

UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS



TESIS DOCTORAL

Metodologías analíticas avanzadas para el aseguramiento de la calidad de complementos alimenticios y la extracción selectiva de compuestos bioactivos

Advanced analytical methodologies for quality assurance of food supplements and selective extraction of bioactive compounds

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

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Resumen / Summary

RESUMEN

El mercado de los complementos alimenticios de origen vegetal (CAV) es un sector dinámico y en continua expansión, potenciado por el estilo de vida de la sociedad moderna, donde estos productos se enfocan a la mejora del rendimiento, la salud o el bienestar del consumidor. Sin embargo, su creciente demanda y las limitaciones en cuanto a su regulación, han proporcionado un entorno idóneo para que dichos productos sean objeto de prácticas fraudulentas que, no sólo afectan de forma negativa a su calidad, generando desconfianza en cuanto a la seguridad de su consumo, sino que además suponen importantes pérdidas económicas para las empresas del sector. En este sentido, resulta imprescindible el desarrollo de nuevas aproximaciones analíticas que contribuyan a una mejor caracterización de la composición de los CAV y evaluación de su calidad. Por otra parte, uno de los principales intereses compartidos por la industria alimentaria y el sector de los nutraceuticos, es la mejora de los procedimientos de obtención y caracterización de nuevos compuestos bioactivos para su empleo como ingredientes funcionales o complementos alimenticios, basado en el empleo de metodologías eficaces y medioambientalmente respetuosas. Así, y dentro de un contexto de economía circular, la extracción de compuestos bioactivos de alto valor añadido se presenta como una alternativa de interés para la revalorización de diversos subproductos agroalimentarios.

En base a lo expuesto, el objetivo principal de esta Tesis Doctoral, se ha centrado en el **desarrollo de nuevas metodologías analíticas para la evaluación de la calidad y genuinidad de CAV y en el desarrollo de procesos de extracción y fraccionamiento de compuestos bioactivos eficaces y medioambientalmente sostenibles**. Para ello, los resultados se han estructurado en dos secciones:

En la **Sección 3**, se recogen los resultados correspondientes al desarrollo de nuevas metodologías (multi)analíticas, basadas en el empleo conjunto de diferentes técnicas cromatográficas [cromatografía de gases (GC), cromatografía de líquidos (LC)] acopladas a espectrometría de masas (MS) o en el análisis directo por dicha técnica (DIMS), para la evaluación de fraudes de calidad en complementos alimenticios de *Coleus forskohlii* y ajo negro. Estas metodologías han permitido detectar fraudes asociados al contenido del compuesto bioactivo declarado, a la adición de sustancias no declaradas en el etiquetado, a la presencia de compuestos bioactivos de origen sintético o a la sustitución de la fuente natural por otra de menor valor económico, entre otros. También se ha demostrado el potencial de las metodologías por DIMS para el control rápido y eficaz de la calidad y la detección de posibles fraudes de composición en CAV.

Por otro lado, en la **Sección 4** se ha considerado la optimización de las condiciones de procesado del ajo envejecido, con el fin de alcanzar un compromiso entre el contenido en

compuestos bioactivos y las deseables propiedades organolépticas del producto. Además, se han desarrollado nuevas metodologías de extracción eficaces y sostenibles, basadas en técnicas avanzadas [asistidas por microondas (MAE) o ultrasonidos (UAE) o empleando líquidos presurizados (PLE)], para su aplicación a ajo negro y a los subproductos derivados de su cultivo como forma de revalorización de los mismos. Por último, se ha explorado el empleo de los disolventes eutécticos profundos naturales (NADESs) como extractantes medioambientalmente seguros y alternativos a los disolventes orgánicos convencionales, para el fraccionamiento selectivo de carbohidratos bioactivos de bajo peso molecular frente a los no bioactivos a partir de mezclas sintéticas o extractos vegetales, consiguiéndose altos porcentajes de enriquecimiento en la mayoría de los casos. Cabe destacar que estos estudios preliminares abren nuevas vías de investigación en este campo, escasamente abordado hasta el momento.

Los resultados incluidos en esta Tesis Doctoral suponen, no sólo una contribución reseñable en el área de la Química Analítica, sino también en el de la Ciencia y Tecnología de los Alimentos y, en particular, en el de los complementos alimenticios e ingredientes alimentarios, de gran interés y repercusión actual para consumidores, empresas productoras y comercializadoras del sector y organismos reguladores.

SUMMARY

The market of plant-based food supplements (CAV) is a dynamic sector in constant expansion, promoted by the lifestyle of the current society, where these products are aimed at improving consumer performance, health or well-being. However, their increasing demand and the limitations of supplement regulation have created an environment ideal for fraudulent practices targeting these products. Such practises not only negatively affect the quality of the products, leading to consumer mistrust, but also result in significant losses for companies of the sector. In this sense, the development of new analytical approaches is essential to achieve a better characterization of CAV composition and to evaluate their quality. On the other hand, one of the main interests shared by the food industry and the nutraceutical sector, is the development of more efficient and environmentally friendly procedures for the extraction and characterization of new bioactive compounds to be used as functional ingredients or food supplements. Thus, within the context of a circular economy, the extraction of high added-value bioactive compounds is presented as an interesting alternative for the revalorization of agri-food by-products.

Regarding the information presented, the main objective of this PhD Thesis has focused on **the development of new analytical methodologies to evaluate the quality and the authenticity of CAV, as well as the development of efficient and eco-friendly extraction and fractionation processes of bioactive compounds**. In order to achieve this objective, the results have been structured into two sections:

In **Section 3**, the results of the development of new (multi)analytical methodologies are presented. These methodologies are based on the combined use of different chromatographic techniques [gas chromatography (GC), liquid chromatography (LC)] coupled to mass spectrometry (MS) or direct analysis by MS (DIMS), for the evaluation of quality frauds in dietary supplements based on *Coleus forskohlii* and black garlic. These strategies have enabled the detection of frauds related to the declared bioactive content, the addition of undeclared substances on the label, the presence of synthetic bioactive compounds or the substitution of the natural source by one of a lower economic value, among others. In addition, the potential of DIMS methods for the fast and efficient quality control of CAV and the detection of frauds related to their bioactive content has been demonstrated.

On the other hand, in **Section 4** the optimization of processing conditions of aged garlic has been considered in order to achieve a balance between the bioactive compound content and the desirable organoleptic properties of the product. Furthermore, new effective and sustainable extraction methodologies have been developed, based on advanced techniques [microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) or pressurized liquid extraction (PLE)], for their application in black garlic and by-products derived from garlic cultivation as an

alternative for their revalorization. Finally, natural deep eutectic solvents (NADESs) have been explored as environmentally safe and alternative extractants to conventional organic solvents, for the selective fractionation of low-molecular-weight bioactive carbohydrates from non-bioactive ones in synthetic mixtures or plant extracts, achieving high enrichment percentages in most cases. It is worth noting that these preliminary studies open new research avenues in this area, which has been scarcely explored to date.

The results presented in this PhD Thesis represent not only a noteworthy contribution to the area of Analytical Chemistry, but also to that of Food Science and Technology, and in particular to the area of food supplements and food ingredients, which are of great interest and impact for consumers, production and commercial companies in the sector, as well as, regulatory institutions.

Glosario de términos

Glosario de términos

1,9-DDF	1,9-dideoxiforskolina 1,9-dideoxiforskolin
aa	Aminoácidos Amino acids
ABTS	Ácido 2,2'-azino-bis-(3-etiltiazolina-bencenosulfónico-6) 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AG	Ajo envejecido Aged garlic
ABG	Ajo negro envejecido Aged black garlic
ATP	Adenosín trifosfato Adenosine triphosphate
CAV	Complementos alimenticios de origen vegetal Plant-based food supplements
CH	Carbohidratos Carbohydrates
ChCl	Cloruro de colina Choline chloride
COS/OSC	Compuestos organosulfurados Organosulfur compounds
cAMP	Adenosín monofosfato cíclico Cyclic adenosine monophosphate
DAD	Detector de matriz de diodo Diode array detector
DES	Disolvente eutéctico profundo Deep eutectic solvent
DIMS	Inyección Directa - Espectrometría de Masas Direct Injection - Mass Spectrometry
DP	Grado de polimerización Degree of polymerization
DPPH	2,2-Difenil-1-picrilhidracilo 2,2-Diphenyl-1-picrylhydrazyl
EI	Impacto electrónico Electronic ionization

(EI)MS	Espectrometría de masas con ionización por impacto electrónico Mass spectrometry with electron impact ionization
ELSD	Detector evaporativo de dispersión de luz Evaporative light scattering detector
ESI	Ionización por electrospray Electrospray ionization
FIA	Análisis por inyección en flujo Flow injection analysis
FOS	Fructooligosacáridos Fructooligosaccharides
FTIR	Espectroscopía de infrarrojo con transformada de Fourier Fourier transform infrared spectroscopy
GC	Cromatografía de gases Gas chromatography
GC-O	Cromatografía de gases - olfatometría Gas chromatography - olfactometry
GS1PC	γ -Glutamyl-S-1-propenil-L-cisteína γ -Glutamyl-S-1-propenyl-L-cysteine
GSAC	γ -Glutamyl-S-alil-L-cisteína γ -Glutamyl-S-allyl-L-cysteine
HBA	Aceptor de enlace de hidrógeno Hydrogen bond acceptor
HBD	Donador de enlace de hidrógeno Hydrogen bond donor
HILIC	Cromatografía de interacción hidrofílica Hydrophilic interaction liquid chromatography
HMDS	Hexametildisilazano Hexamethyldisilazane
HMF	Hidroximetilfurfural Hydroxymethylfurfural
ILs	Líquidos iónicos Ionic liquids
IMC	Índice de masa corporal Body mass index
LC	Cromatografía de líquidos
LC	Liquid chromatography

LMWCs	Carbohidratos de bajo peso molecular Low molecular weight carbohydrates
LRI	Índice de retención lineal Linear retention index
MAE	Extracción asistida por microondas Microwave-assisted extraction
MS	Espectrometría de masas Mass spectrometry
MS/MS	Espectrometría de masas en tandem Tandem mass spectrometry
NADESs	Disolventes eutécticos profundos naturales Natural deep eutectic solvents
NMR	Resonancia magnético nuclear Nuclear magnetic resonance
PLE	Extracción con líquidos presurizados Pressurized liquid extraction
Q	Cuadrupolo simple Single quadrupole
QqQ	Triple cuadrupolo Triple quadrupole
QToF	Cuadrupolo acoplado a tiempo de vuelo Quadrupole time-of-flight
RF	Factor de respuesta Response factor
S1PC	S-1-propenil-L-cisteína S-1-propenyl-L-cysteine
SAC	S-alil-L-cisteína S-allyl-L-cysteine
SAMC	S-alil-mercaptocisteína S-allyl-mercaptocysteine
SFE	Extracción con fluidos supercríticos Supercritical fluid extraction
SPME	Microextracción en fase sólida Solid phase microextraction
SWE	Extracción con agua subcrítica
SWE	Subcritical-water extraction

TFA	Ácido trifluoroacético Trifluoroacetic acid
TMS	Trimetilsilil derivados Trimethylsilyl derivatives
TMSO	Trimetilsililoximas Trimethylsilyloximes
TPC	Contenido total de polifenoles Total polyphenol content
t _R	Tiempo de retención Retention time
UAE	Extacción asistida por ultrasonidos Ultrasounds-assisted extraction
UV	Ultravioleta Ultraviolet



1. Introducción

1. INTRODUCCIÓN

1.1. Compuestos bioactivos

Uno de los principales intereses compartidos por la industria alimentaria y el sector de los nutraceúticos radica en la mejora de los procedimientos de **obtención y caracterización de nuevos compuestos bioactivos** para su empleo como ingredientes funcionales, complementos alimenticios, etc. Los compuestos bioactivos hacen generalmente referencia a compuestos orgánicos de origen natural (*e.g.* metabolitos secundarios) extraídos principalmente de plantas, aunque también de hongos, microorganismos o animales, que presentan algún tipo de actividad biológica (*e.g.* antioxidante, antiinflamatoria, anticancerígena, etc.) en el organismo, dependiendo estos efectos del tipo de compuesto, su dosis o biodisponibilidad. Además de los de **origen natural**, y para satisfacer la creciente demanda de estos compuestos y hacer su comercialización más rentable, es frecuente también la comercialización de compuestos bioactivos de **origen sintético o biotecnológico** (Li, 2015). Por otra parte, y dentro de un contexto de economía circular en el que se pretende maximizar el aprovechamiento de recursos naturales con el mínimo impacto ambiental, está cobrando cada día mayor interés la extracción de compuestos bioactivos de alto valor añadido como forma de **revalorización de diversos subproductos agroalimentarios**, forestales, etc. (Bala *et al.*, 2023; Sarker *et al.*, 2024).

Desde el punto de vista químico, los compuestos bioactivos pueden tener **distinta estructura molecular y actuar a través de diferentes mecanismos de acción**. Algunos de los principales grupos de compuestos bioactivos incluyen a los terpenoides (carotenoides, fitoesteroides, etc.), los compuestos fenólicos (antocianinas, catequinas, flavonoides, isoflavonas, lignanos y taninos), los compuestos organosulfurados (*e.g.* tioles), los carbohidratos prebióticos, etc. (Bernhoft, 2008; Kamiloglu *et al.*, 2020; Urrialde *et al.*, 2022). De entre las distintas fuentes y tipos de compuestos con propiedades bioactivas, en esta Tesis Doctoral se profundizará en el estudio de dos matrices vegetales de origen natural (*Coleus forskohlii* y ajo negro), así como en el estudio de algunos compuestos bioactivos ubicuos en fuentes vegetales como los inosítoles y en el de otros de origen sintético con reconocidas actividades biológicas como la lactulosa.

1.1.1. Compuestos bioactivos presentes en *Coleus forskohlii*

Coleus forskohlii (familia Lamiaceae), también conocida como *Coleus barbatus* o *Plectranthus barbatus* (Figura 1.1.), es una planta perenne originaria de la India pero que en la actualidad se distribuye también por otras áreas geográficas como Egipto, Etiopía o Brasil. Esta planta es ampliamente usada en la medicina tradicional India (*Ayurveda*) para el tratamiento de diversas enfermedades, dadas sus numerosas propiedades bioactivas, entre las que cabe mencionar su actividad antioxidante, analgésica, antiinflamatoria, broncodilatadora o gastroprotectora

(Alasbahi & Melzig, 2010; Bhowal & Mehta, 2017; Kulbat-Warycha *et al.*, 2022; Pullaiah, 2022). Dichas propiedades beneficiosas para la salud se deben a la gran variedad de compuestos bioactivos que presenta y que se encuentran distribuidos a lo largo de toda la planta, aunque principalmente en la raíz. Así, y como se detalla a continuación, los extractos de dicha parte morfológica son ricos, entre otros, en polifenoles, alcaloides y, principalmente, en dos grupos de **diterpenos: abietanos y labdanos** (Kulbat-Warycha *et al.*, 2022).

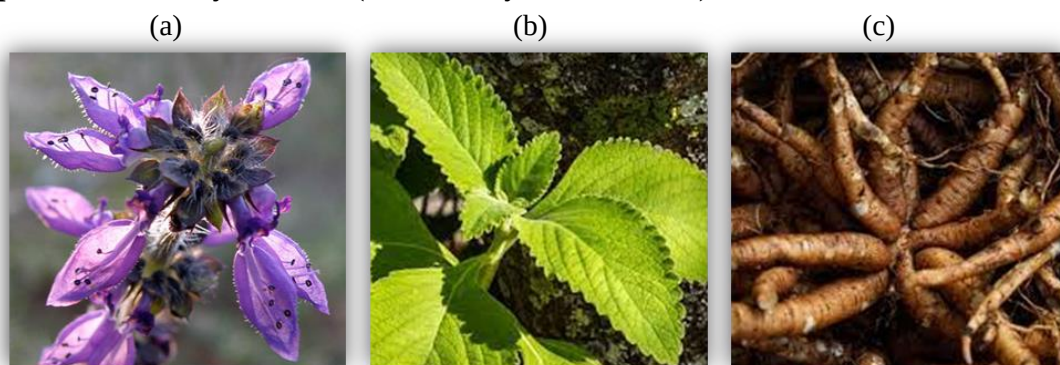


Figura 1.1. *Coleus forskohlii*. (a) Flor; (b) hoja y tallo; (c) raíz.

❖ Forskolina: biosíntesis y mecanismo de acción

La **forskolina** (Figura 1.2.) es un diterpeno tipo labdano y el **principal compuesto bioactivo** presente en la raíz de *C. forskohlii* (0,15-1,5 % *p/p*), no encontrándose en otras especies de *Coleus* (Pateraki *et al.*, 2017).

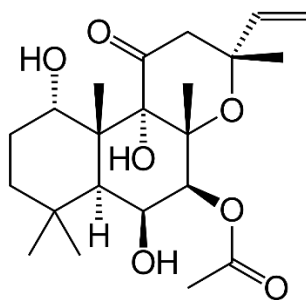


Figura 1.2. Estructura de la forskolina.

Las rutas biosintéticas de los diterpenos tipo labdano son complejas y aún no se han elucidado completamente. En el caso de la forskolina, y tal y como se muestra en la Figura 1.3., el precursor geranilgeranil difosfato (GGPP) forma el 13R-manoil-óxido mediante la actuación de las enzimas diterpeno sintasa (diTPS) de clase 1 y 2. Este óxido da lugar a la 9-desoxi-7-deacetilforskolina que, en última instancia, formará la forskolina mediante procesos enzimáticos adicionales, incluida la acetilación (Pateraki *et al.*, 2014; Forman *et al.*, 2018).

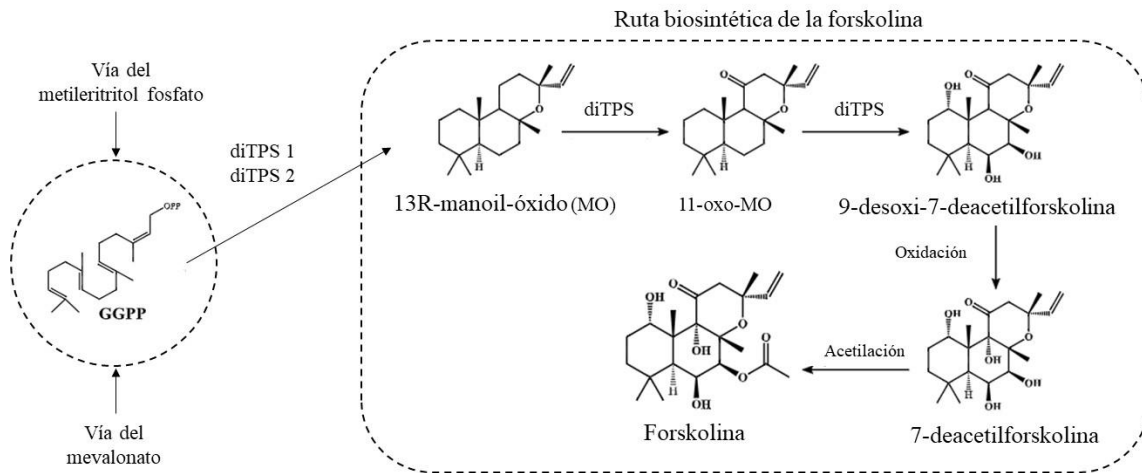


Figura 1.3. Ruta biosintética de la forskolina a partir del precursor universal geranilgeranil difosfato (GGPP). Esquema adaptado de Forman *et al.* (2018).

El principal **mecanismo de acción de la forskolina** implica la activación de la enzima adenilato ciclasa, que tiene un papel crucial en la producción de adenosín monofosfato cíclico (AMPC). Al aumentar los niveles de AMPC, se desencadena una cascada de señalización celular, incluyendo la activación de la proteína quinasa A (PKA), que puede tener diferentes efectos en el metabolismo celular (Figura 1.4.), como la lipólisis (descomposición de grasas), o la influencia en la secreción de insulina, disminuyendo así los niveles de glucosa en sangre (Seamon *et al.*, 1981; Laurenza *et al.*, 1989; Chen *et al.*, 2021).

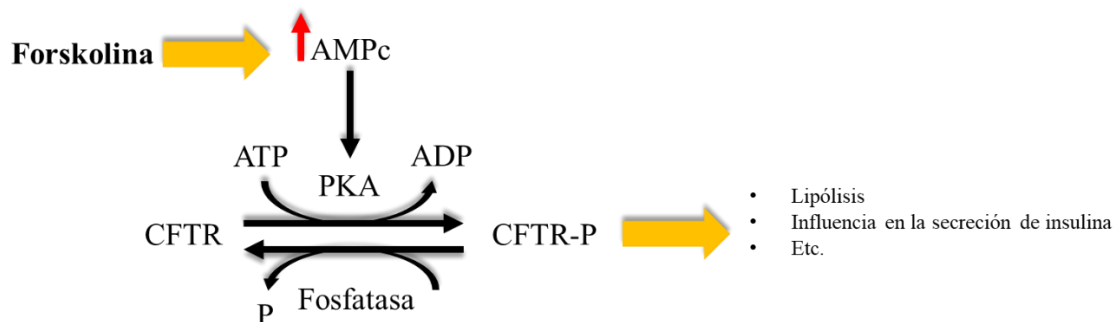


Figura 1.4. Ejemplo del mecanismo de acción de la forskolina. ADP, adenosín monofosfato; ATP, adenosín trifosfato; AMPC, adenosín monofosfato cíclico; PKA, proteína quinasa dependiente de AMPC; CFTR, regulador de la conductancia transmembrana de la fibrosis quística; CFTR-P, CFTR fosforilado. Figura adaptada de Salehi *et al.* (2019).

❖ Otros compuestos bioactivos presentes en *C. forskohlii*

Como ya se ha mencionado, los **diterpenos** son los compuestos mayoritarios en *C. forskohlii*, habiéndose identificado numerosos compuestos de tipo abietano en la hoja y el tallo de esta planta, y de tipo labdano, mayoritariamente en la raíz. Dentro de este último grupo, que incluye numerosos diterpenos con la estructura mostrada en la Figura 1.5., y que se diferencian entre sí por las diferentes sustituciones en las posiciones C-1, C-6, C-7 y C-9, se han identificado, además de la forskolina, hasta 26 compuestos, tal y como se muestra en la Tabla 1.1. La mayoría de ellos, en muchos casos isómeros o diastereoisómeros de la forskolina, se encuentran presentes en menor abundancia que la forskolina, destacando la 7-deacetil forskolina (forskolina D), isoforskolina, 9-deoxiforskolina, 1,9-dideoxiforskolina e isómeros de acetilforskolina (Hebbani Nagarajappa *et al.*, 2016; Bhowal & Mehta, 2017; Pullaiah, 2022). Se han identificado también estructuras diferentes a las mencionadas con otras sustituciones, así como diferentes glicósidos (Alasbahi & Melzig, 2010; Pullaiah, 2022). Muchos de ellos, intermediarios en la ruta de biosíntesis de la forskolina, como la isoforskolina, también presentan bioactividad (*e.g.* propiedades antiinflamatorias) (Yang *et al.*, 2011; Du *et al.*, 2019). Por último, los **compuestos fenólicos y sus derivados** son otro de los grupos de compuestos bioactivos presentes en *C. forskohlii*, habiéndose identificado una gran variedad de ellos (*e.g.* ácidos quínico, 4-hidroxibenzoico, 2-hidroxibenzoico, vanílico, cafeico, etc.) en los extractos de raíz de esta planta (Kulbat-Warycha *et al.*, 2022). Aunque muchos de estos compuestos también tienen un efecto terapéutico, se ha concluido que la forskolina es el principal responsable de la bioactividad de esta especie (Salehi *et al.*, 2019). Por ello, actualmente, existen en el mercado distintos complementos alimenticios basados en extractos de raíz estandarizados en cuanto a su contenido en forskolina, y principalmente enfocados a la reducción de peso, cuya caracterización ha sido escasamente abordada hasta el momento (Arai *et al.*, 2020; Fukiwake *et al.*, 2022).

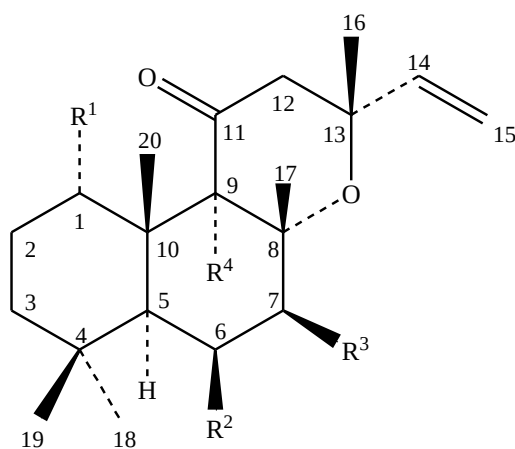


Figura 1.5. Estructura general de los diterpenos tipo labdano presentes en *C. forskohlii*.

Tabla 1.1. Diterpenos tipo labdano identificados en *C. forskohlii*.

R ¹	R ²	R ³	R ⁴	Compuesto
OH	OH	OAc	OH	Forskolina
OH	OH	OAc	H	9-Deoxiforskolina
H	OH	OAc	H	1,9-Dideoxiforskolina
H	OH	OH	H	1,9-Dideoxi-7-deacetilforskolina
H	OH	OH	OH	Deacetil-1-deoxiforskolina
H	OAc	OAc	OH	6-Acetil-1-deoxiforskolina
H	OAc	OAc	H	6-Acetil-1,9-dideoxiforskolina
OAc	OAc	OAc	OH	1,6-Di-O-acetilforskolina (Forskolina A)
OAc	OH	OAc	OH	1-Acetilforskolina (Forskolina B)
OH	OAc	OH	OH	Isoforskolina (Coleonol B, Forskolina C)
H	OAc	OH	H	1,9-Dideoxicoleonol B
OH	OH	OH	OH	7-Deacetilforskolina (Forskolina D)
OAc	OH	OAc	H	Forskolina E
OAc	OH	OAc	OH	Forskolina F
OH	OAc	OAc	H	Forskolina G
OAc	OAc	H	H	Forskolina H
OAc	OAc	OH	OH	Forskolina I
OH	OAc	OAc	OH	Forskolina J (6-O-acetilforskolina)
OAc	OAc	OAc	H	1,6-Diacetoxi-9-deoxiforskolina (Forskolina K)
H	OH	H	H	Forskolina L
H	OH	H	OH	Coleosol
OAc	OH	H	OH	1-Acetoxicoleosol
H	H	H	OH	Coleol
H	H	H	H	Óxido de 11-Oxomanoil
H	OH	α -OAc	H	Coleonol E
H	OAc	α -OH	OH	Coleonol F
OH	OH	α -OAc	H	Deoxicoleonol

1.1.2. Compuestos bioactivos presentes en ajo negro

El ajo (*Allium sativum* L.) es consumido en todo el mundo como alimento, condimento y complemento alimenticio, debido a sus características organolépticas y sus propiedades beneficiosas para la salud, entre las que cabe mencionar su actividad antioxidante, antimicrobiana, anticancerígena, antiinflamatoria, antihiper glucémica y antihipertensiva (Bae *et al.*, 2014; Kuete, 2017). A pesar de estas propiedades, el fuerte aroma y sabor del ajo, junto con la propensión a ocasionar problemas gastrointestinales o reacciones alérgicas en individuos susceptibles, limitan

la aceptación de este producto por el consumidor (Borrelli *et al.*, 2007; Ahmed & Wang, 2021). Con el objetivo de mejorar el perfil organoléptico y aumentar la presencia de compuestos bioactivos, se ha sometido al ajo a diversos tipos de procesado como la desodorización o el envejecimiento (*aging process*), dando lugar éste último a un producto de elevado valor añadido en el sector de la alimentación como es el **ajo negro** (Bae *et al.*, 2014; Liu *et al.*, 2022).

El origen del ajo negro data de hace siglos y, aunque hay discrepancias, la mayoría de los autores coinciden en su origen asiático (Japón, Tailandia y Corea) (Ahmed & Wang, 2021). Aunque en la actualidad su utilización en la gastronomía española es limitada, el interés por este producto se encuentra en alza, empleándose principalmente como ingrediente de platos de alta cocina, y en la industria alimentaria para la elaboración de diferentes productos tales como yogures, quesos, mermeladas, caramelos, etc. En España, la producción de ajo negro comenzó hace algunos años, destacando la localidad de Las Pedroñeras (Cuenca, Castilla La Mancha) donde este vegetal se comercializa bajo el Certificado de Indicación Geográfica Protegida (IGP).

El ajo negro (Figura 1.6.) es un alimento funcional que resulta de someter el ajo fresco a un **proceso de envejecimiento** bajo condiciones controladas de temperatura (60-90 °C) y humedad (70-90 %) durante un período de tiempo comprendido entre 1 y 3 meses (Bae *et al.*, 2014; Xu *et al.*, 2015; Kimura *et al.*, 2017). Dependiendo de las condiciones de envejecimiento, el producto final tiene características diferentes, como distinto grado de pardeamiento del bulbo, que puede ser marrón o incluso negro (cuando el tratamiento térmico es intenso y las temperaturas de producción alcanzan 70-90 °C) (Kang, 2016), así como diferente contenido en carbohidratos, aminoácidos, compuestos fenólicos, etc. No obstante, no hay un método de producción estandarizado, y su elección dependerá de las tradiciones propias de cada región o de las características deseadas del producto final (Ahmed & Wang, 2021). Por otra parte, se han descrito métodos alternativos, menos empleados que el procesado térmico, para la obtención de ajo negro como la **fermentación con probióticos** (*Saccharomyces cerevisiae*, *Monascus pilosus*, *Lactobacillus plantarum*, etc.) (Kim *et al.*, 2016), o la **inmersión de los bulbos de ajo en soluciones hidroalcohólicas** (15-20 % etanol) durante períodos largos de hasta 18 meses (Kanamori *et al.*, 2019; Woo *et al.*, 2022).



