83 ± 0.20[c]
8	0.72 ± 0.01[c]	1.09 ± 0.29[a]	0.23 ± 0.07[b]	0.89 ± 0.14 [a]	57.67 ± 13.25[c]
9	n. d.	0.73 ± 0.05[a]	0.18 ± 0.08[b]	0.72 ± 0.02 [ab]	55.11 ± 4.56[c]
10	n. d.	1.13 ± 0.11[a]	0.21 ± 0.01[b]	0.93 ± 0.05 [ab]	62.53 ± 5.47[c]

n.d.: non detected.
* No duplicate available.
[a, b, c] Different letters within each column are significantly different at $p < 0.05$ when compared using Tukey's test.

SAS conditions are far above the MCP, the mixing of CO_2 with the solvent is produced instantaneously and no liquid-gas interphase occurs, resulting in smaller and more regular particles due to their condensation from a gaseous phase.

Due to the lack of information about phase equilibria of the complex mixtures CO_2 + ethanol + licorice phytochemicals, the $SCCO_2$ and licorice ethanolic solution (LES) flow rates were established with the aim of attaining a homogenous supercritical phase (≈3% mass ethanol) at the pressures and temperatures studied, according to the CO_2 + ethanol binary phase equilibria data (Chang, Day, Ko, & Chiu, 1997; Joung et al., 2001; Knez, Škerget, Ilič, & Lütge, 2008; Reverchon & De Marco, 2011). Indeed, this is an approximation, since the presence of a large number and varied phytochemicals in the supercritical phase may really change the MPC in comparison with that of the binary CO_2 + ethanol.

3.2. Effect of pressure, temperature and concentration of phytochemicals in the licorice ethanolic solution on SAS precipitation yield

Table 1 shows the results obtained by SAS process with LES1 (14.2 mg/ml) at different precipitation pressures and temperatures, reporting the precipitate and oleoresin yields, together with the TPC, TEAC and IC_{50} values. All SAS experiments were carried out by duplicate and the average deviations are given (Table 1).

The SAS pressure and temperature did not present statistically significant differences ($p > 0.05$) on the precipitation yield, but it has to be highlighted that the precipitates obtained in experiments 1 and 2 in Table 1 (both at 313.15 K) were very viscous, with large agglomerates adhered to the precipitation vessel walls, and they were difficult to recover and to quantify their weight and thus, high deviations were obtained. On the other hand, for the rest of experiments reported in Table 1, which were performed at 308.15 K (experiments 3 to 6), solid and dry powders were obtained, and the average deviation of precipitation yields between duplicates was lower than 3.34% (mean deviation of 1.61%). Then, it was observed an important effect of pressure and temperature conditions on the precipitate morphology.

The general trend of precipitates and oleoresins yields vs. pressure can be observed in Fig. 3 for experiments 3 to 6 of Table 1 (LSE1 at 308.15 K). Yield mean values are depicted in the figure as a function of SAS precipitation pressure. The lower pressures (12.5 and 15.0 MPa) brought about higher precipitate yields than oleoresin yields, while the opposite effect occurred at the higher pressures (17.5 and 20.0 MPa). Other authors also observed an increase of precipitation yield at constant temperature when pressure is decreased, as is the case of Osorio-Tobón, Carvalho, Rostagno, Petenate, and Meireles (2016) investigating the SAS precipitation of curcuminoids from an ethanolic turmeric extract, and Visentín, Cismondi, and Maestri (2011) in the study of rosemary ethanolic extracts SAS fractionation as a method to improve carnosic acid recovery. This general trend can be supported by the well-known change of phytochemicals solubility with $SCCO_2$ density. As the pressure in the precipitation vessel increases at constant temperature, the $SCCO_2$ density increases and thus, the solubility of licorice phytochemicals in the supercritical phase also increases, resulting in a decrease of precipitation yield. Then, while lower amounts of solid powders are recovered in the precipitates, larger amounts of oleoresins are recovered from the separator. Furthermore, it can be observed that total recovery of licorice phytochemicals (mass in precipitate and oleoresin/mass feed into SAS process) reached values in the range 58–67% with LES1 (experiments 3–6 in Table 1) which were lower than in the case of LES2 (68–91% Table 2).

Table 2 show the results of SAS precipitation assays when the concentration of the licorice ethanolic solution was 9.6 mg/ml (LES2). Experiments were carried out at 308.15 and 313.15 K and pressures of 15 and 20 MPa. Since in previous experiments SAS pressure and temperature did not affect significantly precipitation yield, no duplicates were accomplished with LES2. Nevertheless, it can be observed that also in this case, at constant temperature, the lower pressures resulted in higher precipitation yields. Dry powders were obtained in all the experiments reported in Table 2, including those assays carried out at 313.15 K. Higher yields were obtained with LES2, being the mean precipitation yield 40.30 ± 10.14%, significantly higher than the yields obtained with LES1 (mean value 26.92 ± 7.16%). Additionally, it can be observed from Tables 1 and 2 that the total yields of SAS process (precipitate + oleoresin) were higher at the lower

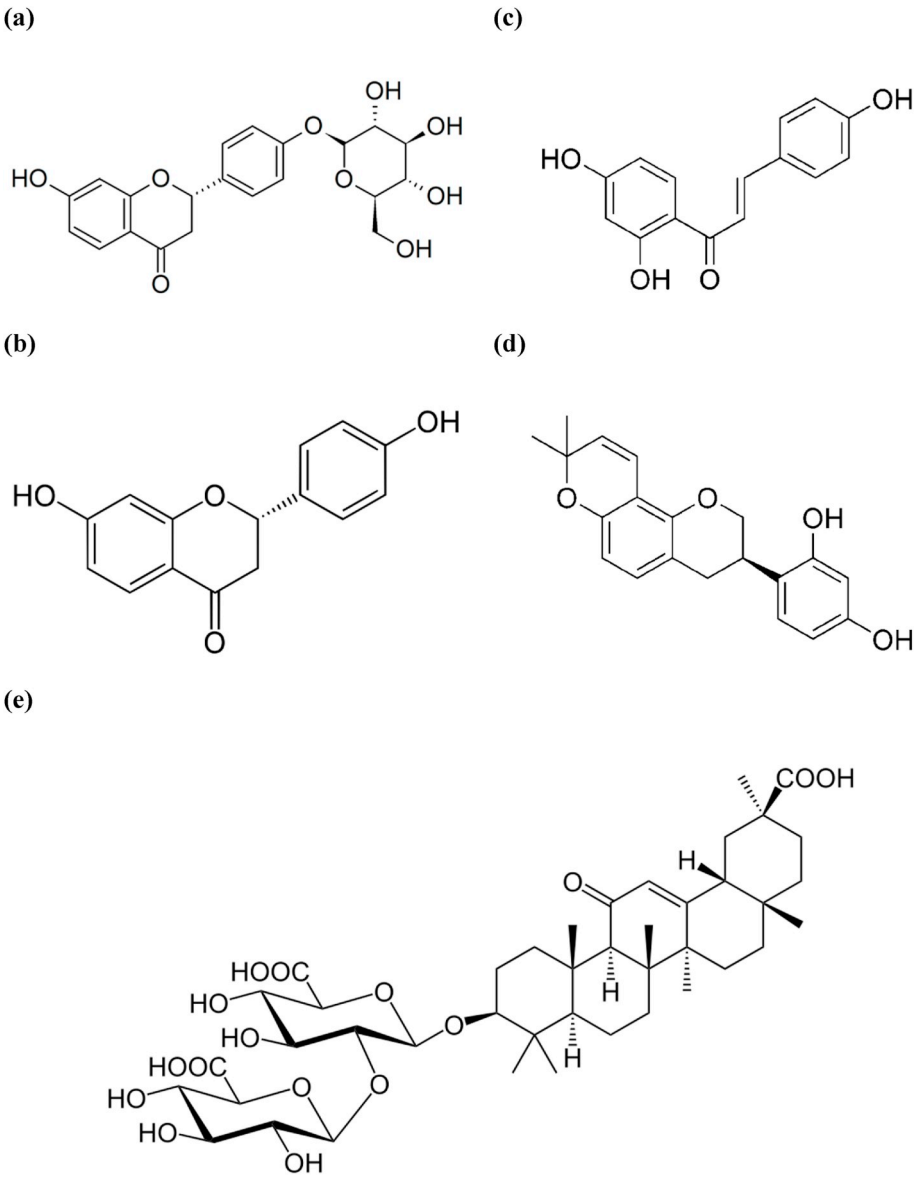

Fig. 5. Chemical structure of (A) liquiritin, (B) liquiritigenin, (C) isoliquiritigenin and (D) glabridin and (E) glycyrrhizic acid.

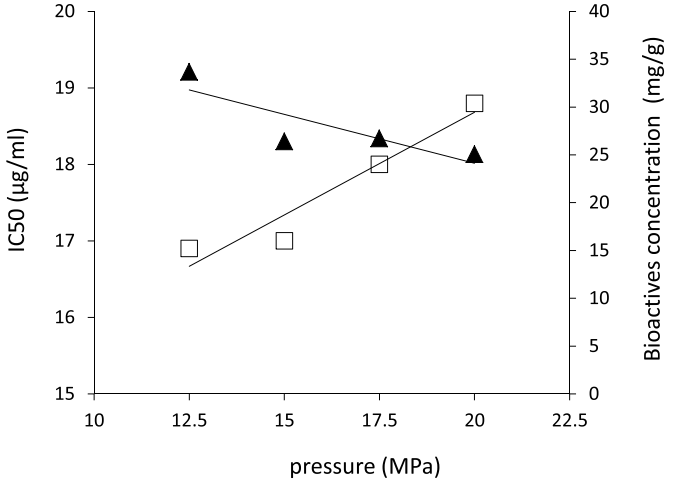

Fig. 6. (□) IC_{50} values and (▲) summation of licorice bioactives concentrations (Table 3) as a function of pressure at 308.15 K and 14.2 mg/ml LES.

concentration of the licorice ethanolic solution used. For example, experiment 7 in Table 2 shows a 1.5-fold increase of process yield (91%) in comparison with its counterpart at 14.2 mg/ml (experiment 4 in Table 1).

The different behavior observed at 313.15 K when using the different licorice ethanolic solutions may be due to an expected higher MCP of LES1 in comparison with the MCP of LES2, as a result of the higher concentration of phytochemicals in LES1. Thus, it is possible that SAS operation conditions were subcritical for LES1 while supercritical for LES2. Furthermore, higher concentration of phytochemicals results in higher solution viscosity and may impair atomization, as reported by (Prosapio, De Marco, & Reverchon, 2018). Then, experiments with LES1 at 313.15 K lead to the coalescence of particles, forming agglomerates, while experiments with LES2 at the same temperature resulted in dry powders.

3.3. Phenolic compounds and antioxidant activity of precipitates and oleoresins

Tables 1 and 2 show the content of total phenolic compounds (TPC) and the antioxidant activity (TEAC and IC_{50} values) of precipitates and

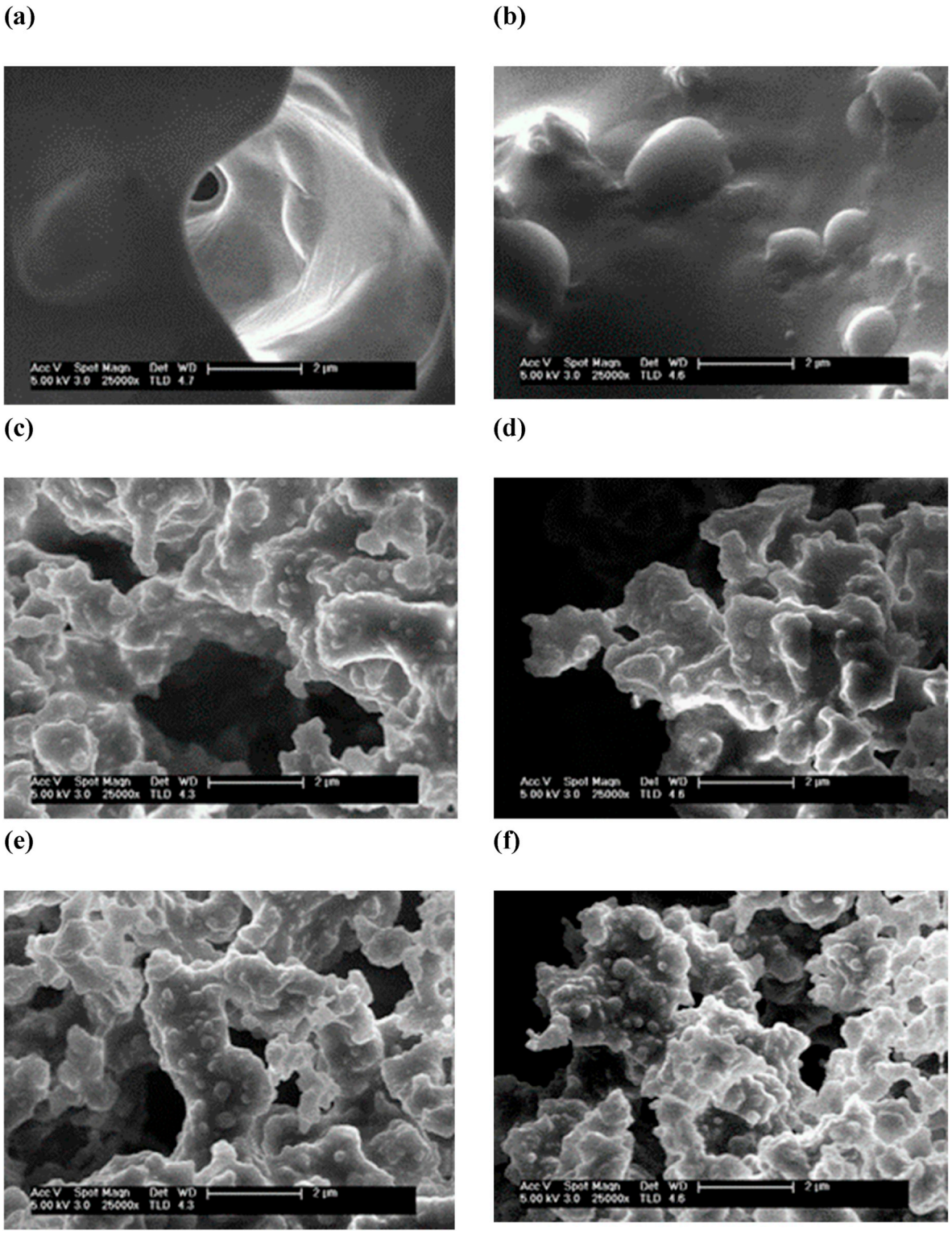

Fig. 7. SEM images (25000 x) of precipitates obtained with LES1 (14.2 mg/ml): (a) to (f), experiments 1 to 6 in Table 1.

oleoresins obtained by SAS process. In the case of precipitates, the ANOVA test demonstrated that SAS pressure did not present statistically significant differences ($p > 0.05$) on TPC, TEAC or IC_{50} values, but SAS temperature statistically affects ($p < 0.05$) the content of TPC in precipitates, being the TPC values lower at 313.15 K (both with LES1 and LES2). In the analysis of oleoresins TPC values, none pressure, temperature or licorice extract concentration demonstrated statistically significant differences ($p > 0.05$). Furthermore, no statistically significant differences were observed between precipitates and oleoresins concerning the content of total phenolic compounds.

Regarding the effect of SAS pressure, temperature and licorice extract concentration on the antioxidant activity (TEAC and IC_{50} values) no statistical differences were observed either in the analysis of precipitates or oleoresins. Nevertheless, significant differences were determined between the antioxidant activity of precipitates and oleoresins. The precipitates TEAC values obtained were in the range

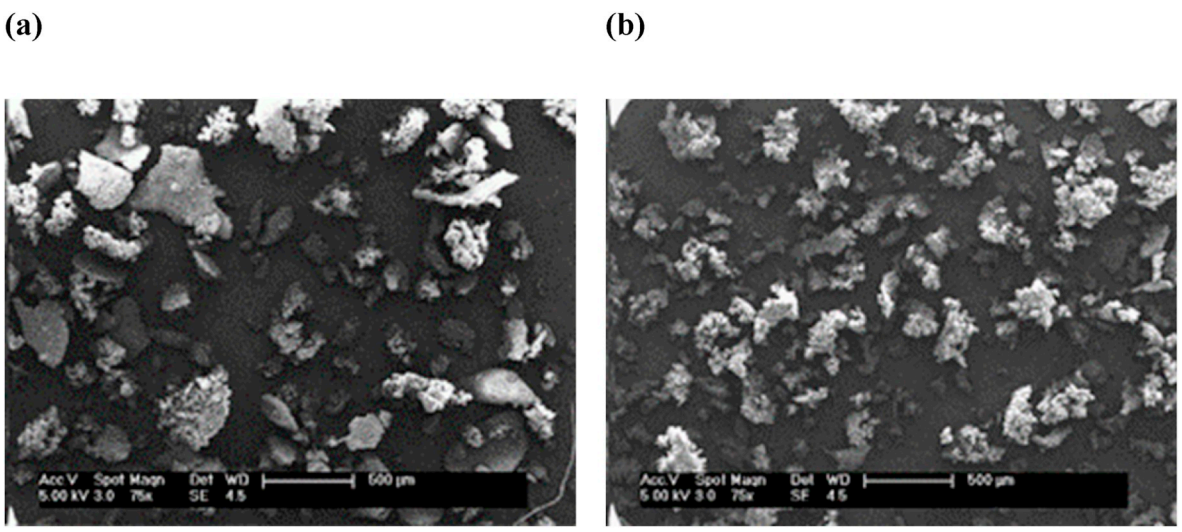

Fig. 8. SEM imagens (75x) of precipitates obtained at 313 K with LES1 (14.2 mg/ml): (a) experiment 1 and (b) experiment 2 of Table 1.

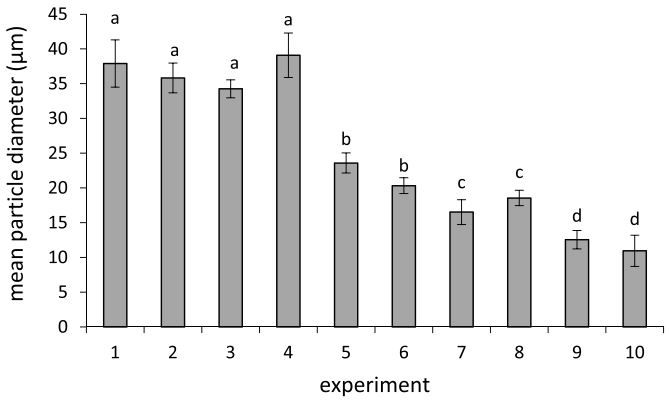

Fig. 9. Mean particle diameters (μm) of SAS licorice precipitates obtained in experiments 1 to 10 of Tables 1 and 2 Different letters within each column are significantly different at $p < 0.05$ (Tukey's test).

0.731 ± 0.039 mg/g, statistically higher than the oleoresins TEAC values (0.486 ± 0.035 mg/g). Consequently, significantly lower IC_{50} values were obtained in precipitates (17.64 ± 1.11 μg/ml) in comparison with IC_{50} values of oleoresin (26.62 ± 1.09 μg/ml).

Since phenolic compounds are substances with recognized antioxidant activity it is generally stated that the higher the TPC the higher the antioxidant activity, that is the higher TEAC values and the lower IC_{50} values. The TEAC and IC_{50} values obtained in precipitates and oleoresins are depicted in Fig. 4 as a function of TPC. In general, as can be observed in Fig. 4(a), there is no clear relationship (e.g. linear relation) between TEAC and TPC values but is apparent that the precipitates presented higher TEAC values than oleoresins for the same TPC concentration. This means that different type and phenolic compounds are present in precipitates and oleoresins, being the phenolic compounds present in the precipitate of greater antioxidant capacity. Accordingly, the IC_{50} values of the precipitates are lower than those corresponding to oleoresins, as can be observed in Fig. 4(b).

The licorice precipitates obtained in this work presented higher antioxidant activity than licorice extracts reported previously in the literature. For example (Somaris E. Quintana, Cueva, et al., 2019), obtained licorice extracts with IC_{50} values in the range 21.9–248.1 μg/ml using $SCCO_2$ extraction at different pressures, CO_2 flow rates and ethanol cosolvent percentages. Furthermore, Vlaisavljević et al. (2018) used a mixture of methanol (70%) - water (30%) enhanced with an ultrasonic bath, and the antioxidant scavenging activity of the licorice extracts obtained were c.a. 72–75% of the values obtained in this work. Finally, Yang, Zhang, Yu, Gao, and Sun (2019) reported IC_{50} values of 85.4–140.4 μg/ml for licorice extracts obtained with a mixture of methanol/water/phosphoric acid (79.5/20/0.5 v/v/v) in an ultrasonic bath.

3.4. SAS fractionation of licorice phytochemicals

As mentioned before, in the case of SAS precipitation of ethanolic plant extracts, the fractionation of its bioactive substances is generally carried out, due to the different solubility of the plant extract components in the supercritical CO_2 + ethanol phase (Villanueva Bermejo et al., 2015; Villanueva-Bermejo et al., 2017).