Figura 1.6. Bulbo y dientes de ajo negro.

Como resultado del envejecimiento del ajo negro, este producto presenta nuevas propiedades fisiológicas, como una mayor actividad antioxidante y antitumoral (Shang *et al.*, 2019; Liu *et al.*, 2022). En general, el contenido en agua y el pH del ajo negro disminuyen significativamente en comparación con el ajo fresco. Sus propiedades organolépticas también se modifican: el fuerte sabor y aroma del ajo fresco se reducen notablemente a consecuencia de la disminución en el contenido de alicina (principal compuesto del ajo fresco responsable de sus propiedades organolépticas), dando lugar a diversos alcaloides y flavonoides con propiedades antioxidantes (Kimura *et al.*, 2017). La composición y bioactividad del ajo cambian también drásticamente durante el envejecimiento. Así, diversas reacciones de pardeamiento no enzimático (*e.g.* reacción de Maillard), caramelización y oxidación química son el origen del sabor dulce de este producto (por el aumento del contenido total de azúcares libres como fructosa, glucosa y sacarosa), de su textura gelatinosa con apariencia pegajosa, así como de su mayor contenido en compuestos bioactivos con propiedades similares o mejoradas respecto al ajo crudo (Bae *et al.*, 2014; Kang, 2016; Kimura *et al.*, 2017; Liu *et al.*, 2022; Yudhistira *et al.*, 2022).

Numerosos estudios en la literatura científica han descrito las diversas y bien establecidas propiedades beneficiosas del ajo negro. La Figura 1.7. destaca, entre otras, su actividad cardioprotectora, sus efectos antioxidantes, antiinflamatorios, inmunomoduladores y hepatoprotectores, entre otros (García-Villalón *et al.*, 2016; Matsutomo *et al.*, 2017; Kanamori *et al.*, 2019; Ahmed & Wang, 2021; Yudhistira *et al.*, 2022). Estas propiedades se atribuyen, tanto a la actividad de los compuestos bioactivos de forma individual, como al efecto sinérgico de todos los compuestos presentes en el ajo negro.

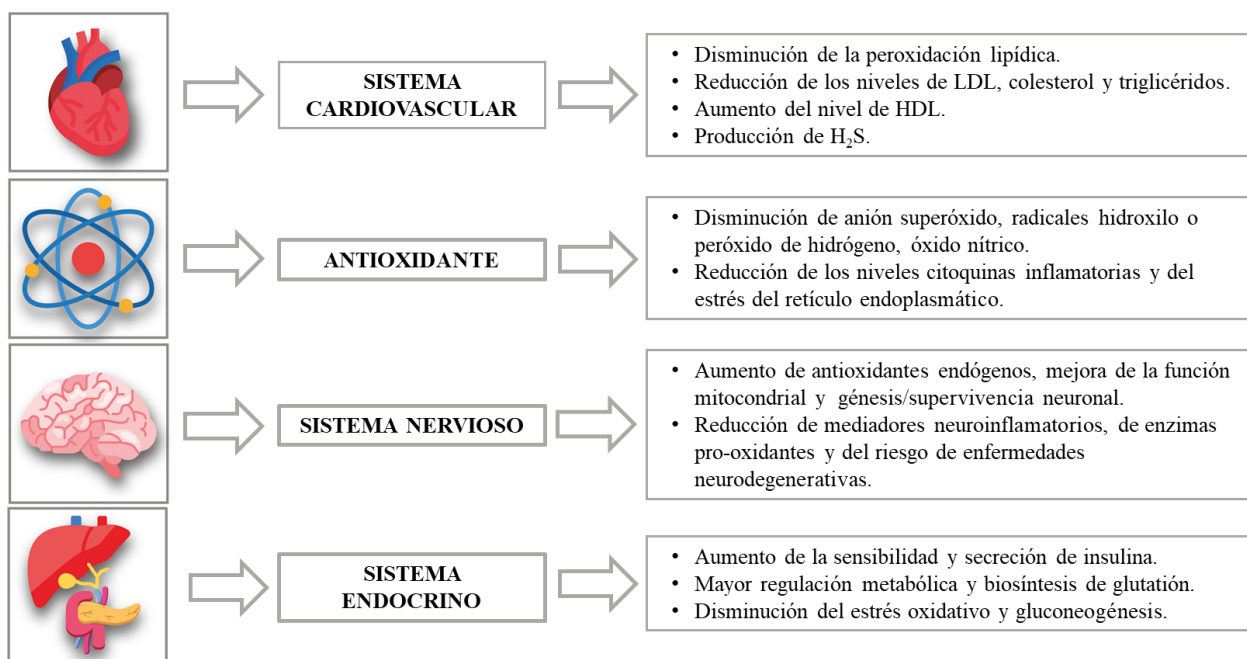


Figura 1.7. Propiedades beneficiosas del ajo negro.

❖ **S-alil-L-cisteína: biosíntesis y mecanismo de acción**

La composición del ajo negro puede variar según las condiciones de procesamiento comentadas en el apartado anterior. Si bien en el ajo fresco encontramos carbohidratos (28-36 %), proteínas (6-8,4 %) y grasas (0,1-0,5 %) (Ahmed & Wang, 2021), son los **compuestos organosulfurados (COS)**, tanto hidrofóbicos (aliína, dialilsulfuro, dialildisulfuro, dialiltrisulfuro, ajoenos, etc.), como hidrofílicos [alicina, S-alil-mercapto-L-cisteína, S-metil-L-cisteína, **S-alil-L-cisteína (SAC)**, γ -glutamyl-S-alil-L-cisteína (γ -GSAC), etc.], los principales responsables de sus propiedades bioactivas. Sin embargo, en el ajo negro, la concentración de los COS hidrofóbicos disminuye considerablemente ya que son muy volátiles y se pierden durante el procesamiento, mientras que aumenta la concentración de algunos COS hidrofílicos como la **SAC, principal responsable de muchas de las bioactividades** del ajo negro y compuesto en base al cual se estandarizan muchos de los complementos alimenticios basados en extractos de este producto.

La **SAC**, es un aminoácido azufrado con estructura similar a la cisteína y soluble en agua. La cantidad de SAC no solo está determinada por la variedad de ajo, sino que también puede verse afectada por diversos factores externos, tales como las prácticas agrícolas aplicadas, las técnicas de manipulación posteriores a la cosecha, las condiciones ambientales y las condiciones de envejecimiento.

La Figura 1.8. muestra las diferentes rutas metabólicas que ocurren durante el procesamiento del bulbo de ajo, tomando como punto de partida el precursor de la SAC, la γ -GSAC. Dicho compuesto azufrado es el más abundante en el ajo fresco y, en condiciones normales, tras la ruptura o triturado del ajo, se produce su S-oxigenación y su deglutamilación, dando lugar a la S-allyl-L-cisteína sulfóxido o aliína. La enzima aliinasa es la responsable de transformar la aliína en alicina, principal compuesto azufrado responsable del aroma del ajo. La alicina es muy inestable, originando diferentes tipos de compuestos azufrados volátiles, como el sulfuro de dialilo, disulfuro de dialilo, o ajoenos. Sin embargo, durante el envejecimiento del ajo, también tiene lugar la formación de SAC tras la hidrólisis enzimática de la γ -GSAC, por acción de la enzima γ -glutamyl transferasa (γ -GTP) (Yudhistira *et al.*, 2022; Rais *et al.*, 2023).

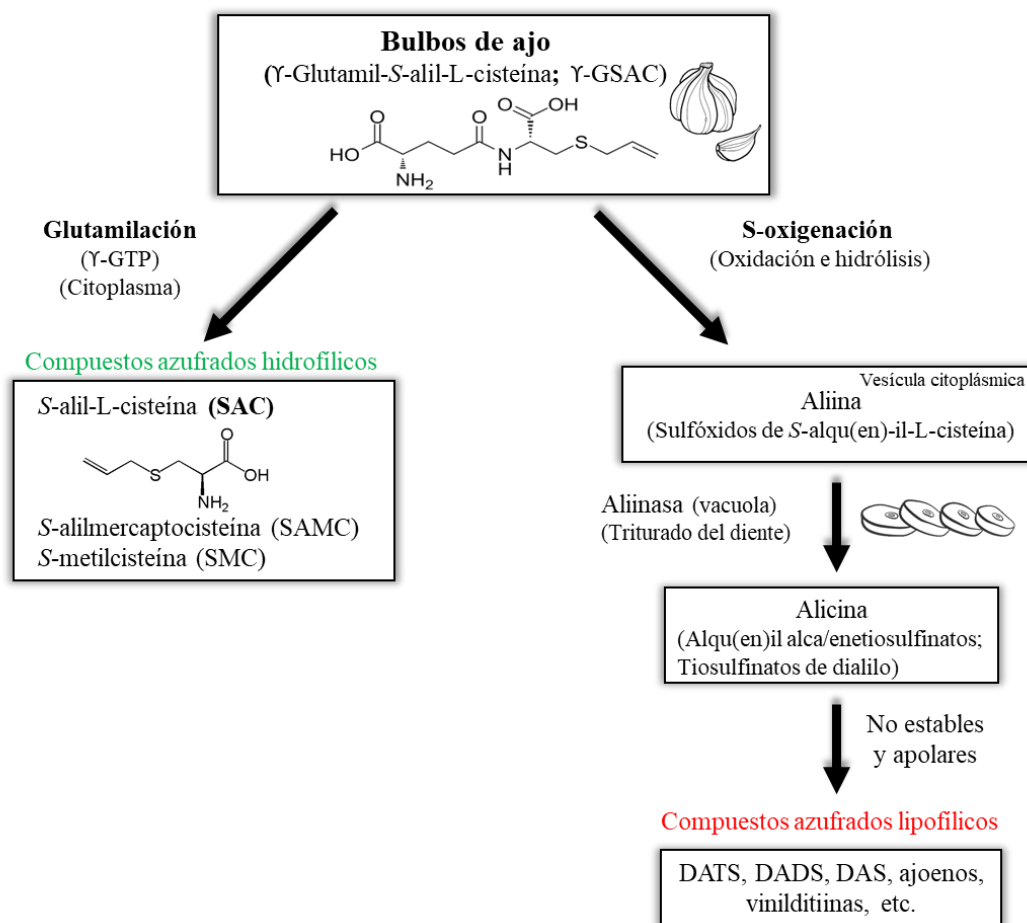


Figura 1.8. Cambios químicos de la γ -glutamyl-S-alil-L-cisteína (γ -GSAC) producidos durante el procesamiento del ajo. Figura adaptada de Yudhistira *et al.* (2022). DATS: trisulfuro de dialilo; DADS: disulfuro de dialilo; DAS: sulfuro de dialilo.

La SAC es capaz de aportar numerosos beneficios relacionados con su actividad antioxidante ya que desempeña un papel clave en diversas funciones fisiológicas relacionadas con el sistema cardiovascular, el sistema endocrino y el sistema nervioso (Colín-González *et al.*, 2012; Ruiz-Sánchez *et al.*, 2020; Rais *et al.*, 2023). La Figura 1.9. muestra los mecanismos antioxidantes asociados a la SAC, así como algunos de los procesos celulares en los que participa:

- Neutralización del anión superóxido ($O_2^{\cdot-}$), el peróxido de hidrógeno (H_2O_2), el radical hidroxilo ($OH^{\cdot}$), el radical peroxinitrito ($ONOO^{\cdot-}$) y el radical peroxilo ($LOO^{\cdot}$) generados en las células neuronales, así como el ácido hipocloroso ($HOCl$) y el oxígeno singlete ($^1O_2$) producidos en las células microgliales (líneas azules).
- Propiedades quelantes sobre los iones Fe^{2+} y Cu^{2+} , evitando así la reacción de Fenton.
- Inhibición de la translocación del NF- κ B al núcleo, evitando la señalización apoptótica (Colín-González *et al.*, 2012).

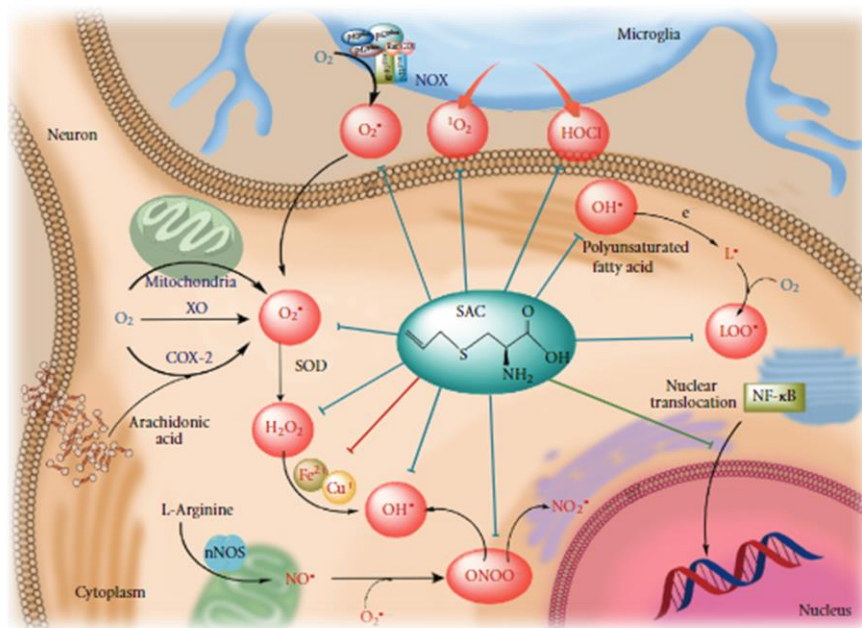


Figura 1.9. Mecanismo antioxidante asociado a la S-alil-L-cisteína. COX-2: ciclooxigenasa-2, NOX: NADPH oxidasa, nNOS: óxido nítrico sintasa neuronal, SOD: superóxido dismutasa, XO: xantina oxidasa. Imagen reproducida de Colín-González *et al.* (2012).

❖ Otros compuestos bioactivos presentes en ajo negro

Además de la SAC, se han descrito en ajo negro otros COS con similar estructura tales como la **S-1-propenyl-L-cysteine (S1PC)**, diastereoisómero de la SAC con reportadas propiedades bioactivas (*e.g.* antioxidante, cardioprotector, etc.) (Kodera *et al.*, 2017). Si bien dicho compuesto bioactivo podría considerarse como un marcador de calidad complementario a la SAC en complementos alimenticios de ajo negro, su determinación en productos basados en esta fuente natural ha sido escasamente abordada hasta el momento (Woo *et al.*, 2022) a pesar de su interés.

Por otra parte, el ajo negro también es un producto rico en **fructooligosacáridos (FOS)** de **tipo inulina**, especialmente los de la familia de la **neo-kestosa** (β -D-fructofuranosil-(2-6)- α -D-glucopiranosil-(1-2)- β -D-fructofuranósido) y la **neo-nistosa** (β -D-fructofuranosil-(2-6)- α -D-glucopiranosil-(1-2)- β -D-fructofuranosil-(1-2)- β -D-fructofuranósido), con mejores propiedades prebióticas en comparación con los FOS convencionales (glucopiranosil-(1-2)- β -D-fructofuranosil-((1-2)- β -D-fructofuranósido)_n). La neo-kestosa y la neo-nistosa proporcionan beneficios para la salud ya que estimulan selectivamente el crecimiento de microorganismos beneficiosos sobre la microbiota intestinal, promoviendo el crecimiento de especies de *Bifidobacterium* y *Lactobacillus* y reduciendo el número de bacterias Gram positivas y Gram negativas dañinas presentes en el colon (*e.g.* especies de *Bacteroides*, *Clostridium*, *Escherichia coli*, *Fusobacterium*, *Salmonella*, *Listeria*, *Shigella*, *Campylobacter* y *Vibrio cholera*) (Nobre *et al.*, 2022).

Por otro lado, la **inulina** (Figura 1.10.), constituyente del ajo fresco (15-20 %), también se encuentra en el ajo negro. Se trata de un polisacárido constituido por monosacáridos de fructosa unidos con enlaces (1→2) y una glucosa terminal, enlazados de forma lineal, ramificada o cíclica. La inulina se emplea en diversas industrias (química, farmacéutica y alimentaria) como sustituto de azúcares y grasas, mejorando propiedades sensoriales y aportando textura. Además, posee propiedades prebióticas y se ha relacionado con mejoras en los índices glicémicos y antioxidantes en personas con diabetes tipo 2, así como con la reducción de peso e índice de masa corporal (IMC) (Tresina *et al.*, 2022; Qin *et al.*, 2023). Durante el envejecimiento del ajo negro aumenta la ruptura de enlaces glucosídicos en la inulina, liberando unidades de fructosa y glucosa. La liberación de monosacáridos confiere un distintivo sabor dulce al producto y hace disminuir su contenido en polisacáridos respecto al ajo fresco.

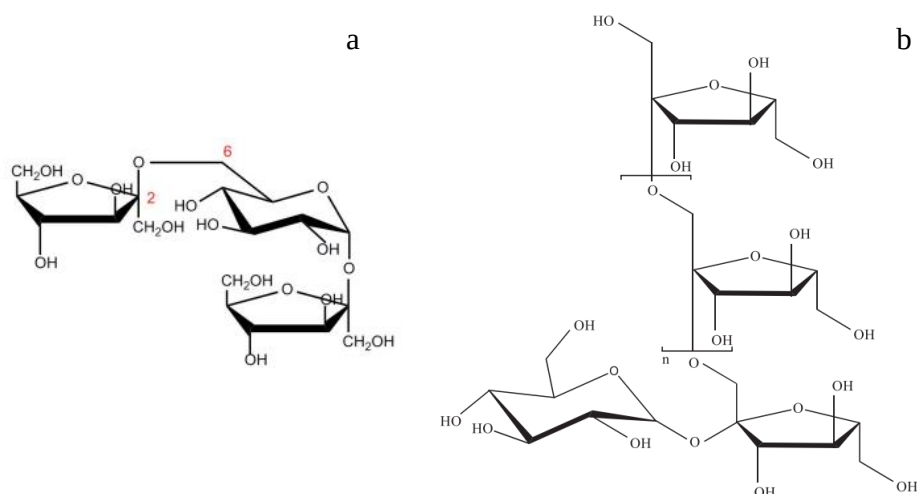


Figura 1.10. Estructura de (a) la neo-kestosa y (b) la inulina.

Los **compuestos fenólicos** son componentes importantes en el ajo negro, destacando los flavanoles y flavonoles. Se ha descrito que su concentración puede aumentar con respecto a la del ajo fresco entre 3 y 5 veces durante el envejecimiento debido, entre otros factores, a su liberación a partir de diversas estructuras supramoleculares que los almacenan, y contribuyendo así a su liberación a partir de la ruptura de estructuras supramoleculares que los almacenan, otorgando una mayor actividad antioxidante de este producto (Choi *et al.*, 2014; Martínez-Casas *et al.*, 2017; Ahmed & Wang, 2021).

Los **ácidos orgánicos**, principalmente el cítrico, están presentes en el ajo negro y contribuyen a la reducción del pH, afectando al sabor, degradando proteínas y polisacáridos, y favoreciendo la estabilidad microbiológica (Ahmed & Wang, 2021). En relación a los **aminoácidos (aa)** presentes en el ajo negro, su contenido es variable, aumentando en las primeras etapas del envejecimiento y reduciéndose en procesos de producción prolongados y/o llevados a cabo a altas

temperaturas. Los aminoácidos esenciales triptófano, histidina, y valina son los más abundantes (Kang, 2016).

El **5-hidroximetilfurfural (5-HMF)** es un antioxidante relevante en el ajo negro, vinculado a su pardeamiento. Su concentración aumenta durante el proceso de envejecimiento, acelerándose cuando las temperaturas son superiores a 60 °C y afectando negativamente a la calidad del ajo cuando sus concentraciones son elevadas (Kimura *et al.*, 2017; Ahmed & Wang, 2021). A pesar de que es conocida la actividad citotóxica, mutagénica y carcinogénica del 5-HMF, aún no existe un límite establecido para el ajo negro por ningún organismo regulador (Chua *et al.*, 2022). De hecho, se han descrito rangos muy amplios para la concentración de este compuesto en ajo negro, alcanzándose en algunos casos los 5 mg g⁻¹, valores del orden de 100 veces superiores al límite permitido en miel (Zhang *et al.*, 2016; Tu *et al.*, 2021; Dursun Capar *et al.*, 2022).

1.1.3. Carbohidratos bioactivos: lactulosa y polialcoholes

Los **carbohidratos (CH)** son uno de los principales constituyentes de los alimentos ya que pertenecen al grupo de nutrientes básicos implicados en la alimentación y en el metabolismo. Es sabido que determinados carbohidratos, además de poseer propiedades nutricionales, presentan propiedades beneficiosas añadidas y, por tanto, pueden formar parte de los alimentos o ser adicionados a ellos como ingredientes alimentarios para la **mejora de distintas características sensoriales, funcionales o tecnológicas**. Algunos de estos carbohidratos bioactivos son los denominados prebióticos, como la lactulosa, y los polialcoholes.

❖ **Lactulosa**

La lactulosa (4-O-β-D-galactopiranosil-D-fructofuranosa) es un disacárido no digerible de origen semisintético. Tiene un sabor muy dulce, más que la lactosa, y aproximadamente un 60-80 % respecto al de la sacarosa. Es soluble en agua y presenta dos estructuras cristalinas en estado puro: anhídrida y trihidratada, siendo la primera la forma comercial.

La lactulosa tiene numerosas aplicaciones tanto en la industria alimentaria como en la farmacéutica. En el sector alimentario se suele utilizar como edulcorante en la producción de helados, postres, etc., gracias a su sabor dulce y textura agradable (Nooshkam *et al.*, 2018). Por otro lado, la lactulosa es un agente humectante y conservante, reduce la humedad y prolonga la vida útil de ciertos alimentos como los productos de panadería y/o confitería. La lactulosa promueve también el crecimiento selectivo de la microbiota intestinal (principalmente Bifidobacterias) y mejora la salud digestiva (Ruiz-Matute *et al.*, 2007; Schuster-Wolff-Bühning *et al.*, 2010; Seki & Saito, 2012; Aït-Aïssa & Aïder, 2014; Guerrero *et al.*, 2015; Nooshkam *et al.*, 2018). En el sector farmacéutico, la lactulosa se destina al tratamiento del estreñimiento,

actuando como laxante osmótico que facilita el paso de las heces por el intestino (Aït-Aissa & Aïder, 2014).

Aunque se encuentra de forma natural en pequeñas cantidades en algunos productos lácteos sometidos a procesos de calentamiento, la lactulosa generalmente se produce a escala industrial mediante un tratamiento térmico a partir de una solución alcalina de lactosa (Figura 1.11.) (Seki & Saito, 2012). Sin embargo, las condiciones alcalinas y de alta temperatura empleadas para la isomerización, sumado a las altas concentraciones de catalizadores necesarias, provocan la creación de numerosos compuestos no deseables, como galactosa, tagatosa, epilactosa, entre otros, que han de ser eliminados previo a su comercialización.

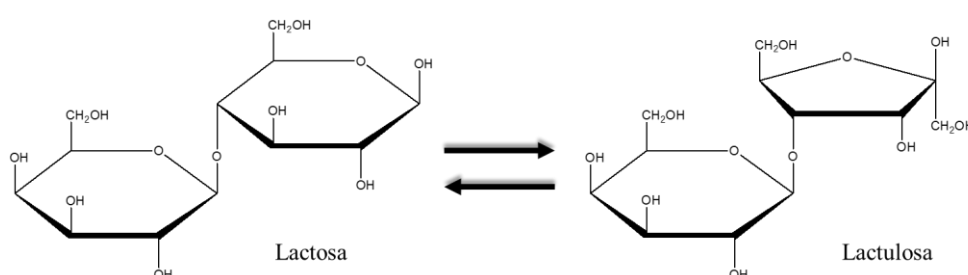


Figura 1.11. Reacción de isomerización de la lactosa.

❖ **Polialcoholes**

Los polialcoholes son alcoholes polihídricos con varios grupos hidroxilo que pueden tener estructura lineal (alditales) o cíclica (ciclitales) (Figura 1.12.).

Los **alditales** son derivados de azúcares obtenidos a partir de procesos químicos, como la reducción de los grupos carbonilo a grupos hidroxilo, entre otros (Angyal, 1984). Entre ellos, destaca el manitol (hexitol), primer alditol cristalino natural descrito y uno de los de mayor importancia en la industria alimentaria debido a su bajo poder calórico (Belitz *et al.*, 2009; Dai *et al.*, 2017). Además de su uso como endulzante de alimentos (tiene entre un 40 y 50 % del poder edulcorante de la sacarosa) e ingrediente dietético, se usa para la cristalización de productos. También presenta aplicaciones en medicina como sustituto del plasma sanguíneo en caso de hemorragias o como diurético osmótico. El manitol se encuentra de forma natural en alimentos de origen vegetal (*e.g.* remolacha, apio, aceitunas, etc.), aunque también se obtiene por reducción catalítica de mezclas de glucosa/fructosa a altas temperaturas y presiones o a partir de la sacarosa, y como subproducto de algunas fermentaciones. En todos estos procesos se precisa la separación posterior del manitol resultante de los otros azúcares presentes en las mezclas de síntesis.

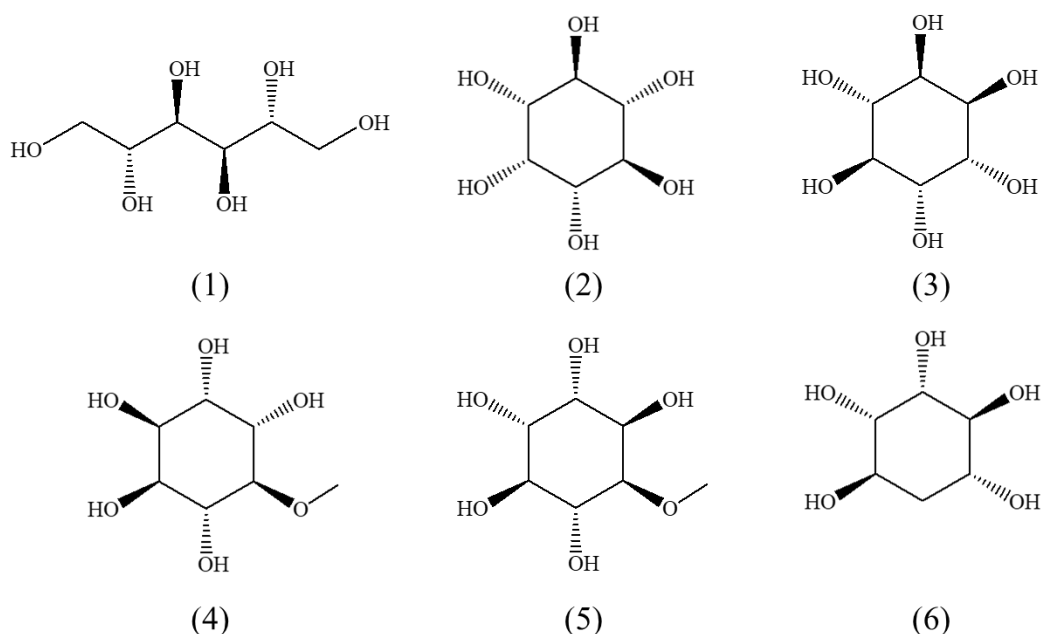


Figura 1.12. Estructura de (1) manitol, (2) *myo*-inositol, (3) *chiro*-inositol, (4) pinitol, (5) quebrachitol y (6) quercitol.

Los polialcoholes cíclicos o **ciclitoles** son compuestos en los que al menos tres de sus carbonos tienen un hidrógeno reemplazado por un grupo hidroxilo. Dentro de este grupo, se encuentran los **inositoles**, isómeros naturales de la glucosa, cuya estructura está constituida por un anillo de seis carbonos, cada uno con un grupo hidroxilo. Los inositoles, principalmente el *myo*- y *chiro*-inositol, así como algunos derivados de estos como los metil-inositoles (*e.g.* pinitol y quebrachitol) y los desoxi-inositoles (*e.g.* quercitol) se encuentran en una gran variedad de fuentes naturales, destacando las de origen vegetal tales como legumbres, soja, maíz, etc. (Clements & Darnell, 1980; Ruiz-Matute *et al.*, 2011; Ruiz-Aceituno *et al.*, 2014). Son muchas las propiedades biológicas atribuidas a los inositoles y sus derivados, principalmente relacionadas con la regulación de los desequilibrios hormonales asociados con el síndrome de ovario poliquístico (Nestler *et al.*, 1999; Siracusa *et al.*, 2022), la utilización de la grasa o eficacia contra la insulinoresistencia (Owczarczyk-Saczonek *et al.*, 2018), entre otras. Los inositoles se pueden obtener mediante procesos de extracción a partir de la fuente natural, aunque también pueden ser sintetizados mediante reacciones de hidrólisis ácida del fitato, fermentación microbiana y biocatálisis enzimática *in vitro* a partir de almidón, celulosa, sacarosa o glucosa (Li *et al.*, 2022).