Table 3 presents some key licorice bioactive compounds (Fig. 5) identified in both, precipitates and oleoresins. In general, no statistical differences can be observed of SAS operating conditions (pressure, temperature and concentration of licorice extract) on the concentration of the key bioactive compounds studied when precipitates or oleoresins are analyzed. Nevertheless, important and statistically significant differences ($p < 0.05$) can be observed between the concentration of liquiritin, glycyrrhizic acid and glabridin in the UAE extract, precipitates and oleoresins. That is, a fractionation of these compounds has been accomplished at the same time that the precipitation process occurred. In general, glabridin and isoliquiritigenin compounds are more abundant in the oleoresins, while liquiritin and glycyrrhizic acid are concentrated in the precipitates. The observed trend may be explained considering the polarity of these compounds, which is related to their chemical structure. The most polar compounds are less soluble in the supercritical phase (CO_2 + ethanol cosolvent) and thus these polar compounds should preferable precipitate. On the contrary, the less polar compounds (more soluble in the supercritical phase) should be preferable recovered in the separator, together with the ethanol cosolvent. Both liquiritigenin and glabridin are the most non-polar compounds identified, with only two hydroxyl groups in their structure. Isoliquiritigenin has a structure similar to liquiritigenin but the latter is a flavanone and isoliquiritigenin is a chalcone (flavanone precursor). The chalcones have the central ring open, so they have a free hydroxyl group that gives it greater polarity compared to the flavone liquiritigenin. Glycyrrhizic acid is a glycosylated terpenoid, and despite its terpenoid part, the glycosylated sugar provides some polarity to this acid, producing its concentration in the precipitate. Finally, liquiritin is the most polar compound of those studied (with 5 hydroxyl groups in its chemical structure) and it is observed that it is most abundant in the precipitate.

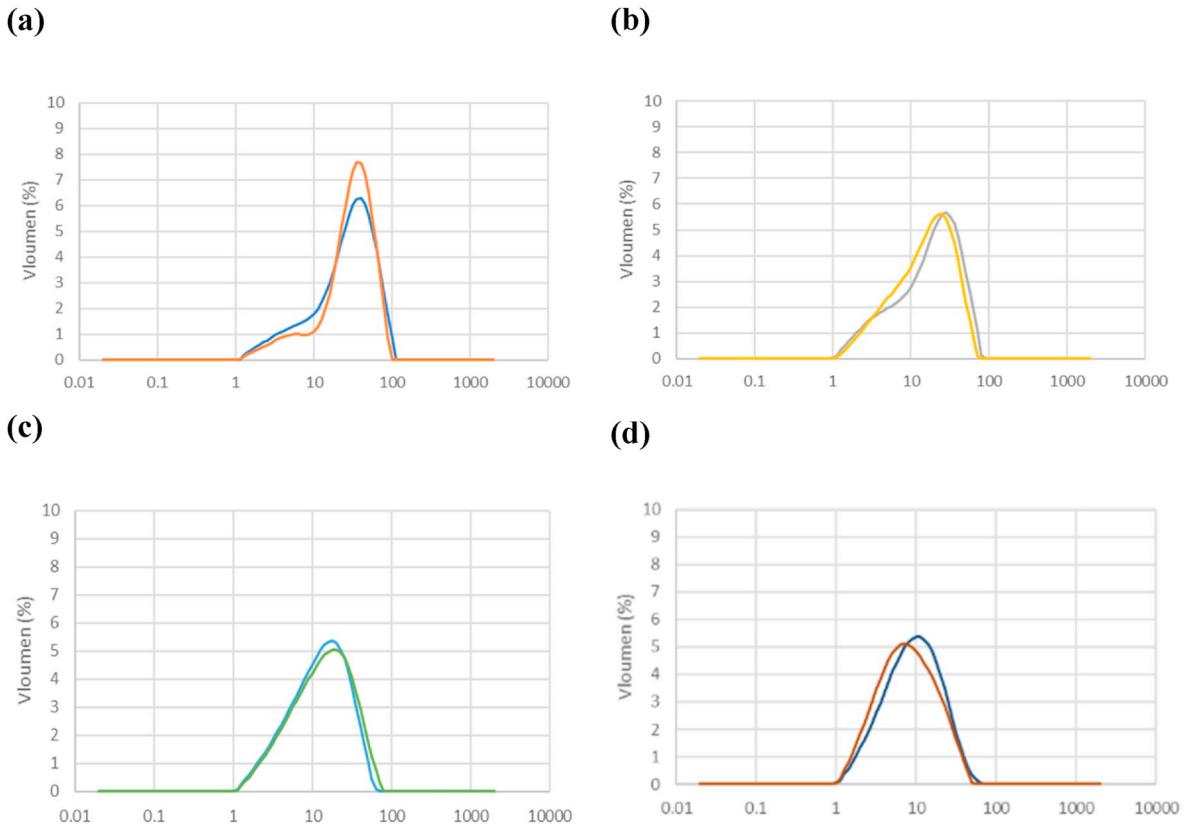

Fig. 10. Particle size distribution (μm) of precipitates obtained at 308.15 K with LES1 (14.2 mg/g). Duplicate experiments 3 to 6 of Table 1: (a) 12.5 MPa; (b) 15 MPa; (c) 17.5 MPa; (d) 20 MPa.

Kaur et al. (2013) pointed out that glabridin and isoliquiritigenin are key compounds responsible for the antioxidant activity of licorice root. Nevertheless, in our study, although oleoresins presented higher concentrations of glabridin than precipitates (2.5–5.3 times higher) the IC_{50} values of oleoresins resulted significantly higher than that of precipitates. This means that, among the bioactives studied in this work, glabridin cannot be identified as the crucial licorice antioxidant compound.

Fig. 6 shows for experiments 3 to 6 (308.15 K and 14.2 mg/ml of the licorice ethanolic solution) the variation with pressure of the precipitate IC_{50} mean values and the summation of the licorice bioactives concentrations (mg/g) determined by HPLC (Table 3). It can be observed in Fig. 6 that the mean IC_{50} values have a tendency to decrease with decreasing pressure, possibly as a result of the increasing sum of the concentration of these bioactives. Then, the precipitates with better antioxidant activity also contain larger amounts of these licorice key bioactives. Therefore, it could be concluded that the bioactive compounds studied in this work influence the antioxidant activity of licorice SAS precipitates.

3.5. Morphology and particle size of precipitates

Taking into account the phase equilibria of the binary CO_2 + ethanol mixture [43–45] the corresponding critical pressures at 308.15 K and 313.15 K are both lower than 10 MPa. Thus, considering the binary mixture, the operating conditions set for all the experiments in Tables 1 and 2 should be above the MCP (supercritical homogeneous phase region of Fig. 2) and no liquid-gas interphase should occur, which could lead to the formation of small and uniform particles. Nevertheless, as stated before, in the case of the precipitation of vegetal extracts, the presence of a large variety of phytochemicals in the organic solution may change significantly the MCP of the supercritical phase. Fig. 7 shows the morphology (SEM imagens) resulted in experiments 1 and 2 of Table 1. As can be clearly deduced from the figure, the morphology obtained in experiments 1 and 2 are very different from those resulted in the rest of experiments. A semi-continuous material is observed, more similar to a gum-resin, with cavities within the aggregates. These images might corroborate that SAS operating conditions in these experiments were below the MCP, probably in the two-phase region of Fig. 2, as a result of the higher temperature and higher concentration of phytochemicals in the ethanolic solution. Fig. 8 show SEM imagens at a lower scale (75x) of the precipitates obtained in experiments 1 and 2, where it can be observed adjoined particles (coalescence phenomenon) in large sizes, especially at 15 MPa (experiment 1).

On the other hand, for the rest of experiments of Fig. 7, particles with similar morphology and micronized size were obtained. Nevertheless, uniform and spherical structures in the precipitates were not obtained probably due to precipitation conditions in the subcritical region (see Fig. 2) since, as mentioned before, uniform and small spherical particles (nanoparticles) are generally obtained at pressures larger than those corresponding to the MCP (Reverchon et al., 2010, 2008; Werling & Debenedetti, 2000).

The mean particle sizes of the precipitates obtained in all the experiments are depicted in Fig. 9. Deviations are in the range 1.1–3.4 μm (< 10%). SAS pressure, temperature and concentration of the ethanolic licorice solution significantly influence the particle size of the precipitates obtained. Furthermore, the particle size distributions of duplicate SAS experiments 3 to 6 are depicted in Fig. 10. At constant temperature (308.15 K) and concentration of the licorice ethanolic solution (14.2 mg/g) the mean particle size of the precipitated powders decreases significantly with pressure, from 34.27 μm at 12.5 MPa to 20.32 μm at 25 MPa. In addition, it could be inferred from Fig. 10 that at the higher pressures the particle sizes are somewhat more heterogeneous. The distribution at the lower pressure (12.5 MPa) is narrower

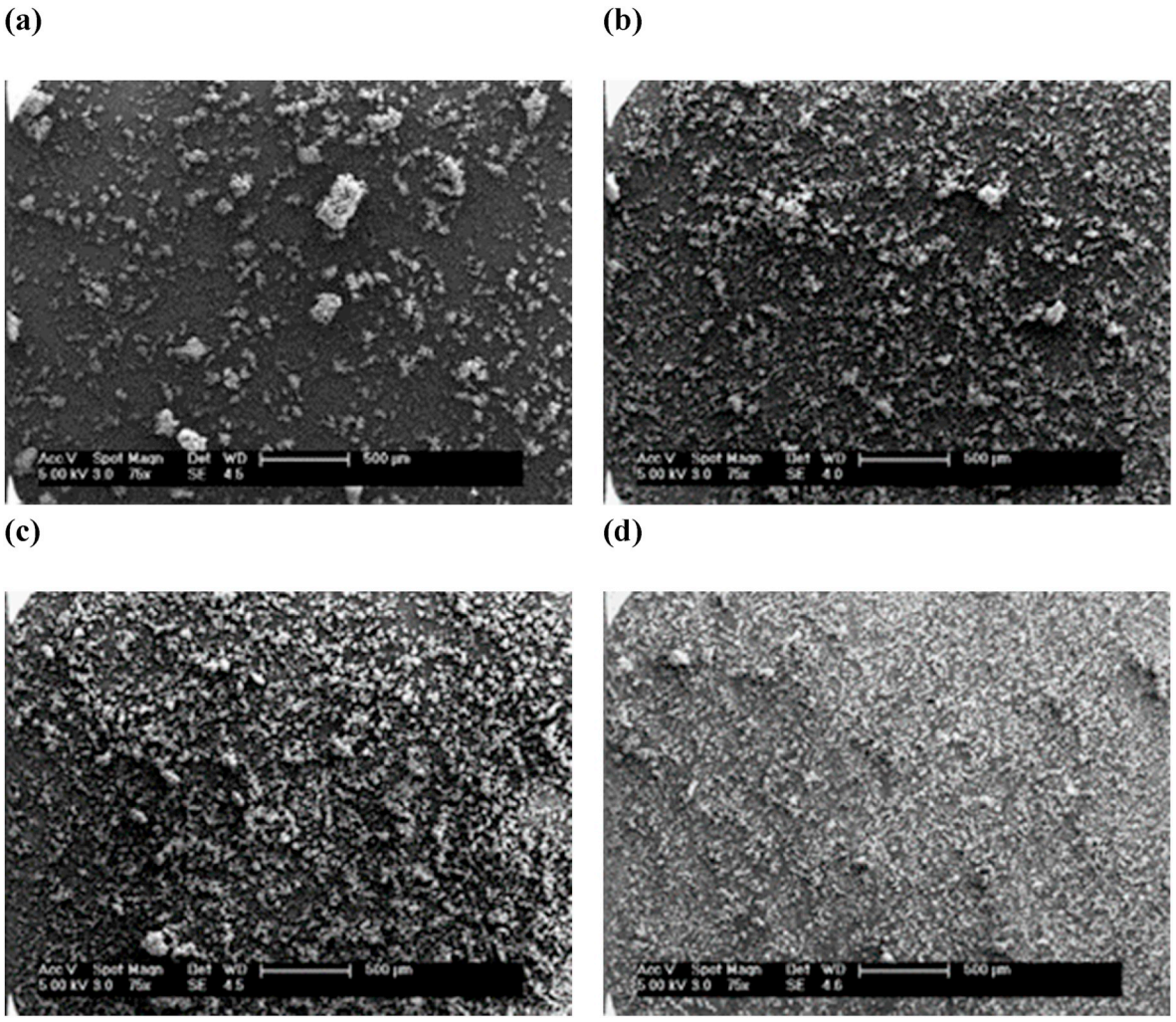

Fig. 11. SEM imagens (75x) for precipitates at 308.15 K: (a) 15 MPa and 14.2 mg/ml (LES1); (b) 15 MPa and 9.6 mg/ml (LES2); (c) 20 MPa and 14.2 mg/ml (LES1); (d) 20 MPa and 9.6 mg/ml (LES2).

and more normal, while increasing pressure the behavior appears as a multi-modal distribution, with significant smaller sizes. This tendency of particle size decrease with an increase in the precipitation pressure is consistent with the analysis published by Werling and Debenedetti (1999) and Martín and Cocero (2004) in their SAS precipitation simulation models. Furthermore, several experimental works confirm this tendency, such as the SAS precipitation of tartaric acid reported by Kröber and Teipel (2002), the *Achilea millefolium* L. ethanolic extract studied by Villanueva-Bermejo et al. (2017), and mango leaf extracts carried out by Guamán-Balcázar et al. (2019).

Additionally, SAS temperature presented statistically significant differences ($p > 0.05$) on the particles sizes, as can be deduced form experiments 7 to 10 in Fig. 9, being the smaller particles obtained at 313.15 K than at 308.15 K. Besides the effect of pressure and temperature on particle size, it can be observed in the SEM imagens presented in Fig. 11 that decreasing the concentration of the licorice ethanolic solution smaller particles are obtained. The lower the concentration of licorice phytochemicals in the ethanolic solution, the more similar MCP of the supercritical phase to that of the binary CO_2 + ethanol, and thus the precipitation conditions established are closer to be in the supercritical homogenous region.

4. Conclusions

The supercritical anti-solvent SAS precipitation of licorice ethanolic solutions produced the fractionation of licorice phytochemicals: dry powders with small aggregate particles together with oleoresin by-product were obtained. SAS pressure and temperature did not affect significantly precipitation yield, while when the concentration of licorice phytochemicals in the ethanolic solution decreased from 14.2 to 9.6 mg/ml, higher precipitation yields were obtained.

In general, no significant influence of pressure, temperature and concentration of the licorice ethanolic solution on the recovery of total phenolic compounds (TPC) was observed either in precipitates or oleoresins. Furthermore, no statistically significant differences were observed between de content of TPC in precipitates and oleoresins. Nevertheless, the precipitates presented better antioxidant activity than the oleoresins (significant higher TEAC values and lower IC_{50} values) for similar concentration of TPC. That is, due to the fractionation caused by SAS technique different type of phenolic compounds resulted in precipitates and oleoresins, being superior antioxidants those present in the precipitates. Since the concentration of glabridin licorice bioactive was up to 5.3 times higher in oleoresins than in precipitates this compound seems not to be the responsible of the observed antioxidant activity. On the other hand, the higher concentrations of liquiritin, liquiritigenin, glycyrrhizic acid, isoliquiritigenin and glabridin (all licorice bioactives studied in this work) in the precipitates, resulted in licorice powders with better antioxidant activity.

Particles with smaller size were obtained with increasing pressure and temperature, and decreasing the concentration of phytochemicals

in the licorice ethanolic solution. Nevertheless, agglomerated particles were obtained, probably due to precipitation conditions in the range below the supercritical multicomponent (phytochemicals + CO_2 + ethanol) mixture critical point. It is highlighted the importance of SAS operating conditions well above the critical point of the supercritical mixture to obtain an adequate morphology with regular and spherical particles.

CRediT authorship contribution statement

Somaris E. Quintana: Investigation, Methodology, Writing - review & editing. **Diego Martín Hernández:** Investigation, Methodology, Writing - review & editing. **David Villanueva-Bermejo:** Writing - review & editing. **Mónica R. García-Risco:** Supervision, Writing - review & editing. **Tiziana Fornari:** Conceptualization, Supervision, Investigation, Writing - review & editing, Validation.

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4.3. PREPARACIÓN DE FORMULACIONES OLEOSAS DE EXTRACTOS VEGETALES MICRONIZADOS UTILIZANDO TECNOLOGÍA DE FLUIDOS SUPERCRÍTICOS.

Este capítulo contiene los resultados obtenidos en relación a la combinación de extractos vegetales bioactivos, micronizados utilizando $SCCO_2$, con materiales lipídicos de diferente tipo. Obviamente, en esta combinación no solo el extracto vegetal aporta su actividad biológica, sino que el propio lípido puede aportar bioactividad, además de actuar como portador. Los resultados obtenidos de estos trabajos se presentan a través de un artículo científico titulado "*Bioactive Ingredients in Oils by Supercritical Anti-solvent Process (BIOSAS)"*, pendiente de ser enviado a publicación, y de una patente europea titulada "*Supercritical carbón dioxide-based methodology to formulate bioactive preparation*", cuya solicitud fue presentada el 15 de abril de 2020, con el número EP No. 20 382 302.6.

Para desarrollar la nueva técnica de formulación, es decir la combinación de un extracto vegetal micronizado con un lípido portador mediante tecnología supercrítica, el primer desafío fue la adaptación del equipo de precipitación supercrítica antisolvente (SAS) disponible (Thar Technologies) para lograr la innovación deseada. La Figura 4.1 muestra las diferencias en la configuración de una unidad SAS y de la nueva técnica desarrollada, denominada BIOSAS, para lograr la incorporación de micro- y/o nanopartículas bioactivas en un medio oleoso.

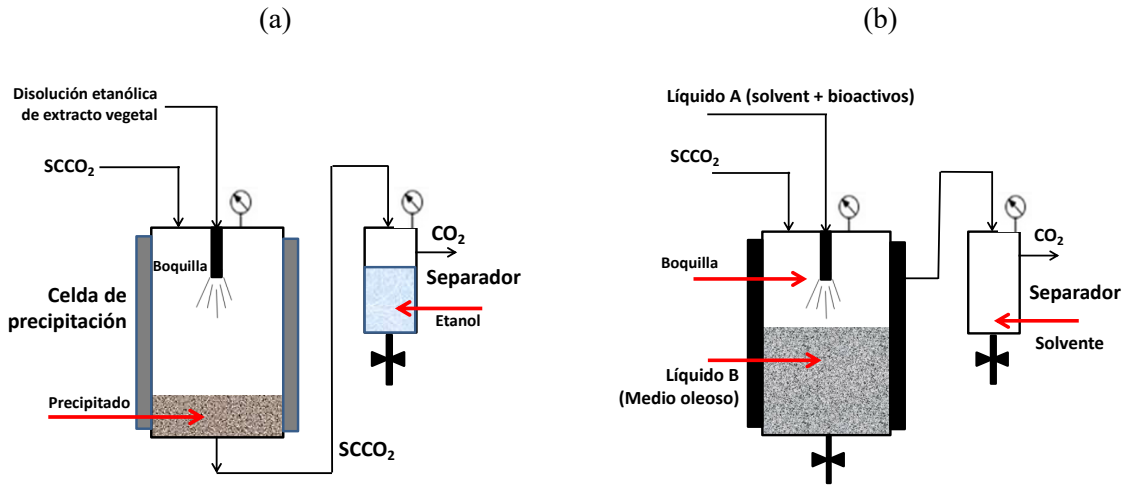

Figura 4.1. Comparación entre la configuración del proceso SAS (a) y la del proceso BIOSAS (b).

En el proceso BIOSAS se elimina el fritado que retiene las partículas sólidas precipitadas en el proceso SAS, y se introduce el medio oleoso dentro de la celda de precipitación, añadiendo una válvula en el fondo de la celda para la descarga del producto (una dispersión de pequeñas partículas de extracto vegetal en el lípido). Así, el CO_2 en condiciones supercríticas se introduce y descarga por la parte superior de la celda de precipitación. Cabe destacar que, si bien en esta tesis el proceso se desarrolló en modo discontinuo, es posible extender su aplicación al modo de operación continua y en estado estacionario, simplemente

alimentando en forma continua a la celda de precipitación los Líquidos A (disolución de extracto vegetal) y B (medio oleoso), así como el $SCCO_2$.

Sin el objetivo de desarrollar un producto nutracéutico específico, se emplearon distintas sustancias y sus disoluciones, así como diversos medios oleosos, para incorporar antioxidantes naturales en aceites, con el único fin de analizar la viabilidad de la técnica, ponerla a punto, y verificar la formación de una dispersión homogénea y estable de micro- y/o nanopartículas bioactivas sólidas en el medio oleoso. Se emplearon los extractos etanólicos de hojas de romero (*Rosmarinus officinalis* L.) y raíz de regaliz (*Glycyrrhiza glabra*), así como un extracto acuoso de *Quillaja saponaria* y un compuesto puro, la quercetina, como sustancia modelo. Como matrices oleosas para dispersar los componentes bioactivos micronizados se utilizaron diversos aceites comestibles, como el aceite de oliva, aceite de argán y aceite de lino.