En general, los procesos de purificación de carbohidratos bioactivos de otros azúcares o de catalizadores que intervienen en su proceso de obtención y que puedan interferir en su bioactividad son complejos y costosos, dada su gran similitud estructural en muchos casos, presentando gran interés la búsqueda de alternativas medioambientalmente seguras y eficaces para el fraccionamiento selectivo de estos compuestos.

1.2. Complementos alimenticios

Los **complementos alimenticios** son productos que contienen uno o más compuestos bioactivos que pueden aportar beneficios nutricionales y/o fisiológicos para la salud. La Directiva 2002/46/CE del Parlamento Europeo (o bien el Real Decreto 1487/2009 del ordenamiento jurídico español) los define como “los productos alimenticios cuyo fin es complementar la dieta normal y consistentes en fuentes concentradas de nutrientes o de otras sustancias que tengan un efecto nutricional o fisiológico, en forma simple o combinada, comercializados en forma dosificada, es decir, cápsulas, pastillas, tabletas, píldoras y otras formas similares (bolsitas de polvos, ampollas de líquido, botellas con cuentagotas, etc.) y que deben tomarse en pequeñas cantidades unitarias”.

Aunque, en principio, dichos complementos alimenticios no serían necesarios si se sigue una dieta equilibrada que aporte todos los nutrientes esenciales para el crecimiento, mantenimiento y funcionamiento del organismo, la realidad demuestra que su consumo se encuentra muy extendido entre la población, principalmente en los países desarrollados. El estilo de vida de la sociedad moderna, que a menudo dificulta una adecuada alimentación, así como la conciencia cada vez mayor de la importancia de los efectos fisiológicos y/o nutricionales asociados a su ingesta para la mejora del rendimiento, la salud o el bienestar (European Public Affairs & Ipsos, 2022), son algunas de las razones que justifican que el sector de los complementos alimenticios se encuentre en continua expansión. Su impacto económico en el año 2022 supuso 164.000 millones de dólares y se espera un crecimiento anual del 9% hasta el año 2030 (ResearchAndMarkets.com, 2021). Además, acontecimientos recientes tales como la pandemia por la COVID-19 favorecieron no sólo el incremento drástico de ventas de complementos alimenticios (Arlauskas *et al.*, 2022), sino también un mayor interés científico por estos productos. En este sentido, la Autoridad Europea de Seguridad Alimentaria (EFSA) recomienda destinar los complementos alimenticios para corregir deficiencias nutricionales, mantener una ingesta adecuada de determinados nutrientes o apoyar funciones fisiológicas específicas (European Food Safety Authority, 2022).

1.2.1. Complementos alimenticios de origen vegetal

Los complementos alimenticios engloban una amplia gama de ingredientes como vitaminas, minerales, aceites de pescado, probióticos, ácidos grasos esenciales, extractos de plantas, etc. De entre ellos, se definen como **complementos alimenticios de origen vegetal (CAV)** aquéllos que consisten en plantas, algas, hongos o líquenes, completos o las partes anatómicas de los mismos, pudiendo presentarse enteros, fragmentados, molturados, o pulverizados, y en preparaciones obtenidas a partir de ellos mediante procesos de extracción, destilación, concentración y fermentación, entre otros (AESAN, 2007).

En la actualidad, los CAV tienen una mayor aceptación entre los consumidores por diferentes motivos, entre los que cabe señalar:

- Percepción natural del producto y de la seguridad para su consumo en comparación con aquéllos de origen sintético.
- Preferencia por productos procedentes de fuentes orgánicas y sostenibles, como los residuos de la industria agrícola, que se perciben como más respetuosos con el medio ambiente y más éticos en términos de producción (Egan *et al.*, 2011).
- Creciente popularidad de dietas veganas y vegetarianas que favorecen la demanda de productos como los CAV que se ajusten a estas elecciones dietéticas (Alcorta *et al.*, 2021).
- Posible sinergia entre los distintos componentes bioactivos de los CAV, lo que les otorga un valor añadido para promover la salud y el bienestar (Yun, 2010; Pezzani *et al.*, 2019).

Todas estas razones justifican no sólo que los CAV sean cada vez más consumidos, sino también la amplia variedad de productos disponibles en el mercado para satisfacer las preferencias del consumidor. Así, se estima que el 18.8 % de los ciudadanos europeos consumen, al menos, un CAV al día, hecho reseñable que coincide con el interés renovado en enfoques holísticos como la terapia nutricional y la medicina alternativa (García-Alvarez *et al.*, 2014).

1.2.2. Legislación de los CAV

Como se ha mencionado previamente, los complementos alimenticios están regulados a nivel europeo por la Directiva 2002/46/CE, que se traspone a la legislación nacional mediante el Real Decreto 1487/2009. Sin embargo, dicha legislación se centra únicamente en la regulación de vitaminas y minerales, así como de las sustancias utilizadas como fuentes de tales nutrientes para su elaboración. Dicho Real Decreto ha sido recientemente modificado por el RD130/2018, donde se incluye un anexo con otras sustancias diferentes de vitaminas y minerales con efecto nutricional o fisiológico que pueden utilizarse en la fabricación de complementos alimenticios. No obstante, los ingredientes botánicos siguen sin estar considerados en dicho listado.

La falta de una normativa europea que contemple requisitos armonizados de calidad y seguridad específicos para los CAV ha hecho que muchos de los países miembros creen sus propias disposiciones nacionales para su regulación, elaborando listas de sustancias que pueden usarse en la elaboración de complementos, lo que conlleva importantes discrepancias en la clasificación de los CAV de unos países a otros. En general, para la comercialización de estos productos, además de información relativa a la composición del producto, se requiere únicamente que el producto sea seguro para el consumo en base a la información bibliográfica y/o aportada por el fabricante (Directiva 2002/46/CE, 2002; Silano *et al.*, 2011).

Por otra parte, cabe destacar que algunos productos basados en extractos de plantas se pueden comercializar como complementos alimenticios o como productos medicinales a base de hierbas, cuyos requerimientos regulatorios difieren notablemente. La mayor flexibilidad en cuanto a los requisitos exigidos para los complementos alimenticios ha marcado una tendencia creciente por parte de las industrias del sector hacia la transferencia de productos registrados como medicinales hacia su comercialización como CAV, o a la aparición de nuevos productos directamente registrados como CAV, lo que está impulsando su mercado.

En cuanto a las declaraciones nutricionales y de propiedades saludables de los complementos alimenticios, reguladas mediante el Reglamento (CE) 1924/2006, cabe señalar que, para ser autorizadas a nivel europeo por la EFSA, han tenido que someterse previamente a un procedimiento en el que se demuestre que están basadas en datos científicos sólidos, y en su mayoría están relacionadas con vitaminas y minerales (EFSA Panel on Dietetic Products Nutrition and Allergies (NDA), 2021; Martini *et al.*, 2019; Kušar & Pravst, 2022). Es de destacar también que en el año 2012 se evaluaron más de 2.000 declaraciones de salud asociadas a productos botánicos (incluidos los complementos alimenticios) y, tras varias opiniones científicas negativas de la EFSA, la UE decidió ponerlas “en espera”, a la expectativa de una consideración adicional sobre cómo proceder con ellas (Document Executive Summary of the Evaluation of the Regulation (EC) N° 1924/2006 on Nutrition and Health Claims, 2020).

Por otra parte, las limitaciones arriba mencionadas en cuanto a la regulación de los CAV, junto a su cada vez mayor demanda por parte del consumidor, han proporcionado un entorno idóneo para que dichos productos sean **objeto de prácticas fraudulentas**. De hecho, se ha reportado un número creciente de alertas relacionadas con la presencia no declarada de plantas tóxicas, laxantes con posibles efectos hepatotóxicos o nefrotóxicos, o incluso de medicamentos, y que han obligado a retirar algunos CAV del mercado por el riesgo que suponían para la seguridad de los consumidores (AESAN, 2022; Ekar & Kreft, 2019). Además, son frecuentes otros tipos de fraudes basados en el reemplazo de la fuente natural declarada por otra de menor valor económico, ya sea por otra especie botánica o por ingredientes de origen sintético (Mena-García *et al.*, 2023). Se han descrito también discrepancias entre la presencia o el contenido de compuesto bioactivo real y el declarado en el etiquetado o la declaración falsa de la relación planta:extracto (Hong *et al.*, 2017). Todos estos fraudes afectan de forma negativa a la calidad de los complementos alimenticios y generan desconfianza en cuanto al consumo seguro de estos productos, además de suponer importantes consecuencias económicas para las empresas del sector, obligadas a establecer protocolos rigurosos y cada día más sofisticados para demostrar la calidad de sus productos.

En base a todo lo expuesto, resulta evidente la necesidad de contribuir a ampliar el conocimiento que actualmente se tiene sobre los CAV y, en este sentido, resulta imprescindible

el desarrollo de nuevas aproximaciones analíticas que permitan una mejor caracterización de su composición.

1.3. Análisis de compuestos bioactivos

El desarrollo de metodologías analíticas que permitan el estudio de la composición, tanto de los productos naturales de los que se extraen los compuestos bioactivos, como de los propios CAV, es fundamental para garantizar la calidad y genuinidad de estos productos y, desde el punto de vista empresarial, contribuir a su diferenciación respecto de la competencia. Debido a la gran **diversidad de compuestos bioactivos** presentes en este tipo de matrices, así como su frecuente presencia en forma de **mezclas complejas**, las técnicas analíticas generalmente elegidas para la caracterización de su composición, así como para la detección de marcadores de calidad y/o autenticidad, son aquéllas que proporcionan una alta resolución y sensibilidad, como la **cromatografía de gases (GC) o de líquidos (LC, incluyendo la cromatografía líquida de ultra-alto rendimiento, UHPLC) acopladas a espectrometría de masas (MS)** (Vaclavik *et al.*, 2014; Guijarro-Díez *et al.*, 2017; Gafner *et al.*, 2023). Independientemente de la técnica de separación empleada, el uso de analizadores de MS de alta resolución y para MS tándem (MSⁿ) resulta ventajoso para la identificación inequívoca de adulterantes y análogos estructurales (Vaclavik *et al.*, 2014).

Estudios previos sobre la evaluación de la calidad de diferentes extractos vegetales y/o CAV (Martello *et al.*, 2007; Damiano *et al.*, 2014; Choi *et al.*, 2015; Mena-García *et al.*, 2021, 2022, 2023) han puesto de manifiesto las ventajas que aporta la aplicación de **estrategias multianalíticas** basadas en el empleo combinado de diferentes técnicas de separación acopladas a MS, junto con un posterior tratamiento quimiométrico de los datos. Sin embargo, la selección de las técnicas y condiciones de operación más apropiadas para cada tipo de extracto o CAV son críticas cuando se pretende realizar una caracterización exhaustiva de su composición o para la detección de los cada día más sofisticados fraudes a los que se ven sometidos, y que a menudo constituyen un reto analítico.

Por otra parte, el desarrollo y validación de metodologías rápidas basadas en **inyección directa-MS (DI-MS)** constituye una alternativa de interés, escasamente abordada hasta el momento, para la clasificación de muestras en aplicaciones relacionadas con el control de calidad y autenticidad de alimentos (Hou *et al.*, 2016; He *et al.*, 2021), no existiendo antecedentes sobre su aplicación a la detección fiable y sensible de fraudes en CAV.

En los siguientes apartados se describen los fundamentos y algunas aplicaciones destacables de las metodologías por GC, LC y DI-MS empleadas en la presente Tesis Doctoral para el análisis,

tanto de los compuestos bioactivos de interés, como para la detección de fraudes de composición relacionados con la calidad y autenticidad de los CAV en estudio.

1.3.1. Cromatografía de gases

El fundamento de la GC implica la volatilización de la muestra en el inyector caliente (250-350 °C) de un cromatógrafo de gases, seguida de la separación de los componentes de la mezcla en una columna con una fase estacionaria y dimensiones determinadas. Los compuestos separados llegan al detector generando una señal, que se registra en forma de cromatograma. De entre los distintos detectores (ionización de llama (FID), captura electrónica (ECD), etc.) que pueden emplearse en GC, a continuación, se describen los dos empleados en esta Tesis Doctoral.

En el caso del acoplamiento **cromatografía de gases-espectrometría de masas (GC-MS)**, los compuestos que eluyen de la columna son dirigidos hacia la cámara de ionización a través de una línea de transferencia, donde son ionizados y fragmentados, generalmente por impacto electrónico (EI) a un potencial establecido de 70 eV. Dichos iones/fragmentos son posteriormente acelerados y dirigidos al analizador, que los separa en función de su relación masa/carga (m/z), siendo el cuadrupolo (Q) el analizador más comúnmente empleado. El registro así obtenido, denominado espectro de masas, consiste en una representación de la intensidad de los iones/fragmentos separados en función de su m/z (Sparkman *et al.*, 2011a, 2011b). La Figura 1.13. muestra el esquema general de un equipo de GC.

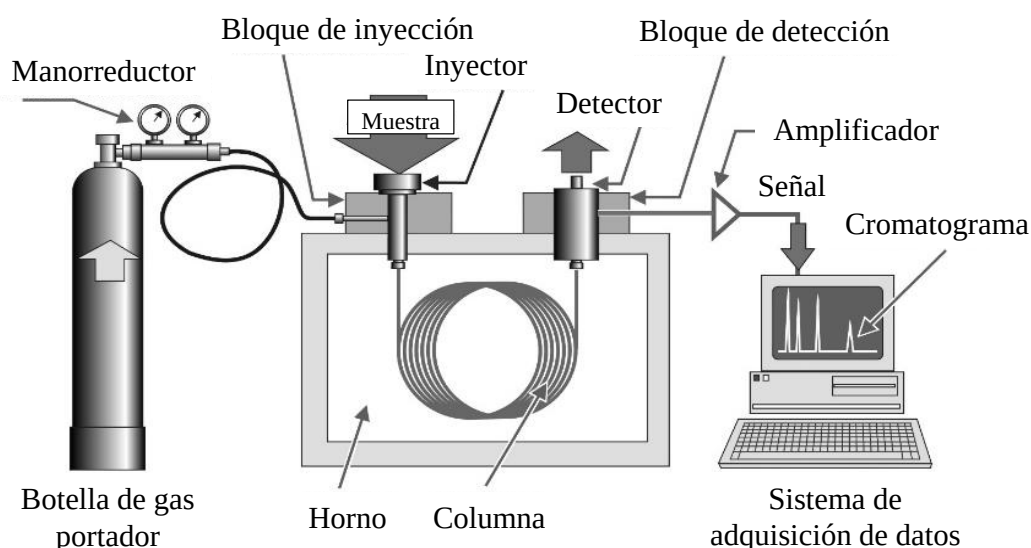


Figura 1.13. Esquema de un cromatógrafo de gases. Figura adaptada del libro “La Espectrometría de Masas en Imágenes” (Esteban,1993).

En el caso de la **cromatografía de gases-olfatometría (GC-O)**, generalmente empleada para determinar los compuestos aromáticamente activos en alimentos y bebidas, se combina el análisis cromatográfico tradicional con la detección sensorial, donde el olfato humano actúa como un detector de gran sensibilidad y selectividad, para estudiar mezclas complejas e identificar los principales compuestos activos responsables del aroma (de-la-Fuente-Blanco & Ferreira, 2020). Para llevar a cabo estos análisis, una persona debidamente capacitada actúa como evaluador o asesor, detectando la presencia de compuestos aromáticamente activos a través de un olfatómetro específicamente diseñado, generalmente un puerto cónico de vidrio adaptado a la forma de una nariz, conectado en paralelo a detectores tradicionales de GC (*e.g.* FID, MS, etc.). El evaluador es capaz de medir diferentes parámetros para cada compuesto detectado: descriptores del aroma, intensidad, duración y tiempo de retención. La variabilidad en la capacidad olfativa de los evaluadores y otros factores como la velocidad de la respiración, estado de ánimo o de salud pueden afectar significativamente la calidad de los datos, por lo que es esencial controlar estos factores para obtener resultados reproducibles. Aun así, para la identificación de los compuestos es necesaria la información aportada por otros detectores y/o mediante el empleo de patrones (Brattoli *et al.*, 2013; de-la-Fuente-Blanco & Ferreira, 2020; Mahmoud & Zhang, 2024).

La GC es la técnica de elección para el análisis de aquellos compuestos que puedan vaporizarse sin descomponerse y, por tanto, la recomendada no sólo para el **análisis de compuestos volátiles** en matrices vegetales (aceites esenciales, plantas, etc.), sino también para el de muestras de interés en los campos alimentario, farmacéutico, cosmético, ambiental y forense, por mencionar algunas de ellas.

La introducción de la muestra en los equipos de GC puede abordarse de distintas formas. Entre ellas, y como alternativa limpia y sensible a la frecuente inyección de extractos líquidos obtenidos mediante el empleo de disolventes orgánicos, los métodos basados en la **Microextracción en Fase Sólida (SPME)** han despertado gran interés en los últimos años para el análisis de la composición aromática de alimentos y plantas (Jalili *et al.*, 2020).

La SPME utiliza una fibra de sílice recubierta con una fase polimérica que, al exponerse a la muestra, es capaz de retener los compuestos volátiles de interés, por un mecanismo (adsorción, reparto, etc.) dependiente de la fibra elegida. Transcurrida esta etapa, la fibra se inserta directamente en el inyector del GC, donde los compuestos retenidos son desorbidos térmicamente y transferidos a la columna cromatográfica para su separación (Anastassiades & Scherbaum, 2005) (Figura 1.14.). La ventaja de aunar en un solo paso la extracción y preconcentración de volátiles mejora significativamente la sensibilidad del análisis, pudiéndose también modificar la selectividad en función de la fibra de SPME empleada. Además, la posibilidad de miniaturización en el análisis de muestras para las que se dispone de una cantidad limitada, así como la

automatización de la SPME-GC son otras de las ventajas asociadas al empleo de esta técnica para el análisis de compuestos volátiles (Dugheri *et al.*, 2022).

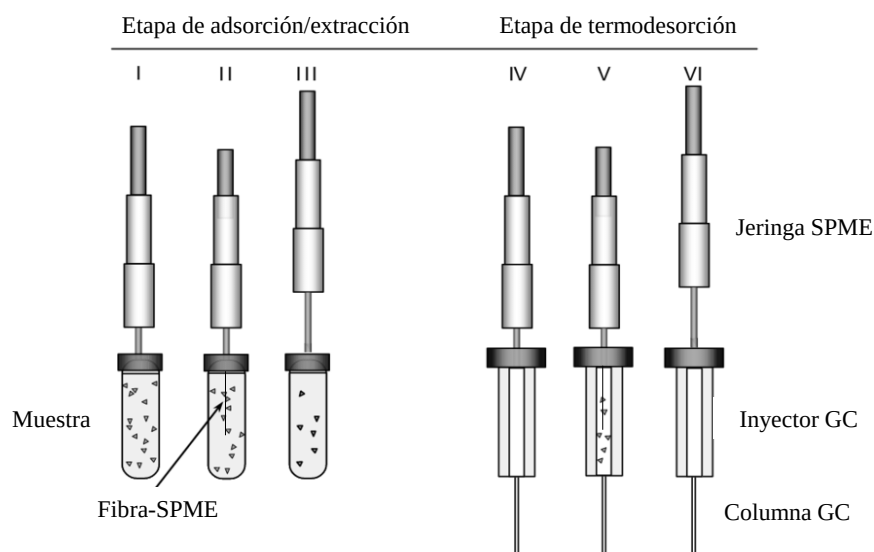


Figura 1.14. Esquema del procedimiento de SPME típico. Figura adaptada de Anastassiades y Scherbaum (2005).

Por otro lado, la GC puede emplearse también para el **análisis de compuestos semivolátiles o de baja volatilidad** como los carbohidratos, ácidos grasos o aminoácidos, si son previamente transformados en derivados volátiles y térmicamente estables a través de una reacción de derivatización adecuada (Sparkman *et al.*, 2011a). En términos generales, la **derivatización** reemplaza los átomos de hidrógeno activos presentes en los analitos (generalmente en grupos funcionales como -OH, -COOH, -SH, -NH o -CONH) por otros grupos que permitan reducir su polaridad y aumentar así su volatilidad. Entre las distintas reacciones de derivatización, una de las más comunes es la sililación, donde los hidrógenos activos de las moléculas se reemplazan por grupos sililo, generalmente trimetilsililo (TMS), formando trimetilsilil éteres. Esta reacción ha sido ampliamente usada para el análisis de compuestos por GC como los carbohidratos neutros (Ruiz-Matute *et al.*, 2011). Sin embargo, el principal inconveniente de estos derivados es que, para un mismo carbohidrato reductor, se pueden obtener hasta 5 configuraciones diferentes en disolución (trimetilsilil derivados de α - y β -furanosa, α - y β -piranosa y cadena abierta), lo que supone una desventaja para el análisis de mezclas complejas. Para resolver esta limitación se suele oximar el carbono anomérico del azúcar en una etapa previa a la sililación (Figura 1.15.), obteniéndose así trimetilsilil oximas de las formas *syn*- (*E*) y *anti*- (*Z*) de cada carbohidrato reductor, lo que facilita su identificación y cuantificación.

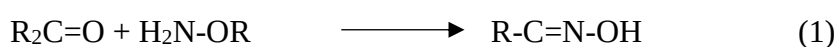


Figura 1.15. Esquema de formación de oximas (1) y trimetilsiliderivados (2).

1.3.2. Cromatografía de líquidos de alta resolución

La cromatografía de líquidos de alta resolución es una técnica de gran versatilidad, empleada generalmente para el análisis de **compuestos polares y/o de alto peso molecular**. En dicha técnica, la separación de los distintos componentes de la muestra tiene lugar haciendo uso de una fase estacionaria, generalmente adherida a un soporte sólido y depositada dentro de una columna, y una fase móvil líquida, que se desplaza a presión a través de la fase estacionaria, arrastrando consigo a los analitos y en la que los mismos deben ser solubles. Los principales constituyentes de un sistema de LC, tal y como se muestra en la Figura 1.16., son: una bomba, una unidad desgasificadora, un inyector, una columna (en el interior de un horno), un detector y un procesador de datos.

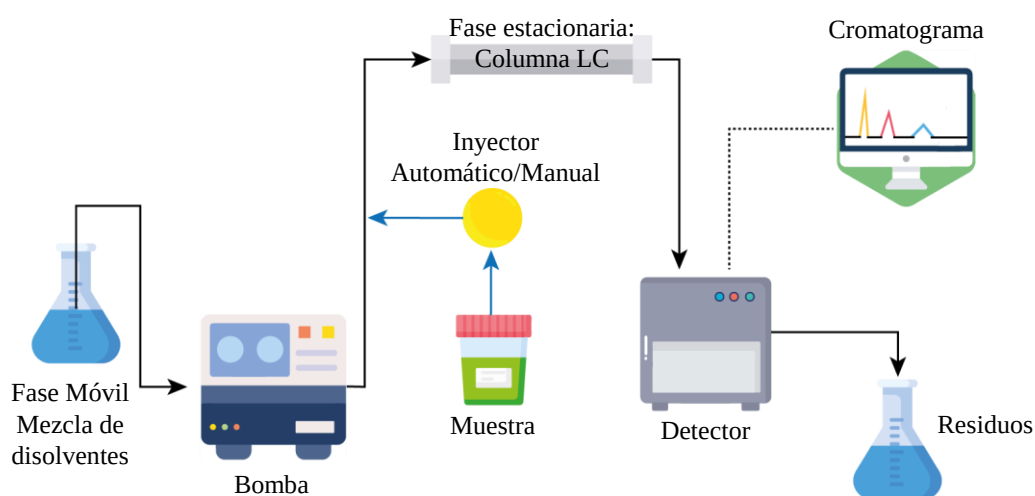


Figura 1.16. Esquema de los principales componentes de un sistema de LC.

La eficacia de la separación de analitos en LC viene determinada por la composición de la fase móvil y de la fase estacionaria, condicionadas a su vez por el mecanismo de separación o **modo de operación** elegido. De entre los distintos modos de operación actualmente disponibles, a continuación, se describen la cromatografía en fase inversa (RPLC) y la cromatografía de interacción hidrofílica (HILIC) por ser los empleados para los análisis por LC llevados a cabo en esta Tesis Doctoral.

- **Cromatografía en fase inversa.** Es el modo de operación más extendido y con mayor número de aplicaciones, al permitir la separación de compuestos de muy distinta naturaleza (*e.g.* proteínas, compuestos fenólicos, carbohidratos, aminoácidos, etc.), de interés en campos tan diversos como el farmacéutico (Yabré *et al.*, 2018), alimentario (Reuhs, 2017) o ambiental (Hussen, 2022), entre otros.

Requiere el empleo de una fase estacionaria no polar (típicamente partículas de octadecilsilano, C₁₈) y una fase móvil polar (generalmente mezclas de agua con metanol,

acetonitrilo o tetrahidrofurano). El mecanismo de retención se basa en las interacciones hidrofóbicas de los analitos con la fase estacionaria, donde los más polares eluyen al principio, mientras que los más hidrofóbicos quedan más retenidos. Además, se pueden añadir aditivos como el ácido trifluoroacético, acético o fórmico que, ajustando el pH de la fase móvil, pueden contribuir a una mejor separación.

- **Cromatografía de interacción hidrofílica.** Este modo de operación se puede describir como una variante de la cromatografía en fase normal, donde se emplean columnas con una fase estacionaria polar (*e.g.* sílice funcionalizada con grupos polares tipo amino, amida o ciano). La fase móvil suele estar constituida por un porcentaje alto de fase orgánica (> 70%), generalmente acetonitrilo, y en menor medida, un disolvente acuoso/tampón. También se suelen emplear aditivos como sales (NH_4OH , $\text{CH}_3\text{COONH}_4$) para mejorar la separación y elución de los analitos. En cuanto a su fundamento, una de las teorías más aceptadas es la del reparto líquido-líquido de los analitos entre la capa de disolvente acuoso adherida a la superficie polar de la fase estacionaria y la fase orgánica. Además, y en función de la fase HILIC empleada, pueden darse otras interacciones secundarias tipo electrostáticas y/o de enlaces de hidrógeno. Todas estas interacciones dan lugar a un orden de elución generalmente opuesto al de la fase inversa.

Aunque la proporción de disolvente orgánico/acuoso juega un papel determinante en la selectividad de la separación, la elección de la fase estacionaria también es crucial para adaptar la química de la columna HILIC a los grupos funcionales presentes en los analitos de interés (Buszewski & Noga, 2012; Reuhs, 2017; Hussien, 2022). Las aplicaciones para esta variante de LC son muy diversas, permitiendo el análisis, tanto de moléculas polares muy pequeñas como nucleósidos, nucleótidos, aminoácidos, monosacáridos, etc., como de moléculas de mayor tamaño (*e.g.* glucanos, glicoproteínas, etc.), cuyo análisis resulta relevante en sectores como el farmacéutico, el de la proteómica y metabolómica, etc. (Buszewski & Noga, 2012).

La cromatografía líquida es la técnica de separación más ampliamente utilizada para la caracterización de extractos vegetales, y sus múltiples posibilidades de acoplamiento a diferentes **detectores** (absorción ultravioleta/visible (UV/Vis), fluorescencia, detector evaporativo de dispersión de luz (ELSD) y MS) le confieren la posibilidad de analizar diferentes clases de metabolitos.

Los **detectores UV/Vis o de fluorescencia** son útiles para la detección de compuestos que contengan grupos cromóforos o fluoróforos, respectivamente, tal es el caso de flavonoides (quercetina o kaempferol), cumarinas, proteínas o ácidos nucleicos. No obstante, su aplicación a otros compuestos como los carbohidratos que carecen de dichos grupos, requiere una etapa de derivatización que puede ser previa o posterior a la separación cromatográfica. Una alternativa para su análisis es la detección mediante **ELSD**, ya que este tipo de detectores no dependen de

las propiedades ópticas del compuesto. El funcionamiento de estos detectores se basa en la nebulización del efluente de la columna por un gas nebulizador (N_2), de tal forma que las gotas obtenidas son transportadas a lo largo de un tubo evaporador (o de deriva) y dirigidas hacia un haz de luz láser, cuya dispersión está relacionada con la concentración de soluto en el eluyente (Dreux & Lafosse, 1995; Reuhs, 2017). Todos estos detectores son útiles cuando ya se tiene una información previa de los compuestos que se pretende analizar o se dispone de patrones que permitan confirmar la identidad de los analitos diana, puesto que no proporcionan una información estructural. Están, por tanto, orientados al denominado análisis dirigido o *target* de los compuestos de interés. Sin embargo, debido a la complejidad de muchas matrices, y a la diversidad estructural de los compuestos bioactivos, el acoplamiento **LC-MS** suele preferirse para la caracterización mejorada, en términos de sensibilidad y selectividad, de dichos compuestos en estudios no dirigidos (*untarget*), sin requerir procedimientos añadidos de derivatización (Cavaliere *et al.*, 2018).

Existen múltiples tecnologías tanto para la ionización como para la separación de los iones, lo que da lugar a los distintos **tipos y configuraciones de espectrómetros de masas** disponibles en el mercado. La fuente de ionización por electrospray (ESI) es la más utilizada para el análisis de macromoléculas biológicas (Kaur *et al.*, 2022), y su fundamento consiste en hacer pasar los analitos junto con la fase móvil a través de un capilar de nebulización a alto voltaje que, junto con un gas de secado, hace que se formen los iones en fase gas. Atendiendo a los tipos de analizadores, el más común es el cuadrupolo (Q) debido, principalmente, a su facilidad de uso, pequeño tamaño y bajo precio. No obstante, existen otros como los analizadores híbridos de cuadrupolo tiempo de vuelo (QToF) o los de triple cuadrupolo (QqQ) que, a pesar de ser más costosos, presentan como ventajas el proporcionar una mayor información estructural, el permitir realizar experimentos de MS en tándem (MS^n) y, en el caso del QToF, la obtención de masas exactas.

Además, se pueden encontrar otras variantes de la MS como la **espectrometría de masas de relaciones isotópicas (IRMS)**. Esta técnica analítica se puede acoplar tanto a GC como a LC, y permite determinar la composición isotópica en hidrógeno, carbono, etc., expresada como valores delta (*e.g.* $\delta^{13}C$, ‰), de compuestos específicos en mezclas complejas. Dado que las relaciones isotópicas varían en función de la fuente u origen de la muestra, éstas resultan muy útiles para la diferenciación de compuestos naturales y sintéticos en estudios orientados a la evaluación de la autenticidad y genuinidad de alimentos como miel, frutas, aceites, especias, etc. (Pianezze *et al.*, 2019; Yücel *et al.*, 2023; Vinothkanna *et al.*, 2024). Sin embargo, a pesar de su potencial, la IRMS no ha sido aplicada hasta el momento a la detección de fraudes de genuinidad en CAV.

1.3.3. Inyección directa-espectrometría de masas

En la actualidad existe una gran demanda de metodologías analíticas rápidas que, no sólo resulten ventajosas en cuanto a eficiencia (mayor número de muestras analizadas por unidad de tiempo) y costes asociados, sino de proporcionar resultados fiables y de calidad. En este sentido, las metodologías basadas en **inyección directa espectrometría de masas (DIMS)**, en las que el tiempo de análisis se reduce significativamente al obviar la etapa de separación cromatográfica, han despertado gran interés en el ámbito del control de calidad, autenticidad de alimentos, etc.

En cuanto a la aplicación de la DIMS al análisis de compuestos volátiles, se han descrito metodologías basadas en distintos sistemas de introducción de muestra acoplados/seguídos de **espectrometría de masas con ionización por impacto electrónico ((EI)MS)**. Así, y entre otros, se han desarrollado y aplicado con éxito métodos por desorción térmica directa acoplada en línea a GC-MS (DTD-GC-MS) para la identificación de quimiotipos y cuantificación de volátiles mayoritarios en muestras de tomillo (Lebrón-Aguilar *et al.*, 2016), o basados en SPME MS para la autenticación de mieles de distinto origen botánico (Quintanilla-López *et al.*, 2022).

Por otra parte, se han optimizado también metodologías basadas en **inyección en flujo acoplada a espectrometría de masas con ionización por electrospray (FIA-(ESI)MS)** que, entre otras aplicaciones, han permitido la clasificación de alimentos con distintos objetivos (Campmajó *et al.*, 2022; Termopoli *et al.*, 2021; Vilà *et al.*, 2022). Sin embargo, la aplicación de esta metodología para garantizar la autenticidad y calidad de CAV es limitada, existiendo solo un trabajo en este campo, enfocado en el análisis de complementos basados en *Scutellaria lateriflora* (Sun & Chen, 2011).

A pesar de sus potenciales ventajas como métodos de cribado rápidos (Figura 1.17.), es necesario señalar que la aplicación de aproximaciones basadas en **FIA-(ESI)MS** requiere siempre una evaluación previa, no siempre abordada en detalle, de sus potenciales limitaciones para el fin analítico pretendido (*e.g.* efecto de interferentes con idéntica masa nominal sobre los resultados del análisis cuantitativo). Además, no son tampoco comunes los estudios en los que se comparan las potenciales ventajas/limitaciones de las metodologías basadas en DIMS con las de las correspondientes aproximaciones cromatográficas (por GC-MS o LC-MS), siendo estos estudios de gran interés para la posible implementación ventajosa de métodos rápidos por FIA-(ESI)MS tanto en laboratorios del sector público como privado.

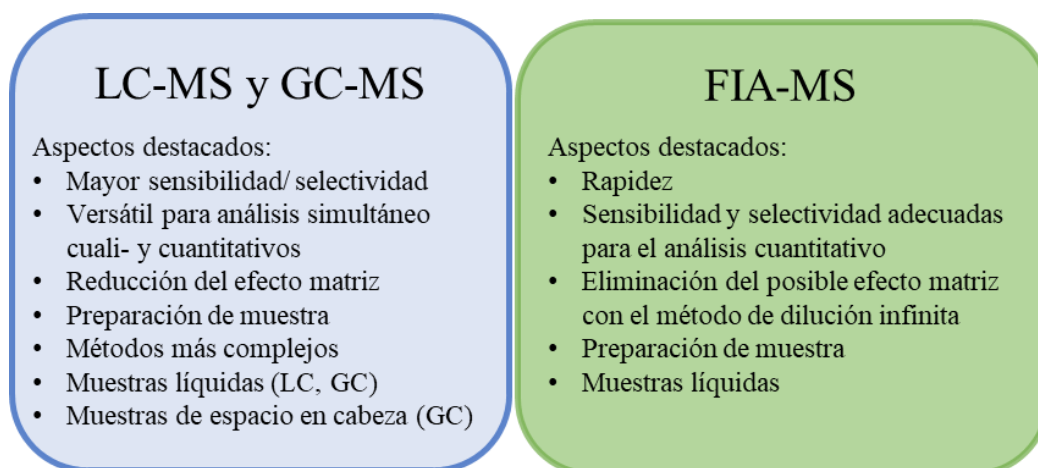


Figura 1.17. Comparación de las características de LC-MS y GC-MS respecto de FIA-MS. Figura adaptada de (Nanita & Kaldon, 2016).

1.4. Obtención de extractos enriquecidos en compuestos bioactivos

Los compuestos bioactivos de matrices vegetales se encuentran presentes en diversas partes de las plantas, desde las hojas y tallos, hasta las raíces y frutos. Se localizan en estructuras celulares específicas como las células de la epidermis, el parénquima, los tejidos vasculares o incluso en compartimentos subcelulares como las vacuolas o los plastos (Wink, 2008). Su presencia puede depender del tipo de planta, de su genética, del ciclo de vida y de las condiciones ambientales.

La recuperación de compuestos bioactivos a partir de la matriz vegetal considerada requiere un proceso de **extracción** eficaz, rentable económicamente y medioambientalmente sostenible, que puede abordarse de distintas formas. Además, para facilitar la extracción, la matriz vegetal suele someterse a un pretratamiento que tiene como objetivo la destrucción de la pared vegetal, facilitando así la liberación de los compuestos bioactivos y aumentando la eficiencia de su extracción (Krakowska-Sieprawska *et al.*, 2022). Dependiendo de la naturaleza de la muestra, el pretratamiento puede incluir el secado, la liofilización, la fermentación o la hidrólisis enzimática (Oliveira *et al.*, 2016; Ebrahimi *et al.*, 2022). No obstante, resulta determinante considerar el tipo y características específicas de los compuestos a extraer, ya que, durante su procesamiento, dichos analitos pueden experimentar cambios en su composición química irreversibles (oxidación, hidrólisis, etc.) (Renard *et al.*, 2017; Krakowska-Sieprawska *et al.*, 2022).

La obtención a escala industrial de extractos ricos en compuestos bioactivos para su uso como CAV generalmente se lleva a cabo por métodos de **extracción convencional sólido-líquido (SLE)** a partir de partes específicas de la matriz vegetal seleccionada. Para ello, la matriz sólida elegida se sumerge en un disolvente adecuado, polar o no polar, en función de los analitos a extraer, bajo unas determinadas condiciones de temperatura y tiempo, con agitación constante. A

continuación, se procede a la separación de la fase líquida mediante una etapa de centrifugación, decantación o filtración (Lefebvre *et al.*, 2021). El rendimiento de este tipo de extracción suele ser bajo, por lo que es conveniente repetir el proceso hasta que el analito de interés se haya recuperado en su práctica totalidad (Poojary *et al.*, 2017). En este sentido, la SLE presenta una limitada eficacia de extracción, además de implicar largos tiempos y emplear, en muchos casos, grandes volúmenes de disolventes orgánicos volátiles. Por esta razón, en los últimos años han surgido técnicas de extracción avanzadas, más eficaces y sostenibles, como la **extracción asistida por microondas** (MAE) (Altemimi *et al.*, 2017; Lefebvre *et al.*, 2021; López-Salazar *et al.*, 2023) o **por ultrasonidos** (UAE) (Altemimi *et al.*, 2017; Kumar, 2017; Lefebvre *et al.*, 2021; Shen *et al.*, 2023), la **extracción con líquidos presurizados** (PLE) (Amador-Luna *et al.*, 2023; Lefebvre *et al.*, 2021) o **con fluidos supercríticos** (SFE), etc. como alternativas ventajosas y prometedoras para la obtención de extractos bioactivos de alto valor añadido. La optimización de las condiciones de extracción en estas técnicas puede permitir alcanzar mayores rendimientos para los compuestos de interés, a menudo con mayor selectividad y en tiempos más cortos (García-Sarrió *et al.*, 2018; Mena-García *et al.*, 2019, 2020). A continuación, se detallan los fundamentos, características y ventajas de las técnicas utilizadas en esta Tesis Doctoral (UAE, MAE y PLE).

Extracción asistida por ultrasonidos

La extracción de compuestos bioactivos mediante UAE se basa en la combinación del efecto térmico, mecánico y, el principal y más abundante, el fenómeno de la cavitación. El efecto térmico se refiere al proceso en el cual la energía vibratoria del ultrasonido (US) se absorbe por el medio y se convierte en calor, provocando un aumento en la temperatura de dicho medio. Este valor calorífico está influenciado por factores como la potencia ultrasónica, el tiempo de exposición y las propiedades del medio (Qiu *et al.*, 2020; Shen *et al.*, 2023). Por otro lado, el efecto mecánico describe cómo, tras la aplicación de US a un medio, se induce la vibración de partículas dentro de dicho medio, generando compresiones y expansiones. Esto resulta en un movimiento mejorado de las partículas, acelerando así el proceso de transferencia de masa (Wen *et al.*, 2018). Por último, la cavitación ultrasónica se refiere al fenómeno por el que las ondas ultrasónicas provocan vibraciones y acumulación de energía en pequeñas burbujas (núcleos de cavitación) dentro de un líquido. Cuando la energía supera un umbral, estas burbujas colapsan bruscamente, liberando una significativa cantidad de energía. El colapso de estas burbujas genera temperaturas (5000 K) y presiones extremadamente altas (100 MPa), acompañadas de una rápida tasa de enfriamiento. Además, se generan intensas fuerzas hidrodinámicas que pueden dar lugar a la formación de radicales libres y aumentar la presión y temperatura (Wen *et al.*, 2018; Qiu *et al.*, 2020; Shen *et al.*, 2023).

En general, los dispositivos de UAE consisten en baños y sondas, como muestra la Figura 1.18. y se diferencian en el tipo de transductores y la forma en la que generan y reciben las ondas

ultrasónicas. Los primeros, son más económicos, muy fáciles de manejar y permiten alcanzar altos rendimientos de extracción, aunque ofrecen menor intensidad ultrasónica con respecto a las sondas ultrasónicas. Los factores que afectan a la extracción son la potencia del equipo, frecuencia de US, tamaño de partícula de la muestra, tiempo de extracción, volumen de disolvente, temperatura del baño y la posición de la muestra en el baño. En comparación con el baño de ultrasonidos, la sonda proporciona generalmente una mayor eficacia de extracción, ya que se sumerge directamente en la solución para reducir la resistencia a la transferencia de masa. Sin embargo, en esta modalidad es más difícil el control de la temperatura, que aumenta más rápido durante el proceso de extracción y puede dar lugar a la degradación de algunos de los compuestos de interés. Los factores que hay que tener en cuenta para estos dispositivos son la amplitud, temperatura, forma (importante para el efecto de cavitación) y diámetro de la sonda así como las propiedades químicas de los analitos y muestra (Santos & Capelo, 2007; Shen *et al.*, 2023).

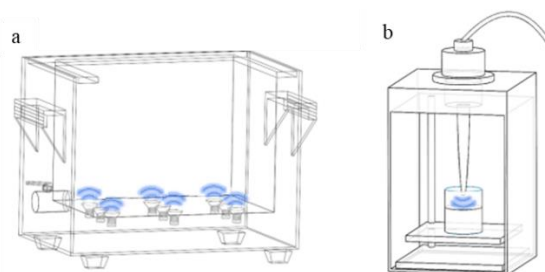


Figura 1.18. (a) Baño y (b) sonda de ultrasonidos.

Esta técnica ha sido ampliamente utilizada para la extracción de compuestos de diferente naturaleza, desde compuestos fenólicos (Rodsamran & Sothornvit, 2019), flavonoides (Yang *et al.*, 2019), antocianinas (Ochoa *et al.*, 2020), cannabinoides y terpenos (Addo *et al.*, 2022), hasta almidón o proteínas a partir de frutas, semillas, residuos de la industria agrícola, etc. (Görgüç *et al.*, 2019; Shen *et al.*, 2023).

Extracción asistida por microondas

La MAE (Figura 1.19.) se basa en la irradiación con microondas electromagnéticas de mezclas sólidas o líquidas para inducir su calentamiento, el cual dependerá de la constante dieléctrica de sus componentes. Por ejemplo, compuestos polares como el agua con una constante dieléctrica elevada (78,5 a 25 °C) son capaces de absorber energía y emitirla, mientras que los disolventes apolares, como el hexano (1,87 a 25 °C) son insensibles a las microondas. La temperatura es el principal factor que afecta a este tipo de extracción, junto con otros como la potencia, el tiempo, la relación entre la cantidad de muestra y el volumen de disolvente, etc. La optimización de estas condiciones es determinante para mejorar los rendimientos de extracción (Lefebvre *et al.*, 2021).

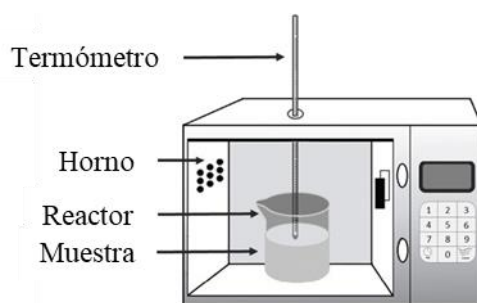


Figura 1.19. Dispositivo de extracción asistida por microondas.

El proceso de MAE, se lleva a cabo a una frecuencia comprendida entre 300 MHz - 300 GHz, y se basa en la absorción selectiva de la energía de microondas por parte de moléculas polares, lo que conduce a un calentamiento rápido y uniforme de la muestra, resultando en una extracción más eficiente y en menor tiempo de los compuestos de interés. A escala laboratorio se trabaja con una frecuencia de 2,45 GHz, mientras que a escala industrial la frecuencia empleada es en torno a 915 MHz. Los fenómenos que tienen lugar en este tipo de extracción son la conducción iónica y la rotación dipolar. En el primer caso, las moléculas cargadas migran, encontrándose otras moléculas cargadas también sujetas al efecto del campo electromagnético. La rotación dipolar sucede a causa de la adaptación de las moléculas dipolares a la alternancia del campo eléctrico. La fricción de las moléculas en movimiento y las colisiones entre los dipolos causan el aumento de temperatura (Zhang *et al.*, 2011; López-Salazar *et al.*, 2023). Tal y como sucede en la UAE, la temperatura puede favorecer o no la extracción, ya que puede beneficiar a ciertos compuestos y degradar otros. Así, la extracción selectiva de compuestos por MAE se debe principalmente al control de la temperatura (inducida por la irradiación), y está influenciada por la composición del disolvente o la matriz vegetal (Lefebvre *et al.*, 2021). A diferencia de la SLE o UAE, donde la obtención de compuestos intracelulares depende de procesos de permeación y solubilización, en MAE todo el volumen de la muestra es calentado desde el interior y no necesita estar en contacto con una superficie caliente para la conducción de calor (Li *et al.*, 2013; López-Salazar *et al.*, 2023).

Entre las distintas aplicaciones de esta técnica, se ha descrito su empleo exitoso para la extracción eficaz de compuestos fenólicos o polisacáridos bioactivos a partir de frutas, vegetales o algas (Mena-García *et al.*, 2019), inositoles e inulina de brácteas externas de alcachofa (Ruiz-Aceituno *et al.*, 2016), pectinas de piel de pomelo (Liew *et al.*, 2016) o polifenoles presentes en residuos de madera de manzano (Moreira *et al.*, 2017), entre otros ejemplos.

Extracción con líquidos presurizados (PLE)

Esta técnica se basa en el empleo de disolventes a elevadas presiones (500-3000 psi) y temperaturas (40-200 °C), de manera que estos se mantienen en estado líquido durante todo el

proceso de extracción. Si el disolvente empleado es agua, se la denomina extracción con agua subcrítica (SWE). Generalmente, en estas extracciones, la muestra sólida se encuentra empaquetada en una celda donde tiene lugar la extracción durante un periodo corto de tiempo (5-30 min). Bajo estas condiciones de presión, la constante dieléctrica del disolvente, y con ella su polaridad, disminuyen bruscamente según aumenta la temperatura. Además, la tensión superficial del disolvente disminuye, de forma que su viscosidad es menor, lo que favorece la solubilidad de los analitos en el disolvente y, por tanto, su recuperación. Otras de las ventajas de este tipo de extracción son la reducción del volumen de disolvente empleado y del tiempo de extracción. Sin embargo, en general, no es una técnica de extracción adecuada para la obtención de compuestos termolábiles, aunque una adecuada selección de disolvente, temperatura, presión y tiempo puede permitir su extracción (Ajila *et al.*, 2011).

En PLE se puede trabajar tanto en modo estático como dinámico. La Figura 1.20. muestra la configuración general de estos dispositivos para ambos modos de operación. En la configuración dinámica, el disolvente es bombeado de manera continua a través de la celda de extracción. Por el contrario, en el modo estático, el proceso consiste en uno o más ciclos de extracción, reemplazándose el disolvente de la celda de extracción en cada ciclo sucesivo. En general, y previo a la extracción, la muestra ha de ser sometida a procesos de molienda o triturado y homogeneización, para disminuir el tamaño de partícula y maximizar la superficie de contacto con el extractante, facilitando una mayor velocidad y difusión del analito a través de los poros de la matriz (Alvarez-Rivera *et al.*, 2020). Se suelen emplear agentes dispersantes o deshidratantes (arena de mar, tierra de diatomea) para favorecer la distribución uniforme de la muestra y contribuir al llenado de la celda, reduciendo así el consumo de disolventes.

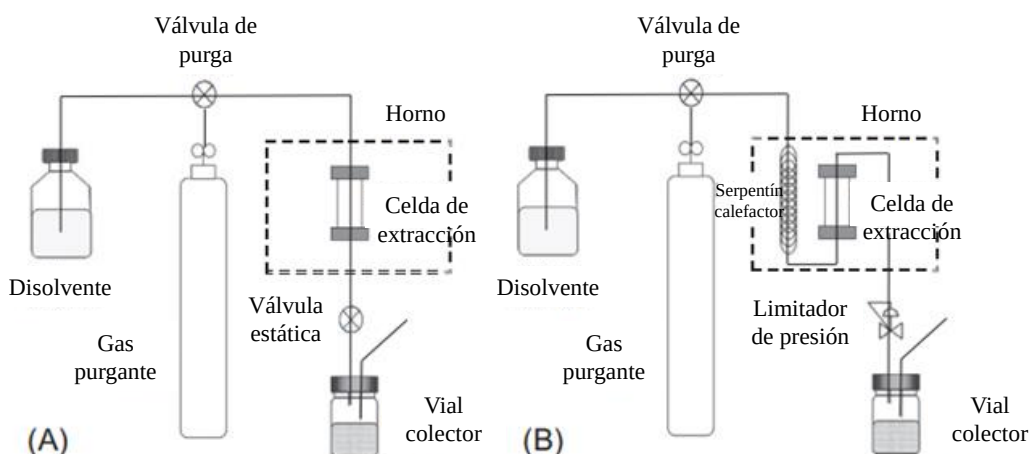


Figura 1.20. Configuración del sistema de PLE: dinámica (a) y estática (b). Figura reproducida de Álvarez-Rivera *et al.* (2020).

Esta técnica ha sido propuesta para la extracción de una gran variedad de compuestos bioactivos procedentes de alimentos, plantas y residuos agrícolas (Álvarez-Rivera *et al.*, 2020), pudiéndose mencionar, entre otras, su aplicación a la extracción de compuestos fenólicos a partir de cáscaras de cacahuete desgrasadas (Bodoira *et al.*, 2017) o de la pulpa de uva (*Vitis vinifera* L. cv. Syrah) (Pereira *et al.*, 2019), ciclitos procedentes de alfalfa (Al-Suod *et al.*, 2019) o piñones (Ruiz-Aceituno *et al.*, 2014), oligosacáridos prebióticos de ginseng brasileño (Vardanega *et al.*, 2017), etc.

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2.Objetivos, metodología y plan de trabajo

2. OBJETIVOS, METODOLOGÍA Y PLAN DE TRABAJO

2.1. Objetivos

En los últimos años, el mercado de los CAV ha experimentado una gran expansión, en gran medida debido al cada vez mayor interés por el consumo de productos naturales que, junto a una dieta equilibrada y ejercicio físico moderado, contribuyen a promover un mejor estado de salud. Además, y desde el punto de vista empresarial, el mercado de los CAV es un sector muy dinámico y sujeto a tendencias, lo que demanda el continuo desarrollo de nuevos productos por parte de las empresas del sector para satisfacer la demanda del consumidor. En este sentido, el reciente desarrollo de muchos de estos productos, su diversidad, junto con las limitaciones actuales de su regulación, hacen que los CAV sean frecuentemente objeto de una gran variedad de prácticas fraudulentas relacionadas con su composición, y que pueden incluso afectar a la salud del consumidor. En este sentido, la presente Tesis Doctoral pretende, por un lado, responder a la demanda de **metodologías (multi)analíticas mejoradas**, basadas tanto en aproximaciones que combinen los datos obtenidos por GC-MS y LC-MS, como en métodos rápidos por FIA-(ESI)MS, que permitan la caracterización de la composición de extractos vegetales/CAV y **aseguren su calidad y autenticidad**.

Por otra parte, el **desarrollo de nuevos CAV** debe contemplar, no sólo el **empleo de fuentes naturales ricas en compuestos bioactivos**, sino también el empleo de **metodologías eficaces y medioambientalmente respetuosas** para la obtención de los correspondientes extractos. En este sentido, la aplicación de métodos basados en técnicas de extracción avanzadas (UAE, PLE, MAE, etc.) y de disolventes verdes (NADESs) puede ser una alternativa de interés para la obtención de CAV, no sólo de mayor calidad, sino más sostenibles. Si, además, dichos métodos son aplicados a la **revalorización de residuos agroalimentarios**, la pretendida sostenibilidad es todavía mayor.

Por último, y como se ha mencionado previamente, los procedimientos actualmente empleados para la obtención de compuestos bioactivos para su aplicación como ingredientes funcionales, nutraceúticos, etc. demandan disponer de metodologías para su **fraccionamiento** que aúnen **eficacia y sostenibilidad**, siendo los basados en NADESs una opción potencialmente ventajosa para tal fin.

Teniendo en cuenta estas consideraciones previas, la presente Tesis Doctoral, enmarcada dentro del Proyecto “Metodologías multianalíticas avanzadas para el aseguramiento de la calidad y revalorización de complementos alimenticios de origen vegetal” financiado por la Comunidad de Madrid para la realización de Doctorados Industriales (IND2020/BIO-17409), tiene **un doble objetivo general**: (i) desarrollar nuevas metodologías analíticas para la evaluación de la calidad y genuinidad de complementos alimenticios de *C. forskohlii* y ajo negro y (ii) desarrollar procesos

eficaces y medioambientalmente respetuosos para la extracción y fraccionamiento de compuestos bioactivos.

Para la consecución de este doble objetivo general, se propusieron los siguientes **objetivos específicos**:

Objetivo general 1:

1.1. Desarrollar metodologías multianalíticas para la caracterización de la composición de extractos de referencia y detección de posibles fraudes en complementos alimenticios de *C. forskohlii* y ajo negro.

1.2. Desarrollar métodos por FIA-(ESI)MS para la detección rápida y fiable de fraudes en dichos CAV.

1.3. Evaluar la posible implementación en la empresa Pharmactive Biotech Products S.L.U. de las metodologías analíticas desarrolladas para la mejora en el control rutinario de calidad de extractos / CAV.

Objetivo general 2:

2.1. Optimizar las condiciones de envejecimiento del ajo para simultáneamente proporcionar muestras ricas en compuestos bioactivos y con una calidad organoléptica mejorada que puedan ser empleadas para la obtención de nuevos complementos de ajo negro.

2.2. Desarrollar nuevos métodos de obtención de extractos de ajo negro enriquecidos en compuestos bioactivos mediante técnicas de extracción avanzadas (MAE) y comparar su eficacia respecto a las técnicas convencionales.

2.3. Revalorizar los subproductos derivados del cultivo de ajo como fuente de compuestos bioactivos mediante la optimización y aplicación de técnicas de extracción avanzadas (PLE y UAE).

2.4. Evaluar el empleo de NADESs para la extracción y el fraccionamiento selectivo de carbohidratos bioactivos (mono-/disacáridos y polialcoholes) en función de su estructura química.

2.5. Analizar el potencial de explotación por Pharmactive Biotech Products S.L.U. de los extractos generados en el objetivo general 2.

2.2. Metodología y plan de trabajo

Para alcanzar el doble objetivo general planteado en esta Tesis Doctoral, se ha establecido el plan de trabajo mostrado en la **Figura 2.1.**, en el que los objetivos generales 1 y 2 se describen en las **secciones 3 y 4**, respectivamente. Cada una de estas secciones se encuentra a su vez dividida en distintas subsecciones que se corresponden con los artículos (publicados o en proceso de publicación) derivados de los resultados obtenidos en esta Tesis Doctoral y cuya metodología se detalla a continuación:

Sección 3 (prefacio 3.1.). Desarrollo de nuevas metodologías (multi)analíticas para la evaluación de la calidad y genuinidad de complementos alimenticios de origen vegetal (CAV).

Sección 3.2. Estrategia multianalítica para la detección de fraudes en complementos de *C. forskohlii*.

- Desarrollo y validación de métodos por GC-MS y LC-MS para la determinación de forskolina y otros compuestos presentes en complementos de *C. forskohlii*.
- Caracterización por GC-MS y LC-MS de extractos de raíz de *C. forskohlii*.
- Identificación de compuestos desconocidos y confirmación de estructuras por LC-MS/MS.
- Aplicación de los métodos mediante GC-MS y LC-MS a distintos complementos de *C. forskohlii* para la determinación de su contenido en forskolina y comparación con el contenido declarado en el etiquetado.
- Evaluación de la calidad y genuinidad mediante la identificación de discrepancias entre los perfiles cromatográficos de los extractos de referencia y los de complementos de *C. forskohlii*.

Sección 3.3. Análisis por Inyección en Flujo-Espectrometría de Masas para la detección rápida de fraudes en complementos alimenticios de *Coleus forskohlii*.

- Empleo de un diseño experimental Box-Behnken para la optimización de diferentes parámetros de la fuente de ionización del espectrómetro de masas para el análisis del compuesto bioactivo forskolina.
- Desarrollo y validación de un método rápido por FIA-(ESI)MS para determinar forskolina en complementos de *C. forskohlii*. Comparación con el método por LC-MS.
- Evaluación de la calidad: comparación del contenido en forskolina experimental con el declarado en el etiquetado para los complementos de *C. forskohlii* comerciales analizados.

Sección 3.4. Estudio comparativo basado en LC-MS y FIA(ESI)MS para la cuantificación de S-alil-L-cisteína en complementos de ajo negro.

- Optimización mediante un diseño experimental Box-Behnken de los parámetros de operación en (ESI)MS para la determinación de SAC.
- Desarrollo y validación de métodos por FIA-(ESI)MS y por LC-MS para la cuantificación de dicho compuesto bioactivo.
- Aplicación de ambas metodologías a complementos de ajo envejecido comerciales: (i) comparación del contenido en SAC determinado experimentalmente [por FIA-(ESI)MS y por LC-MS] y declarado en el etiquetado. Identificación de las ventajas y desventajas de ambas técnicas para la cuantificación de SAC y para la evaluación de la calidad de complementos de ajo envejecido.
- Identificación de discrepancias entre los perfiles obtenidos por LC-MS para extractos de referencia y complementos comerciales de ajo envejecido. Identificación de potenciales fraudes de genuinidad.
- Análisis por LC-IRMS de patrones naturales y sintéticos de SAC y de fracciones purificadas por extracción en fase sólida (SPE) de extractos de complementos de ajo envejecido. Aplicación a la confirmación de fraudes relacionados con el uso de compuestos bioactivos de origen no natural.

Sección 4 (prefacio 4.1.). Desarrollo de procesos de extracción y fraccionamiento de compuestos bioactivos eficaces y medioambientalmente respetuosos.

Sección 4.2. Cambios en el contenido en compuestos bioactivos y en el perfil aromático del ajo envejecido según las condiciones de envejecimiento.