Cuando el Líquido A es una disolución etanólica de compuestos bioactivos con polaridad moderada, su inyección a través de la boquilla y el contacto de las microgotas formadas con el $SCCO_2$ produce partículas sólidas de estos compuestos bioactivos, debido a la completa eliminación del etanol de las microgotas por disolución en la fase supercrítica. Parte de estas micro- y/o nanopartículas se disuelven en el aceite (Líquido B) mientras que otras se dispersan en el medio oleoso en forma homogénea, sin aglomerarse, y manteniendo su pequeño tamaño.

Cuando el Líquido A es una disolución acuosa de compuestos bioactivos de alta polaridad, debido a la escasa solubilidad del agua en $SCCO_2$, son las mismas microgotas formadas las que se disuelven en el medio oleoso, generando una microemulsión, en la que la fase acuosa dispersa contiene las sustancias bioactivas polares.

En resumen, en este tercer capítulo se presenta el desarrollo y aplicaciones de una nueva técnica para formular dispersiones homogéneas de pequeñas partículas de sustancias bioactivas, polares y medianamente polares, en matrices oleosas mediante tecnología supercrítica (BIOSAS).

4.3.1. Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process (BIOSAS)

The Journal of Supercritical Fluids (Pendiente de envío)

Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process (BIOSAS)

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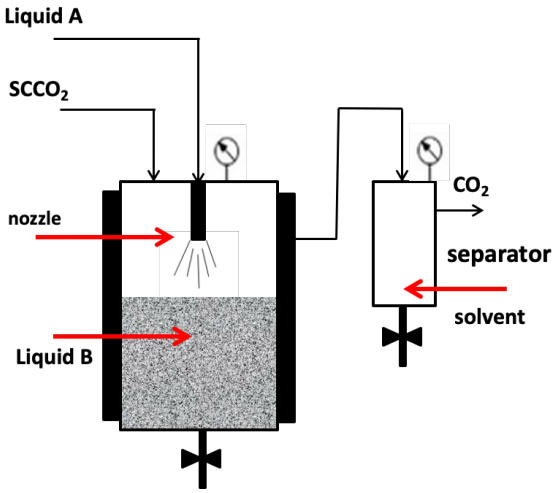
Graphical abstract



Highlights

- Method for homogeneous dispersion of plant bioactives in lipid matrix
- Normal nanoparticle size distribution was obtained
- $SCCO_2$ inert and low temperature conditions contribute to lipid and bioactives protection
- Quercetin, saponins, rosemary and licorice phytochemicals were incorporated in edible oils.

Abstract

This work presents the novel method named Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process (BIOSAS) to formulate homogenous dispersions of bioactive substances in oily matrix. The method is based on the carbon dioxide supercritical anti-solvent (SAS) technique to produce small drops of a solution (containing the bioactives) in the presence of a lipid substance.

Ethanolic solutions were used in the case of moderate polar bioactives, such as quercetin, as model pure substance, and complex mixtures (extracts) of rosemary and licorice phytochemicals. In these cases, ethanol was completely removed from the droplets by SAS effect and solid bioactive particles with mean diameters below 1μm were homogenously dispersed in the lipid matrix (olive, argan and flax oils). On the other hand, aqueous solutions were used to dissolve high polar bioactives, such as saponins. In this case the water droplets produced were high-pressure mixed with the lipid phase containing an emulsifier (lecithin) and stable emulsions were obtained.

Based on SAS technique, BIOSAS permits the control of particle size distribution and can be used to formulate lipid-based nutraceuticals or food supplements in an inert atmosphere and at low temperatures an thus, the method may contribute to reduce the oxidation and/or thermal degradation of both the bioactive substances and lipid matrix.

Keywords: Supercritical antisolvent precipitation; bioactive extracts; oily dispersions; nutraceuticals; emulsions; particle size distribution.

1. Introduction

Many of bioactive components were identified and isolated from vegetables, fruits, legumes, oils, nuts, and whole grains and have shown numerous beneficial effects on human health including antioxidant, anti-inflammatory, antibacterial, antitumor and immunomodulatory activities. The incorporation of these bioactive molecules into functional food ingredients and nutraceuticals, preserving their biological activity, attaining high bioavailability, and thus efficacy, is a challenging task due to the usual poor stability and/or low solubility of these biomolecules.

The main disadvantage of traditional formulation methods, such as those using encapsulating biopolymers (single emulsion, double emulsion, coacervation processes), electrospinning or spry drying, is the use of organic solvents where most of them are toxic. These solvents must be completely eliminated from the bioactive designed system before their intake, what demand heating steps that can lead to the degradation of the bioactive compounds.

Innovative methodologies for the formulation of bioactive molecules from vegetal matrix include the use of supercritical carbon dioxide ($SCCO_2$) technology [1,2]. The application of supercritical fluids enables the control of the size and morphology of the bioactive particles and thus several methods have been developed for the synthesis of micro and nano-particles based on spray processes using $SCCO_2$ either acting as a solute, a solvent or an antisolvent. In the literature, different type of food components, such as proteins, lipids, carbohydrates and other minor components were formulated using $SCCO_2$ technologies to target specific properties and functionality.

For example, the Particles from Gas-Saturated Solutions (PGSS) method is a well-known process in which the $SCCO_2$ acts as a solute [3]. The process can make particles of materials that can absorb $SCCO_2$ at high concentrations. The supercritical fluid is dissolved in the molten material (e.g. a polymer or a liquid-suspended solution) and the high-pressure mixture is rapidly depressurized through a nozzle leading to particle formation by precipitation. PGSS is especially useful for the impregnation of active ingredients in polymer matrices.

In the case of Rapid Expansion of Supercritical Solutions (RESS) or Crystallization from Supercritical Solutions (CSS) applications, the particle formulation is carried out using $SCCO_2$ acting as solvent. These techniques are adequate when the biomolecules have high solubility in the supercritical fluid [4,5]. The biomolecules are dissolved in $SCCO_2$ and the high-pressure solution is rapidly depressurized through a nozzle, to lead the precipitation of the biomolecules at low pressure.

Special attention has been carried out in last decades in supercritical particle formation technologies in which $SCCO_2$ acts as an antisolvent, with more than 10 diverse approaches developed [1]. For example, in Supercritical Antisolvent (SAS) approach, the biomolecules have low solubility in $SCCO_2$ but high solubility in a liquid solvent which in turn is high soluble in $SCCO_2$. A solution containing the biomolecules is sprayed through a nozzle into a chamber containing $SCCO_2$ that acts as an antisolvent [6]. The rapid contact between the two phases generates supersaturation of the droplets, and results in fast nucleation and growth of solid particles. Another example in which $SCCO_2$ acts as an antisolvent is the Supercritical Fluid Extraction of Emulsions (SFEE) [7,8]. SFEE combines the efficiency of the supercritical fluid extraction with the ability of water in oil (w/o) or oil in water emulsions (o/w) processes to obtain nanoparticles. Basically, the $SCCO_2$ extracts the oil-organic phase in which it is solubilized the water-insoluble drug. The drug remains therefore in a suspension that is established by using a surfactant. This technique provides particles with low concentration (ppm) of the organic solvent.

These supercritical particle formation technologies have been intensively applied in the area of pharmacy, while natural or food materials-based applications have been more limited although are growing currently due to their great potential. Lipids (β-sitosterol, soy lecithin, milk fat, cocoa butter), proteins (gelatin, lysozyme, zein) and carbohydrates (lactose, chitosan, β-glucan), are examples of food-related applications of $SCCO_2$ particle formation technologies [1]. Indeed, the majority of recent studies are focused on minor component ingredients present in plant materials.

For example, CSS was used to encapsulate anthocyanins extracted for jabuticaba in order to increase and retain the pigments stability, protecting this compound from environmental conditions [9] and vanillin was micronized by RESS process [10] without altering its properties and holding its crystalline form. Furthermore, $SCCO_2$ has been utilized as antisolvent (SAS process) in a wide variety of process, such as the

micronization of vitexin, an apigenin flavone glucoside found in the passion flower [11], the precipitation of high catechin/low caffeine powder from green tea [12], phenolic compounds from yarrow [13] rosemary [14] or licorice [15] among others.

There are numerous challenges circumvented with the different supercritical particle formation processes described and applied in the literature. Nevertheless, none of them attain the supercritical dispersion of micronized particles in lipid matrix, leading to a high-performance formulation technique to combine the bioactivity of minor plant-based compounds and lipids, improving bioavailability and efficacy.

In this work a new supercritical approach named Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process (BIOSAS) to incorporate bioactive compounds in a lipid matrix is presented. The method is based in SAS precipitation method to produce small drops of a solution (containing the bioactives) in a high-pressure cell, in which the lipid matrix was placed. The antisolvent effect causes the production of micro or nanoparticles which are energetically mixed with the oil.

Quercetin, rosemary and licorice phytochemicals dissolved in ethanol were homogenously dispersed in the lipid matrix (olive, argan and flax oils). Also, stable emulsions were obtained when saponin aqueous solutions were mixed with the lipid phase (olive oil), containing an emulsifier (lecithin).

BIOSAS permits the homogeneous inclusion of non-polar and polar biomolecules in small particle sizes and can be used as a novel technique to formulate lipid-based nutraceuticals or food supplements in an inert atmosphere and at low temperatures and thus, circumventing the oxidation and/or thermal degradation of the biomolecules.

2. Materials and methods

2.1.Chemicals

CO_2 (N38) was supplied from Carburos Metálicos (Madrid, Spain). Ethanol (99.5 % purity) was purchased from Panreac (Barcelona, Spain). Carnosic acid (> 97% purity) was from Cymit (Cymit Química S.L., Barcelona, Spain). From Sigma–Aldrich (St. Louis, MO, USA), 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one,3,3′,4′,5,6-Pentahydroxyflavone – Quercetin (> 95 % purity), quillaja bark (20% saponins), L-α-lecithin, egg yolk, highly purified soy bean lecithin and rosmarinic acid

(≥ 98% purity) were purchased. Orthophosphoric acid (85% purity) was purchased from Scharlab S.L. (Sentmenat, Spain) and acetonitrile (99,8% purity) from Macron (Poland). Extra virgin oil, virgin flax and argan oils were purchased in local markets.

2.2. Preparation of bioactive solutions

2 g of quercetin was dissolved in 200 mL of ethanol to obtain the quercetin ethanolic solution, and 2 g quillaja bark saponins were dissolved in 200 mL of milli-Q water to produce the saponin water solution.

In the case of plant phytochemicals, rosemary (*Romarinus officinalis* L.) leaves and licorice (*Glycyrrhiza glabra* L.) roots were obtained from Murciana herbalist's (Murcia, Spain) and were ground using a Premill 250 hammer mill (Lleal S.A., Granollers, Spain). Then, ultrasound assisted extraction (Branson Digital Sonifier 550 model, Danbury, USA) with ethanol was accomplished. The extractions were carried out with a sample/ethanol ratio of 1:10 (w/v) for 15 min at constant temperature (25 °C). The liquid extracts were rotary evaporated under vacuum until the concentrations of solid solutes were 17 mg/mL and 10 mg/mL, respectively for the rosemary and licorice ethanolic solutions. These values were set according to previous studies concerning the SAS precipitation of ethanolic rosemary solutions [14] and licorice ethanolic solutions [15] to produce dry micronized powders.

2.3. Supercritical antisolvent precipitation of biomolecules in oily matrix (BIOSAS)

Figure 1 shows the scheme of BIOSAS process. The solution containing the bioactive substances (Liquid A) is pumped through a nozzle into a mixing vessel, in which the oily matrix (Liquid B) was placed. Simultaneously, supercritical carbon dioxide ($SCCO_2$) is pumped into the vessel at high pressure and mild temperature.

When Liquid A is an ethanolic solution of bioactive substances, since the solvent is high soluble in the supercritical fluid, the mass transfer of $SCCO_2$ into the ethanolic droplets produced by the nozzle, together with the evaporation of ethanol into the supercritical phase, lead to the supersaturation of the solutes dissolved in the droplets and produces their precipitation and simultaneous dispersion into Liquid B (oily phase). That is, solid micro- and/or nano-particles of bioactives are produced, induced by the $SCCO_2$

158 antisolvent (SAS) effect and then, these particles are homogeneously dispersed in the oily
159 matrix placed into the vessel. The supercritical outlet stream, which contains CO_2, ethanol
160 and eventually some amount of bioactives not dispersed, is decompressed and recuperated
161 in a separator.

162 When Liquid A is a water solution, the low solubility of water in $SCCO_2$ hinder the
163 antisolvent effect and thus the droplets (containing the bioactives) are mixed at high
164 pressure conditions with the oily phase (Liquid B) producing water-oil (W/O) emulsions,
165 providing if necessary the addition of a suitable amount of emulsifier in the oil. In this
166 case, BIOSAS performance is similar to that of a high-speed homogenizer, and the
167 addition of emulsifiers to Liquid B favors the stability of the formed emulsions.

168 BIOSAS process was performed using the supercritical technology equipment Thar
169 SF2000 (Thar Technology, PA, USA) (Figure 2) with some modifications. The
170 equipment comprises two high-pressure pumps for feeding, respectively, the supercritical
171 $SCCO_2$ and Liquid A, and a stainless-steel mixing vessel (273 mL) and separator (500
172 mL) with independent control of temperature and pressure. The vessel is equipped with a
173 101.6 μm inner diameter nozzle for the injection of Liquid A. $SCCO_2$ and Liquid A
174 streams are fed from the top in co-current manner (coaxial nozzle). A manual high-
175 pressure valve is located at the bottom of the vessel to collect the oil with the dispersed
176 solutes once BIOSAS process is completed. The supercritical outlet stream flows out of
177 the vessel from the top. The equipment is coupled with a CO_2 recirculation system,
178 including a demister unit, a filter, a condenser and a CO_2 storage tank.

179 $SCCO_2$ was pumped into the mixing vessel, where 50-100 g of oil was previously placed,
180 until pressure (10-25 MPa) and temperature (308.15 or 313.15 K) conditions were
181 attained. Then, the Liquid A was pumped for 10-60 min into the vessel at 1.6-3.2 mL/min,
182 while maintaining a constant flow of $SCCO_2$ (50 g/min). In all the experiments, once the
183 pumping of Liquid A through the nozzle was finished, $SCCO_2$ was pumped during
184 additional 5-15 min. During the process, the separator was kept at 0.1 MPa and 313.15
185 K.

186 Particularly, in the case that Liquid A is an ethanolic solution, the depressurization of the
187 supercritical stream in the separator produced the ethanol precipitation together with those
188 substances which were not dispersed in Liquid B (i.e. solutes which are soluble in the

189 $SCCO_2$-ethanol supercritical phase). This material was recovered from the separator and
190 was rotary evaporated under vacuum until an oleoresin-type product was obtained.
191 Finally, the vessel was depressurized, and the oily dispersion or W/O emulsion of
192 bioactive substances was recovered by the high-pressure valve placed at the bottom of
193 the mixing vessel. Products were kept at 298 K under darkness until analysis.

194 Table 1 shows all the experiments carried out in this work to investigate BIOSAS
195 practicability, varying the lipid matrix (Liquid B) and the pumped bioactive solution
196 (Liquid A). Pressure and temperature, Liquid A and $SCCO_2$ flows, together with the
197 amount of Liquid B placed in the mixing vessel and concentration of bioactives in Liquid
198 A are given in the table.

199 **2.4. Assessment of BIOSAS dispersion yield**

200 In the case of ethanolic solutions, the BIOSAS dispersion yield was evaluated as the total
201 mass of phytochemicals incorporated in the lipid matrix (Liquid B) related to the mass of
202 phytochemicals introduced in the system by pumping the ethanolic solution (Liquid A).
203 The total mass of phytochemicals dispersed in the lipid matrix was estimated as the
204 difference between the mass pumped and the mass of solids recovered in the separator
205 (oleoresin), which was obtained by evaporation of the ethanol solvent. Thus, it is assumed
206 that no loss of phytochemicals was produced (e.g. material detained in the tubing or
207 dragged out of the system with the decompressed CO_2). Furthermore, in the case of
208 rosemary solution, two key bioactives (carnosic acid and rosmarinic acid) were identified
209 and quantified in the lipid matrix and in the oleoresin, in order to check mass balance and
210 verify the hypothesis assumed.

211 **2.5.HPLC analysis**

212 Carnosic acid and rosmarinic acid were identified and quantified in the case of BIOSAS
213 rosemary samples following the procedures describes by Vicente et al., [16] using a
214 HPLC Prominence-i LC-2030C 3D Plus (Shimadzu) equipped with a RP-C18 (250 × 4.6
215 mm; 3 μm) chromatography column. The mobile phase consisted of 0.1 % of phosphoric
216 acid in water (solvent A) and acetonitrile (solvent B) applying the following gradient: 0–
217 8 min, 77 % A, 8–25 min, 25 % A, 25–40 min 25 % A and the 40–45 min 77 % A. Initial
218 conditions were gained in 5 min. The flow rate was constant at 0.7 mL/min. Injection

volume was 20 µl and the detection was accomplished using a diode array detection system, storing the signal at a wavelength of 230, 280 and 350 nm.

In the case of quercetin BIOSAS dispersion, HPLC analysis was carried out as previously described by Wei et al. [17]. A Prominence-i LC-2030C 3D Plus (Shimadzu) equipped with a quaternary solvent delivery system, an autosampler and DAD detector and RP-C18 (250 × 4.6 mm; 3 µm) was used. The column temperature was set at 25 °C. The mobile phase consists of acetonitrile (A) and 0.026% aqueous H3PO4 (v/v), applying the following gradient elution: at 0–20 min, 20%–25% A, 20–30 min, 25%–34% A, 30–50 min, 34%–50% A, 50–60 min, 50%–60% A and 60–80 min, 60% A. After 5 min, the initial conditions were achieved. The flow rate was 0.7 mL/min and was kept constant during analysis. Injection volume was 20 µl and detection was accomplished at 254, 280 and 370 nm.

2.6.Morphology and particle size analysis

A confocal laser scanning microscope (Zeiss LSM 710, Carl Zeiss Micro imaging GmbH, Germany) was used to visualize the phytochemicals particles microstructure dispersed in oil. Phytochemicals particles were identified by simple polarized light. Carl Zeiss Plan-Apochromat 40× oil-immersion objective lens was employed to view each dispersion samples. Digital image files in 512 × 512-pixel resolution were recorded with the Zen LSM software (Carl Zeiss Micro imaging GmbH, Germany).

The size distribution of phytochemicals dispersed in the oils was measured by Zetasizer analyzer (Malvern Zetasizer Nano ZS, from Malvern Instruments Ltd – UK). The instrument was attached with a He-Ne laser lamp (0.4 mW) at wavelength of 633 nm. Measurements were carried out at 25 °C in insulated chamber using dynamic light scattering technique. In the case of emulsions, particle size and particle size distributions were measured by light scattering with a laser diffraction system Mastersizer 2000 (Malvern Instruments Ltd., Malvern, UK), equipped with a wet dispersion unit.

2.7.Stability of the bioactive-rich oily dispersions

The physical destabilization of the oily dispersions against time was evaluated by measuring the settling behavior using the multiple light technique scattering (MLS) (Turbiscan Lab Expert, Formulaction, France) in which a pulsed near-infrared light

source with a wavelength of 850 nm is forced to pass through a suspension maintained at rest into a glass tube. In this work 8-10 mL of samples were measured; the data were recorded every 5 minutes for 2 h, 48 h after preparation.

Backscattering and Turbiscan stability index (TSI) were used to quantify the stability of samples:

$$TSI = \sum_{l} \frac{\sum_{h} |scan_{l}(\mathrm{h}) - scan_{l-1}(\mathrm{h})|}{H}$$

Where, h and H is the selected height and the total height of sample respectively. This parameter accounts for all processes that occur in the sample (particle coalescence and settling processes). A higher TSI value indicates a less stable dispersion.

For emulsions the zeta-potential of emulsions were measured at 25 ºC using a Zetasizer analyzer (Malvern Zetasizer Nano ZS, from Malvern Instruments Ltd – UK). Before measurement, the sample were diluted at 5 % in distillated water.

2.8. Thermal properties

Thermal properties of samples were characterized by using differential scanning calorimetry (DSC-Q200 TA Instruments, USA). The samples were heated from 25 °C to 200 °C using an aluminum crucible with a pierced lid with a 1.0 mm diameter orifice and approximately 4 mg of each sample under an air flow rate of 150 mL/min at a heating rate of 10 °C/min.

3. Results and Discussion

Table 1 shows the BIOSAS conditions applied in all experiments carried out in our study. It should be pointed out that no optimization of BIOSAS process was particularly intended in this work for any specific study-case, but a good number of BIOSAS process applications were envisioned in order to demonstrate the potentials of this novel supercritical procedure.

3.1. BIOSAS dispersion of quercetin in oil

The dispersion of a pure bioactive compound (quercetin) in oil was carried out first to assess the feasibility of a simple BIOSAS process. The conditions were 20 MPa, 313.15 K, with $SCCO_2$ and quercetin solution (QUE) flows of 50 g/min and 2 mL/min, respectively. These operating conditions attain a homogenous supercritical CO_2 + ethanol phase at 313.15 K and guarantee the complete elimination of ethanol from the mixing vessel [18–20]. The concentration of quercetin in QUE was 10 mg/mL, and flax oil (57 g) was the oily matrix placed into the mixing vessel. The QUE solution was pumped for 45 min.

The resulted concentration of quercetin in the flax oil was 1.55 ± 0.05 % mass (HPLC analysis). High quercetin dispersion yield (quercetin in oil / quercetin pumped into the mixing vessel) was obtained, since no quercetin was possible to identify in the ethanol recovered in the separator. The solubility of quercetin in a medium chain triacylglycerol mixture at 393.15 K is 1.77 ± 0.29 mg/g [21]. This value is considerably lower than the amount dispersed by BIOSAS process in high molecular weight triacylglycerols at noticeably lower temperature (> 15 mg/g). Several commercial products are available in the market with encapsulated quercetin that recommend doses intake of 500-1000 mg/day, what would be equivalent to an intake of 30-70 mL/day of the quercetin-enriched flax oil obtained by the BIOSAS process.

Figure 3 show the backscattering spectra of the lipid dispersions 72 h after BIOSAS production (Figure 3.a), the optical microscope image of the quercetin particles dispersed in the flax oil (Figure 3.c) and the particle size distribution determined by light scattering (Figure 3.d). As it can be observed in Figure 3.a good stability of the oily dispersion can be assessed after 72 h of production, presenting values lower than 0.1. The TSI parameter evaluates the stability of the sample by measuring its light intensity variations from the bottom to the top. The representation of TSI values vs. time of the flax oil + quercetin dispersion is illustrated in Figure 3.b. During the studied time, the TSI values do not change significantly with time and were lower than 0.1.

Furthermore, quercetin nano-particles were homogeneously dispersed in flax oil, with a normal particle size distribution and a mean particle size of 739.9 ± 188.4 nm (Figures 3.b and 3.c). Therefore, the BIOSAS process allowed the homogeneous and stable

304 spreading of moderate polar compound within a non-polar matrix under the form of nano-
305 particles. The dispersions obtained were stored in beaker tubes at ambient temperature
306 and showed good stability after a week of production: no sediments and/or particle
307 agglomerations were visually observed.

308 **3.2. BIOSAS dispersion of rosemary bioactive extract in oils**

309 The flow rates of $SCCO_2$ and rosemary ethanolic solution (RES) were, respectively, 50
310 g/min and 1.6 mL/min. The concentration of rosemary phytochemicals in RES was 17
311 mg/mL. Experiments were carried out with three different oils (Table 1) placed into the
312 mixing vessel: olive oil (50 or 100 g), argan oil (50 g) and flax oil (50 g) at different
313 pressures (12, 15 and 20 MPa) and 313.15 K. The RES solution was pumped for 60 min
314 in all experiments.

315 In this case, while some phytochemicals of RES were precipitated and dispersed in the
316 oil, some other phytochemicals (those soluble in the supercritical CO_2 + ethanol phase)
317 were recovered with the ethanol cosolvent in the separator. In this way, two different
318 fractions were obtained from RES, an oily product with some rosemary bioactives
319 dispersed (RES-LP: lipid product) and a viscous fraction (RES-OP: oleoresin product)
320 which was recovered from the separator after evaporation of ethanol.

321 Table 2 shows the amount (mg) of rosemary phytochemicals recovered in RES-OP after
322 ethanol evaporation. According to these values, it can be estimated that rosemary
323 phytochemicals in the form of micro- and nano-particles were dispersed in olive oil in the
324 range 0.15 % to 1.84 % mass, while in the case of argan and flax oil these values were,
325 respectively, 2.73% and 2.82 %. Thus, the dispersion of rosemary bioactives in argan oil
326 or flax oil was considerably larger than the in the case of olive oil. Under the conditions
327 studied, the dispersion yields (phytochemicals in oil / phytochemicals pumped) attained
328 in RES-LP were in the range of 4.72 to 56.50 % in the case of olive oil, while yields c.a.
329 85 % were obtained for argan and flax oil.

330 For comparative purposes with the BIOSAS process, some experiments were performed
331 to dissolve the rosemary extract in olive oil at 313.15 K for 4 hours with continuous
332 stirring. In this case, less than 0.5 % mass of the extract was possible to dissolve in the
333 olive oil, after what precipitation of the extract occurred. Therefore, although no

optimization was intended to carry out, BIOSAS permitted almost a 4-fold increase of a stable incorporation of rosemary phytochemicals in olive oil, in comparison with traditional dissolution process.

Table 3 shows the concentration of rosmarinic acid and carnosic acid determined in the RES-LP samples obtained with olive oil, with values in the range of 0.05-0.23 mg rosmarinic acid and 0.03-0.21 mg carnosic acid per gram of olive oil. Moreover, it was determined c.a. 0.40 mg/g of rosmarinic acid and 4.90 mg/g of carnosic acid dispersed in argan oil, and 0.51 mg/g of rosmarinic acid and 6.39 mg/g of carnosic acid in flax oil. Then, the concentrations of these key rosemary antioxidants in argan oil or flax oil were considerably higher than the values obtained in the case of olive oil. According to EFSA [21] the dietary exposure of carnosic acid estimated for adults would be between 500-1500 mg per day. Considering an intake of 50 g of any of the oils per day, the carnosic acid daily intake is below the toxic limits established by EFSA. Rosmarinic acid itself exhibits low toxicity (LD_{50} in mice is 561 mg/kg for intravenous administration) and is not mutagenic but there are insufficient toxicity data from which a safe dose could be derived [22].

Table 3 also shows the concentration of rosmarinic acid and carnosic acid resulted from the HPLC analysis of the oleoresins (RES-OP) recovered in the separators. Taking into account the total mass of rosmarinic acid and carnosic acid quantified in the RES-LP and RES-OP samples, and in order to satisfy the mass balance of these substances, the concentration of these substances in the ultrasound rosemary extract should be 0.8-1.4 % mass for rosmarinic acid and 6.1-7.6 % mass for carnosic acid. These values are quite in accordance with those determined by HPLC analysis in the ultrasound rosemary extract (0.93 % mass of rosmarinic acid and 6.77 % mass of carnosic acid). Thus, the mass balance was satisfactory verified and the hypothesis that no losses of phytochemicals were produced was reasonable confirmed for these substances.

The dispersion yields of rosmarinic acid and carnosic acid (mass dispersed in the oil / mass pumped into the mixing vessel) were calculated and are given in Table 3. Rosmarinic acid yields (44-82 %) were significantly higher than carnosic acid yields (2-10 %). This result can be explained considering the higher solubility of carnosic acid in $SCCO_2$, particularly when ethanol is present as cosolvent [23], in comparison with rosmarinic acid which is a high-polar substance almost insoluble in $SCCO_2$. While

366 carnosic acid is solubilized in the supercritical stream flowing out of the mixing vessel to
367 the separator, rosmarinic acid is precipitated and dispersed in the oil placed in the mixing
368 vessel. Thus, rosmarinic acid is rather recovered dispersed in the lipid matrix while
369 carnosic acid is preferably recovered in the separator (oleoresin product). That is,
370 BIOSAS process can attain in one step the fractionation and incorporation of selective
371 biomolecules of plant extracts in lipid matrix.

372 Figure 4 shows the microscopic imagen and particle size distribution of the rosemary
373 dispersions obtained in the experiments 5 (olive oil), 8 (argan oil) and 9 (flax oil) of Table
374 1. A homogeneous distribution of RES particles in the oils was achieved in all cases. The
375 sizes of RES particles dispersed in the lipid matrix were below1 μm, and in the case of
376 olive oil the samples obtained at 15 MPa presented the lower average size (397.85 ± 0.06
377 nm). Yet, larger sizes were observed with argan oil and flax oil, being the average size
378 1081 ± 224.6 and 621.91 ± 132.8, respectively.

379 The dispersions obtained showed high stability after 48 h of production, since no particle
380 migration by creaming or sedimentation and/or particle agglomeration was visually
381 observed. Furthermore, the physical stability of the oil dispersions was characterized by
382 Multiple Light Scattering. Figure 5 show the backscattering profile of the olive oil
383 dispersion obtained in experiment 5 (50 g oil) as a function of the storage time and the
384 length of the container (a tube). This technique can detect, at early stage, the occurrence
385 of different simultaneous destabilization mechanisms before they could be observed by
386 the naked-eye. The backscatter profiles depicted in Figure 5 did not show significant
387 changes in intensity indicating that the dispersions were stable until the time of analysis
388 (48 h after production). Furthermore, the TSI values for this sample (Figure 5.b.) do not
389 change significantly during time of analysis and values were lower than 0.1. Similar
390 behavior was observed for the other rosemary dispersions.

391 Thermal properties of the rosemary phytochemicals dispersed in olive oil were studied by
392 differential scanning calorimetry (DSC). This technique offers a measure, as a function
393 of temperature, of the heat flow (loss or gain) resulting from physical or chemical changes
394 within a sample. It is usually used to characterize the crystallization and melting point
395 behavior, giving information about polymorphism and/or crystal assembling. Figure 6
396 show the thermal behavior of the RES-olive oil dispersions obtained in experiments 4 and
397 5 of Table 1, in comparison with the pure olive oil. All the samples presented endothermic

peaks and the thermal behaviors of both dispersions were very similar to that of olive oil, indicating similar thermal stability. The peak melting temperature of the dispersions 4 and 5 were 268.44 and 269.12 K, respectively, slightly lower than the melting point of olive oil (270.17 K) (Figure 6). This reduction in the peak melting temperature can be related to a less ordered structure, since lower amount of energy is required to breakdown the internal connections of the lipid matrix, indicating a more imperfect structure.

3.3 BIOSAS dispersion of licorice bioactive extract in edible oils

In order to extend the analysis of the viability of BIOSAS to incorporate plant phytochemicals to lipid matrix, the dispersion of a licorice root ethanolic extract was investigated. The flow rates of $SCCO_2$ and licorice ethanolic solution (LES) were, respectively, 50 g/min and 3.2 mL/min. The concentration of licorice phytochemicals in LES was 10 mg/mL. Experiments were carried out with 70 g of three different oils (Table 1) placed into the mixing vessel: argan oil, flax oil and olive oil. Process temperature, pressure and time are given in Table 1 for each of the four experimental assays accomplished. In all cases, successful dispersion of licorice phytochemicals in the oils was achieved, and high stability after 48 h of production was visually observed. It was estimated that c.a. 0.16, 0.26 and 0.18% of licorice bioactive microparticles were dispersed in argan, flax and olive oil, respectively. BIOSAS dispersion yields (licorice phytochemicals in oil / licorice phytochemicals pumped into the mixing vessel) were in the range 7-20 %.

As an example, Figure 7 shows the microscopic imagen and particle size distribution of the licorice root dispersions obtained in the case of flax oil (experiment 12 in Table 1). The sizes of LES particles dispersed in the lipid matrix were normal distributed, moderately narrow and monomodal in appearance, with average particle size of 451.9 ± 75.22 nm. Furthermore, the backscatter profiles depicted in Figure 8 did not show significant changes in intensity indicating that the dispersions were stable until the time of analysis (48 h after production). In the case of licorice phytochemicals dispersions, the TSI values show somewhat higher variations with time of analysis, but also in these case values were always satisfactorily low (TSI < 0.13) indicating good stability (Figure 8.b.). Similar results were observed for the rest of LES dispersions obtained.

3.3.BIOSAS emulsions

In the case of high polar bioactives, such as saponins, water was the solvent used to produce Liquid A. Since water has low solubility in $SCCO_2$, the micro- and/or nano-droplets of the solvent (water) containing the bioactives are dispersed in the lipid matrix (Liquid B) and thus, a W/O emulsion is obtained, being the water phase rich in the bioactives. To favor the emulsion formation a food emulsifier (lecithin) was added to the lipid matrix.

The incorporation of an aqueous extract of *Quillaja saponaria* (QWS) in olive oil was carried out at 20-25 MPa, 313.15 K and 50 g/min of $SCCO_2$. The QWS (10 mg/g) was pumped at 1.6 mL/min for 10 min, while 70 g of olive oil was placed into the mixing vessel to achieve a c.a. 20:80 W/O emulsion. 1.00 % and 1.25 % of lecithin was added to olive oil to flavor the emulsification. All the QWS pumped was emulsified with the lipid matrix and thus, the concentration of quillaja extract in the olive oil after BIOSAS process was 0.23 % mass.

Figure 9 show the optical microscope imagens of the emulsions and Figure 10 the droplet size distribution. Droplet sizes are rather homogeneous and are in the range 10-200 μm (Table 4). The mean droplet sizes obtained, together with the zeta potential values after 48 h of production, are given in Table 5. The zeta potential values are close to -40 mV in all cases, indicating good stability of the emulsions obtained. Regarding the effect of pressure and lecithin content, Figure 11 shows a decrease in the mean droplet size with the decrease of lecithin content at 20 MPa, while the opposite effect was observed at 25 MPa. Despite more experiments are necessary to confirm this tendency, it seems that the effect of the emulsifier strongly depends on the mixing pressure used.

4. Conclusions

A novel supercritical procedure BIOSAS to disperse bioactive substances in oily matrix is presented for the first time. The method accomplishes SAS technique in the presence of a lipid matrix. In this way the micronized bioactives are homogeneously incorporated in the lipid substance.

BIOSAS was successfully applied to disperse both pure bioactive substances (e.g. quercetin) and complex mixtures of bioactives (e.g. plant extracts). The solutions to pump

458 the bioactives into the system may contain a solvent high-soluble in $SCCO_2$ (e.g. ethanol)
459 in which case solid micro- and nano-particles of bioactives are homogeneously dispersed
460 in the oily matrix with high stability. Actually, it was demonstrated that BIOSAS yield
461 varied considerably with the type of lipid matrix and bioactive solution used, being
462 significantly higher the yields attained in the dispersion of rosemary bioactives in several
463 edible oils (olive, flax and argan oils) in comparison with the dispersion of licorice
464 bioactives in the same oils. Furthermore, as proved for the case of applying BIOSAS to
465 rosemary extract, the process conditions (pressure, temperature, flows, process time, etc.)
466 can be used to improve dispersion yield.

467 On the other hand, the alternative to formulate W/O emulsions via BIOSAS method was
468 presented for the first time, achieving the incorporation of saponins to a lipid matrix.
469 Thus, BIOSAS can also attain the dispersion of high-polar biomolecules in different oils,
470 providing the addition of an emulsifier to favor the stability of the W/O emulsion
471 produced. Further research and applications concerning this BIOSAS alternative are
472 currently under development.

473 In summary, in this work it was validated BIOSAS supercritical technique. Based in SAS
474 process, BIOSAS permits the control of particle sizes and permits the formulation of
475 bioactive lipid-based products, such as nutraceuticals, functional food ingredients or food
476 supplements. Besides the micronized form attained for the dispersion of the bioactive
477 substances in the oil, BIOSAS was developed using only green solvents, in non-oxidized
478 conditions and low temperatures, and thus, the method is particularly adequate to reduce
479 degradation of both the bioactive substances and lipid matrix.

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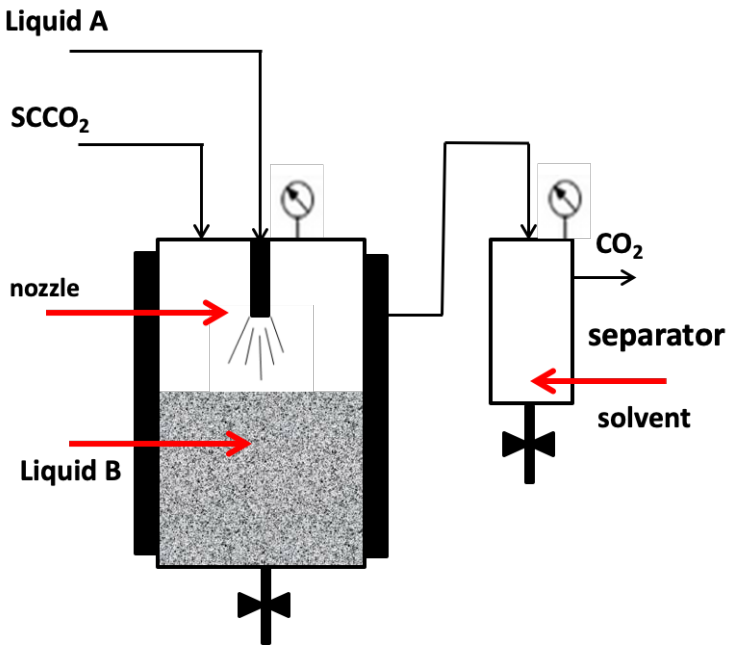
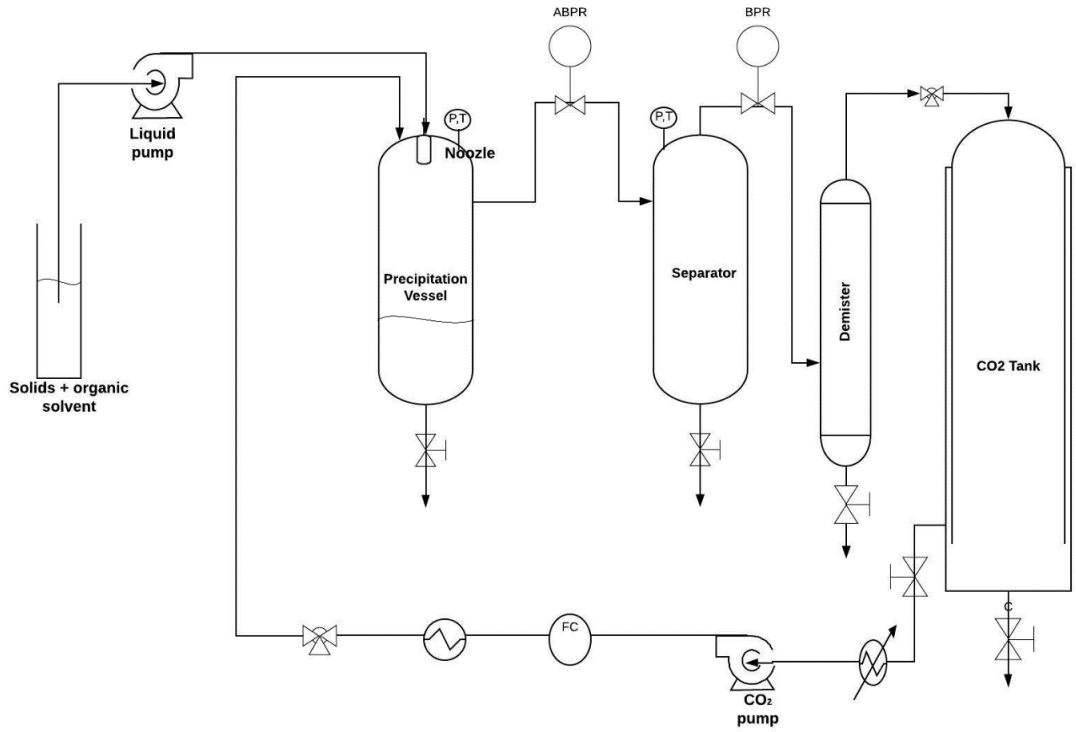


573

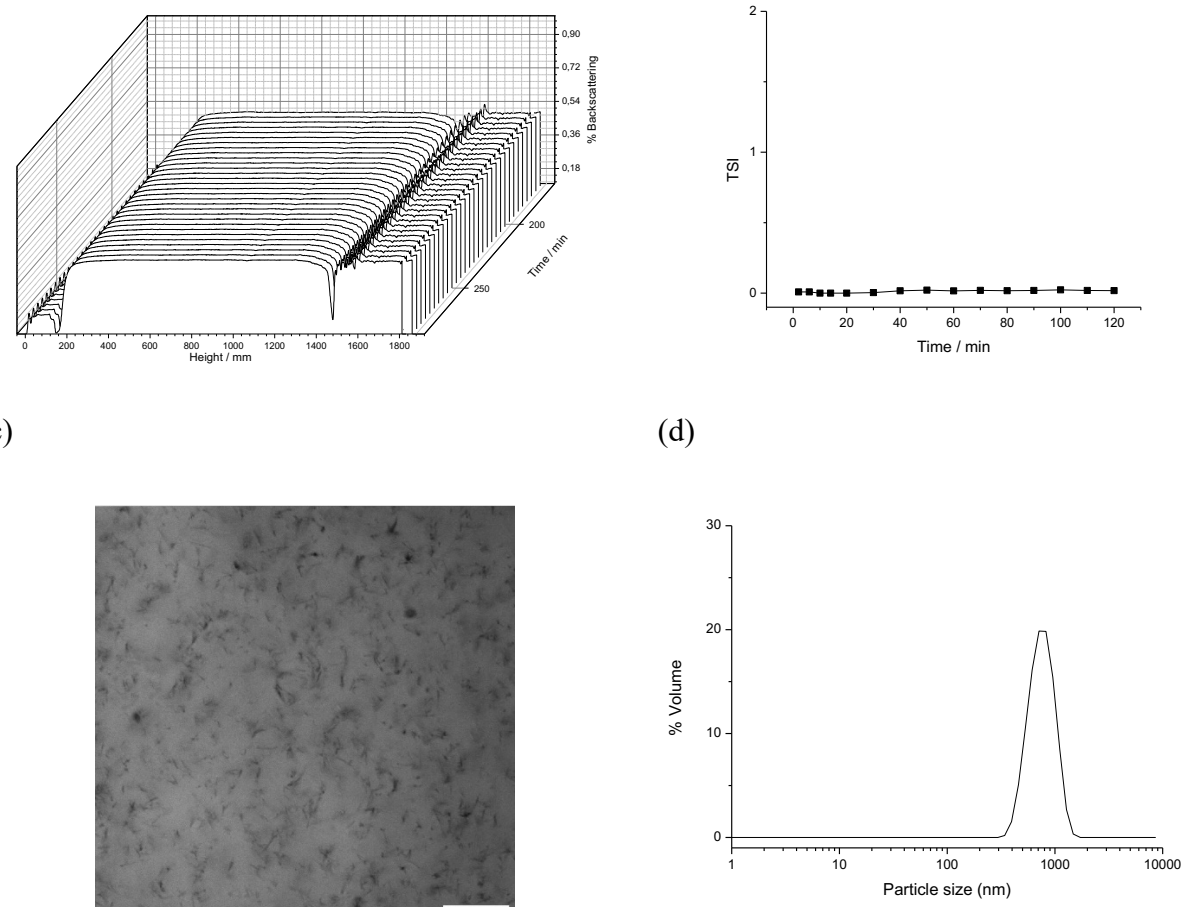
574 **Figure 1.** Scheme of BIOSAS process.