- Obtención mediante la tecnología ABG Cool-Tech™ de muestras de ajo envejecido con distintas condiciones de procesamiento (tiempo de envejecimiento: 0-40 días, con/sin triturado de muestra durante el envejecimiento).
- Análisis por LC-MS de las muestras de ajo fresco y ajo envejecido previamente obtenidas: cuantificación de bioactivos (SAC y S1PC) e identificación de otros compuestos típicos del ajo.
- Selección de condiciones de operación en SPME-GC-MS para la evaluación de los cambios en el perfil volátil de las muestras de ajo sometidas a distintas condiciones de envejecimiento. Comparación con la composición volátil de ajo fresco y de complementos de ajo negro comerciales.
- Identificación mediante SPME GC-O de los principales compuestos responsables del aroma en muestras de ajo envejecido seleccionadas en base a su mayor contenido en

bioactivos y mejor perfil aromático. Comparación de resultados con los de ajo fresco y con los de un complemento de ajo negro comercial.

Sección 4.3. Extracción asistida por microondas para la obtención de extractos multifuncionales de ajo envejecido.

- Desarrollo y validación de métodos analíticos mediante HILIC-ELSD y LC-MS para el análisis de COS, CH y aa del ajo negro.
- Selección de disolventes para la extracción de compuestos bioactivos.
- Comparación de las técnicas de SLE, MAE y UAE para la obtención de extractos de ajo negro ricos en compuestos bioactivos.
- Selección de la técnica de extracción más eficaz y empleo de un diseño experimental Box-Behnken para la optimización de las condiciones de operación que maximicen la extracción de compuestos bioactivos de ajo negro.
- Aplicación de la metodología previamente optimizada a diferentes muestras de ajo negro comerciales.

Sección 4.4. Valorización de subproductos de ajo mediante el empleo de técnicas avanzadas de extracción.

- Selección del subproducto más adecuado para la obtención de extractos ricos en compuestos bioactivos (COS, CH y aa) a partir de distintos residuos de la planta de ajo (raíz, pieles, tallo y hojas).
- Empleo de un diseño experimental Box-Behnken y de un diseño factorial de 3 niveles para la optimización de las condiciones experimentales en la extracción por UAE y PLE, respectivamente, de compuestos bioactivos a partir del subproducto previamente seleccionado como más prometedor.
- Aplicación de las metodologías UAE y PLE optimizadas a diversas muestras del subproducto a revalorizar.

Sección 4.5. Empleo de disolventes eutécticos profundos naturales para la solubilización y el fraccionamiento selectivo de carbohidratos de bajo peso molecular.

- Síntesis y caracterización de NADESs basados en cloruro de colina.
- Estudio de la solubilidad de mono- y disacáridos en distintos NADESs. Evaluación del efecto de la temperatura en la solubilidad de los carbohidratos.
- Evaluación de la influencia de la estructura química de los carbohidratos (aldosa/cetosa; grado de polimerización) en su solubilidad.
- Estudio de la degradación de carbohidratos disueltos en NADESs.
- Preparación de mezclas binarias aldosas/cetosos. Evaluación de la separación selectiva de carbohidratos bioactivos respecto de los no bioactivos.

- Síntesis de lactulosa mediante isomerización de la lactosa y análisis de la mezcla resultante mediante HILIC-MS.
- Evaluación de la separación selectiva de los carbohidratos presentes en la mezcla de síntesis.

Sección 4.6. Uso de disolventes eutécticos profundos basados en cloruro de colina para la separación selectiva de polialcoholes bioactivos

- Estudio de la solubilidad de polialcoholes lineales y cíclicos en distintos NADESs.
- Preparación de mezclas binarias polialcohol/monosacárido en NADESs.
- Evaluación de la separación selectiva de polialcoholes y otros LMWCs presentes en las mezclas binarias.
- Estudio del efecto de los NADESs para la extracción selectiva de polialcoholes de matrices vegetales (apio y algarroba).

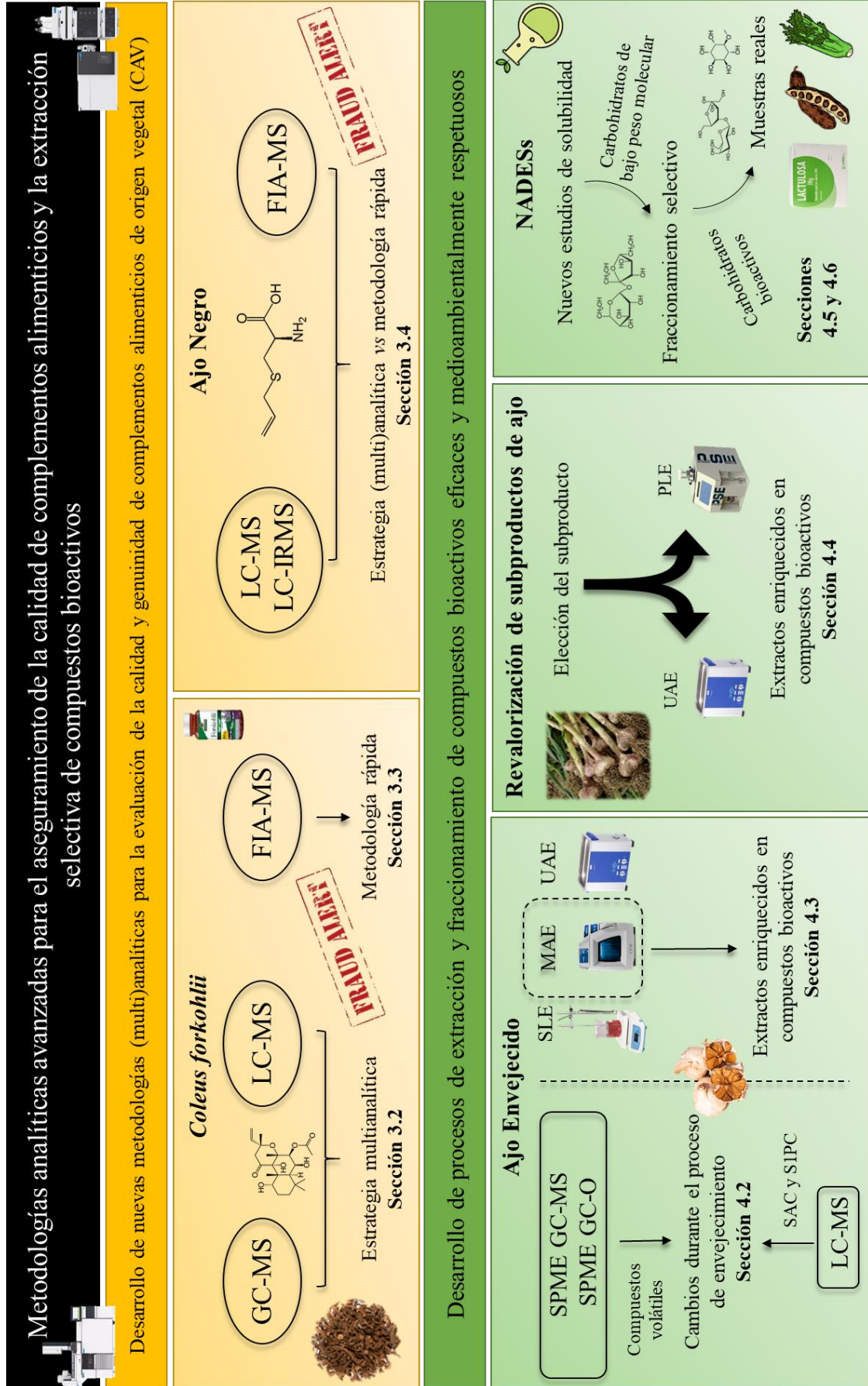


Figura 2.1. Plan de trabajo.



3. Desarrollo de nuevas metodologías (multi)analíticas para la evaluación de la calidad y genuinidad de complementos alimenticios de origen vegetal (CAV)

3. DESARROLLO DE NUEVAS METODOLOGÍAS (MULTI)ANALÍTICAS PARA LA EVALUACIÓN DE LA CALIDAD Y GENUINIDAD DE COMPLEMENTOS ALIMENTICIOS DE ORIGEN VEGETAL (CAV)

3.1. Prefacio

3.1.1. Calidad, seguridad y tipos de fraudes en CAV

Como se ha comentado previamente, los CAV son un objetivo preferente para el fraude debido a su elevado valor de venta, su creciente popularidad en el mercado y las limitaciones derivadas de su regulación, que se ven agravadas por el comercio por Internet, una de sus principales vías de venta (Lachenmeier *et al.*, 2013; Mena-García & Ruiz-Matute, 2018). A pesar de que los complementos que incluyen vitaminas/minerales continúan siendo la categoría más popular entre los usuarios, el consumo general de productos de origen vegetal ha experimentado un notable aumento en los últimos años. De hecho, en 2018, un 41% de los consumidores afirmaron haber tomado CAV en los últimos 12 meses, lo que representa un incremento del 13% respecto a 2013 (CRN Consumer Survey, 2018).

Las autoridades reguladoras de todo el mundo son cada vez más exigentes en cuanto a las determinaciones necesarias para garantizar la composición y autenticidad de las fuentes naturales que se van a utilizar, tanto en medicamentos a base de plantas, como en complementos alimenticios. Aunque los requisitos varían de un país a otro y algunas autoridades reguladoras son más activas que otras, la Organización Mundial de la Salud (OMS) ha recalcado en su nueva estrategia mundial (Estrategia de la OMS sobre Medicina Tradicional: 2014-2023) la necesidad de realizar pruebas de calidad y garantizar una normativa adecuada como prioridad clave para que los consumidores puedan incluir con seguridad estos productos en sus opciones de atención sanitaria (Organización Mundial de la Salud, 2013).

Cuando se habla de fraude, los CAV se encuentran en la cima de los complementos alimenticios objeto de prácticas fraudulentas (Begg & Wheatley, 2021). En algunos casos, dichos fraudes pueden llegar a comprometer la seguridad del consumidor, puesto que se ha descrito la presencia de ingredientes no declarados, como hierbas tóxicas (Charen & Harbord, 2020), derivados de plantas con posibles efectos adversos sobre la salud (*e.g.* hepatotoxicidad, nefrotoxicidad) y fármacos sintéticos (Hamidi, 2023). De hecho, ya hay numerosos estudios que revelan este tipo de prácticas en CAV destinados a la pérdida de peso, la disfunción eréctil o al aumento de energía (Esposito *et al.*, 2023; Hamidi, 2023). Así, Moreira *et al.* (2013) detectaron la presencia de laxantes y diuréticos (declarados o no declarados) en 8 de 26 productos basados en fórmulas vegetales y comercializados como productos naturales, mientras que Reeuwijk *et al.* (2014) identificaron distintos ingredientes farmacológicamente activos (sibutramina, fenolfaleína, sildenafil, entre otros) en 20 de 24 CAV destinados a la pérdida de peso.

Para poder identificar los CAV objeto de fraude y adoptar las medidas necesarias para garantizar su control y vigilancia, es importante conocer los distintos tipos de prácticas fraudulentas (Figura 3.1.) que se suelen cometer:

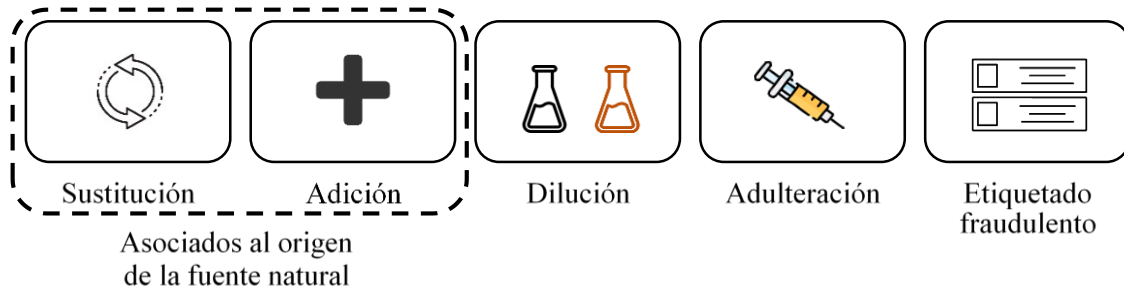


Figura 3.1. Tipos de fraude cometidos en CAV.

- **Asociados al origen de la fuente natural.** Podemos encontrar fraudes por:
 - **Sustitución.** Reemplazo de la planta indicada en el etiquetado por una más económica o de menor calidad. En este caso, la escasez o el elevado coste de la planta con propiedades bioactivas provoca su sustitución por otras fuentes más económicas que afectan a la calidad y autenticidad y pueden comprometer la seguridad del producto final (Mena-García & Ruiz-Matute, 2018).
 - **Adición.** Incorporación de una planta de menor valor y/o de menor calidad (*e.g.* que contenga menor concentración de principio activo) para aumentar la capacidad de suministro del producto y abaratar los costes de producción.
- **Dilución.** Aumento de la cantidad del producto disponible debido a la dilución de un extracto o producto con una mayor cantidad de disolvente o mediante la adición de antiagregantes/excipientes (*e.g.* carbohidratos de bajo y alto peso molecular como maltosa o maltodextrinas) (Gafner *et al.*, 2023). En ocasiones, se adicionan partes de la planta de menor valor distintas de la declarada (Osman *et al.*, 2019).
- **Adulteración.** Adición de una sustancia natural o sintética no autorizada o no declarada para modificar los atributos del producto, como el color o los efectos terapéuticos.
- **Etiquetado fraudulento.** Táctica consistente en etiquetar incorrectamente el producto para venderlo a un mayor coste. Esto incluye el anuncio de dosis más altas, afirmaciones no veraces sobre la naturaleza del producto o la escritura incorrecta de la formulación. Generalmente, los etiquetados incorrectos persiguen un fraude económico; no obstante, la clasificación errónea de especies, la confusión de nombres o la permisividad en el intercambio de fuentes naturales también pueden desencadenar errores en el etiquetado de CAV (Gafner *et al.*, 2023). Aquí se incluyen las declaraciones falsas de numerosas sustancias sintéticas como bioactivos de origen natural.

Si bien muchos de estos tipos de fraudes se han detectado en CAV procedentes de distintas fuentes naturales como la garcinia (Hong *et al.*, 2017; Mena-García *et al.*, 2022a, 2022b), el ginseng (Hong *et al.*, 2017), la alcachofa (Mena-García *et al.*, 2021) o la cúrcuma (Y. H. Wang *et al.*, 2013), entre otros, existen muy limitados antecedentes para las matrices contempladas en la presente Tesis Doctoral, el **ajo negro** y *C. forskohlii*, por lo que su estudio se considerará en esta sección.

3.1.2. Metodologías para la evaluación de la calidad de los CAV

La gran diversidad de potenciales fraudes en CAV, así como la complejidad de las matrices vegetales empleadas para su obtención, que generalmente incluyen no sólo compuestos bioactivos con distinta estructura y concentración, sino también compuestos interferentes con su bioactividad, hacen que su detección no sea una tarea fácil y, en muchos casos, suponga un gran reto analítico.

En la actualidad, se ha comprobado que el empleo de **estrategias multianalíticas** que permitan combinar los resultados obtenidos por distintas técnicas, como GC-MS y LC-MS, es un alternativa prometedora a los análisis dirigidos, para la caracterización de muestras complejas y detección de fraudes en complementos alimenticios (Martello *et al.*, 2007; Damiano *et al.*, 2014; Choi *et al.*, 2015; Mena-García *et al.*, 2021, 2022b, 2023). La información que aporta cada técnica puede ser única y complementaria a la otra, lo que proporciona un mayor conocimiento sobre la composición de la muestra objeto de estudio. En el caso de los CAV, la aplicación de este tipo de estrategias puede permitir, además de la clasificación de muestras de acuerdo a su calidad/genuinidad, postular uno o más marcadores de genuinidad que faciliten, en última instancia, la detección de fraudes. Es de destacar que existen muy pocos trabajos enfocados en la evaluación de la calidad de los complementos basados en *C. forskohlii* y ajo negro, y ninguno ha sido llevado a cabo mediante estrategias multianalíticas.

Respecto al *C. forskohlii*, la LC acoplada a diferentes detectores, como UV (Schaneberg & Khan, 2003), ELSD (Virgona *et al.*, 2010) o MS (Zhang *et al.*, 2009; Hebbani Nagarajappa *et al.*, 2016; He *et al.*, 2019; Singh & Suryanarayana, 2019) ha sido empleada por numerosos autores para el análisis de **forskolina** (su principal compuesto bioactivo) y/o sus derivados en muestras de raíz. En cuanto a la GC-MS, se ha aplicado para el análisis de extractos, tanto polares como apolares, de la raíz, tallos y hojas de esta planta, pudiéndose identificar distintos compuestos bioactivos como terpenoides, flavonoides, taninos y alcaloides (Rajkumar & Malathi, 2015; Ibrahim *et al.*, 2018). Sin embargo, escasos trabajos han contemplado el análisis de forskolina por esta técnica (Inamdar *et al.*, 1980). A pesar de todos estos estudios, los productos obtenidos a partir de dicha planta (incluyendo los CAV) no están suficientemente caracterizados. Schaneberg y Khan (2003) analizaron por LC-DAD el contenido en forskolina de 17 CAV basados en extracto

de *C. forskohlii* (estandarizados entre el 1-20% en forskolina, o sin estandarización). De acuerdo a este estudio, el 70% de los productos cumplían o superaban (en algún caso, hasta 5 veces más) el porcentaje de forskolina declarado en la etiqueta. Estas discrepancias son relevantes ya que, hasta el momento, la dosis segura de este compuesto establecida puede variar, por ejemplo, de la afección a tratar (asma: 10 mg de forskolina/día; obesidad/síndrome metabólico: 25 mg de forskolina/día) (www.drugs.com/npp/forskolin.html). Arai *et al.* (2020) y Fukiwake *et al.* (2022) desarrollaron métodos basados en LC-DAD y LC-MS, respectivamente, para el análisis de forskolina en CAV de *C. forskohlii*, llegando a conclusiones similares en ambos estudios. Así, se identificaron discrepancias en cuanto al contenido en forskolina, cuyo valor experimental varió significativamente respecto al declarado (hasta 300 veces), tanto por exceso como por defecto. Para aquellos complementos con contenidos muy bajos del compuesto bioactivo se cuestionó el potencial efecto beneficioso sobre la salud de su consumo, mientras que para aquellos productos cuya dosis diaria superaba los 1000 mg de extracto de *C. forskohlii* se advirtió de la posible incidencia de efectos adversos como la diarrea (Nishijima *et al.*, 2019). Sin embargo, todos estos estudios únicamente se centran en la evaluación de posibles discrepancias en cuanto al contenido en forskolina y no se contemplan otras prácticas fraudulentas a las que pueden verse sometidos estos CAV. Para este último objetivo, el empleo de estrategias multianalíticas, como las basadas en el empleo conjunto de la información obtenida por LC-MS y GC-MS, sería de gran interés pues permitiría, no sólo la caracterización exhaustiva de la composición de estas muestras, sino también la identificación, entre los compuestos detectados, de potenciales marcadores de calidad y/o genuinidad.

La otra matriz seleccionada en esta Tesis Doctoral ha sido el **ajo negro**, empleado como fuente natural de complementos ricos en su principal compuesto bioactivo, la **SAC**. Su composición ha sido ampliamente investigada a través de diferentes técnicas analíticas como GC-MS y, sobre todo, LC-MS. En el caso de GC-MS, el análisis de compuestos no volátiles como la SAC requiere de una etapa previa de derivatización que, generalmente, suele abordarse mediante la síntesis de sus *tert*-butildimetilsilil- (TBDMS) (Kanamori *et al.*, 2019) o metilcloroformiato- (MCF) (Wang *et al.*, 2007) derivados. En este sentido, la LC ofrece ventajas respecto a la GC ya que, generalmente, no precisa una etapa de derivatización, reduciéndose así el tiempo de preparación de la muestra y el consumo de reactivos necesarios para su análisis. Son varios los detectores que se han empleado hasta el momento para el análisis de los compuestos bioactivos del ajo negro, como los de UV/Vis, fluorescencia (previa derivatización) o MS. De entre ellos, los métodos por LC-UV son probablemente los más populares, si bien presentan desventajas, no sólo asociadas a la limitada absorción de la SAC (208 nm), sino también por las posibles interferencias causadas por la coelución con otros compuestos que absorban a la misma longitud de onda (Bae *et al.*, 2012; Lee *et al.*, 2014). Por estos motivos, para la cuantificación de la SAC es preferible la

elección de metodologías basadas en LC-MS, incluyendo el uso de analizadores para MSⁿ, que, aunque suponen un mayor coste de análisis, confieren mayor especificidad y sensibilidad (Lee *et al.*, 2014; Kim *et al.*, 2016; Woo *et al.*, 2022).

Aunque la composición de los extractos de ajo negro y los efectos fisiológicos beneficiosos derivados de su consumo están ampliamente descritos (Choi *et al.*, 2014; Ryu & Kang, 2017; Shang *et al.*, 2019; Tedeschi *et al.*, 2022), existe una demanda de conocimiento sobre la composición de los CAV de ajo negro, así como de información fiable y contrastada sobre el cumplimiento de los niveles de estandarización en SAC declarados en productos comerciales, y que actualmente suelen ser muy variables (entre el 0.1 y el 0.5% de SAC) o incluso no se especifica. En este sentido, y para dar respuesta a la demanda de las empresas del sector de los nutraceúticos y de los organismos encargados de su regulación, resulta esencial el desarrollo de metodologías analíticas que permitan, tanto el análisis mejorado de SAC, como la caracterización multicomponente de CAV de ajo negro, objetivos que contribuirían favorablemente al aseguramiento de la calidad y autenticidad de estos productos y que han sido escasamente abordados hasta el momento. Por otra parte, recientemente está cobrando interés la determinación cuantitativa diferencial en extractos y complementos de ajo negro de la SAC y la **S1PC** (diastereoisómero bioactivo de la SAC cuyo contenido no suele declararse y resulta muy variable según el proceso de envejecimiento del ajo empleado), con vistas a la propuesta de nuevos marcadores de calidad y genuinidad. Sin embargo, hasta el momento, los estudios que abordan esta temática son muy limitados (Woo *et al.*, 2022).

Por último, otra de las demandas en cuanto al análisis de CAV, tanto de ajo negro como de *C. forskohlii*, es la necesidad de disponer de **métodos rápidos** que permitan la determinación fiable de los compuestos bioactivos declarados (SAC y forskolina, respectivamente). Aunque los métodos espectrofotométricos son implementados como procesos de rutina por su accesibilidad y rapidez, son generalmente métodos poco selectivos y sensibles y la fiabilidad de los resultados es, en ocasiones, dudosa. Entre otras técnicas de DI-MS, la **FIA-MS** ha surgido como una aproximación prometedora para este propósito. Sin embargo, su aplicación requiere una optimización detallada de las condiciones de operación más adecuadas para cada tipo de muestra y analito de interés, así como la consideración de las posibles limitaciones de este tipo de aproximaciones asociadas al posible efecto matriz o a la existencia de compuestos interferentes.

En base a estos antecedentes, el objetivo general de esta sección se ha centrado en el desarrollo de nuevas metodologías analíticas, incluyendo estrategias multianalíticas y métodos rápidos, para la evaluación de la calidad/autenticidad de complementos alimenticios basados en *C. forskohlii* y en ajo negro.

Así, la **Sección 3.2.** incluye el artículo titulado “Development of a multianalytical strategy for detection of frauds in *Coleus forskohlii* supplements” de Jiménez-Amezcuca *et al.*, publicado en *Journal of Chromatography A* 1676 (2022) 463198. En este trabajo se ha desarrollado una estrategia multianalítica novedosa, basada en GC-MS y LC-MS, para evaluar la autenticidad y calidad de complementos alimenticios basados en extractos de raíz de *C. forskohlii*.

La **Sección 3.3.** se compone del artículo titulado “Flow Injection Analysis–Mass Spectrometry for the fast detection of frauds in *Coleus forskohlii* food supplements” de Jiménez-Amezcuca *et al.* enviado para su publicación en *Journal of Chromatography A*. En esta ocasión se ha desarrollado un método analítico rápido basado en FIA-MS para la cuantificación de forskolina en complementos alimenticios estandarizados en este compuesto, que se ha aplicado a la detección de posibles fraudes de composición.

La **Sección 3.4.** recoge el artículo “A comparative study of LC-MS and FIA-(ESI)MS for quantitation of S-allyl-L-cysteine in aged garlic supplements” de Jiménez-Amezcuca *et al.*, publicado en *Foods* 13 (2024), 2645, en el que se comparan las metodologías desarrolladas y validadas por LC-MS y FIA-(ESI)MS para su aplicación al análisis cuantitativo de SAC y para la evaluación de la calidad/genuinidad de complementos comerciales de ajo envejecido.

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3.2. Desarrollo de una estrategia multianalítica para la detección de fraudes en complementos de *C. forskohlii*

Development of a multianalytical strategy for detection of frauds in *Coleus forskohlii* supplements

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Development of a multianalytical strategy for detection of frauds in *Coleus forskohlii* supplements

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ABSTRACT

A new multianalytical methodology based on gas chromatography (GC) and liquid chromatography (LC) coupled to mass spectrometry (MS) has been proposed to evaluate frauds affecting the composition of *Coleus forskohlii* root supplements (FKS). After optimization and validation of chromatographic methods, 24 FKS were analyzed. Forskolin, their main bioactive component, was only found in 50% of the FKS evaluated (in the 0.032–17.1% range), with 27% of these supplements showing concentrations of this bioactive lower than those declared in their labels. Application of this methodology also proved to be successful for the detection of frauds regarding the replacement of *C. forskohlii* by other vegetable sources (green tea, soy leaves and a plant of the Berberidaceae family) in 17% of supplements analyzed. A study on stability of forskolin under accelerated conditions allowed to rule out its degradation as responsible for the lack of this bioactive or other natural constituents in 25% of FKS evaluated. It can be concluded that the multianalytical methodology here developed is an advantageous alternative to address the wide diversity of frauds affecting these supplements.

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1. Introduction

The consumption of food supplements, especially those containing plant ingredients, has increased in recent years, mainly due to their accessibility via online market and the fact that society perceives them as natural products to overcome nutritional deficiencies, maintain an adequate intake of nutrients, or to support specific physiological functions [1]. However, food supplements are not subjected to a strict legal framework like prescription drugs are and could be target of frauds. In this sense, the replacement of the natural source by other(s) of less economic value, the undeclared addition of pharmacological products, the discrepancy of the presence and/or quantity of the declared bioactives or the non-controlled addition of synthetic bioactive components have been detected in some supplements [2–4].

Coleus forskohlii (also known as *Coleus barbatus* or *Plectranthus barbatus*) is a perennial plant from the Lamiaceae family, native from India and distributed all over different countries (Egypt, Arabia, Ethiopia, Brazil, etc.) [5]. In Indian traditional medicine

(Ayurveda), it is applied for treating several disorders due to its antioxidant, antiaging, analgesic, antiinflammatory, bronchodilator, gastroprotective and anthelmintic properties [6–8] and its root extracts are currently commercialized as food supplements.

Roots of *C. forskohlii* are rich in bioactive metabolites such as flavonoids and mainly diterpenoids. Forskolin, a labdane-type diterpenoid, is the main bioactive component of roots (approximately 0.15–1.5 % w/w) and it has exhibited positive effects on asthma, hypertension, heart disease, diabetes and obesity, among others [9]. The major mechanism of action of forskolin is based on the adenylyl-cyclase (AC) enzyme activation. This diterpenoid activates various isoforms of AC, resulting in the increase in intracellular cyclic adenosine 3',5'-monophosphate (cAMP), which is a transmitter of intracellular signals that modulates and affects the activity of many cell enzymes. In some diseases, such as obesity, in which the cAMP levels are reduced, forskolin could play an important role, by increasing these levels and triggering the adipocyte lipolysis and the loss of fat in cells [10,11].

Several techniques have been proposed for extraction (e.g., hydrotronic extraction, microwave-assisted extraction, three-phase partitioning) and isolation (e.g., silica gel column chromatography, charcoal column chromatography, immunoaffinity) of forskolin

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from *C. forskohlii* roots to be used in food supplements. Different purities and recoveries were reported depending on the selected technique [12]. At present, the maximum safe dose of forskolin is not clearly established, and *C. forskohlii* supplements (FKS) are marketed with standardized levels of this bioactive within a wide range (from 1 to 20% forskolin) or even with no mention of its content.

The composition of *C. forskohlii* root extracts has been widely studied either by gas chromatography coupled to mass spectrometry (GC-MS) [6,8,13] or by high performance liquid chromatography coupled to MS (LC-MS) [14]. However, studies regarding the characterization of FKS aimed to evaluate their quality/authenticity are very limited and only focused on the evaluation of potential discrepancies between the declared and experimentally-determined content of forskolin [15,16]. The characterization of other constituents of FKS, which could be used as quality markers, or the detection of additional fraudulent practices have not been previously addressed in these samples. To that aim, in this manuscript, a new multi-analytical strategy based on GC-MS and LC-MS analyses of *C. forskohlii* root supplements has been evaluated for the first time.

2. Materials and methods

2.1. Reagents and samples

Analytical standards of fructose, galactose, glucose, sucrose, chiro-inositol, maltose, myo-inositol, pinitol, sedoheptulose, 1,9-dideoxyforskolin (1,9-DDF), forskolin and phenyl- β -D-glucoside (internal standard, I.S.) were obtained from Sigma Aldrich (St. Louis, MO, US). Derivatization reagents including hydroxylamine chloride, anhydrous pyridine, hexamethyldisilazane (HMDS) and trifluoroacetic acid (TFA) were also acquired from Sigma Aldrich.