575

576 **Figure 2.** Schematic diagram of equipment used to accomplish BIOSAS process. ABPR:
577 Automatic back pressure regulator, BPR: manual back pressure regulator, P: manometer,
578 T: temperature probe, FC: flowmeter.

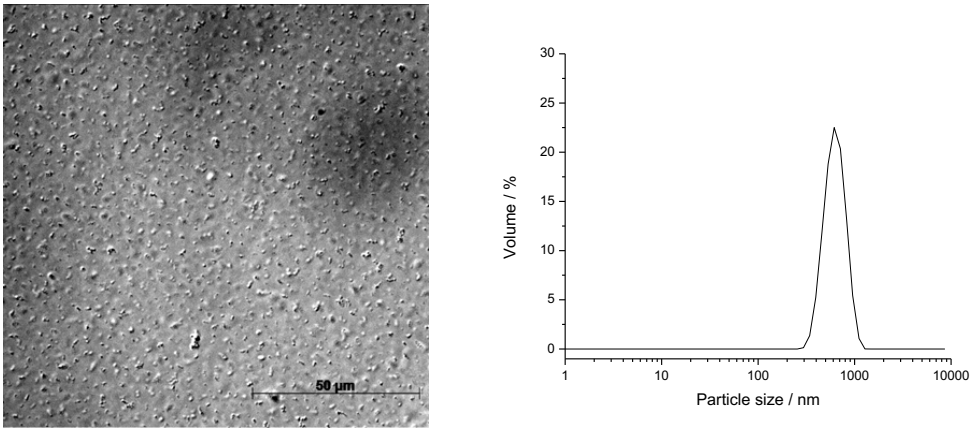
(a) (b)



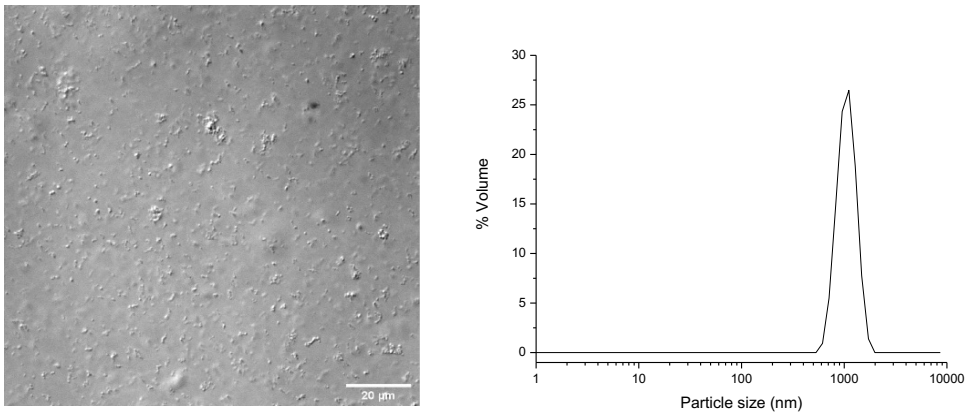
(c) (d)

579 **Figure 3.** Backscattering spectra of the lipid dispersions (a), turbiscan stability index (b),
580 microscopic imagen (c), and particle size distribution (d) of quercetin nano-particles
581 dispersed in flax oil after 72 h of BIOSAS production (experiment 1 in Table 1).

(a)



(b)



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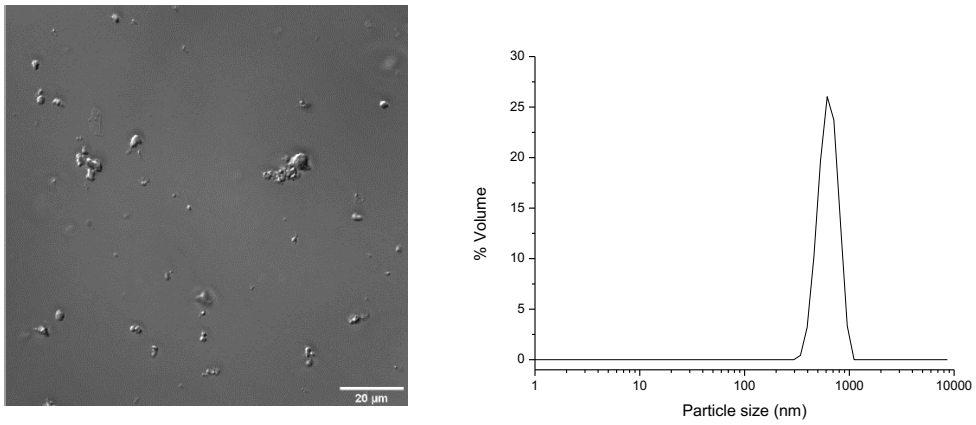


582 **Figure 4.** Microscopic imagen and particle size distribution of rosemary phytochemicals
583 dispersed in (a) olive oil, (b) argan oil and (c) flax oil (experiments 2, 8 and 9 in Table 1).

(a) (b)

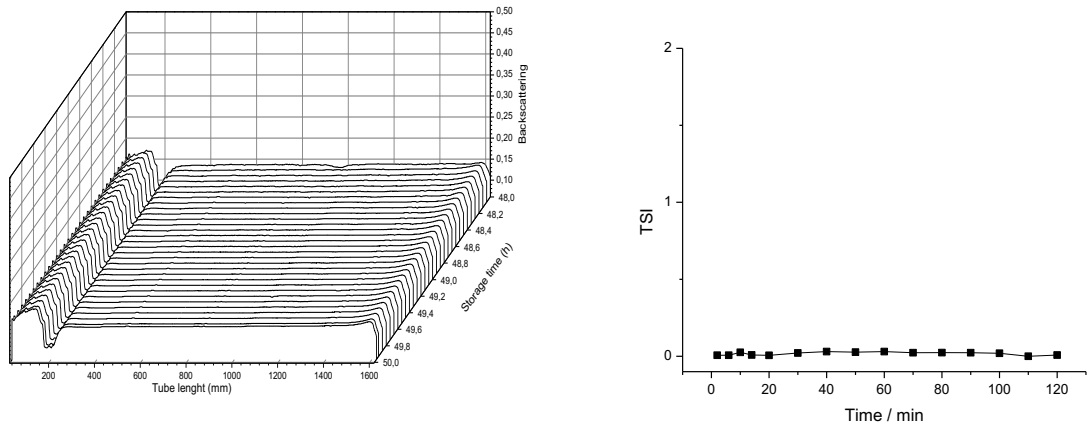
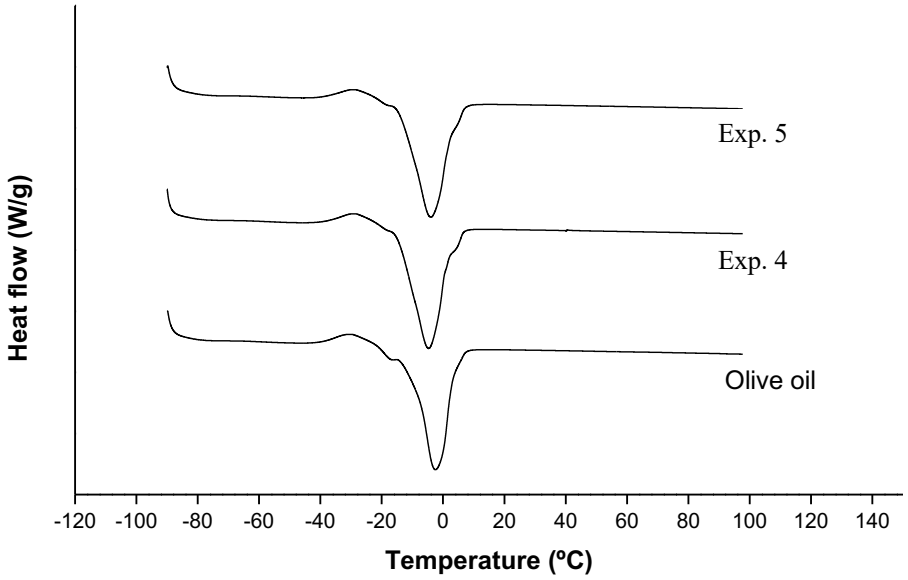


Figure 5. Backscattering spectra (a) and turbiscan stability index (b) of the lipid dispersions of rosemary extract obtained at 15 MPa and 313 K after 72 h of BIOSAS production (experiment 5 in Table 1) (a) and turbiscan stability index (b).

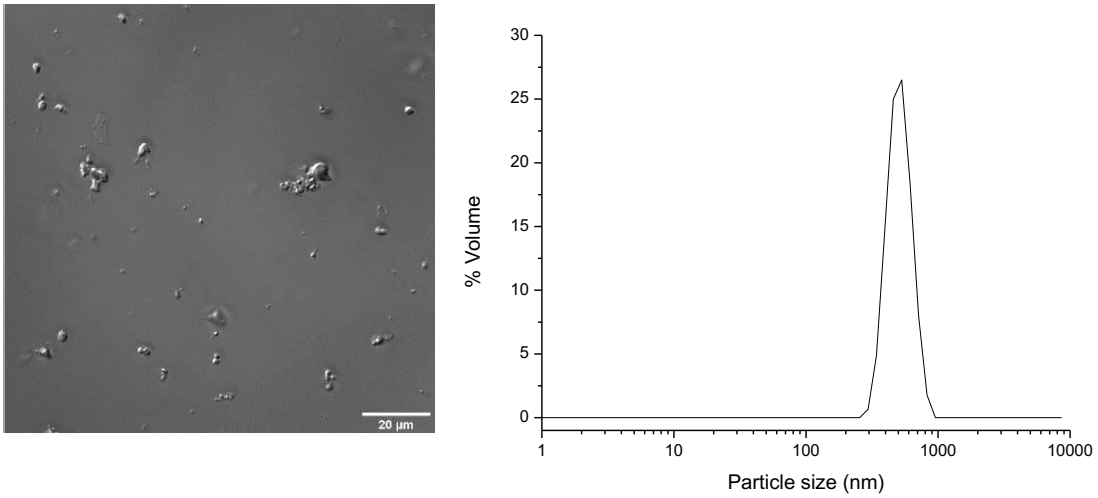
584



585

586 **Figure 6.** Melting curves (obtained by DSC) of olive oil and RES dispersions at 15 MPa
587 and 313 K. Exp. 4: 100 g olive oil; Exp. 5: 50 g olive oil.

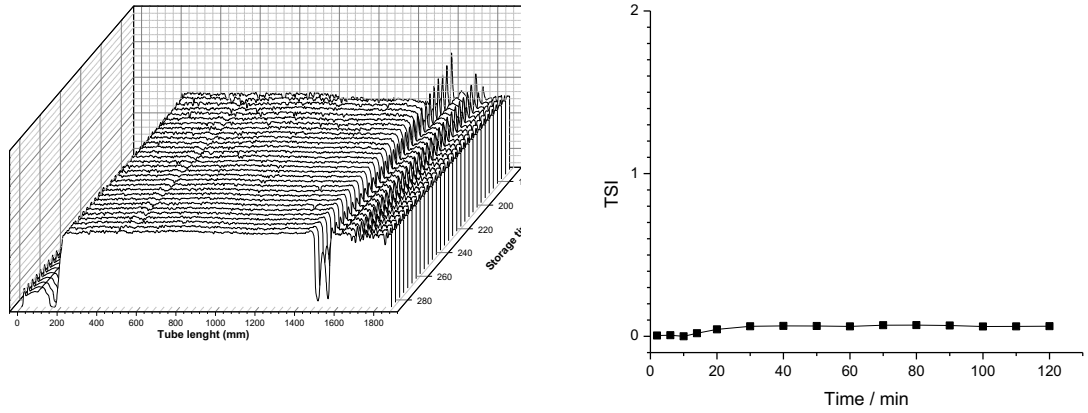
(a) (b)



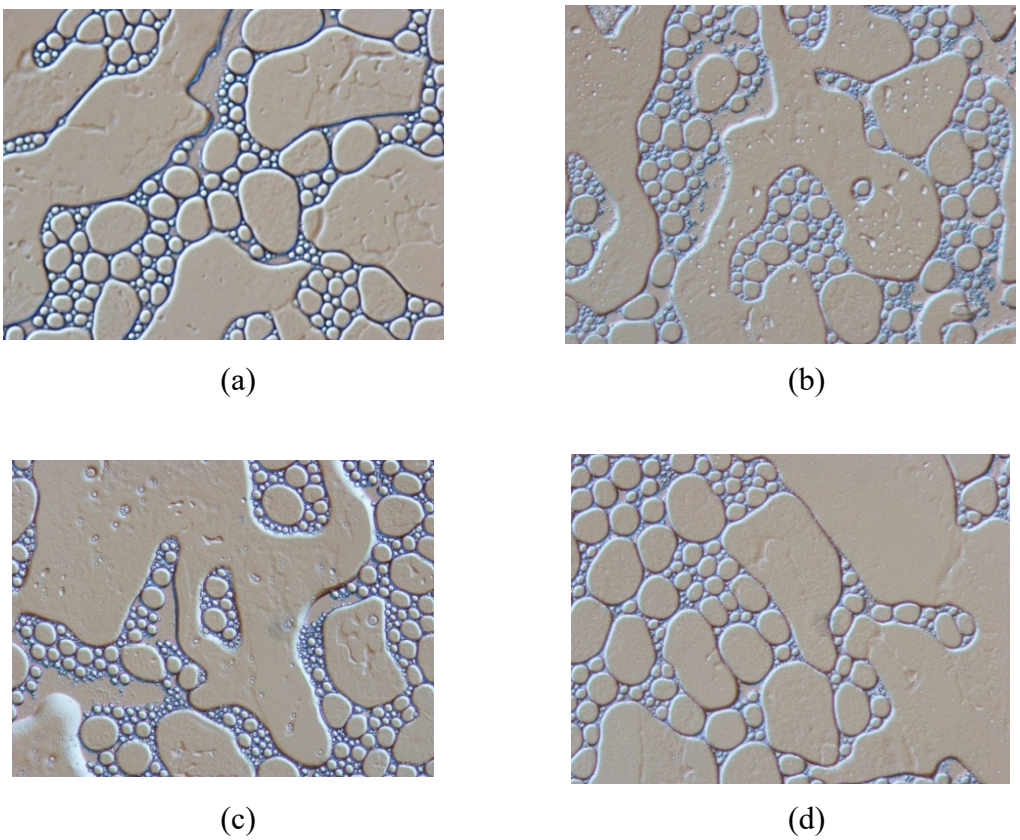
588 **Figure 7.** Microscopic imagen and particle size distribution of licorice root nanoparticles
589 dispersed in flax oil obtained at 20 MPa and 313.15 K after 48 h of BIOSAS production
590 (experiment 12 in Table 1).

591

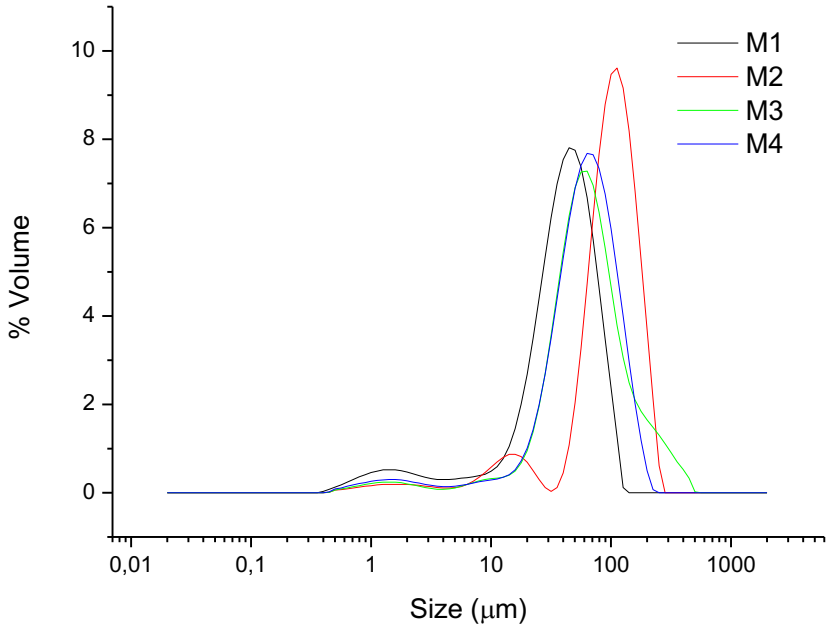
(a) (b)



592 **Figure 8.** Backscattering spectra (a) and turbiscan stability index (b) of licorice root
593 extract dispersed in flax oil obtained at 20 MPa and 313.15 K after 48 h of BIOSAS
594 production (experiment 12 in Table 1)

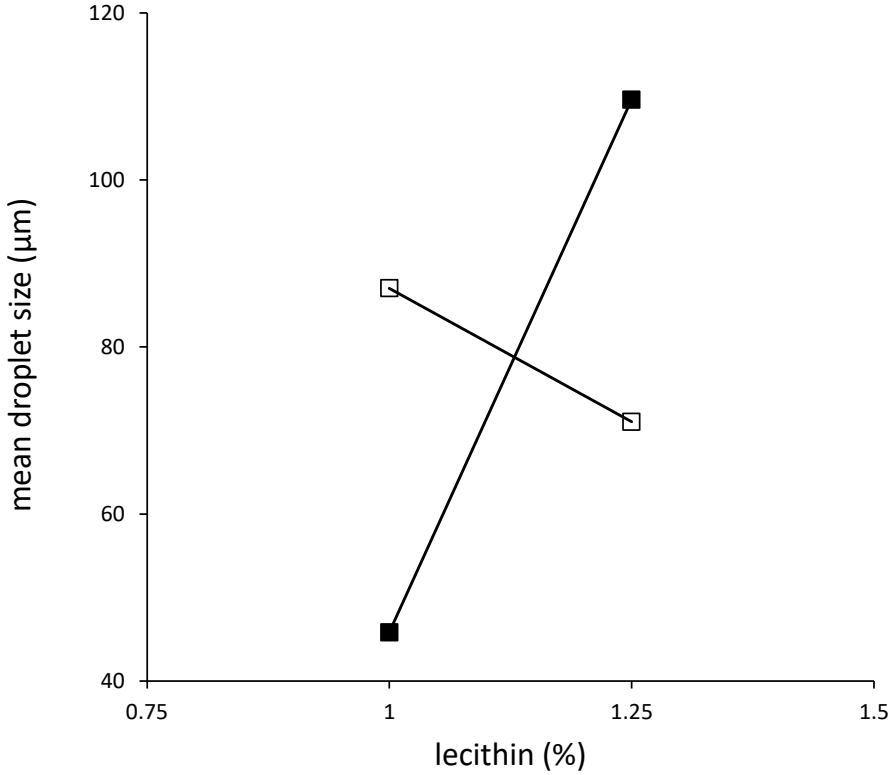


595 **Figure 9.** Optical microscope imagens of 20:80 W/O emulsions of *Quillaja saponaria*
596 aqueous extract 48 h after their production: (a) 20 MPa and 1% lecithin, (b) 20 MPa and
597 1.25% lecithin, (c) 25 MPa and 1% lecithin and (d) 25 MPa and 1.25% lecithin.



598

599 **Figure 10.** Drop size distribution of 20:80 W/O emulsions of *Quillaja saponaria* aqueous
600 extract 48 h after their production: (M1) 20 MPa and 1% lecithin, (M2) 20 MPa and
601 1.25% lecithin, (M3) 25 MPa and 1% lecithin, (M4) 25 MPa and 1.25% lecithin.



602

603 **Figure 11.** Effect of pressure and emulsifier (lecithin) in the mean droplet size of 20:80
604 W/O emulsions containing saponins produced by BIOSAS: (■) 20 MPa; (□) 25 MPa.

605 **Table 1.** BIOSAS process conditions applied in all cases studied in this work: QUE: quercetin; RES: rosemary ethanolic solution; LES: licorice
606 ethanolic solution; QWS: quillaja water solution.

No.	Liquid A	Liquid A (mg/mL)	Liquid B	Liquid B (g in vessel)	P (MPa)	T (K)	Liquid A flow (mL/min)	Time (min)
1	QUE	10	flax oil	57	20	313.15	2.0	45
2	RES	17	olive oil	100	12	313.15	1.6	60
3	RES	17	olive oil	50	12	313.15	1.6	60
4	RES	17	olive oil	100	15	313.15	1.6	60
5	RES	17	olive oil	50	15	313.15	1.6	60
6	RES	17	olive oil	100	20	313.15	1.6	60
7	RES	17	olive oil	50	20	313.15	1.6	60
8	RES	17	argan oil	50	20	313.15	1.6	60
9	RES	17	flax oil	50	20	313.15	1.6	60
10	LES	10	argan oil	70	15	308.15	3.2	45
11	LES	10	argan oil	70	20	313.15	3.2	30
12	LES	10	flax oil	70	20	313.15	3.2	30
13	LES	10	olive oil	70	20	313.15	3.2	57
14	QWS	10	olive oil (1.00% lecithin)	70	20	313.15	1.6	10
15	QWS	10	olive oil (1.25% lecithin)	70	20	313.15	1.6	10
16	QWS	10	olive oil (1.00% lecithin)	70	25	313.15	1.6	10
17	QWS	10	olive oil (1.25% lecithin)	70	25	313.15	1.6	10

607

608 **Table 2.** BIOSAS yield (phytochemicals in Liquid B / phytochemicals in Liquid A pumped) and concentration of phytochemicals (% mass) in the
609 lipid matrix (Liquid B): QUE: quercetin; RES: rosemary ethanolic solution; LES: licorice ethanolic solution; QWS: quillaja water solution.

No.	Liquid A	Liquid B	Phytochemicals recovered in the separator (mg)	BIOSAS yield (%)	Phytochemicals concentration in Liquid B (% mass)
1	QUE	flax oil	n. d.	-	1.58
2	RES	olive oil	1310	19.73	0.32
3	RES	olive oil	1555	4.72	0.15
4	RES	olive oil	1240	24.02	0.39
5	RES	olive oil	1195	26.78	0.87
6	RES	olive oil	1160	28.92	0.47
7	RES	olive oil	710	56.50	1.84
8	RES	argan oil	265	83.76	2.73
9	RES	flax oil	220	86.52	2.82
10	LES	argan oil	1330	7.64	0.16
11	LES	argan oil	850	11.46	0.16
12	LES	flax oil	780	18.75	0.26
13	LES	olive oil	1700	6.80	0.18
14	QWS	olive oil (1.00% lecithin)	-	-	0.23
15	QWS	olive oil (1.25% lecithin)	-	-	0.23
16	QWS	olive oil (1.00% lecithin)	-	-	0.23
17	QWS	olive oil (1.25% lecithin)	-	-	0.23

610 n.d.: no detected

611 **Table 3.** Rosmarinic acid (RA) and carnosic acid (CA) determined in RES-LP (lipid product) and recovered in RES-OP (oleoresin product) after
612 BIOSAS dispersion in olive oil.

Exp.	RES-LP (lipid product)				RES-OP (oleoresin product)				RA dispersion yield (%)	CA dispersion yield (%)
	RA (mg/g oil)	CA (mg/g oil)	RA (mg)	CA (mg)	RA (mg/g oleoresin)	CA (mg/g oleoresin)	RA (mg)	CA (mg)		
2	0.10	0.07	10.0	7.0	3.8	90.0	5.0	117.9	66.8	5.6
3	0.12	0.04	6.0	2.0	4.8	80.3	7.5	156.7	44.6	1.3
4	0.14	0.09	14.0	9.0	5.0	78.0	6.2	96.7	69.3	8.5
5	0.21	0.20	10.5	10.0	4.4	83.4	5.3	99.7	66.6	9.1
6	0.15	0.05	15.0	5.0	7.4	95.7	8.6	111.0	63.6	4.3
7	0.23	0.21	11.5	10.5	3.4	124.3	2.4	88.3	82.7	10.6

613

Table 4. Droplet sizes distribution (μm) of quillaja - olive oil W/O emulsions obtained by BIOSAS process.

Exp.	d(0.1)	d(0.5)	d(0.9)
15	10.694	42.953	83.132
16	23.681	106.487	184.224
17	26.397	65.383	176.887
18	24.304	64.366	128.661

Table 5. Mean droplet diameter and zeta potential after 48 h of the BIOSAS production of quillaja - olive oil W/O emulsions.

Exp.	Mean droplet size (µm)	Zeta potential (mV)
14	45.83	-43.50 ± 0.53
15	109.62	-43.37 ± 2.54
16	87.04	-43.23 ± 2.01
17	71.04	-39.93 ± 1.80

4.3.2. Supercritical carbon dioxide-based methodology to formulate bioactive preparation

Patente solicitada

MINISTERIO
DE INDUSTRIA, ENERGÍA
Y TURISMO

Oficina Española
de Patentes y Marcas

Acknowledgement of receipt

We hereby acknowledge receipt of your request for grant of a European patent as follows:

Submission number	300361106	
Application number	EP20382302.6	
File No. to be used for priority declarations	EP20382302	
Date of receipt	15 April 2020	
Your reference	903 955	
Applicant	Universidad Autónoma de Madrid	
Country	ES	
Title	Supercritical carbon dioxide-based methodology to formulate bioactive preparations	
Documents submitted	package-data.xml	ep-request.xml
	application-body.xml	ep-request.pdf (5 p.)
	SPECEPO-1.pdf\903 955 Description.pdf (24 p.)	SPECEPO-2.pdf\903 955 Claims.pdf (3 p.)
	SPECEPO-3.pdf\903 955 Abstract.pdf (1 p.)	SPECEPO-4.pdf\903 955 Figures.pdf (11 p.)
	OLF-ARCHIVE.zip\903 955 Text to file.pdf	f1002-1.pdf (2 p.)
Submitted by	CN=Gustavo Fuster 26814	
Method of submission	Online	
Date and time receipt generated	15 April 2020, 19:58:59 (CEST)	
Official Digest of Submission	87:3D:50:E9:88:2E:5D:12:B3:E3:BE:1D:08:6F:0A:EA:66:B6:2D:91	

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Request for grant of a European patent

For official use only		
1 Application number:	MKEY	
2 Date of receipt (Rule 35(2) EPC):	DREC	
3 Date of receipt at EPO (Rule 35(4) EPC):	RENA	
4 Date of filing:		

5 Grant of European patent, and examination of the application under Article 94, are hereby requested. ☒

Request for examination in an admissible non-EPO language: Se solicita el examen de la solicitud según el artículo 94.

5.1 The applicant waives his right to be asked whether he wishes to proceed further with the application (Rule 70(2)) ☐

Procedural language: en

Filing Language: en

6 Applicant's or representative's reference: 903 955

Filing Office: ES

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10-1 State of residence or of principal place of business: Spain

14.1 The/Each applicant hereby declares that he is an entity or a natural person under Rule 6(4) EPC. ☒

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Inventor(s)

23 Designation of inventor attached ☒

24 Title of invention

Title of invention: Supercritical carbon dioxide-based methodology to formulate bioactive preparations

25 Declaration of priority (Rule 52) and search results under Rule 141(1)

A declaration of priority is hereby made for the following applications

25.2 Re-establishment of rights

Re-establishment of rights under Article 122 EPC in respect of the priority period is herewith requested for the following priority/priorities

25.3 The EPO is requested to retrieve a certified copy of the following previous application(s) (priority document(s)) via the WIPO Digital Access Service (DAS) using the indicated access code(s):

Request	Application number:	Access Code

25.4 This application is a complete translation of the previous application ☐

25.5 It is not intended to file a (further) declaration of priority ☒

26 Reference to a previously filed application

27 Divisional application ☐

28 Article 61(1)(b) application ☐

29 Claims

Number of claims: 19

29.1 ☒ as attached

29.2 ☐ as in the previously filed application (see Section 26.2)

29.3 ☐ The claims will be filed later

30 Figures

It is proposed that the abstract be published together with figure No.

31 Designation of contracting states

All the contracting states party to the EPC at the time of filing of the European patent application are deemed to be designated (see Article 79(1)).

32 Different applicants for different contracting states

33 Extension/Validation

This application is deemed to be a request to extend the effects of the European patent application and the European patent granted in respect of it to all non-contracting states to the EPC with which extension or validation agreements are in force on the date on which the application is filed. However, the request is deemed withdrawn if the extension fee or the validation fee, whichever is applicable, is not paid within the prescribed time limit.

33.1 It is intended to pay the extension fee(s) for the following state(s):

33.2 It is intended to pay the validation fee(s) for the following state(s):

34 Biological material

38 Nucleotide and amino acid sequences

38.1 The description contains a sequence listing. ☐

38.2a The sequence listing is attached in computer-readable format in accordance with WIPO Standard ST.25 (Rule 30(1)). ☐

38.2b The sequence listing is attached in PDF format ☐

Further indications

39 Additional copies of the documents cited in the European search report are requested

Number of additional sets of copies:

40 Refund of the search fee under Article 9(2) of the Rules relating to Fees is requested ☐

Application number or publication number of earlier search report:

42 Payment

Method of payment: Credit Card

The fees and costs indicated under Fee selection will be paid by credit card within a month after filing.

Currency: EUR

43 Refunds

Any refunds should be made to EPO deposit account: 28120316

Account holder: Hoffmann Eitle S.L.U.

Fees	Factor applied	Fee schedule	Amount to be paid
001 Filing fee - EP direct - online	1	125.00	125.00
002 Fee for a European search - Applications filed on/after 01.07.2005	1	1 350.00	1 350.00
015 Claims fee - For the 16th to the 50th claim	0	245.00	0.00
015e Claims fee - For the 51st and each subsequent claim	0	610.00	0.00
501 Additional filing fee for the 36th and each subsequent page	4	16.00	64.00
Total:		**EUR**	**1 539.00**

44-A Forms

		Details:	System file name:
A-1	Request		as ep-request.pdf
A-2	1. Designation of inventor	1. Inventor	as f1002-1.pdf

44-B Technical documents

		Original file name:	System file name:
B-1	Specification	903 955 Description.pdf Description	SPECEPO-1.pdf
B-2	Specification	903 955 Claims.pdf 19 claims	SPECEPO-2.pdf
B-3	Specification	903 955 Abstract.pdf abstract	SPECEPO-3.pdf
B-4	Specification	903 955 Figures.pdf 11 figure(s)	SPECEPO-4.pdf
B-9	Pre-conversion archive	903 955 Text to file.pdf	OLF-ARCHIVE.zip

44-C Other documents

Original file name: System file name:

45

General authorisation:

46 Signature(s)

Place:	**Madrid**
Date:	**15 April 2020**
Signed by:	**Gustavo Fuster 26814**
Association:	**HOFFMANN EITLE S.L.U.**
Representative name:	**Gustavo Fuster**
Capacity:	**(Representative)**

Form 1002 - 1: Public inventor(s)

Designation of inventor

User reference: 903 955
Application No:

Public

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	The applicant has acquired the right to the European patent:	As employer

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Date:	**15 April 2020**
Signed by:	**Gustavo Fuster 26814**
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Capacity:	**(Representative)**

CAPÍTULO 5

DISCUSIÓN GENERAL

5. DISCUSIÓN GENERAL

Los organismos vegetales producen una gran diversidad de compuestos químicos. Una parte de éstos se forman siguiendo las vías del metabolismo secundario y desempeñan una importante función en las interacciones entre las plantas y el medio que las rodea. En las últimas décadas se ha intensificado el interés científico en demostrar *in vitro* e *in vivo* las actividades biológicas de estas sustancias. Estos estudios contribuyen a comprender el uso, desde tiempos remotos, de plantas y hierbas como conservantes alimentarios y/o como preparados de la medicina tradicional, y han lanzado una carrera, no solo científica sino también empresarial, dentro del área de la alimentación y salud, para el desarrollo de nuevos ingredientes, complementos alimenticios y/o nutracéuticos de uso específico para la salud. En los últimos 10 años, más de 22000 artículos científicos (Scopus) has sido publicados con investigaciones vinculadas a los extractos de plantas, se han solicitado unas 5000 patentes de nuevos nutracéuticos, y se han constituido más de 30 empresas de base tecnológica en España con el objetivo general de producir nuevos productos de uso para la salud de origen natural (https://empresite.eleconomista.es).

Esta Tesis Doctoral contribuye al estudio de las diversas aplicaciones de los extractos vegetales en el campo alimentario. Por un lado, se analizan nuevas fórmulas para preparar recubrimientos comestibles, con el objetivo de prolongar la vida útil de los alimentos y, por otro lado, se contribuye al desarrollo, implementación y validación de una nueva técnica de formulación de ingredientes, que permite combinar compuestos bioactivos con lípidos portadores. Esta nueva técnica, además de lograr la mezcla de los compuestos bioactivos con lípidos, acomete simultáneamente la reducción de tamaño a micro- y nanopartículas, con el objetivo de favorecer la biodisponibilidad y la absorción de los principios activos, y potenciar el efecto beneficioso a nivel biológico. En los siguientes apartados se discuten críticamente los resultados alcanzados en los trabajos, presentados en los capítulos anteriores en forma de publicaciones científicas.

5.1. PREPARACIÓN DE RECUBRIMIENTOS COMESTIBLES CON EXTRACTOS VEGETALES

En la elaboración de recubrimientos comestibles utilizando extractos vegetales se utilizaron cuatro procedimientos de producción con alto potencial para la recuperación de ingredientes bioactivos a partir de plantas: hidrodestilación (HD) e hidrodestilación asistida con ultrasonidos (HD-UAE), con el objetivo de obtener el aceite esencial de la matriz vegetal, la extracción sólido-líquido asistida con ultrasonidos (UAE), para obtener extractos con compuestos químicos de mayor polaridad, y la extracción con fluidos supercríticos (SFE) empleando dióxido de carbono como fluido supercrítico y etanol como co-solvente, como técnica innovadora alternativa a los métodos anteriores.

En el trabajo titulado: "*Comparison between essential oils and supercritical extracts into chitosan-based edible coatings on strawberry quality during cold storage*" (The Journal of Supercritical Fluids, en revisión) se estudia la aplicación de extractos naturales obtenidos

mediante SFE para el desarrollo de recubrimientos comestibles, y se compara su uso con el de los aceites esenciales. Si bien existen muchos trabajos en la bibliografía vinculados a la preparación de recubrimientos comestibles con aceites esenciales, el uso de extractos supercríticos ha sido menos investigado, y no existe actualmente trabajos que comparen la aplicación de ambos tipos de extractos. El análisis de la actividad antioxidante y antimicrobiana (propiedades esenciales de un buen recubrimiento para la conservación de alimentos) de los extractos procedentes de cinco matrices vegetales (romero, tomillo, regaliz, artemisia y centaura), empleando HD, HD-UAE y SFE, permitió seleccionar dos plantas (romero y tomillo) como las más interesantes y adecuadas para desarrollar el estudio comparativo. Si bien los extractos supercríticos de raíz de regaliz también resultaron muy activos, esta planta no fue incluida en el estudio comparativo debido a la escasa recuperación del aceite esencial por HD y HD-UAE.

En general, el contenido de compuestos fenólicos fue determinante en la capacidad antioxidante de los extractos. Los compuestos fenólicos son reconocidos por su actividad antioxidante, por lo que este resultado era de esperar. A mayor concentración de compuestos fenólicos en el extracto, mayor su actividad antioxidante. Los valores de ABTS de todos los extractos y fracciones analizadas se correlacionaron adecuadamente con los valores de TPC, con un coeficiente de regresión lineal de $R^2 = 0,837$. Los aceites esenciales, obtenidos por HD y HD-UAE, presentaron bajo contenido de compuestos fenólicos en comparación con los extractos SFE, a excepción del aceite esencial de tomillo con valores de TPC de 270 - 330 mg GAE/g, hasta 6 veces mayores que los extractos SFE. No obstante, es importante explicar que en la SFE los contenidos de compuestos fenólicos variaron significativamente con las condiciones de extracción, resultando el uso de etanol como co-solvente y/o el fraccionamiento por descompresión en cascada, dos alternativas adecuadas para aumentar el contenido de compuestos fenólicos en los correspondientes productos. Concretamente, el mayor contenido de compuestos fenólicos se observó para los extractos supercríticos de romero empleando etanol como co-solvente (174 mg GAE/g), y la segunda fracción por descompresión en cascada (167 mg GAE/g), seguido por el extracto de raíz de regaliz obtenido por fraccionamiento en cascada, concretamente 159 mg GAE/g en la fracción obtenida en el segundo separador, el extracto de tomillo y en mucha menor proporción los extractos de artemisia y centaura (< 27 mg GAE/g).

En relación a la actividad antimicrobiana, excepto en el caso de la centaura, tanto los extractos obtenidos por hidrodestilación como los extractos supercríticos de las plantas estudiadas presentaron actividad frente a bacterias Gram positivas (*S. aureus*) y Gram negativas (*E. coli*). En general, los productos obtenidos por HD o HD-UAE contienen una mayor concentración de terpenoides volátiles, algunos de los cuales con reconocida actividad antimicrobiana (borneol, eucaliptol, timol, carvacrol) (Liu y col., 2017; Olasupo y col., 2003). Así, los valores de IC_{50} de los productos HD y HD-UAE estuvieron contenidos en el rango 0,05-2,12 mg/mL, siendo menores que los correspondientes a los extractos supercríticos (0,76-2,93 mg/mL).

Considerando los resultados obtenidos en relación a las actividades biológicas estudiadas de los extractos supercríticos, y comparando los altos rendimientos de la SFE frente a la hidrodestilación, se concluye que la tecnología supercrítica puede ser apropiada para la producción de extractos vegetales (especialmente de romero, tomillo y regaliz) con buena actividad antioxidante y antimicrobiana, para ser empleados en la formulación de recubrimientos alimentarios comestibles. Para comprobarlo, como se mencionó anteriormente, se seleccionaron ciertos extractos, concretamente los extractos supercríticos de romero (40 °C y 15 MPa) y de tomillo (40 °C, 15 MPa y 10% de etanol como co-solvente) y sus aceites esenciales (HD) para la preparación de los recubrimientos y su aplicación en fresas, como modelo de un alimento muy susceptible al deterioro por crecimiento microbiano.

Los recubrimientos utilizados para las fresas se elaboraron a base de quitosano con la adición en distintas proporciones (1 y 5 % en peso) de extracto supercrítico o de aceite esencial, de romero o tomillo, siguiendo el mismo protocolo. El almacenamiento se llevó a cabo a 4 °C durante 10 días, y se observaron ciertos parámetros para determinar el efecto de los distintos recubrimientos en la conservación de las bayas. Si bien los recubrimientos comestibles producidos con aceites esenciales exhibieron con el tiempo una pérdida de peso algo mayor, resultaron más eficaces en retrasar el aumento de pH, disminuyendo el efecto de maduración del fruto por consumo de ácidos orgánicos, y preservaron mejor el contenido de los compuestos fenólicos de las fresas (especialmente los recubrimientos elaborados con aceite esencial de romero). No obstante, cabe destacar que los recubrimientos con extracto supercrítico de romero presentaron la menor pérdida de peso en las frutas, en comparación con todos los tratamientos ensayados, y también fueron muy eficaces para conservar el contenido de compuestos fenólicos de las bayas. En relación al color de la fruta, no se encontraron efectos importantes en relación con ambos tipos de recubrimientos.

Si bien en términos generales los recubrimientos con aceites esenciales funcionaron mejor que los preparados con extractos SFE, en comparación con la muestra control (fresas sin recubrimiento alguno) los extractos supercríticos combinados con quitosano mostraron capacidad de mejorar los parámetros de la maduración de las fresas, las que exhibieron un desarrollo fúngico significativamente menor que en el caso de la muestra control. Es decir, comparando con la muestra control, es posible concluir un efecto protector de los extractos supercríticos de romero y tomillo para ser empleados como recubrimientos comestibles. No obstante, sería interesante explorar la eficacia de estos extractos en otro tipo de alimento, debido a que, aunque en menor medida que los aceites esenciales, los extractos supercríticos también aportan el sabor y aroma característico de la planta.