FKS from different brands (FKS1-FKS24) were purchased online in different websites (Amazon, Aliexpress and iHerb). **Table S1** of Supplementary Material shows the different formulations (tablets and capsules) and composition of FKS under study, as declared in the labelling. All supplements were analyzed prior to their expiration date.

C. forskohlii roots were acquired from Plantaromed (Spain). Samples were ground to fine particles in an IKA A10 basic mill (IKA-Werke, Germany), sieved through a 500 μ m mesh and stored in closed containers at room temperature and protected from light until extraction. Soy leaves, green tea and a berberine supplement were acquired in local shops (Madrid, Spain).

2.2. Extraction procedure

To evaluate the authenticity of FKS, reference extracts from *C. forskohlii* roots were obtained and analyzed. Methanol was selected as a polar solvent to re-dissolve FKS or to extract polar bioactives, whereas heptane was used as solvent for non-polar compounds. Ultrasound assisted extraction of *C. forskohlii* roots (100 mg) and FKS (60 mg) with these solvents (1 mL) was carried out using an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) at 25 °C for 5 min. In both cases, extracts were immediately centrifuged at 4401 g for 10 min and diluted (1:2–1:160, v/v) as required. Unless otherwise specified, all experiments were performed in triplicate.

2.3. Stability of forskolin

Aliquots (0.2 mL) of the methanolic extracts of *C. forskohlii* root and FKS1 were evaporated to dryness in a miVac Duo Concentrator from GeneVac Ltd. (Ipswich, UK) and stored at 50 °C for 12

weeks. Samples were taken at 0, 1, 2, 3, 4, 8 and 12 weeks of storage and then analyzed by GC-MS as indicated below. Experiments were carried out in duplicate.

2.4. Derivatization of methanolic extracts

Trimethylsilyl oximation was selected as derivatization procedure prior to GC-MS analysis of methanolic extracts. For this purpose, 0.1 mL of phenyl- β -D-glucoside (1 mg mL⁻¹) used as I.S. were added to an eppendorf with 0.15 mL of root / FKS extracts. Samples were dried in a rotatory evaporator at 40 °C and further subjected to derivatization.

The two-step derivatization (oximation+silylation) procedure was carried out as previously described by Ruiz-Aceituno et al. [17]. For oxime formation, dried samples were treated with 350 μ L of 2.5% hydroxylamine chloride in pyridine at 75 °C for 30 min. Silylation was then carried out using 350 μ L of HMDS and 35 μ L of TFA by heating at 45 °C for 30 min. All derivatized samples were centrifuged at 4401 g for 10 min and the supernatants further injected into the GC-MS system.

2.5. GC-MS analysis

Derivatized and non-derivatized samples (from methanol and heptane extracts, respectively) were analyzed in a 6890 N gas chromatograph coupled to a 5973 single quadrupole (Q) mass spectrometer; both from Agilent Technologies (Palo Alto, CA, USA). Chromatographic analyses were carried out on a Zebron ZB-1 capillary column (30 m $\times$ 0.25 mm i.d.; 0.25 μ m film thickness; Phenomenex, CA, USA), using helium at $\sim$ 1 mL min⁻¹ as carrier gas.

Different operating conditions (oven programs and injection temperatures) were assayed. Several initial/ending oven temperatures and ramps were evaluated to provide the best separation of target compounds for both derivatized (methanolic extracts) and non-derivatized (heptane extracts) samples. Injections (1 μ L) were carried out in split mode (1:15) at a temperature of 300 °C. The MS detector was operated in electron impact (EI) mode at 70 eV, scanning the 50–650 *m/z* range. The transfer line was set at 280 °C and the ionization source at 230 °C. For data acquisition and analysis, MSD ChemStation software (Agilent Technologies) was used.

Identifications were carried out by comparison of experimental spectra with data from mass spectral libraries (Wiley, NIST), and were further confirmed by using linear retention indices (*I*^L), calculated from retention data of suitable *n*-alkanes (from C₁₀ to C₄₀) analyzed under identical chromatographic conditions.

The internal standard method was used for quantitation. Standard solutions over the expected concentration range in the samples under study were used to calculate the response factor (RF) relative to the internal standard (phenyl- β -D-glucoside for derivatized compounds; octadecane and docosane for underivatized compounds). All analyses were carried out in triplicate.

2.6. LC-MS analysis

Analysis of forskolin and its derivatives in *C. forskohlii* food supplements was performed on two LC-MS instruments.

The first one, used for the qualitative and quantitative analysis of all FKS, was a 1260 Infinity II Prime LC System, including an autosampler, a quaternary pump, a thermostated column compartment and a diode array detector, coupled to a 6125 single quadrupole mass detector (Agilent Technologies, Santa Clara, CA, US) provided with an electrospray ionization (ESI) source. Optimization of electrospray source parameters was performed by infusion of a forskolin standard solution under both positive and negative polarities. Different values for fragmentor voltage (50–150 V) and nebulizing gas (N₂, 99.5% purity) pressure (207–345 kPa) were

also considered. Nitrogen drying gas flow rate and temperature were set at 12 L min⁻¹ and 300 °C, while capillary voltage was set at 3000 V. Analyses were carried out in SCAN mode (50–2000 *m/z* range). Data acquisition and processing were performed using OpenLAB CDS Software (v.2.19.20, Agilent Technologies).

The second equipment was used to identify unknown structures of FKS1, FKS12, FKS18 and FKS19. It was an Agilent 1100 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a Maxis II quadrupole-time of flight (QToF) mass spectrometer (LC-QToF MS, Bruker, Massachusetts, US) provided with an ESI interface working in positive-ion mode. The electrospray voltage was set at 3.5 kV and the drying gas temperature at 200 °C. Nitrogen (99.5% purity) was used as nebulizer (2.0 bar) and drying gas (6 L min⁻¹), while nitrogen of higher purity (99.999%) was used as the collision gas. Optimization of ion transmission into the analyzer was performed by infusing the default test mixture. Full scan mass spectra were recorded in the 50–3000 *m/z* range with external calibration. MS/MS experiments were performed using broad banding collision, with 30 eV as collision energy. Data acquisition and processing were performed using Data Analysis v.4.4.200 software (Bruker).

Chromatographic separation was carried out by using a Luna C₁₈ column (100 mm × 2.1 mm, 3 μm; Phenomenex, Cheshire, UK) operating at 0.22 mL min⁻¹ and thermostated at 30 °C. Different binary gradients consisting of water (eluent A) and acetonitrile (eluent B), both with 0.1% formic acid, were assayed for the optimization of LC-MS method. Injection volume was set as 5 μL.

Compound identification in FKS was based on chromatographic retention and MS data and it was confirmed, when possible, by co-injection of the corresponding commercial standards. For further confirmation of these identifications and for characterization of unknowns, MS/MS data were used. Comparison of experimental MS/MS patterns and data provided by Metlin database (Metabolite and Chemical Entity Database, The Scripps Research Institute, San Diego, CA) was also carried out. When analytical standards were not available, identifications were considered as tentative.

Quantitative analysis of forskolin and its derivatives in FKS by LC-MS was performed in triplicate using an external standard calibration curve of forskolin. Prior to quantitation, matrix effect was evaluated by quantifying forskolin in FKS1 extract diluted in methanol at different ratios (1:1–1:500, v/v).

2.7. Validation of analytical methods

Different parameters were considered for validation of the optimized GC-MS and LC-MS methods. Reproducibility was measured in terms of intra- and inter-day precision by derivatizing (when required) and analysing diluted FKS1 sample (1:40, v/v) under optimal conditions within the same day (*n* = 5) or in 5 consecutive days, respectively. Linearity of the responses was evaluated in the 0.0005–1 mg mL⁻¹ range. Goodness of fitting of calibration curves was evaluated using their correlation coefficients. Recovery was calculated in triplicate after spiking this sample with a known amount of forskolin standard. Limits of detection (LOD) and quantitation (LOQ) were calculated for forskolin as three and ten times the signal to noise ratio (*S/N*), respectively.

2.8. Nuclear magnetic resonance analysis

Nuclear Magnetic Resonance (NMR) analysis of food supplements (FKS4, FKS5, FKS13–16), was accomplished using an Agilent SYSTEM 500 NMR spectrometer (¹H 500 MHz, ¹³C 125 MHz), equipped with a 5-mm HCN cold probe. NMR spectra were recorded at 25 °C, using D₂O as solvent. Chemical shifts of ¹H (δ_H) and ¹³C (δ_C) in parts per million were determined relative

to internal standards of sodium [2, 2, 3, 3-²H₄]-3-(trimethylsilyl)propanoate in D₂O (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) in D₂O, respectively. One-dimensional (1D) NMR experiments (¹H and ¹³C{¹H}) were performed using standard pulse sequences.

2.9. Statistical analysis

Statistica 7.0 program (StatSoft, Inc. Tulsa, OK, USA) was used for statistical analysis of data. The compliance between experimental and declared values of forskolin and concentrations determined by GC-MS and LC-MS were assessed by *t*-test for independent samples (*p* < 0.01). Significance (*p* < 0.05) of differences for results obtained in other studies (e.g., forskolin stability, matrix effect, etc) was determined by the analysis of variance (ANOVA, Tukey test).

3. Results and discussion

Three analytical methods (by GC-MS for non-polar and polar extracts, and by LC-MS for polar extracts) were developed in this work with a double objective: (i) to select the most appropriate method to determine the forskolin content and (ii) to obtain a multi-component authenticity profile of these supplements to identify potential frauds affecting *C. forskohlii* supplements.

3.1. Optimization of analytical methods

3.1.1. Sample preparation

Sample preparation was evaluated prior to the optimization of chromatographic methods. Solvents of different polarities were assayed for the extraction/redissolution, not only of forskolin, but also of other *C. forskohlii* root and FKS components. Heptane was used as a non-polar solvent and as a more environmentally safer alternative to benzene which had been applied in previous studies for forskolin extraction [18]. Regarding polar solvents, water is the most universal and greenest solvent; however, solubility of forskolin in this solvent is low [12]. Both methanol and ethanol were shown to be appropriate solvents to extract this bioactive (0.69 and 0.60 mg g⁻¹, respectively) together with other constituents from *C. forskohlii* roots. However, as methanol provided an improved performance, it was selected for further studies. These results were in good agreement with those published by Singh and Suryanarayana [7], who reported methanol as the optimal solvent for extraction of forskolin.

Special attention was paid to the derivatization process required for the GC-MS analysis of non-volatile and thermolabile polar compounds such as carbohydrates present in methanolic extracts. In this sense, and although forskolin and other terpenoids do not form oximes and give rise to a single TMS derivative, trimethylsilyl oximation was considered as it provides only two peaks per reducing sugar (corresponding to the *syn* (*E*) and *anti* (*Z*) forms) and, therefore, a better resolution among the different compounds is achieved.

3.1.2. Operating conditions

GC-MS conditions were optimized with the aim of achieving the best resolution of FKS components. For the analysis of heptane extracts, the best separation among the different compounds was obtained when the oven temperature was programmed from 50 to 270 °C at 6 °C min⁻¹, then to 310 °C at 10 °C min⁻¹ for 15 min.

Regarding GC-MS analyses of methanolic extracts, initial temperature was set at 150 °C, and different ramps to increase the temperature up to 310 °C were assayed. Low resolution values were obtained between 1,9-DDF and phenyl-β-D-glucoside, used as internal standard (*R*_s = 0.84), and between forskolin and an unknown compound with *m/z* ion 545 (*R*_s = 0.07) using 6 °C min⁻¹

Table 1
Validation of GC-MS and LC-MS methods for the analysis of forskolin in methanolic extracts.

	GC-MS	LC-MS
Calibration curve	$y = 0.3157x - 0.1470$ $R^2 = 0.993$	$y = 2 \cdot 10^9 x + 1 \cdot 10^6$ $R^2 = 0.994$
Linear range (mg mL ⁻¹)	0.05–1.00	0.0005–0.01
Intra-day precision (RSD %)	3.06	1.17
Inter-day precision (RSD %)	3.50	1.81
Recovery (%)	101 (6) ^a	96 (6)
LOD/LOQ (ng mL ⁻¹)	186.03 (21.77) / 620.11 (72.58)	23.86 (1.67) / 79.54 (5.57)

^a Standard deviation in parenthesis.

to 270 °C and then 10 °C min⁻¹ to 310 °C (**Fig. S1** of Supplementary material). On the contrary, good resolution values ($R_s = 1.59$ and $R_s = 1.31$, respectively) were achieved with 8 °C min⁻¹ to 225 °C followed by 10 °C min⁻¹ up to 310 °C. This temperature was held for 10 min.

As regards LC-Q MS analyses, different binary gradients of water (eluent A): acetonitrile (eluent B), both with 0.1% formic acid, were assayed. The best conditions for the separation of forskolin and its derivatives were as follows: from 20% to 70% B in 25 min, then to 85% B in 3 min and finally to 90% B in 2 min. This percentage was kept for 5 min and initial conditions were resumed in 1 min. Regarding MS, forskolin could be detected in both positive and negative polarity modes as previously observed by Cuthbertson et al. [19]. However, peak areas were fifteen times higher in positive mode, which was selected for further experiments. High intensity quasimolecular ions were found at 433 and 375 *m/z*, corresponding to $[M+Na]^+$ and $[M+H-2H_2O]^+$, respectively. Different fragmentor voltages and nebulizing gas pressure values were tested. While no significant differences were observed in forskolin response between 276 and 345 KPa, lower values were obtained at 204 KPa. Regarding fragmentor voltages, the highest response was found at 100 V followed by 150 V. Considering these results, 100 V and 276 KPa were selected as optimal conditions. Although forskolin was detected by the three methods, considering its low solubility in non-polar solvents [7], heptane extracts were not considered for the quantitative analysis of this bioactive. As regards the analysis of methanolic extracts, a linear response was obtained for forskolin by both GC-MS and LC-Q MS methods (**Table 1**). Good values for intra-day (3.06 and 1.17%) and inter-day (3.50 and 1.81%) precision and recovery (101% and 96%) were found for these methods, respectively. Moreover, no significant differences at the 99% confidence level were found in the forskolin content of FKS determined by both methods (e.g., 17.2 (SD 0.5) by GC-MS and 16.5 (SD 0.4) mg g⁻¹ by LC-MS for FKS1). However, considering the higher sensitivity of LC-Q MS (LOD : 23.86 ng mL⁻¹, LOQ : 79.54 ng mL⁻¹) vs. GC-MS (LOD : 186.03 ng mL⁻¹, LOQ : 620.11 ng mL⁻¹), this technique was selected for the quantitative determination of forskolin and its derivatives. These values were lower than those found in previously developed methods to determine FKS quality (HPLC-ELSD 0.95 µg mL⁻¹ [16] and RPLC-PDA 1.5 µg mL⁻¹ [15]). Non-significant differences in forskolin content were found at the different concentration levels evaluated and, therefore, matrix effect was discarded.

3.2. Forskolin content of FKS

Firstly, the quality of FKS was evaluated in terms of forskolin content, by agreement or mismatch with the declared values. All supplements were analyzed by the optimized LC-Q MS method, which resulted in the detection of forskolin at levels greater than the LOQ in only 50% of them (Group 1, **Fig. S2** of Supplementary Material). Traces of this compound were also detected in FKS2 and FKS7 samples (Group 2), while the presence of this bioactive was

not detected in the remaining 42% of the supplements under study (Groups 3 and 4).

The percentages of forskolin in FKS determined by LC-Q MS are shown in **Table 2**. Values ranged from 0.032% in FKS9 to 17.1% in FKS24. Similar percentages (ranging 1–20%) were found by Schaneberg and Khan [15] in the analysis by LC with a photodiode array detector of some commercial products. Although most of the forskolin concentrations experimentally determined were in good agreement with these contents, the *t*-test statistical analysis revealed that concentrations of 27% FKS under study were significantly lower than those stated in labelling. It is worth noting the case of FKS1, which showed a forskolin percentage four times lower than the content declared. Forskolin concentration was not specified in supplement FKS9, but the extremely low value experimentally determined for this bioactive questions its effectiveness for reported health benefits.

3.3. Study of authentication profiles

Once the quality of FKS attending to their forskolin concentration was evaluated, a comprehensive characterization of FKS1–FKS24 supplements was done based on the information provided by the three chromatographic methods previously optimized (**Section 3.1.**) to determine the authenticity of their natural source. In this sense, the different chromatographic profiles of FKS were compared with those of a laboratory-made reference *C. forskohlii* root extract.

3.3.1. Non-polar extracts

Similar GC-MS profiles of heptane extracts were observed for supplements containing forskolin (Group 1, **Fig. S2**). **Fig. 1** shows the GC-MS profile of the FKS1 heptane extract as an example. Apart from forskolin, its derivative 1,9-DDF was identified from GC-MS data (I^T and mass spectrum) of the corresponding commercial standard. Other diterpenoids such as 9-deoxyforskolin (9-DE), 6-acetyl-7-deacetylforskolin (isoforskolin), *trans*-ferruginol, abieta-8,11,13-triene and sugiol were tentatively identified in most of these supplements by comparison of their I^T and mass spectra with those found in the NIST database. These compounds were also found in the reference root extract (see GC-MS profile of **Fig. S3A**) and their presence had been previously described in *C. forskohlii* roots by other authors [20,21]. Borneol and bornyl acetate, present in the root extract, were also found in some samples. The presence of all these compounds could confirm the genuineness of these supplements. Other terpenoids present in roots, previously described in the literature [21], were not detected, probably due to processing conditions of this matrix employed for the manufacture of food supplements. Sterols, decyl acetate and ethyl 4-ethoxybenzoate were also detected; however, their presence was not detected in the root reference extract.

On the contrary, supplements FKS7 and FKS9 only showed the presence of abieta-8,11,13-triene (peak 5), *trans*-ferruginol (peak 6), 1,9-DDF (peak 7) and sterols (peak10). Alkanes from triacosane to

Table 2

Contents (mg g⁻¹) of forskolin in FSK determined by LC-MS and values declared in their corresponding labels. Standard deviation in parenthesis ($n = 3$).

Supplement	Experimental	Declared
FKS1	16.5 (0.4)*	76
FKS2	tr**	26
FKS3	44.2 (2.4)	41
FKS6	71.8 (0.05)*	87
FK7	tr	-
FKS8	102.8 (2.4)	85
FKS9	0.32 (0.004)	-
FKS10	20.1 (1.1)	22
FKS11	19.9 (2.2)*	32
FKS20	110 (1)	98
FKS21	62.8 (0.1)	57
FKS22	132.5 (3.5)	113
FKS23	69.4 (1.3)	67
FKS24	171 (5)	200

* Significant differences ($p < 0.01$) between the experimental and declared contents of forskolin.

**tr: traces ($< LOQ$).

***-: non declared.

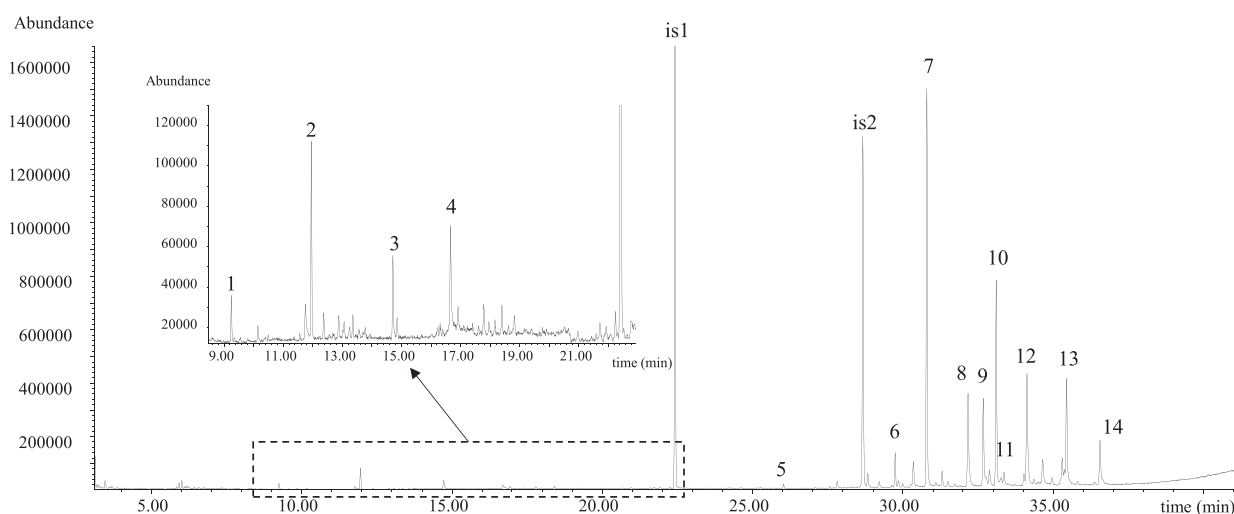


Fig. 1. GC-MS profile of FKS1 heptane extract. 1. borneol, 2. bornyl acetate, 3. decyl acetate, 4. ethyl *p*-ethoxybenzoate, 5. abieta-8,11,13-triene, 6. *trans*-ferruginol, 7. 1,9-DDF, 8. 1,9-DDF related compound, 9. 9-DE, 10. sterols, 11. sugiol, 12. sterols, 13. forskolin, 14. isoforskolin; is1 and is2: internal standards.

heptatriacontane were detected in FKS4, while characteristic compounds of *C. forskohlii* were not found. No peaks were detected in the heptane extracts of the rest of the food supplements analyzed (FKS2 and groups 3 and 4), with the exception of caffeine in FKS17 and FKS18.

3.3.2. Polar extracts

GC-MS profiles. In general, the GC-MS profiles of methanolic extracts of FKS containing forskolin (Group 1 and 2, **Fig. S2**) were characterized by two types of compounds: carbohydrates and/or diterpenoids (e.g., FSK1 in **Fig. 2A**), also found in *C. forskohlii* root extract (**Fig. S3B**). Supplements of group 1, except for FKS1 and FKS9, only contained diterpenoids, while in samples of group 2 only the characteristic carbohydrates were detected.

In group 1, FKS carbohydrates would have been probably eliminated during their manufacture as a consequence of the purification followed for their enrichment in diterpenoids. In addition to forskolin and 1,9-DDF, other characteristic compounds of *C. forskohlii* such as forskolin D, 9-DE and isoforskolin were also tentatively identified in these FKS (mass spectra can be found in **Fig. S4**). Moreover, different unknown compounds with retention times (t_R between 14.5 and 18 min, labelled with asterisks in **Fig. 2A**) and mass spectra (**Fig. S4**) compatible with diterpenoids were

found in these supplements. Some of these peaks were also detected in the methanolic reference root extract at very low levels (**Fig. S3B**). Other compounds, such as sucrose and piperine (0.12 mg g⁻¹) were detected in FKS10, probably coming from black pepper, whose addition is declared on its label. *N*-acetyl-tyrosine (t_R 10.41 min; 70.0 mg g⁻¹), declared as ingredient in FSK10, was also found in FKS21. Moreover, maltose and maltotriose were also found in FKS1 and FKS6, probably arising from the declared addition of maltodextrins and cornstarch, respectively, as ingredients in supplement formulation.

Different monosaccharides including hexoses, such as fructose, glucose, galactose and mannose, and heptoses, such as sedoheptulose, were detected in supplements FKS1, FKS9 and those of group 2. These carbohydrates were also found in the root reference extract (see **Fig. S3B**), confirming their genuine origin. As shown in **Table 3**, high contents of sedoheptulose (14.1–50.6 mg g⁻¹), as compared to the total concentration of monosaccharides (1.9–5.8 mg g⁻¹), were present in these supplements. High levels of this carbohydrate, which is known to play an important role in the cyclic regeneration of *D*-ribulose for carbon dioxide fixation in plant photosynthesis [22], have been previously described in leaves of different varieties of *Coleus* [23] and also in roots of different

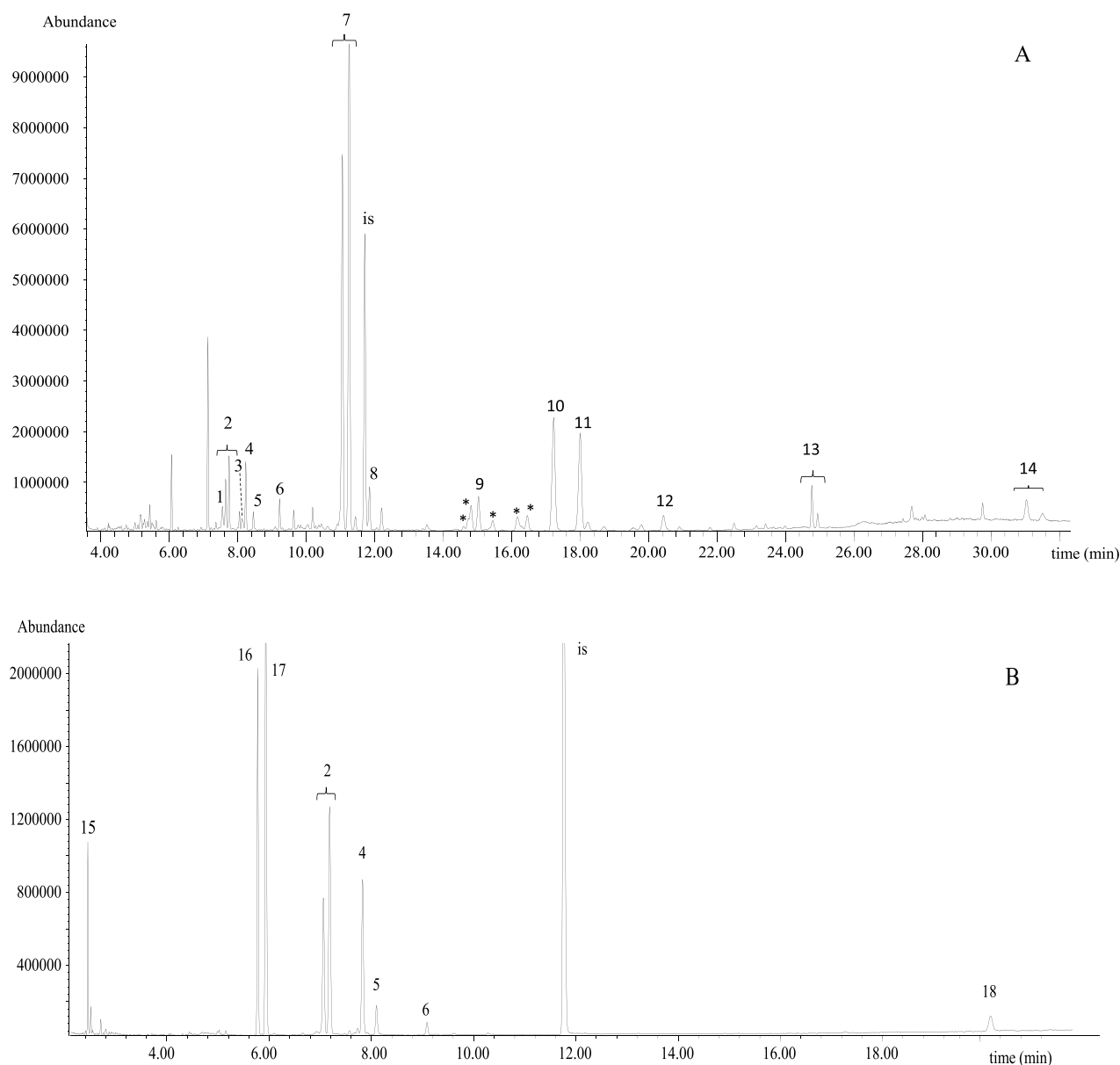


Fig. 2. GC-MS profiles of FKS1 (A) and FKS12 (B) methanolic extracts. 1. mannitol, 2. fructose, 3. galactose *E* + mannose *E*, 4. glucose *E*, 5. glucose *Z* + galactose *Z* + mannose *Z*, 6. *myo*-inositol, 7. sedoheptulose, 8. 1,9-DDF, 9. forskolin D, 10. 9-DE, 11. forskolin, 12. isoforskolin, 13. maltose, 14. maltotriose, 15. malic acid, 16. quinic acid, 17. *vibo*-quercitol, 18. sucrose, * unknowns.

vegetable samples such as rhodiola (*Sedum roseum*, [22]) and carrot (*Daucus carota* L., [24]).

Regarding food supplements that did not contain forskolin (groups 3 and 4, Fig. S2), different GC-MS profiles were obtained. All supplements from group 3 showed the presence of different compounds non characteristic of *C. forskohlii* roots. In this sense, in FKS12, apart from carbohydrates (glucose fructose, *myo*-inositol and sucrose), *vibo*-quercitol, quinic acid and malic acid were identified (Fig. 2B). *vibo*-Quercitol (also known as viburnitol) is a deoxyinositol previously described in different oak species [25], while malic and quinic acids are carboxylic acids ubiquitous in plant kingdom [26,27], but not previously detected in *C. forskohlii*. Similarly, caffeine, gallic acid, epicatechin and catechin, characteristic compounds of green tea [28], were detected in FKS17 and FKS18 (Fig. S5A), while pinitol, ononitol and *chiro*-inositol, typical of soy bean leaves [29], were identified in FKS19 (Fig. S5B). The presence of these compounds in supplements of group 3, not detected

in *C. forskohlii* roots extract, together with the no similarity with the typical carbohydrate and terpenoid profile experimentally determined for this reference extract, pointed at the presumed substitution of the natural source declared in these supplements.

On the other hand, group 4 (Fig. S2) consisted of supplements that only contained small amounts of carbohydrates (glucose, fructose, maltose and/or maltotriose) or those in which no compound was detected by this chromatographic technique.

LC-MS profiles. A similar profile was observed for all the food supplements containing forskolin (groups 1 and 2). Fig. 3A shows the LC-Q MS profile of FKS1 as an example. Apart from forskolin and 1,9-DDF, different diterpenoids such as deacetylforskolin (forskolin D), deoxyforskolin isomers (i.e., 9-DE, 1-acetoxycoleosol), forskolin isomers (isoforskolin and 1-acetyl-7-deacetylforskolin), acetyl-forskolin isomers (forskolin B and 6-acetylforskolin) and carnolic acid were tentatively identified by LC-QToF MS (Table S2).

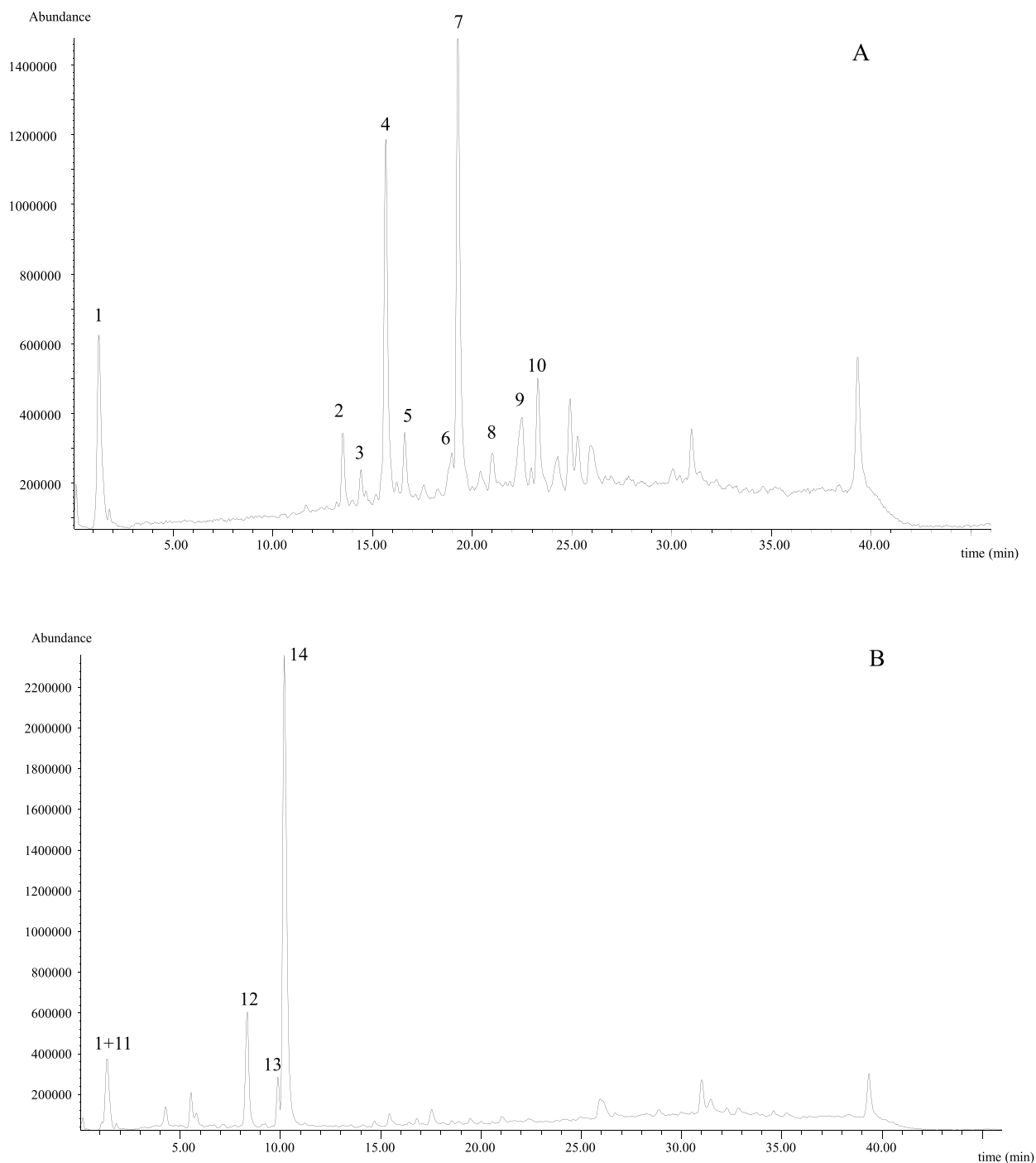


Fig. 3. LC-MS profiles of FKS1 (A) and FKS12 (B) methanolic extracts. 1. mono-, di- and trisaccharides, 2. forskolin D, 3. 9-DE, 4. isoforskolin, 5. 1-acetoxycoleosol, 6. 1-acetyl-7-deacetylforskolin + forskolin B, 7. forskolin, 8. deoxyforskolin isomer, 9. 1,9-DDF+6-acetylforskolin, 10. carnolic acid, 11. quercitol, 12. jatrorrhizine, 13. palmatine, 14. berberine.

Most of these assignments were in good agreement with those previously described by Zhang et al. [30] in *C. forskohlii* plants. These compounds were also identified in the *C. forskohlii* reference extract (Fig. S3C), confirming thus the genuine origin of these supplements. Concentrations of these compounds in FKS under study can be found in Table 4. Isoforskolin was the most abundant diterpenoid, apart from forskolin, with the highest values in FKS24 and FKS1 (17.7 and 14.4 mg g⁻¹, respectively), followed by 1-acetoxycoleosol (7.0 and 4.5 mg g⁻¹ for FKS24 and FKS22, respectively).

LC-Q MS analyses of FKS5, FKS13-FKS16 only revealed the presence of one peak eluting at the beginning of the chromatogram

(1.25 min) corresponding to the coelution of carbohydrates from DP1 ([M+Na]⁺ = 203) to DP5 ([M+Na]⁺ = 851). The presence of these compounds in supplements could mainly arise from excipients such as maltodextrins, corn starch or cellulose used for their manufacturing, although their presence was only declared in FKS5 and FKS13.

As previously found by GC-MS, other compounds atypical of *C. forskohlii* reference extract but characteristic of other plant sources were also detected in FKS12, FKS17, FKS18 and FKS19. MS/MS patterns obtained by LC-QToF MS and the use of Metlin database allowed the tentative identification of jatrorrhizine, palmatine and berberine in FKS12 (Fig. 3B). These compounds are characteristic

Table 3
Concentrations (mg g^{-1}) of carbohydrates detected by GC-MS in methanolic extracts of FKS. Standard deviation in parenthesis ($n = 3$).

Identification	t_R (min)	I^T	FKS1	FKS2	FKS3	FKS4	FKS5	FKS6	FKS7	FKS9	FKS10	FKS12 [§]	FKS13	FKS16	FKS17	FKS18	FKS19 [#]
Mannitol	7.54	2003	0.33 (0.05)	0.14 (0.01)	-	-	-	-	0.13 (0.01)	0.47 (0.08)	-	-	-	-	0.042 (0.08)	0.033 (0.03)	0.053 (0.06)
Fructose	7.65/ 7.74	2013 / 2022	1.7 (0.2)	1.15 (0.05)	-	-	-	-	0.96 (0.04)	4.1 (0.5)	-	3.8 (0.5)	0.82 (0.09)	-	2.1 (0.2)	1.49 (0.08)	6.9 (0.3)
Galactose	8.12	2058	0.44 (0.02)	-	-	-	-	-	tr [*]	0.09 (0.01)	-	0.33 (0.01)	0.35 (0.01)	-	-	-	0.83 (0.04)
+Mannose																	
Glucose	8.24 / 8.45	2069 / 2089	1.21 (0.09)	0.76 (0.04)	-	0.57 (0.03)	0.80 (0.03)	0.57 (0.02)	1.14 (0.04)	1.6 (0.2)	-	2.0 (0.2)	4.8 (0.4)	0.44 (0.02)	6.5 (0.3)	4.4 (0.3)	8.0 (0.3)
myo-	9.22	2158	0.27 (0.03)	0.13 (0.01)	-	-	-	-	0.085 (0.004)	0.55 (0.09)	-	0.08 (0.01)	-	-	0.76 (0.05)	0.64 (0.05)	0.34 (0.02)
Inositol	11.04/	2310 /	25.0 (1.9)	14.1 (2.5)	-	-	-	-	19.0 (0.7)	50.6 (6.3)	-	-	-	-	-	-	-
Sedoheptulose	11.24	2324	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	20.96	2741	-	-	1.10 (0.08)	-	-	-	-	-	1.63 (0.07)	1.12 (0.06)	-	-	14.0 (0.9)	12.2 (1.1)	97.7 (4.5)
Maltose	24.77/	2974/	1.1 (0.1)	tr	-	-	3.2 (0.5)	1.4 (0.1)	1.71 (0.09)	0.99 (0.09)	-	-	12.0 (0.5)	-	14.3 (0.7)	12.2 (1.0)	3.09 (0.15)
Maltotriose	24.94	2992	0.79 (0.06)	-	-	-	4.8 (0.3)	0.89 (0.04)	2.1 (0.8)	0.63 (0.07)	-	-	7.7 (0.2)	-	3.6 (0.5)	3.5 (0.6)	10.1 (0.6)

* tr: traces.

§ vibo-quercitol: 3.3 (0.4) mg g^{-1} .# chiro-inositol: tr, pinitol: 4.9 (0.3) mg g^{-1} and ononitol: 0.54 (0.03) mg g^{-1} .**Table 4**
Concentrations (mg g^{-1}) of forskolin derivatives detected by LC-MS in methanolic extracts of FKS. Standard deviation in parenthesis ($n = 3$).

Identification	t_R (min)	FKS1	FKS2	FKS3	FKS6	FKS7	FKS8	FKS9	FKS10	FKS11	FKS20	FKS21	FKS22	FKS23	FKS24
Forskolin D	13.53	1.51 (0.03)	tr [*]	-	0.62 (0.02)	tr	tr	tr	-	tr	0.2 (0.1)	1.5 (0.1)	0.23 (0.01)	tr	1.39 (0.09)
9-DE	14.43	0.74 (0.01)	- ^{**}	0.6 (0.1)	0.06 (0.02)	-	tr	tr	-	-	tr	0.3 (0.1)	-	tr	1.0 (0.3)
Isoforskolin	15.67	14.40 (0.01)	tr	3.7 (0.2)	4.18 (0.03)	tr	2.61 (0.17)	0.22 (0.01)	0.35 (0.03)	0.340 (0.002)	11.2 (0.2)	7.3 (0.1)	2.99 (0.01)	2.6 (0.2)	17.7 (0.1)
1-	16.65	1.37 (0.09)	-	2.0 (0.1)	tr	-	3.02 (0.14)	tr	0.82 (0.06)	tr	3.73 (0.07)	2.6 (0.1)	4.54 (0.02)	2.17 (0.09)	7.0 (0.5)
Acetoxycycloleol	21.02	0.81 (0.01)	tr	0.25 (0.01)	0.090 (0.001)	tr	tr	0.11 (0.01)	tr	tr	0.57 (0.03)	0.54 (0.04)	2.8 (0.1)	tr	-
Deoxyforskolin															
Isomer															
19-	22.50	2.07 (0.03)	tr	1.8 (0.1)	1.99 (0.01)	tr	2.26 (0.03)	0.02 (0.0005)	0.52 (0.01)	0.570 (0.001)	3.05 (0.09)	2.51 (0.03)	2.6 (0.2)	1.6 (0.11)	7.57 (0.09)
DDF+6-acetylforskolin															

* tr: traces ($< LOQ$).** -: non detected ($< LOD$).

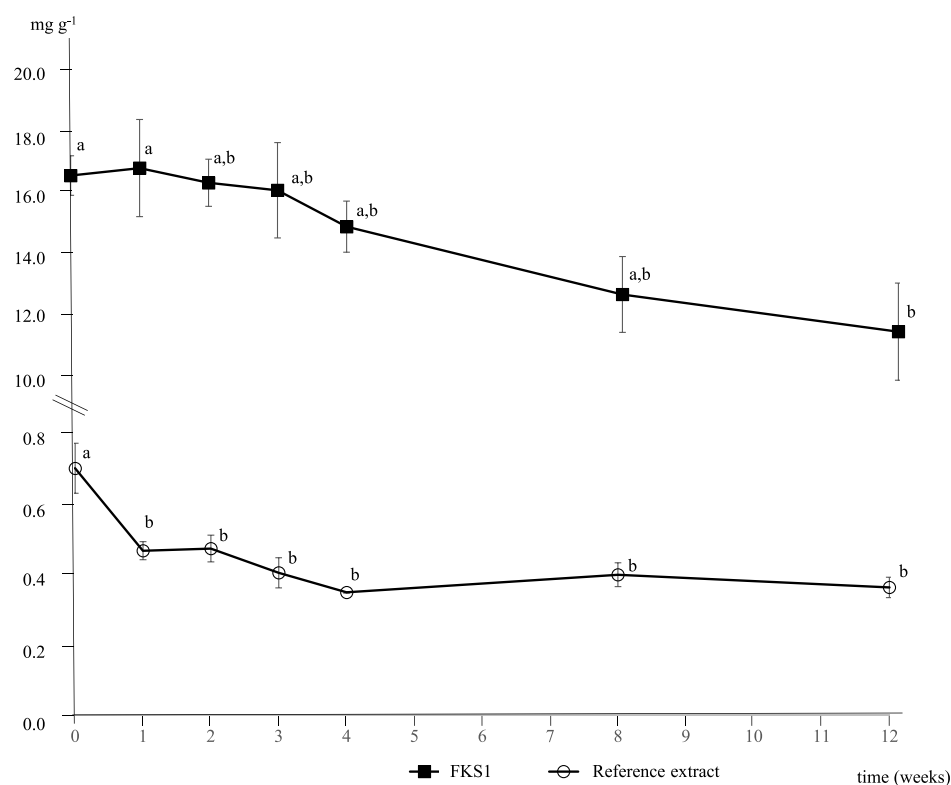


Fig. 4. Concentration of forskolin in FKS1 and reference root extract during storage at 50 °C for 12 weeks. Different letters indicate significant ($p < 0.05$) differences in forskolin concentration between storage times.

of several Chinese herbal medicines [31] such as *Coptis chinensis* Franch and *Tinospora cordifolia* [32]. Similarly, theanine, theobromine, epicatechin, catechin, theaflavin and epigallocatechin gallate were identified by LC-QToF MS in FKS17 and FKS18, characteristic of green tea [28]; and genistin, daidzin, glycitin, daidzein and glycitein in FKS19, characteristic of soy bean [33] (Fig. S6A, B). Assignations of all these compounds, and their presumable implication in *C. forskohlii* frauds were also confirmed by the analysis of laboratory-made genuine extracts of these plant sources (green tea and soy bean leaves, considering also the identifications carried out by GC-MS) and a commercial berberine supplement of certified origin.

Finally, and in agreement with GC-MS data, the LC-Q MS analysis of 25% of supplements analysed (Group 4) evidenced the very limited number (or even the absence) of compounds detectable by this chromatographic technique. In order to characterize their composition, these samples were subjected to ¹³C-NMR and ¹H-NMR analysis, revealing the presence of glucooligosaccharides of high molecular weight (Fig. S7), which are only described as excipients in only some of them (e.g., FKS5 and FKS13).

3.4. Stability of forskolin with storage

Considering the low percentage of supplements containing forskolin, a stability study of this compound was carried out to evaluate its potential degradation after manufacturing and during the commercialization period before the expiration date. For this purpose, the *C. forskohlii* reference extract and FKS1 were stored under accelerated conditions (50 °C, 12 weeks). Fig. 4 shows the changes in forskolin concentration for these two samples along the studied period. Although a slight decreasing trend was observed in forskolin content of FKS1, only significant differences were found at the end of the storage period (12 weeks) compared to control

and 1 week samples. On the contrary, statistically significant differences were found in forskolin concentration for 1 week onwards compared to control. Despite forskolin degradation might occur at some extent with inappropriate storage of FKS, the variability here observed in terms of absolute concentration would not support the lack of this bioactive observed for several FKS.

4. Conclusions

The proposed multi-analytical strategy here optimized in terms of chromatographic and MS operating parameters, based on the combined use of GC-MS and LC-MS data, has been shown to be advantageous over single analytical techniques to evidence not only frauds regarding the content of forskolin, but also related to the undeclared addition of other plant sources in *C. forskohlii* supplements.

The application of this novel methodology has revealed that a high percentage of the *C. forskohlii* supplements analyzed do not comply with the declarations made on their labelling. These results highlight the need for a more reliable control of these products by the competent authorities and the usefulness of the methodology proposed in this study to detect these frauds.

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.

CRediT authorship contribution statement

Ignacio Jiménez Amezcua: Formal analysis, Methodology, Validation, Investigation, Writing – original draft. **Sergio Rivas Blas:**

Methodology, Validation, Writing – original draft. **Marina Díez Municio:** Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing. **Ana Cristina Soria:** Data curation, Investigation, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **Ana Isabel Ruiz Matute:** Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. **María Luz Sanz:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Supplementary materials

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3.3. Análisis en flujo-Espectrometría de Masas para la detección rápida de fraudes en complementos alimenticios de *Coleus forskohlii*

Flow Injection Analysis–Mass Spectrometry for the fast detection of frauds in *Coleus forskohlii* food supplements

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Flow Injection Analysis–Mass Spectrometry for the fast detection of frauds in *Coleus forskohlii* food supplements

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Abstract

Coleus forskohlii supplements (FKSs) are widely consumed for their obesity-preventing effects, mainly related to forskolin, its main bioactive compound. However, these supplements are susceptible to adulteration and various types of frauds have been described. Therefore, the development of rapid and sensitive methodologies is of great interest in order to determine the quality of these products.

In this work a new method by Flow Injection Analysis–Mass Spectrometry (FIA-MS) has been developed and validated for the detection of potential frauds in FKSs. A Box-Behnken experimental design was used for the optimization of MS parameters under both positive and negative polarity modes of the electrospray ionization source. The best results were obtained for positive polarity mode, 300 °C of nebulization gas temperature, a fragmentor voltage of 149 V and a pressure of 30 psi, using 1% of 1 mM sodium acetate in the carrier. When 22 FKS were analysed by the optimised FIA-MS method, 45% of the samples were found to be free of forskolin. There was also a high percentage (69 %) of mismatches between the quantified forskolin content and that declared on the FSK labels. In general, results were comparable with those obtained by LC-MS. Thus, it has been proved that FIA-MS is a fast, cost-effective and simple tool to evaluate the authenticity of FKSs.

Keywords: *Coleus forskohlii*; food supplements; quality; mass spectrometry; frauds

1. Introduction

Coleus forskohlii is a perennial plant of the Lamiaceae family, commonly used in traditional Indian medicine for its anti-obesity, antioxidant and analgesic properties, among others. The root of *C. forskohlii* is rich in bioactive compounds such as labdane-type diterpenes, highlighting forskolin [1,2]. This molecule, together with its isomer isoforskolin, present in minor amounts in *C. forskohlii* roots, are associated, among others, with activities that favor weight reduction since it promotes adipocyte lipolysis [3–6]. As a result, *C. forskohlii* extracts are currently widely marketed as herbal dietary supplements for obesity control.

Although herbal food supplements are perceived by the population as a safe and natural alternative to chemical products to achieve beneficial effects on human health, in recent years, they have been target of several fraudulent practices [7,8]. In particular, in *Coleus forskohlii* food supplements (FKSs), frauds related to the substitution of the natural source by another of lower economic value (e.g. soy leaves, green tea and a plant of the Berberidaceae family), the mismatch between the detected and declared amount of forskolin and even the absence of this bioactive component, have been detected [9].

Liquid chromatography (LC) with photodiode array detector (DAD) has been used for the evaluation of potential discrepancies between the declared and experimentally-determined content of forskolin in FKSs [10]. More recently, Jimenez-Amezcu et al. [9] have developed a new multianalytical strategy based on the use of gas chromatography (GC) and LC coupled to mass spectrometry (MS) to detect several FKS frauds. Although these methodologies have been found to be successful in detecting frauds, the development of faster analytical methods, with similar accuracy and sensitivity to ensure the quality of these food supplements, is of great interest when the analysis of a high number of samples is required. In this sense, flow injection analysis (FIA)-MS is a high-throughput technique in which the sample is directly injected into a carrier and transferred into the atmospheric pressure interface of the mass spectrometer [11]. This technique has shown high potential for the rapid detection of frauds related to the content of bioactives in several products such as tea [12], black cohosh [13], red wine, paprika or olive oil [14,15], considering its robustness and sensitivity [16]; however, to the best of our knowledge, it has not been applied to detect frauds in FKSs.

Then, in this work a new methodology using FIA-MS has been developed and validated for the rapid quality evaluation of *C. forskohlii* root supplements. Forskolin and isoforskolin contents determined by this method have been compared with those obtained by LC-MS and those declared in labels.

2. Materials and methods

2.1. Reagents and samples

An analytical standard of forskolin ($\geq 98\%$) was obtained from Sigma Aldrich (St. Louis, MO, US). *C. forskohlii* supplements from different brands (FKS1-FKS22) were purchased online in different websites (Amazon, Aliexpress and iHerb) and herbal shops.

Methanol and acetonitrile of HPLC-MS grade were acquired from Scharlau (Barcelona, Spain) while the ultrapure water was distilled and purified using the Milli-Q Advantage A10 system from Millipore (Bedford, MA, USA).

2.2. Sample preparation

Methanol (1 mL) was added to each FKS (0.1 g) for the extraction of bioactives using an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) operating at 25 °C for 5 minutes, 37 Hz of frequency and 30% of power. Extracts were immediately centrifuged at 4401 *g* for 10 min, the supernatant was collected, diluted (1:50-1:500, v/v) as required and frozen until analysis.

2.3. FIA-MS

FIA-MS was performed on a 1260 Infinity II LC system, including an autosampler, a quaternary pump and a diode array detector, coupled to a 6125 single quadrupole mass detector (Agilent Technologies, Santa Clara, CA, US) provided with an electrospray ionization (ESI) source. Optimization of ion transmission into the analyzer was performed by both infusing the calibration mixture and injecting a forskolin standard solution (0.01 mg mL⁻¹). FIA-MS experiments were carried out by using 80:20 (v/v) of acetonitrile and water, both acidified with 0.1% formic acid (v/v), at a flow rate of 0.22 mL min⁻¹ as carrier. In addition, the ionization of forskolin was evaluated by adding different percentages (0.1, 1 and 5%) of 1 mM sodium acetate to this carrier. The temperature of elution was set at 30 °C and 5 µL of sample were injected. Nitrogen drying gas was set at a flow rate of 12 L min⁻¹ and capillary voltage at 3000 V. Other MS parameters [nebulizing gas temperature (*T*, °C), fragmentor voltage (*F*, V) and nebulizing gas (N₂ 99.5% purity) pressure (*P*, psi)] were optimized under both positive and negative polarity modes of ESI using a Box-Behnken experimental design. Experimental ranges were selected as follows: *T* = 150-300 °C, *F* = 40-300 V, and *P* = 20-60 psi. Each experiment was carried out in triplicate. Response surface methodology (RSM) was used for calculation of the coefficients to maximize forskolin response (peak areas) in positive and negative (*R*_{f(+)} and *R*_{f(-)}) ionization modes, and to estimate the statistical significance of the regression coefficients. The quadratic model proposed was:

$$R_f = \beta_0 + \beta_1 T + \beta_2 F + \beta_3 P + \beta_{1,1} T^2 + \beta_{2,2} F^2 + \beta_{3,3} P^2 + \beta_{1,2} TF + \beta_{1,3} TP + \beta_{2,3} FP + \varepsilon$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for i^{th} factors, $\beta_{i,j}$ the coefficients for interaction between the factors i and j and ε is the error. R_f was estimated by multiple linear regression (MLR) using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA).

FIA-MS analyses were simultaneously performed in SCAN mode (50–2000 m/z range) and in selected ion monitoring (SIM) mode, for the $[M-H]^-$ 409 and $[M+Na]^+$ 433 m/z ions of forskolin adducts. Data were acquired and processed using HP Chemstation Rev. A.07.01 software (Agilent Technologies, Palo Alto, CA, USA).

Quantitative analysis of forskolin was performed in triplicate using an external standard calibration curve (0.0005–0.01 mg mL⁻¹). Reproducibility was measured in terms of intra- and inter-day precision by analyzing FKS9 sample under optimal conditions within the same day ($n = 5$) or in 5 consecutive days, respectively. Limits of detection (LOD) and quantitation (LOQ) were calculated for forskolin as three and ten times the signal to noise ratio (S/N), respectively. Recovery was also calculated in triplicate after spiking this sample with three different known amounts of forskolin standard (0.001–0.002 mg). Matrix effect was also evaluated by quantifying forskolin in FKS9 extract diluted in water at different ratios (1:50–1:1000, v/v) and also by comparing the slope of forskolin calibration curve with that obtained after spiking the FSK9 with different known concentrations of this compound.

2.4. Liquid chromatography-mass spectrometry (LC-MS)

FKSs were analysed by LC-MS using the previously mentioned 1260 Infinity II Prime LC System coupled to the 6125 mass spectrometry detector (Agilent Technologies). Chromatographic separation was carried out by using a Luna C18 column (100 mm × 2.1 mm, 3 μ m, Phenomenex, Cheshire, UK) operating at 0.22 mL min⁻¹ and thermostated at 30 °C. A binary gradient of water (eluent A): acetonitrile (eluent B) with 0.1% formic acid and 1% of 1 mM sodium acetate in both eluents was used as follows: from 20% to 70% B in 25 min, then to 85% B in 3 min and finally to 90% B in 2 min, keeping this percentage for 5 min and back to initial conditions in 1 min. MS operating conditions were those previously optimised in section 2.3. MS analyses were carried out in SCAN (50–2000 m/z range) and SIM ($[M+Na]^+$ 433 m/z ion of forskolin adduct) modes. Data were processed using HP Chemstation Rev. A.07.01 software (Agilent Technologies). Quantitative analysis of forskolin was performed in triplicate using an external standard calibration curve (0.0005–0.01 mg mL⁻¹).

2.5. Statistical analysis

Statistica 7.0 software (StatSoft, Inc. Tulsa, OK, USA) was used for statistical analysis. Significance ($P < 0.05$) of differences in the experimental and declared values of forskolin, as well

as the concentrations determined by FIA-MS and LC-MS, was determined by the analysis of variance (ANOVA, Tukey Test).

3. Results and discussion

3.1. Optimization of the FIA-MS method

Optimization of mass spectrometric conditions were required before the analysis of FKs by FIA-MS to maximize the ionization of forskolin. For this purpose, ionization in both positive and negative polarity and SCAN mode was firstly evaluated. While a single adduct was detected in negative mode ($[M-H]^-$ 409), several ions corresponding to the sodium and potassium adducts ($[M+Na]^+$ 433 and $[M+K]^+$ 449) and to the consecutive loss of different molecules of water ($[M+H-H_2O]^+$ 393, $[M+H-2H_2O]^+$ 375, $[M+H-3H_2O]^+$ 357) and acetyl groups ($[M+H-2H_2O-CH_2CO]^+$ 333, $[M+H-3H_2O-CH_2CO]^+$ 315, $[M+H-4H_2O-CH_2CO]^+$ 297) were obtained in positive mode (**Figure 1A**). These fragments were in good agreement with the fragmentation pathway of forskolin proposed by He et al. (2019) [17].

Mobile phase additives have often been used to improve the formation efficiency for specific adducts [18]. The chemical interactions generated in the ESI source cause changes in the geometry of ion-ligands, resulting in more intense and/or unique fragment ions, although other ionization processes or unpredictable fragmentation, such as cluster ion formation, may occur, requiring the optimization of the conditions [19]. Therefore, in an attempt to favor the formation of the most abundant ion in positive polarity mode (m/z 433, corresponding to $[M+Na]^+$), different percentages (0.1-5 %) of 1 mM sodium acetate were considered in the composition of the carrier. While 0.1 % sodium acetate was not enough to prevent the formation of other adducts, only negligible abundances of ions 297 and 375 were observed with 1 and 5% of 1 mM sodium acetate. Considering that high salt concentrations in MS can produce ion suppression and/or a decrease in ion desolvation and/or transmission, resulting in a lower response [20], 1% of 1 mM sodium acetate was selected for further studies (**Figure 1B**). As expected, no changes in the forskolin mass spectrum at negative polarity mode were detected using sodium acetate in the carrier.

Operating (ESI)MS conditions (T , F and P) were optimized following a Box-Behnken experimental design to maximize SIM(+) ($[M+Na]^+$ 433 m/z) and SIM(-) ($[M-H]^-$ 409 m/z) responses ($R_{f(+)}$ and $R_{f(-)}$, respectively). Taking into account the flow rate, the boiling point of the carrier and the thermal stability of forskolin, a range from 150 °C to 300 °C was selected to optimize T . Regarding fragmentor voltages, it has been described that, in general, high voltages can help the transmission of ions and thus, the intensity of the response, but fragmentation can also occur [21]. Therefore, fragmentor values in this study were varied from 40 V to 300 V to find the optimal voltage providing the highest abundance of forskolin $[M+Na]^+$ ion and the lowest

of corresponding fragment ions. Finally, nebulizing gas pressure values from 20 to 60 psi, commonly selected when optimizing this parameter [22], were also evaluated.