Por otro lado, considerando los resultados obtenidos en relación a la actividad antioxidante y antimicrobiana de los extractos de raíz de regaliz, así como la escasez de datos bibliográficos sobre la actividad antimicrobiana de sus extractos supercríticos, se estudió la extracción con $SCCO_2$ de la raíz de regaliz, la evaluación *in vitro* de la actividad antioxidante de los extractos y, por primera vez, se determinó *in vitro* la actividad antimicrobiana de las fracciones obtenidas. Los resultados componen el trabajo titulado "*Antioxidant and Antimicrobial Assessment of Licorice Supercritical Extracts*" (Journal Industrial Crops &

Products 139 (2019) 111496) donde se analiza tanto el efecto del uso de etanol como co-solvente como el fraccionamiento en cascada (utilizando un sistema de descompresión de dos celdas) en la composición y en la actividad antioxidante y antimicrobiana de los productos obtenidos. El objetivo fue estudiar la posibilidad de obtener fracciones con propiedades bioactivas mejoradas, en comparación con los resultados obtenidos hasta el momento.

En general, los extractos supercríticos de regaliz obtenidos en este trabajo presentaron una mejor actividad antioxidante en comparación con otros extractos de la literatura, producidos utilizando disolventes líquidos (metanol, cloroformo, etanol, agua) y diferentes técnicas. No obstante, aunque se evidenció una tendencia a una mejor actividad antioxidante cuanto mayor es el contenido de compuestos fenólicos, no se encontró una correlación lineal entre los valores TEAC y TPC de los distintos extractos y/o fracciones producidas. Los mayores contenidos de compuestos fenólicos se obtuvieron al utilizar etanol como co-solvente, aumentando los valores de TPC en forma lineal con la cantidad de etanol utilizada, hasta unos 180 mg GAE/g a 30 MPa, 40 °C y 20% de etanol como co-solvente. Este contenido de TPC es unas 4 veces mayor que los obtenidos con 80% etanol por extracción asistida por microondas (Karami y col., 2013; Karami y col., 2015) y 8, 4 y 2 veces mayor que los obtenidos por extracción Soxhlet utilizando, respectivamente, agua (Gupta y col., 2016), etanol (Visavadiya y col., 2009) y metanol (Hejazi y col., 2017). También, el extracto obtenido a 15 MPa, 40 °C con 20 % de etanol como co-solvente es el que presentó la mejor actividad antioxidante.

Por otro lado, los extractos obtenidos con etanol presentaron una actividad antimicrobiana baja o incluso nula contra bacterias Gram positivas (*S. aureus*) y Gram negativas (*E. coli*). Por el contrario, la extracción con $SCCO_2$ puro a alta presión (30-40 MPa) con un fraccionamiento en línea dio lugar a una fracción (primer separador a 15 MPa) con un bajo contenido de compuestos fenólicos pero con una buena actividad antimicrobiana, mientras que la fracción recogida en el segundo separador (≈ 5 MPa) presentó buena actividad antioxidante. En general, *S. aureus* fue más resistente (IC_{50} mayores) que *E. coli* al efecto antimicrobiano de los extractos y/o fracciones supercríticas de raíz de regaliz, resultado que coincide con los presentados por Chandra y Gunasekaran, (2017) en el estudio de extractos de raíz de regaliz obtenidos por extracción sólido-líquido con un disolvente apolar (cloroformo).

Así, en este estudio se destaca la capacidad de la técnica de fraccionamiento supercrítico, aplicado a la raíz de regaliz, para separar selectivamente las sustancias del extracto, y obtener fracciones con propiedades biológicas diferentes, antimicrobianas o antioxidantes. Cabe destacar también que se identificaron y cuantificaron los compuestos característicos de la raíz de regaliz (liquiritina, liquiritigenina, ácido glicirrícico, isoliquiritigenina y glabridina) en todos los extractos y fracciones supercríticas obtenidas. Mientras que se observó que la presencia de algunos de estos compuestos podía tener relación con la actividad antioxidante de la muestra (por ejemplo, glabridina), no se encontró relación alguna entre la actividad antimicrobiana y alguno de estos compuestos.

Con el objetivo de comparar los extractos supercríticos de raíz de regaliz con los obtenidos mediante disolventes más polares, se llevó a cabo la extracción asistida por

ultrasonidos (UAE) de la planta, utilizando disolventes de diferente polaridad (etanol, metanol y sus mezclas con agua). En el trabajo titulado *"Preparation and characterization of licorice-chitosan coatings for postharvest treatment of fresh strawberries*" (Enviado al Journal Applied Science) se evaluó el efecto de la temperatura y de la polaridad de los disolventes en la composición y actividad antioxidante y antimicrobiana de los extractos obtenidos. Los extractos más activos se utilizaron para elaborar recubrimientos comestibles, y se evaluaron sus propiedades reológicas, así como su efecto en la conservación de las características fisicoquímicas y microbiológicas de fresas.

El mayor contenido de TPC se alcanzó en las muestras obtenidas empleando etanol como disolvente, con contenidos entre a 144 mg GAE/g (25 ºC) y 163 mg GAE/g (50 ºC), seguido por los extractos obtenidos con metanol. También, se evidenció que a mayor contenido de TPC, mayor la actividad antioxidante del extracto, pero menor o incluso nula la actividad antimicrobiana contra bacterias Gram positivas (*S. aureus*) y Gram negativas (*E. coli*). Por el contrario, los extractos obtenidos combinando etanol o metanol con 50 % v/v de agua, presentaron bajos valores de TPC, menor actividad antioxidante y mayor actividad antimicrobiana en comparación con los extractos obtenidos con los disolventes puros. En resumen, se concluye que se obtuvo un mayor contenido de TPC con disolventes de menor constante dieléctrica, es decir menor polaridad, y fueron los que presentaron mejor actividad antioxidante. En cambio, los extractos con menor contenido de TPC y que resultaron mejores antimicrobianos se obtuvieron con los disolventes de mayor polaridad.

En general, la temperatura de extracción afectó a la concentración de los compuestos bioactivos de la raíz de regaliz, siendo que las muestras obtenidas a 50 ºC presentaron una mayor concentración de biomoléculas que las obtenidas a 25 ºC. Por otro lado, los extractos etanólicos de raíz de regaliz presentaron un alto contenido de glabridina, seguido de ácido glicirrícico, mientras que lo opuesto se observó en los extractos obtenidos con metanol: agua y etanol: agua. En todos los casos se encontraron bajas concentraciones de liquiritigenin, isoliquiritigenin y liquiritin. La extracción de ácido glicirrizico (el componente más polar de los identificados por cromatografía de líquidos) aumentó considerablemente en función de la polaridad (constante dieléctrica) del disolvente utilizado, mientras que se observó el efecto contrario para la glabridina (componente menos polar). Teniendo en cuenta los resultados del análisis de la actividad antioxidante y antimicrobiana, podría concluirse que, mientras la glabridina contribuye a la actividad antioxidante de la raíz de regaliz (tal como se observó en el caso de los extractos supercríticos), el ácido glicirrícico contribuye a su actividad antimicrobiana. Estos resultados coinciden con lo que ha sido observado en la bibliografía tanto en relación a la actividad antimicrobiana Irani y col., 2010; Thakur y col., 2016; Wang y col., 2015) como a la actividad antioxidante (Chandra y Gunasekaran, 2017; Vaya y col., 1997; Vlaisavljević y col., 2018) de la raíz de regaliz.

El extracto UAE de raíz de regaliz obtenido con etanol: agua (50:50) se seleccionó para la formulación de los recubrimientos comestibles, por presentar un balance adecuado entre sus propiedades antioxidantes y antimicrobianas. Este extracto contiene 86 mg GAE/g y valores de IC_{50} contra *E. coli* y *S. aureus* de 0.84 y 1.43 mg/mL, respectivamente, valores similares a los

de los extractos supercríticos que mostraron tanto actividad antioxidante como antimicrobiana (por ejemplo, la fracción obtenida en el primer separador a 30 MPa y 40 °C, con 70 mg GAE/g e IC_{50} de 0.76 y 1.49 mg/mL). Así, se elaboraron tres soluciones formadoras de recubrimientos, una a base de quitosano, y las otras dos a base de quitosano con la adición de 1 y 5 % en peso del extracto UAE de regaliz seleccionado. Se estudiaron por primera vez en esta tesis las propiedades reológicas de estos recubrimientos, puesto que, si bien la presencia en el recubrimiento de las sustancias bioactivas propias de la matriz vegetal suele mejorar sus propiedades como conservantes, también pueden modificar las propiedades físicas de las soluciones formadoras de las películas. Los efectos de la fuerza viscosa del fluido, la tensión superficial y la densidad de estas soluciones, son determinantes para una buena formación de la película protectora (recubrimiento) (Chen y col., 2016).

Todas las soluciones formadoras de recubrimientos exhibieron un comportamiento de fluido no newtoniano. La solución a base de quitosano presentó un comportamiento tipo pseudo-plástico ("*shear thinning*") caracterizado por uniones intermoleculares que se rompen más rápidamente que su velocidad de deformación, con el aumento de la velocidad de cizalla. La adición de extracto de raíz de regaliz al quitosano provocó una disminución en el valor del esfuerzo cortante y un cambio de comportamiento de dilatancia ("*shear thickening*"), probablemente debido al agrupamiento de partículas y una transición de orden a desorden, provocado por la presencia de los fitoquímicos del regaliz en la solución.

Por otro lado, todas las soluciones elaboradas presentaron una buena capacidad para la formación de películas. En particular, la adición de fitoquímicos de regaliz aumentó las propiedades elásticas (G') y viscosas (G'') de la película, siendo más viscosa con mayor adición de extracto. Así, las películas que contienen compuestos bioactivos de la raíz de regaliz mostraron mayor flexibilidad en comparación con la película de quitosano, permitiendo una mejor aplicación como recubrimiento de las fresas, y lográndose una mejor extensibilidad superficial sobre las muestras, formando recubrimientos adelgazantes con mayor uniformidad y adherencia, resultando en una mejora en la apariencia física de las fresas.

En relación a los parámetros vinculados a la maduración de las frutas, todos los recubrimientos redujeron la pérdida de peso de la fruta en comparación con las muestras sin recubrimiento, siendo más eficaces los recubrimientos de quitosano con extracto de regaliz. Los valores de pH de las muestras no cambiaron significativamente durante el almacenamiento y, aunque hubo diferencias en el día 1 (aplicación del recubrimiento), no se observaron diferencias significativas entre las fresas recubiertas en el día 10 (pH medio de 4,05 ± 0,10) mientras que se obtuvo un valor de pH significativamente más alto para la fruta sin recubrir (4,50 ± 0,20). Los valores de acidez se redujeron en todos los tratamientos (fresas recubiertas y sin recubrir) debido a la maduración y a la naturaleza de los ácidos orgánicos de esta fruta. No obstante, esta disminución en la acidez no indujo un cambio significativo en el pH.

Además, desde el punto de vista microbiológico, los recubrimientos con compuestos bioactivos de raíz de regaliz resultaron mejores que los recubrimientos formados únicamente con quitosano. El porcentaje de descomposición por hongos fue mayor en las muestras control,

seguido de las muestras de quitosano, mientras que las fresas con recubrimientos con extracto de regaliz no presentaron descomposición fúngica alguna en el tiempo de almacenamiento. Esta mayor acción antimicrobiana de los recubrimientos con regaliz se verificó con el recuento de microorganismos, en el cual, las fresas sin recubrimiento presentaron los valores más altos de UFC/g de mesófilos, psicrofilos y levaduras, mientras que las muestras con extracto de regaliz presentaron valores prácticamente nulos en el tiempo de almacenamiento. En resumen, la adición de fitoquímicos de regaliz para preparar el recubrimiento comestible permitió mejorar las propiedades antimicrobianas del quitosano. También, es importante destacar que los recubrimientos con extracto de regaliz redujeron la pérdida de los compuestos fenólicos de las fresas, en comparación con la muestra control o con las fresas recubiertas con quitosano. Así, la adición de extracto de regaliz permitió extender la vida útil de las fresas en un periodo de tiempo mayor al alcanzado por Pavinatto y col. (2020) empleando películas de quitosano, o Muley y Singhal (2020) empleando quitosano con conjugados de proteína de suero.

5.2. MICRONIZACIÓN DE EXTRACTOS VEGETALES MEDIANTE TECNOLOGÍA DE FLUIDOS SUPERCRÍTICOS

La tecnología supercrítica antisolvente (SAS) empleando $SCCO_2$ como antisolvente constituye un procedimiento para la micronización de sustancias, con el objetivo de producir partículas regulares de pequeñas dimensiones, difíciles de obtener con procedimientos tradicionales (secado por aspersión, liofilización, evaporación de solvente, coacervación). Estas sustancias micronizadas, en forma de polvo, con tamaños incluso dentro de la escala nanométrica, se utilizan frecuentemente para la preparación de fármacos y fórmulas nutracéuticas. En el caso de utilizar la tecnología SAS para la micronización de un extracto de origen vegetal (una mezcla multicomponente de sustancias con diferentes polaridades y volatilidades) durante el proceso se produce un fraccionamiento de esta mezcla: mientras que las sustancias más polares y de menor volatilidad precipitan en forma de polvo, las menos polares y más volátiles (más solubles en la fase supercrítica) no precipitan y, en cambio, se recogen como subproducto del proceso en el separador (Figura 1.5). Así, en diferentes condiciones de operación se obtienes productos diferentes, no sólo en relación al tamaño y forma, sino también en cuanto a la composición de precipitado y subproducto. Ambos factores son muy importantes en la formulación de nutracéuticos a partir de extractos vegetales.

En esta tesis se estudió la precipitación SAS de dos extractos vegetales, los extractos etanólicos de romero (*Rosmarinus officinalis L.*) y raíz de regaliz (*Glycyrrhiza glabra L.*), ambos obtenidos por extracción asistida con ultrasonidos. Estos estudios dieron lugar a los trabajos titulados, respectivamente, "*Supercritical antisolvent particle precipitation and fractionation of rosemary (Rosmarinus officinalis L.) extracts*" (Journal of CO_2 Utilization, 34 (2019) 479 – 489) y "*Fractionation and precipitation of licorice (Glycyrrhiza glabra L.) phytochemicals by supercritical antisolvent (SAS) technique*" (Journal LWT-Food Science and Technology 126 (2020) 109315)

En la precipitación SAS, la selección de las condiciones de operación en comparación con el punto crítico de la mezcla CO_2 + etanol + sustancia a precipitar, ejerce una influencia

clave en el tamaño y la morfología de las partículas precipitadas, desde la obtención de aglomerados de gran tamaño y/o partículas huecas, hasta partículas homogéneas de tamaño nanométrico. Mientras que en el caso de una sustancia pura el punto crítico puede que sea conocido, en el caso de un extracto vegetal multicomponente no existen datos disponibles en la bibliografía respecto del punto crítico de la mezcla (PCM). No obstante, en el desarrollo experimental del proceso SAS para los extractos de romero y raíz de regaliz se llevó a cabo un análisis, sobre la base de los puntos críticos de la mezcla binaria CO_2 + etanol, el que permitió observar tendencias y conclusiones interesantes.

Cabe destacar que, en el caso de la precipitación SAS del romero, los trabajos de la bibliografía se han referido al análisis del fraccionamiento de sus compuestos antioxidantes (ácido rosmarínico, ácido carnósico, carnosol), con el objetivo de obtener fracciones altamente bioactivas. Sin embargo, el efecto de las condiciones del proceso sobre la morfología y la distribución de tamaño de partícula de los precipitados no ha sido examinado detalladamente. Por otro lado, en relación a la raíz de regaliz, el estudio del fraccionamiento y precipitación SAS se presenta por primera vez en el contexto de esta tesis.

El rango de las condiciones de operación para los extractos de romero y raíz de regaliz se seleccionaron en función de la experiencia previa del grupo de investigación utilizando el mismo equipo experimental y otras sustancias y matrices vegetales (Villanueva-Bermejo y col., 2017). Se realizaron experimentos en un rango de presión de 8-20 MPa, temperaturas de 35 - 60 °C y caudal másico de $SCCO_2$ entre 50 y 60 g/min. No obstante, no se logró en todas las condiciones ensayadas la precipitación en forma de polvo para los dos tipos de extractos, demostrando la sensibilidad del método al tipo de material vegetal utilizado.

En el caso del romero, el extracto etanólico contenía 16,04 mg/mL de sustancias sólidas; para el estudio de la raíz de regaliz se utilizaron dos concentraciones de extracto diferentes (14,2 mg/mL y 9,6 mg/mL) con el objetivo de analizar la influencia de este parámetro en el proceso SAS. En ambos casos, se obtuvieron dos fracciones: un polvo seco en la celda de precipitación, con partículas de tamaño micro- y nanométrico (precipitado), y un líquido oleoso muy viscoso (oleorresina) obtenido después de eliminar el etanol del producto recuperado del separador. Como se mencionó anteriormente, este fraccionamiento de los compuestos del extracto se puede explicar en términos de su polaridad y volatilidad, determinada por la estructura química de las sustancias. Aquellas más polares son menos solubles en la fase supercrítica (CO_2 + etanol) que abandona la celda de precipitación, por lo que tienden a precipitar y se recuperan en la celda de precipitación. En cambio, los compuestos menos polares, tienden a solubilizarse en la fase supercrítica y, por lo tanto, se recuperan en el separador junto con el co-solvente licuado. Asimismo, la volatilidad de las sustancias influye en esta separación, siendo que, cuanto mayor es su volatilidad, mayor es la tendencia a abandonar la celda de precipitación junto con la fase supercrítica.

En el proceso SAS de romero se observó en las oleorresinas altas concentraciones de los componentes del aceite esencial de romero (alcanfor, eucaliptol, borneol), favorecida por la alta volatilidad de los monoterpenos que componen el aceite esencial de la planta en

comparación con otros compuestos terpenoides de mayor peso molecular. Asimismo, se evidenció una separación selectiva de los principales antioxidantes del romero (ácido rosmarínico, ácido carnósico y carnosol) y que el fraccionamiento de estos compuestos se efectuó según la solubilidad de estas sustancias en la fase supercrítica (ácido rosmarínico << carnosol < ácido carnósico), llegando a duplicarse la concentración de ácido rosmarínico en los precipitados y la concentración de ácido carnósico y carnosol en las oleorresinas. Los resultados obtenidos en el fraccionamiento SAS de los principales compuestos bioactivos del romero, siguieron las mismas tendencias publicadas en trabajos anteriores (Sánchez-Camargo y col., 2016; Visentin y col., 2012; Visentín y col., 2011): el ácido rosmarínico se recuperó en el precipitado (alcanzando valores de 40 mg/g), mientras que el ácido carnósico se encontró en las dos fracciones, pero más concentrado en la oleorresina (hasta 79 mg/g en los precipitados y 300 mg/g en las oleorresinas). Los valores publicados son similares a los obtenidos en esta tesis. Por ejemplo, Visentín y col. (2011) obtuvo concentraciones de ácido carnósico de 18,4 - 55,1 mg/g en la celda de precipitación y 28,2 - 330 mg/g en el separador. Posteriormente, estudiando la encapsulación SAS de estos compuestos con un polímero, Visentin y col. (2012) obtuvo concentraciones entre 6,7 - 4,0 mg/g de ácido rosmarínico y de 18 - 181 mg/g de ácido carnósico en los precipitados. Por su parte, Sánchez-Camargo y col. (2016), en la precipitación SAS de un extracto de romero obtenido con etanol y agua, obtuvieron concentraciones de 9,89 - 67,66 mg/g de ácido rosmarínico y de 13,74 - 87,07 mg/g de ácido carnósico en el precipitado, siendo necesaria la liofilización para obtener el producto precipitado en forma de polvo seco.

En cuanto al contenido total de compuestos fenólicos (TPC) en las fracciones obtenidas, las muestras que presentaron altos valores de TPC también presentaron altos valores TEAC, es decir una alta capacidad antioxidante. No obstante, en cuanto al contenido de TPC, aunque la diferencia entre los valores correspondientes a la oleorresina y el precipitado de un mismo ensayo no fue significativa, las oleorresinas presentaron una mayor capacidad antioxidante que los precipitados. Así, mientras que los valores de TPC y TEAC en los precipitados fueron, respectivamente, en los rangos de 103-177 mg GAE/g y 448 - 975 µmol Trolox /g, las oleorresinas presentaron un rango similar de valores TPC (120 - 221 mg GAE/g) y significativamente mayores valores TEAC (664 - 1376 µmol Trolox /g). Posiblemente, esto pueda deberse al tipo de compuesto fenólico presente en cada producto y, en este sentido, cabe remarcar que los diterpenos fenólicos del romero (ácido carnósico, carnosol), componentes antioxidantes mayoritarios del romero, se concentraron preferentemente en las oleorresinas.

Como regla general los precipitados de romero con mejor actividad antioxidante se obtuvieron a bajas presiones y altas temperaturas, mientras que justo lo opuesto se evidenció en las oleorresinas. Teniendo en cuenta el valor TEAC del extracto de romero original (661,84 ± 0,25 µmol Trolox/g) es notable el incremento obtenido en las oleorresinas, con valores de hasta 1376 µmol Trolox/g, lo que demuestra el potencial de la técnica SAS para fraccionar extractos vegetales y mejorar así sus actividades biológicas.

En cuanto al estudio de microestructura de los polvos de romero precipitados, en este trabajo se presenta el análisis de tamaño de partícula en función de las variables del proceso. Se obtuvieron partículas en el rango de 0,62 a 21,06 µm, siendo más pequeñas a mayor presión

y menor temperatura de operación. La morfología y la distribución de tamaño de estas partículas resultaron más o menos homogéneas, en función de las condiciones de operación. A bajas presiones (9 - 10 MPa) las partículas fueron delgadas, alongadas y aglomeradas, observándose morfologías variables. A mayores presiones (15 - 20 MPa) las partículas fueron mucho más esféricas y con una distribución de tamaño más homogénea.

En el caso de la precipitación SAS de extracto etanólico de raíz de regaliz, hubo muchos intentos fallidos especialmente a temperaturas mayores que 40 °C, en los que no fue posible obtener partículas secas o se formaron grandes aglomerados, pegados a las paredes y al fritado de la celda de precipitación. De hecho, la precipitación de partículas se logró en un rango muy estrecho de temperaturas (35 - 40 °C) a presiones entre 12,5 y 20 MPa. No obstante, en este estudio, se incluyó como variable de proceso la concentración de extracto en la disolución etanólica (9,6 mg/mL y 14,2 mg/mL).

Los resultados obtenidos permitieron evidenciar que, en el estrecho rango de temperaturas en las que se logró la formación de partículas secas, la presión y la temperatura no afectaron sustancialmente el rendimiento de precipitación (masa extracto precipitado / masa extracto procesado). No obstante, la concentración del extracto etanólico tuvo un efecto significativo, lográndose un mayor rendimiento de precipitación a menor concentración de extracto en la disolución etanólica. Al igual que en el caso de la precipitación SAS de romero, la recuperación de fitoquímicos fue mayor en los precipitados que en las oleorresinas. También, precipitados y oleorresinas resultaron con contenidos similares de compuestos fenólicos totales, pero contrariamente al proceso SAS de romero, en el caso del regaliz, la actividad antioxidante fue significativamente mejor en los precipitados que en las oleorresinas.

La identificación y cuantificación de los principales compuestos del regaliz (liquiritina, liquiritigenina, ácido glicirrícico, isoliquiritigenina y glabridina) en las fracciones, permitió analizar el fraccionamiento de estos compuestos. Los precipitados presentaron un mayor contenido de liquiritina y ácido glicirrícico, mientras que las oleorresinas presentaron un mayor contenido de glabridina e isoliquiritigenina. Teniendo en cuenta que la concentración de glabridina fue hasta 5,3 veces mayor en las oleorresinas que en los precipitados, los resultados conducen a concluir que este compuesto no parece ser el principal responsable de la actividad antioxidante observada. Este resultado se contradice con el efecto positivo del contenido de glabridina sobre la actividad antioxidante de los extractos (supercríticos o UAE) de regaliz, señalado anteriormente. No obstante, cabe destacar que mientras los extractos supercríticos y UAE de regaliz contienen concentraciones de liquiritina (otra sustancia del regaliz reconocida por su actividad antioxidante) en un rango de 0,6-1,2 mg/g, los precipitados SAS contienen concentraciones del orden de 8,0-9,6 mg/g. Es decir, la mejor actividad antioxidante de los precipitados SAS de regaliz en comparación con las oleorresinas, probablemente se deba al alto contenido de liquiritina.

Por otro lado, los valores IC_{50} de los precipitados fueron en el rango de 17,64 ± 1,11 µg/mL, con diferencias significativas respecto del extracto original (20,40 ± 0,99 µg/mL), pero no menores que los alcanzados con condiciones óptimas en la extracción y fraccionamiento

supercrítico de la raíz de regaliz, los que alcanzaron valores de 8 - 10 µg/mL. Además, estos valores de IC_{50} de los extractos supercríticos fueron inferiores a los obtenidos utilizando la extracción asistida con ultrasonidos y mezclas de metanol-agua (11,5 - 206,52 µg/mL) según publicaron Vlaisavljević y col. (2018) o utilizando mezclas de metanol, agua y ácido fosfórico (85,4-140,4 µg/mL) según Yang y col. (2019), lo que confirma la mejor capacidad antioxidante que se puede obtener de la extracción directa de la raíz de regaliz con $SCCO_2$ y, también, un mayor efecto de la glabidrina en comparación con la liquiritina en la capacidad antioxidante de los extractos de regaliz.

En cuanto al tamaño de las partículas, de forma similar al caso del proceso SAS de romero, las partículas de menor tamaño se obtuvieron al aumentar la presión, con valores del orden de 20 - 35 µm. No obstante, en el proceso SAS de regaliz se observó un efecto de la temperatura contrario al caso de romero, puesto que la disminución de temperatura resultó en partículas secas de mayor tamaño, aunque se debe tener en cuenta que el rango de temperaturas explorado en el proceso SAS de raíz de regaliz fue muy estrecho.

Por otro lado, una menor concentración de fitoquímicos en la disolución etanólica de regaliz también resultó en partículas más pequeñas y, además, más regulares. De hecho, a la mayor concentración de extracto (14,2 mg/mL) y mayor temperatura (40 °C) ensayadas, solo se obtuvieron precipitados a altas presiones (15-20 MPa) y los precipitados obtenidos tuvieron la forma de aglomerados, más parecidos a un material continuo (tipo gomorresina) y con cavidades dentro de los agregados. Es decir, además de comprobar que a temperaturas mayores que 40 °C no fue posible obtener precipitados secos, se observó un cambio radical en la morfología de los precipitados de raíz de regaliz al reducir la temperatura de 40 °C a 35 °C. Para analizar este efecto tan evidente, y diferente al caso del proceso SAS de romero, se llevó a cabo un análisis de la morfología y tamaño de partículas obtenidos en relación a las condiciones de temperatura y presión de operación SAS, comparadas con el punto crítico de la mezcla supercrítica CO_2 + etanol (PCM) representando el punto crítico de la mezcla CO_2 + etanol + fitoquímicos.

Para ambos extractos estudiados, se ha verificado que, a temperatura constante, el aumento de la presión favorece la obtención de precipitados secos, con partículas más esféricas, pequeñas (nanopartículas) y regulares. De acuerdo a la bibliografía (Reverchon y col., 2010; Reverchon y De Marco, 2011) este es el resultado cuando las condiciones de operación se encuentran por encima pero cerca del PCM (ver Figura 1.6), es decir en la región supercrítica homogénea de la mezcla multicomponente. En cambio, cuando las condiciones de operación del proceso SAS están por debajo del PCM (condiciones subcríticas), se obtienen micropartículas expandidas (huecas) y con formas esféricas más irregulares cuando el proceso opera dentro de la región de fase homogénea, mientras que se producen partículas irregulares y aglomerados (posiblemente debido al disolvente residual que no se ha podido eliminar completamente por efecto anti-solvente) cuando el proceso opera dentro de la región heterogénea de dos fases (líquido + vapor).

En este sentido, cabe destacar que la variabilidad del tipo y cantidad de fitoquímicos del extracto que se disuelven en la fase supercrítica y abandonan la celda junto con el CO_2 y el etanol (fitoquímicos que no precipitan) influye significativamente en el PCM. Por lo tanto, en condiciones iguales de operación, los resultados para diferentes matrices vegetales pueden ser muy diferentes, debido a los diferentes PCM. En los trabajos desarrollados en esta tesis, mientras que a 40 °C y presiones de 15-20 MPa se obtuvieron precipitados secos de romero, con partículas pequeñas y esféricas, los precipitados de raíz de regaliz fueron aglomerados irregulares.

Considerando el efecto de fraccionamiento en la precipitación SAS de extractos vegetales, y las fracciones obtenidas, con actividad antioxidante mejorada respecto del extracto original, tanto para el caso de romero (oleorresinas) como para regaliz (precipitados), se puede concluir la utilidad de esta técnica para producir ingredientes de destacada actividad biológica, con aplicación potencial en nutracéuticos y productos alimenticios de uso para la salud. Se destaca la importancia de determinar condiciones del proceso SAS adecuadas, por encima del PCM (supercríticas), para obtener precipitados con una morfología adecuada, con partículas regulares, esféricas y de tamaño nanométrico.

5.3. PREPARACIÓN DE FORMULACIONES OLEOSAS DE EXTRACTOS VEGETALES MICRONIZADOS UTILIZANDO TECNOLOGÍA DE FLUIDOS SUPERCRÍTICOS

El empleo de algunas moléculas bioactivas en la industria alimentaria se encuentra limitado debido a su baja solubilidad en medios acuosos, sensibilidad al pH, fácil degradación en el ambiente gástrico y, en definitiva, una escasa biodisponibilidad. Así, se han desarrollado sistemas de entrega, en los que se incorpora el principio activo en medios micro- o nano-estructurados, en combinación con diversas matrices, generalmente a base de proteínas, carbohidratos y/o lípidos. En particular, las matrices lipídicas han demostrado ser sistemas de entrega muy efectivas, para proteger el principio activo durante la digestión y facilitar su absorción una vez llegado al intestino delgado. Recientemente, muchos avances se están llevando a cabo para formular micro-emulsiones, liposomas, nano-micelas. El reto es evitar la degradación del compuesto bioactivo y/o controlar adecuadamente el tamaño de las partículas.

Dentro de las alternativas vinculadas a la tecnología de fluidos supercríticos se encuentran varias técnicas de micronización. Sin embargo, ninguna de estas consigue simultáneamente, en una sola etapa, la reducción de tamaño del principio activo y la dispersión de las partículas micronizadas en una matriz lipídica. En los trabajos realizados durante el desarrollo de esta tesis, se investigó e implementó una nueva técnica para combinar lípidos con principios activos, basada en la tecnología supercrítica de precipitación anti-solvente (SAS). Los resultados dieron lugar al trabajo titulado "*Bioactive Ingredients in Oils by Supercritical Anti-Solvent Process (BIOSAS)"* (pendiente de ser enviado para su publicación) y la patente titulada "*Supercritical carbon dioxide-based methodology to formulate bioactive preparations"* (solicitud europea presentada el 15 de abril de 2020).

La nueva metodología fue desarrollada en escala piloto, en modo semi-continuo, pero es viable su implementación como una operación continua, lo que da lugar a un procedimiento de alta eficacia para producir nutracéuticos basados en la combinación de lípidos (portadores, aunque también pueden aportar su propia actividad) con principios activos puros o mezclas complejas, como es el caso de los extractos vegetales. El proceso se denominó BIOSAS (*Bioactive Ingredients in Oils by SAS process*) porque al formarse micropartículas del ingrediente bioactivo en una celda de precipitación similar al SAS, estas se ponen en contacto con un lípido a alta presión, resultando en la disolución y/o dispersión de las mismas, dependiendo de la naturaleza química y del tamaño de las partículas formadas.

Se estudiaron diferentes casos, desde un compuesto puro como la quercetina dispersada en aceite de lino, hasta extractos naturales (mezclas complejas de fitoquímicos) como los extractos etanólicos de romero y regaliz dispersados en una variedad de aceites comestibles (oliva, argán y lino), y también se analizó el uso de un extracto acuoso de quillaja en aceite de oliva. Teniendo como objetivo validar el método, es decir no se ha buscado una combinación bioactivo-lípido particular, ni tampoco optimizar los productos a través de las condiciones de operación, se demostró la viabilidad de la formación de dispersiones en un rango de presiones de 12-20 MPa, temperaturas de 35 °C y 40 °C, y concentraciones del ingrediente bioactivo en la disolución entre 10 y 17 mg/mL. Los rendimientos (masa bio-partículas en el lípido / masa bio-partículas procesadas) fueron muy variables, en el rango de 5-86%, dependiendo del sistema bioactivo-lípido estudiado.

En la aplicación a un compuesto puro, se obtuvo una dispersión estable de quercetina en aceite de lino a 40 °C y 20 MPa, con una concentración de quercetina de 15,5 $\pm$ 0,5 mg/g, con un tamaño medio de partícula de 739,9 $\pm$ 188,4 nm, y un rendimiento prácticamente del 100%. Una dosis de 500 mg/día de quercetina podría administrarse con la ingesta de 30 mL (unas dos cucharadas) de esta dispersión. Cabe mencionar que la solubilidad de la quercetina en triglicéridos de cadena media es tan solo de 1,77 ± 0,29 mg/g a más de 100 °C.

En el caso de la aplicación de BIOSAS a extractos naturales (en este caso, extractos de raíz de regaliz y de romero) el proceso acomete en un solo paso el fraccionamiento del extracto, la formación de pequeñas partículas, y la dispersión de las mismas en un lípido. Además de la dispersión, se obtiene un subproducto en el separador, recuperado después de la eliminación de etanol, que en el caso de regaliz y romero se presentó como una fracción viscosa, tipo oleorresina (similar al caso de la precipitación SAS). En todos los ensayos las micropartículas dispersas en el aceite fueron estables, presentando un índice de estabilidad Turbiscan inferior a 0,1. El tamaño medio de las partículas fue siempre inferior a 1 μm, la recuperación fue muy variable, así como la concentración de fitoquímicos en el aceite. En el proceso BIOSAS de la raíz de regaliz se logró dispersar un 1,6, 2,6 y 1,8 mg/g de micropartículas bioactivas en aceite de argán, lino y oliva, respectivamente. Para el caso del romero, se obtuvieron dispersiones de hasta 18,4 mg/g de micropartículas de romero en aceite de oliva, 27,3 mg/g en aceite de argán y 28,4 mg/g en aceite de lino. Cabe mencionar que, a 40 °C y con constante agitación, no es posible incorporar de manera estable (sin que se produzca precipitación) más de 5 mg/g de extracto de romero en aceite de oliva.

Cuando el extracto es de naturaleza muy polar y, por lo tanto, se disuelve adecuadamente en agua, el proceso BIOSAS obtiene la dispersión en el medio oleoso de las microgotas que se forman por la expansión de la disolución acuosa en la boquilla. La baja solubilidad del agua en $SCCO_2$ impide el efecto antisolvente, entonces las microgotas no se secan completamente, y el resultado es que son enérgicamente mezcladas en la celda de precipitación con el medio oleoso, que contiene un emulsificante. Así, se producen emulsiones agua en aceite (w/o), en las que las microgotas de agua dispersadas contienen los ingredientes bioactivos polares del extracto vegetal. La incorporación del extracto acuoso de *Quillaja saponaria* en aceite de oliva, es un ejemplo de la técnica BIOSAS para producir micro-emulsiones. Se logró la incorporación de 2,3 mg/g de extracto de quillaja en aceite de oliva, las emulsiones se mantuvieron estables, presentado un tamaño de gota de 10-200 μm, dependiendo principalmente de presión de operación del proceso supercrítico. Este método supercrítico de producir micro-emulsiones, estudiado e implementado en esta tesis, demanda aún un mayor desarrollo, pero prevé muchas y diferentes aplicaciones en el campo de la tecnología de alimentos.

En resumen, BIOSAS es un nuevo proceso basado en la precipitación supercrítica antisolvente, que puede implementarse en operación continua, para la incorporación de biomoléculas polares y no-polares en forma de partículas secas o pequeñas gotas de tamaño micro- y/o nanométrico, útil para preparar formulaciones nutracéuticas, ingredientes, suplementos alimenticios, o alimentos. La técnica se desarrolla en atmosfera inerte y a bajas temperaturas, aspectos característicos de los procesos supercríticos, previniendo la oxidación y/o la degradación térmica de las biomoléculas.

CAPÍTULO 6

CONCLUSIONES

6. CONCLUSIONS

The most relevant conclusions of this thesis are presented according with the two partial objectives proposed:

(i) Preparation of edible coatings using plant extracts with antioxidant and antimicrobial properties

- Plant extracts of rosemary, thyme, licorice, centaury and mugwort were obtained by supercritical extraction (SFE) with significant higher yields than the products obtained from hydrodistillation (essential oils). Rosemary, thyme and licorice exhibited the best combination of antioxidant and antimicrobial activities.
- Strawberries coated with chitosan and 1% or 5% of supercritical rosemary or thyme extracts presented lower weight loss and fungal decay in comparison with uncoated berries. Nevertheless, in general, the strawberries coated with chitosan and essential oils presented lower spoilage and better preservation of the phenolic compounds. Furthermore, no significant effect of both types of coatings on the berries color appearance was established.
- Supercritical extracts exhibited positive preservation effect on strawberries in comparison with control (uncoated strawberries) and can be produced with yields 10 times higher than essential oils.
- Licorice antimicrobial extracts were obtained using polar solvents (mixtures of methanol or ethanol with water) producing extracts with high content of glycyrrhizin. Antioxidant extracts were obtained with solvents of lower dielectric constant (ethanol) and resulted with high glabridin concentration. Accordingly, the use of ethanol cosolvent in licorice SFE increased the concentration of glabridin and the antioxidant activity of the extracts.
- The addition of licorice root extract to chitosan decreased the shear stress values of the coating solutions and increased the viscous properties of the film, becoming more flexible and allowing better physical properties when applied on strawberries. Additionally, licorice phytochemicals improved the antimicrobial properties of chitosan and better preserved the physicochemical properties and phenolic compounds of strawberries.

(ii) Formulation of antioxidant nutraceuticals

- Supercritical anti-solvent (SAS) precipitation of ethanolic plant extracts permitted the simultaneous fractionation and precipitation of phytochemicals and resulted in a dry powder of small (micro and/or nano) particles and an oleoresin by-product.
- A selective SAS separation of rosemary antioxidants was observed, increasing the concentration of rosmarinic acid in the precipitate (up to 45 mg/g) and carnosic acid and carnosol in the oleoresin (up to 300 and 40 mg/g, respectively). Furthermore, better antioxidant activity was obtained in the oleoresins in comparison with precipitates,

probably due to the large concentration of carnosic acid obtained in the oleoresins, with duplicated TEAC values in comparison with the original ethanolic extract.

- The SAS fractionation of an ethanolic licorice extract produced precipitates with higher antioxidant activity than oleoresins (probably due to high liquiritin content) despite similar concentration of total phenolic compounds were determined.
- Both in rosemary and licorice SAS process, agglomerated particles were obtained due to precipitation conditions probably below the supercritical multicomponent (CO_2 + ethanol + phytochemicals) mixture critical point. Smaller particles and more homogeneous size distribution were obtained at higher SAS pressure and by decreasing the concentration of phytochemicals in the ethanolic solution.
- A novel supercritical procedure to disperse bioactive substances in oils by SAS technique (BIOSAS) was developed and validated. BIOSAS was successfully applied to micronize and disperse pure bioactive substances (quercetin) and complex mixtures of bioactives (rosemary and licorice plant extracts) in edible oils.
- BIOSAS yield varied considerably with the type of bioactive solution and the lipid matrix used. Stable dispersions were obtained and, in comparison with solubility data, significant higher yields were attained in the case of the incorporation of quercetin and rosemary phytochemicals in edible oils.
- BIOSAS is an alternative to formulate w/o emulsions, dispersing micro-droplets of high-polar biomolecules aqueous solutions in oils. An aqueous solution of saponins were dispersed in olive oil using an emulsifier to favor the stability of the w/o emulsion produced.
- Using only green solvents, BIOSAS permits the control of particle size in the formulation of bioactive lipid-based products, under low temperatures and non-oxidative conditions.

Then, the following outcomes can be highlighted as general conclusions:

➔ SFE extracts are not better than essential oils in the preservation of strawberries, but they are effective in comparison with uncoated fruit and can be produced in larger scale. Thus, SFE extracts are potentially useful in the elaboration of edible plant-based coatings.

➔ SFE and UAE licorice root extracts can be produced with good antioxidant and antimicrobial activities and be applied in the elaboration of edible coatings. The coatings combining chitosan with licorice root extracts possess better rheological properties and preservation efficacy in comparison with coatings elaborated with chitosan solely.

➔ SAS precipitation of rosemary and licorice ethanolic extracts allowed the fractionation of phytochemicals and the production of fractions with improved biological properties, such as better antioxidant capacity in the licorice precipitates and in the rosemary oleoresins.

➔ The BIOSAS technique, developed in this thesis, is an alternative to incorporate bioactive molecules in lipids, is the form of small and homogeneous particles and/or droplets, and is useful to elaborate nutraceuticals, food ingredients or supplements, using only green solvents, under non-oxidative conditions and low temperatures, reducing the degradation of both the bioactive substances and the lipid matrix.

CAPÍTULO 7

BIBLIOGRAFÍA

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