Table 1 shows the results obtained in the 15 experiments performed. For the same ionization conditions, the intensity of forskolin response obtained in positive mode was noticeably higher than that in negative mode, as reported in a previous work [9]. In addition, for this ionization mode, great variations in the response were observed when changing ESI conditions (**Figure S1-S3**). In general, high temperatures of nebulizing gas increased forskolin response (**Figure S1**), and thermal decomposition of the molecule was not detected, except for the experiment carried out at 300 °C, 300 V and 40 psi, which provided a lower intensity signal. Regarding fragmentor voltages, in general, lower intensity values of forskolin were detected at high voltages; this effect was even more drastic in negative mode (**Figure S2**). Intensity also slightly decreased when nebulizing pressure increased (**Figure S3**).

The coefficients of the quadratic model proposed were calculated using a response surface methodology and the statistical significance of the regression coefficients was estimated. As for $R_{f(+)}$ model, F^2 and T were the most statistically significant factors at the 95% confidence level ($P < 0.05$) and the quadratic regression equation was:

$$R_{f(+)} = -4.58477 \cdot 10^6 + 54032.6 F + 21392.4 T - 115.424 F^2 - 65.7267 FT$$

This quadratic model explained 75% of the variability of the $R_{f(+)}$ response. The three-dimensional response surface plot of this equation is shown in **Figure S4A**. In general, high temperatures and intermediate fragmentor voltages enhanced forskolin response. In agreement with this, optimal conditions to maximize forskolin response were 300 °C of nebulization gas temperature, a fragmentor voltage of 149 V and a pressure of 30 psi.

Regarding $R_{f(-)}$, the fragmentor voltage (F) was the most significant parameter ($P < 0.05$) to maximize this response, followed by F^2 and P . In negative ionization mode, medium fragmentor voltages provided the highest forskolin response, and, contrary to positive ionization mode, a negligible effect of temperature was observed. The regression equation of this model was $R_{f(-)} = 179149.0 + 1185.58 F - 1803.97 P - 5.12644 F^2$ which explained 70% of the variability of $R_{f(-)}$ (three-dimensional response surface plot is shown in **Figure S4B**). According to this model, optimal conditions were $T = 272$ °C, $F = 116$ V and $P = 20$ psi.

Once selected the optimal conditions for each polarity, external calibration curves were done. A linear response over the concentration range evaluated (0.0005 to 0.01 mg mL⁻¹) was obtained for forskolin both in positive and negative ionization modes, with good correlation coefficients ($R^2 = 0.998$ and $R^2 = 0.990$, respectively). Good values for intra- and inter-day precision were also obtained for both ionization modes (**Table 2**). Regarding sensitivity, LOD and LOQ values

of forskolin were lower in positive mode (0.09 and 0.29 ng mL⁻¹) than in negative mode (2.20 and 7.34 ng mL⁻¹) and noticeably lower than those previously reported by a LC-(ESI) MS method (LOD: 23.86 ng mL⁻¹, LOQ: 79.54 ng mL⁻¹) [9].

Considering that the main drawback of FIA-(ESI)MS analyses of complex mixtures is the potential matrix effect that could induce ion suppression, leading to underestimation of the actual forskolin content, an evaluation of this effect in the analysis of *C. forskohlii* supplements was carried out. As experimentally determined, matrix effect was ruled out for dilutions above 1:500 v/v in positive ionization mode and above 1:300 v/v in negative ionization mode. The potential ion suppression effect was also evaluated by quantifying the diluted FKS9 sample supplemented with different known amounts of forskolin standard. Good recovery values (97%) of forskolin were obtained for both ESI⁺ and ESI⁻ data (**Table 2**). Moreover, the relationship between the slopes of spiked and non-spiked calibration curves (108%), confirmed that no matrix effect occurred.

Therefore, on the basis of the higher response and higher sensitivity provided for quantitation of forskolin, positive ionization mode was selected for further experiments.

3.2. Analysis of *C. forskohlii* supplements

C. forskohlii supplements were analysed by the optimized FIA-(ESI⁺)MS method which allowed the joint quantitation of both forskolin and isoforskolin isomers. Despite its high sensitivity, these compounds were not detected in 9 out of 22 supplements analysed (45% of samples; FKS5, FKS15-22). **Figure 2** shows, as an example, the MS signal corresponding to SCAN and SIM ([M+Na]⁺) acquisition modes for three of the supplements analysed (FKS5, FKS9 and FKS15). While *m/z* ion at 433 was clearly found in FKS9, no signal could be detected above the LOD in FKS5 and FKS15 (**Figure 2A**). This fact questions both their authenticity of these supplements and their intended beneficial effect on health. In the case of FKS5, only *m/z* ions at 365 and 527, compatible with di- and trisaccharides ([M+Na]⁺ adducts), commonly used as additives in supplements, were found (**Figure 2B**). Similar results were observed for FKS15 and FKS17-20. However, sample FKS15, showed a more complex profile (**Figure 2B**). Apart from ions compatible with carbohydrates, other *m/z* ions at 195, 139 and 295 were found. LC-MS analysis of this sample allowed the tentative identification of these compounds as caffeine ([M+H]⁺ 195) and catechins ([M+H]⁺ 139 and 295) which could indicate the presence of tea in this sample. FKS21 also showed the presence of these compounds indicating its potential fraudulent origin.

Table 3 shows the concentrations of forskolin and isoforskolin found in all other FKS. Concentration values ranged from 0.0002 to 38.6 mg per capsule, highlighting the extremely low

values detected for supplements FKS1, FKS2, FKS10, FKS12 and FKS14, that also call into question the beneficial effect on health associated with their consumption.

These experimental forskolin and isoforskolin contents determined by FIA-MS were compared with those provided by LC-MS analysis under here optimized MS conditions. As it can be shown (**Table 3**), most of the results obtained by these two techniques were in good agreement, confirming the usefulness of the FIA-(ESI⁺)MS method here developed for the fast joint determination of these two bioactives in FKSs.

In comparison with forskolin values declared in FKS labels, significant differences were observed in 69% of supplements containing this bioactive compound. In general, the experimental contents were lower than those declared, despite the fact that forskolin and isoforskolin were determined together by the FIA-(ESI⁺)MS method. In fact, some of these differences were extremely high (e.g. FKS1, FKS10, FKS12 and FKS14). In a previous work [7], the degradation of forskolin during product storage was ruled out as the cause of the large differences found between the detected and declared forskolin concentration of some FSKs [7], so such differences could be due to the intentional addition of lower amounts of extract in these supplements to increase economic profit.

4. Conclusions

The new optimized and validated FIA-(ESI⁺) MS method has allowed the joint quantitation of both forskolin and isoforskolin, reducing significantly the analysis time. This method has proven to be an excellent tool to evaluate the authenticity of different *C. forskohlii* supplements. It offers a fast, cost-effective, simple and reliable alternative for producers and regulatory authorities to quickly identify different types of fraud in these products. This approach could be used to detect fraudulent practices in other herbal supplements, such as mislabelling, either due to lower concentrations of declared bioactives or even their absence.

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Table 1. Box-Behnken experimental design for optimization of the (ESI)MS conditions in the analysis of forskolin. $R_{f(+)}$ and $R_{f(-)}$ responses (peak areas) obtained in positive and negative ionization modes, respectively.

T (°C)	F (V)	P (psi)	$R_{f(+)}$ ([M+Na] ⁺ 433)	$R_{f(-)}$ ([M-H] ⁻ 409)
150	40	40	1310730	62052
150	170	20	2158982	121540
150	170	60	1991752	99727
150	300	40	1937637	914
225	40	20	1697528	279441
225	40	60	1486669	147271
225	170	40	3719681	148965
225	170	40	3722693	166841
225	170	40	3780937	184718
225	300	20	1509377	1414
225	300	60	1012359	1259
300	40	40	2945505	96079
300	170	20	5991308	267698
300	170	60	3584550	133201
300	300	40	1009072	1549

345 **Table 2.** Validation of FIA-(ESI)MS methods for the analysis of forskolin in methanolic extracts.

	ESI ⁺	ESI ⁻
Calibration curve	$y = 1.6E9x + 4.9E5$ $R^2 = 0.998$	$y = 2.8E6x + 7.3E3$ $R^2 = 0.99$
Linearity range (mg mL ⁻¹)	0.0005-0.01	0.0005-0.01
Recovery (%)	97 (5)*	97 (10)
LOD/LOQ (ng mL ⁻¹)	0.09 (0.006) / 0.29 (0.02)	2.20 (0.18) / 7.34 (0.62)
Precision intraday (% RSD)	0.5	4.9
Precision interday (% RSD)	5.5	8.8

346 * Standard deviation in parenthesis.

Table 3. Contents (mg per capsule) of forskolin in *C. forskohlii* supplements determined by FIA-(ESI⁺)MS and LC-(ESI⁺)MS.

Code	FIA-(ESI ⁺)MS	LC-(ESI ⁺) MS			Declared
		isoforskolin	forskolin	total	
FKS1	0.007 (0.002) ^{*a}	0.007 (0.002)	0.005 (0.001)	0.012 (0.002) ^b	80 ^c
FKS2	0.073 (0.006) ^a	0.035 (0.0004)	0.031 (0.0004)	0.066 (0.002) ^a	-
FKS3	14.0 (0.5) ^a	tr ^{**}	13.5 (0.8)	13.5 (0.8) ^a	12.5 ^b
FKS4	11.6 (0.5) ^a	tr	9.9 (0.6)	9.9 (0.6) ^a	10 ^a
FKS6	9.1 (0.2) ^a	1.1 (0.04)	9.6 (0.2)	10.7 (0.2) ^a	10 ^a
FKS7	24.5 (0.09) ^a	1.2 (0.4)	23.3 (1.8)	24.4 (2.2) ^a	25 ^a
FKS8	38.6 (0.8) ^a	tr	39.1	39.1 (0.4) ^a	50 ^b
FKS9	3.88 (0.08) ^a	2.4 (0.04)	1.53 (0.02)	3.9 (0.1) ^a	25 ^b
FKS10	0.00034 (0.00003) ^a	-	-	-	10 ^b
FKS11	1.1 (0.1) ^a	0.26 (0.1)	0.25 (0.04)	0.51 ^b	10 ^c
FKS12	0.0002 (0.00001) ^a	-	tr	-	1.5 ^b
FKS13	11.4 (0.3) ^a	tr	11.7 (0.9)	11.7 (0.9) ^a	12.5 ^a
FKS14	0.002 (0.00005) ^a	-	-	-	2 ^b

* Standard deviation in parenthesis (n=3);

^{a-c} Different letters within the same row mean significant ($P < 0.05$) differences;

^{**} tr: traces.

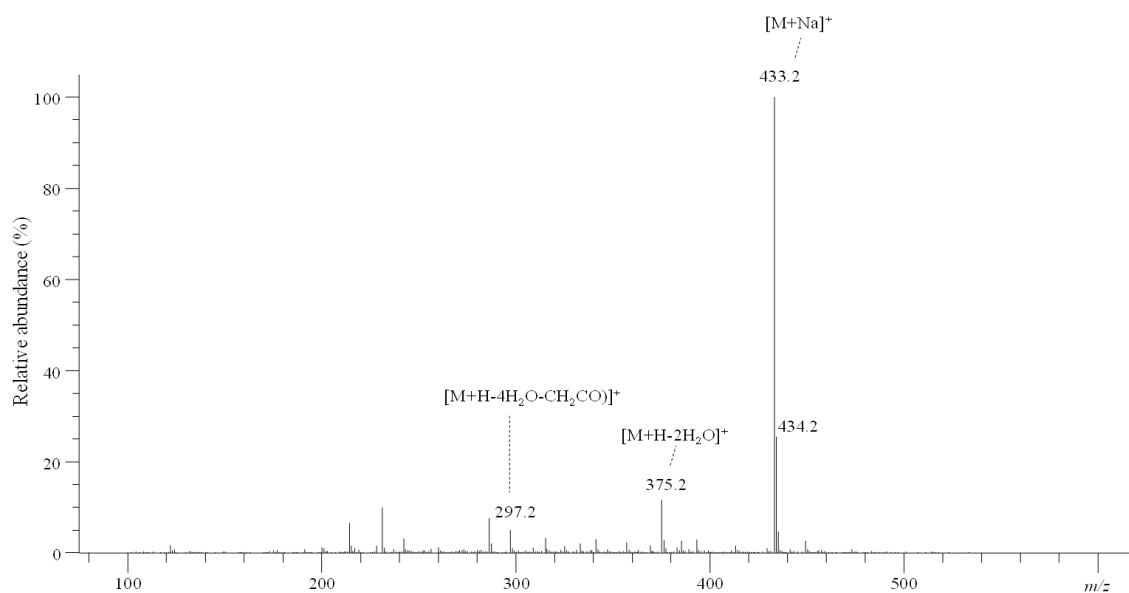
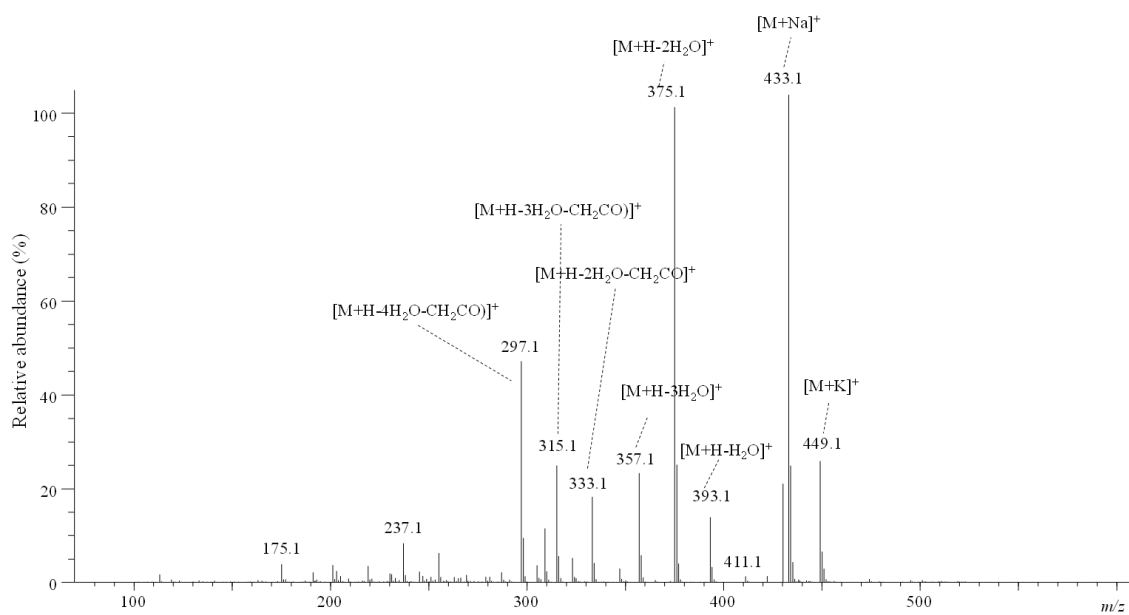


Figure 1. Mass spectra of forskolin without (A) and with (B) 1 mM sodium acetate (1%) as component of the carrier.

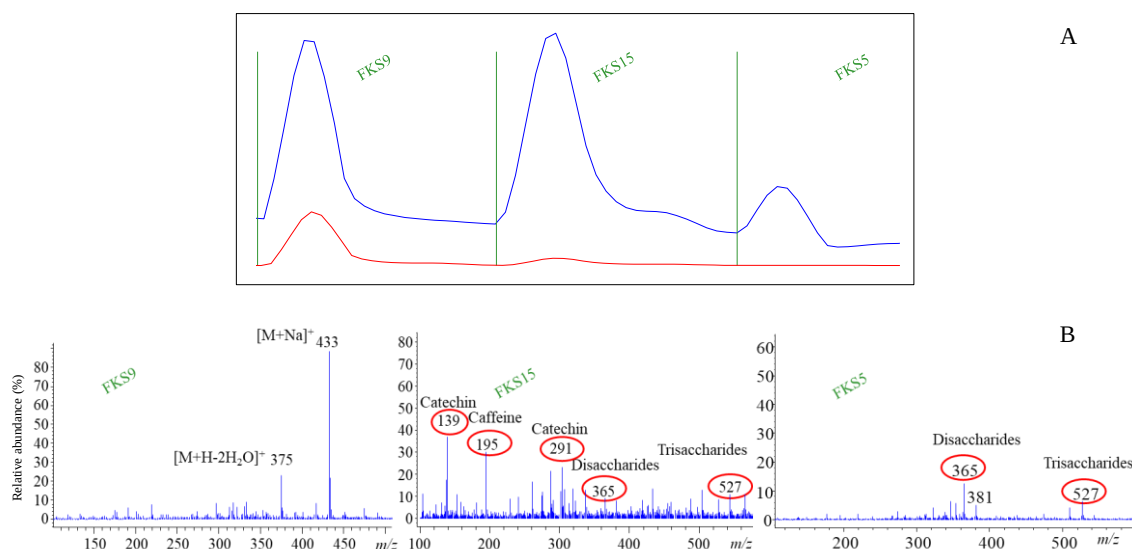


Figure 2. FIA-(ESI⁺)MS analysis of three *C. forskohlii* supplements (FKS9, FKS15 and FK5):
A) SCAN profile (blue line) and SIM ([M+Na]⁺ 433 m/z, red line) signal; B) MS spectra obtained for each FKS.

3.4. Estudio comparativo basado en LC-MS y FIA-(ESI)MS para la cuantificación de S-alil-L-cisteína en complementos de ajo negro

A comparative study of LC-MS and FIA-(ESI)MS for quantitation of S-allyl-L-cysteine in aged garlic supplements

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Article

A Comparative Study of LC-MS and FIA-(ESI)MS for Quantitation of S-Allyl-L-Cysteine in Aged Garlic Supplements

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Abstract: The increasing consumption of food supplements demands the development of improved analytical methodologies to ensure their quality and authenticity. In this paper, two new approaches, liquid chromatography coupled to mass spectrometry (LC-MS) and flow injection analysis-(electrospray ionization) mass spectrometry (FIA-(ESI)MS), were optimized and validated for their application in the quantitative analysis of bioactive S-allyl-L-cysteine (SAC) in commercial aged garlic supplements (AGS). Although both methodologies were found to be useful for the sensitive and precise quantitation of SAC, the LC-MS approach allowed the differential determination of SAC and its bioactive diastereoisomer, S-1-propenyl-L-cysteine (SIPC), together with the identification of a number of organosulfur compounds typical of garlic. Mass fingerprints by FIA-(ESI)MS were proposed as an advantageous alternative to LC-MS analysis when the fast (4 min/sample) screening of AGS for their SAC content is intended, as in applications aimed at high-throughput quality control or standardization. Finally, the results gathered by the application of these two methodologies evidenced the highly variable composition of commercial AGS, as well as the identification of a number of potential composition frauds affecting their genuineness and benefits on health.

Keywords: food supplements; aged garlic; S-allyl-L-cysteine (SAC); LC-MS; FIA-(ESI)MS; frauds; quality; genuineness



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1. Introduction

Awareness of the proven benefits of a healthy lifestyle on quality of life and life expectancy has noticeably increased over the last few years. In this sense, the concern about the rising number of health problems associated with aging and lifestyle-related diseases (e.g., cardiovascular affection, overweight, obesity), particularly in so-called developed countries, has promoted the consumption of food supplements that, together with a balanced diet and regular physical exercise, may contribute to good health status.

Among a large number of natural sources, plant-based dietary supplements are in high demand by consumers as they are commonly perceived as harmless alternatives to synthetic drugs, providing bioactives with a wide spectrum of physiological effects [1,2]. Moreover, the easy accessibility of these products, not only through traditional stores but also through the online market, has contributed to expanding their consumption and increasing the economic impact associated with their production and commercialization. However, the limited availability of some sources, scarcely developed legal frameworks, and shortcomings of the current official protocols for ensuring their standardization and quality have given rise to a number of alerts and fraudulent practices involving food supplements [3]. Among others, discrepancies regarding the presence and content of the declared bioactives [4], the undeclared use of synthetic bioactive compounds [5], the replacement of the declared natural source with a cheaper alternative [2], and the addition of pharmacologically active compounds to enhance bioactivity or counteract undesired side effects have been reported for some supplements [6].

Garlic (*Allium sativum* L.) is widely used as a condiment in traditional cuisine [7,8], and its beneficial health properties have been considered in the development of a number of garlic-based supplements with different formulations (e.g., oils, extracts, and dehydrated garlic powder). However, the strong smell and taste of garlic, as well as its difficult digestion, may negatively affect consumer acceptance of these products. This has led to the development and marketing of supplements based on deodorized garlic and the use of alternative sources, such as black garlic, with better sensory characteristics.

Black garlic is typically obtained by aging raw garlic bulbs at high temperatures (60–90 °C) under controlled humidity (70–90%) for 1–3 months [9–12]. This thermal processing induces non-enzymatic browning reactions (e.g., Maillard reaction), caramelization, and chemical oxidation, resulting in a product with a brown or even black color (depending on the processing conditions), sticky jelly-like texture, sweet flavor, and improved bioactive properties [8,13] compared to raw garlic. Other aging processes less commonly used in the food supplement industry involve the soaking of fresh garlic in hydroalcoholic solutions (e.g., 15–20% ethanol) at room temperature for extended periods of time (at least 18 months) [14] or fermentation of garlic with probiotics (*Saccharomyces cerevisiae*, *Monascus pilosus*, *Lactobacillus plantarum*, etc.) [15–18]. These alternative processes have been reported to preserve or even increase the concentration of garlic bioactives [15,17,18] and give rise to lower levels of unwanted compounds resulting from sugar degradation, such as hydroxymethylfurfural (HMF) [19].

Numerous studies have described the diverse and well-established bioactive properties of aged black garlic, including cardiovascular, antioxidant, anti-inflammatory, anticancer, immunomodulatory, hepatoprotective, neuroprotective, hypolipidemic, and hypoglycemic activities, among others [8,13,20–22]. These bioactivities have been reported to be mainly attributed to the organosulfur compounds (OSC) present in black garlic. Among them, S-allyl-L-cysteine (SAC), a water-soluble sulfur-containing amino acid arising from γ -glutamyl-S-allyl-L-cysteine (γ -GSAC) hydrolysis by γ -glutamyl transpeptidase enzyme [8,23], is the major OSC in aged black garlic. Its content, 4.5–10 times higher as compared to that of fresh garlic [12,24], is usually considered for standardization and valorization of commercial aged garlic supplements (AGS).

A number of analytical methodologies based on gas chromatography coupled with mass spectrometry (GC-MS) or liquid chromatography (LC) with different detectors have been reported for SAC analysis. Approaches based on GC-MS require prior derivatization of SAC, and tert-butyldimethylsilyl (TBDMS) derivatives have been shown to be advantageous for this purpose [14,25]. LC-UV methods allow the direct analysis of SAC, but the limited UV absorption of this bioactive compound, together with the common presence of spectral interferences associated with co-extracted compounds, hamper their use for the reliable quantitation of SAC in black garlic extracts [24,26]. In this sense, the advantages regarding the separation of SAC from other interferences and the sensitivity and selectivity of detection provided by reverse-phase LC-MS methods support their common use for SAC quantitation [14].

Flow injection analysis–mass spectrometry (FIA-MS) fingerprinting has recently been described as a high-throughput alternative to chromatographic methods for applications dealing with food classification and authentication [27–31]. However, FIA-MS approaches have not yet been applied to the analysis of AGS. Thus, in the present paper, the performance of two analytical methods by LC-MS and FIA-MS, after their corresponding optimization and validation, will be compared for the intended quantitation of SAC in commercial AGS. The advantages and limitations of both methodologies as fast and cost-effective approaches for the reliable quantitation of this bioactive, as well as for the assessment of AGS quality and genuineness, will also be evaluated here.

2. Materials and Methods

2.1. Reagents and Samples

Analytical standards (purity > 99%) of HMF, S-1-propenyl-L-cysteine (S1PC), and γ -GSAC were acquired from Sigma-Aldrich (St. Louis, MO, USA), and SAC from Tokyo Chemical Industry (Zwiindrecht, Belgium). Milli-Q water was obtained from an Advantage A10 system (Millipore, Bedford, MA, USA), while methanol (MeOH) and formic acid were acquired from Fisher Chemical (Newington, NH, USA).

Aged garlic supplements (AGS1-24) under study were purchased online from different websites and analyzed prior to their expiration date. Table S1 of the Supplementary Material shows their different formulations and compositions, as declared in the labeling.

Reference aged black garlic powdered extract Abg+[®] (AGRef, $\geq 0.1\%$ SAC) was provided by Pharmactive Biotech Products S.L.U. (Madrid, Spain), and was obtained using the Abg Cool-Tech[™] registered methodology.

2.2. LC-MS Analysis

2.2.1. Sample Preparation

Ultrasound-assisted extraction of AGS and AGRef (100 mg) with 1 mL of Milli-Q water (Millipore, Bedford, MA, USA) was carried out ($n = 3$) using an ultrasonic bath (37 kHz) (Elma Schmidbauer GmbH, Singen, Germany) set at 40 °C for 25 min. Extracts thus obtained were further centrifuged at 4400 $\times g$ for 5 min, and the supernatants were filtered through 0.2 μm teflon filters (Interchim, Montluçon, France) and diluted (1:10–1:50, v/v), as required. All extracts were frozen (−20 °C) and protected from light until analysis.

2.2.2. Optimization of LC-MS Operating Conditions

Qualitative and quantitative analyses of SAC and other AGS components were performed on an Agilent 1100 series system, including an autosampler, a quaternary pump, a thermostated column compartment, and a diode array detector coupled to a 6125 single quadrupole mass spectrometry detector (Agilent Technologies, Santa Clara, CA, USA) provided with an electrospray ionization (ESI) source. According to Jiménez-Amezcu et al. [12], chromatographic separation was carried out using an InfinityLab Poroshell 120 Bonus-RP column (150 mm $\times$ 4.6 mm, 2.7 μm ; Agilent Technologies) thermostated at 30 °C, and flow rate was set at 0.4 mL min^{−1}. A binary gradient consisting of water (eluent A) and MeOH (eluent B), both containing 0.1% formic acid, was used as the mobile phase. The injection volume was set at 5 μL .

Optimization of ESI parameters, considering both positive (ESI⁺) and negative (ESI[−]) polarities, was performed by infusion of a SAC standard solution (0.01 mg mL^{−1}) under the conditions provided by a Box–Behnken experimental design (15 experiments in randomized order, Table S2 of Supplementary Material). Three independent variables were considered in the experimental design: the nebulizing gas pressure (N₂, 99.5%) (P), nebulizing temperature (T), and fragmentor voltage (FV). The experimental ranges evaluated for each of these variables were $P = 20$ –60 psi, $T = 150$ –300 °C, and $FV = 40$ –300 V.

Response surface methodology (RSM) (StatGraphics Centurion XVI software, Statistical Graphics Corporation, Rockville, MD, USA) was used to calculate the coefficients to maximize the response variable (SAC peak area) under positive and negative polarities ($R_{\text{SAC}(+)}$ and $R_{\text{SAC}(-)}$, respectively) and their statistical significance, as well as to estimate the prediction errors (estimation standard error (SSE) and mean absolute error (MAE)). The quadratic model proposed was as follows:

$$R_{\text{SAC}} = \beta_0 + \beta_1 P + \beta_2 T + \beta_3 FV + \beta_{1,1} P^2 + \beta_{2,2} T^2 + \beta_{3,3} FV^2 + \beta_{1,2} P \cdot T + \beta_{1,3} P \cdot FV + \beta_{2,3} T \cdot FV + \varepsilon, \quad (1)$$

where β_0 was the intercept, β_i were the first-order coefficients, $\beta_{i,i}$ were the quadratic coefficients for the i^{th} factor, $\beta_{i,j}$ were the coefficients for the interaction between factors i and j , and ε was the error. Under optimal P , T , and FV , capillary voltage (CV) was also optimized (3000–4000 kV) for a flow rate (F) set at 12 L min^{−1}.

Mass spectra were acquired in SCAN mode (100–1000 m/z) and in SIM mode, registering the quasimolecular ion of SAC under positive (m/z 162 = $[M + H]^+$), and negative (m/z 160 = $[M - H]^-$) polarities. Data acquisition and processing were performed using HP Chemstation Rev. B.04.02 software (Agilent Technologies, Santa Clara, CA, USA).

Compound identification was based on chromatographic retention and MS data and was confirmed, when possible, by co-injection of the corresponding commercial standards. Quantitative analysis of SAC was carried out in triplicate using an external standard calibration curve (0.0001–0.05 mg mL⁻¹).

2.3. Optimization of FIA-(ESI)MS Analysis

Mass spectral fingerprinting of SAC was carried out using the system previously described in Section 2.2.2., by replacing the chromatographic column with a polyetheretherketone (PEEK) tube (105 × 0.17 mm). FIA was used for the direct injection of the sample (5 µL for 4 min) into the carrier and further transmission into the ESI interface of the mass spectrometer. A hydroalcoholic mixture consisting of H₂O:MeOH (93:7, v/v), both with 0.1% formic acid, was employed as the carrier. The flow rate was set at 0.4 mL min⁻¹.

The ESI interface was operated in SIM mode under the polarity and operating conditions previously optimized in Section 2.2.2. All the analyses were performed in triplicate, and quantitation was performed following an external standard calibration curve of SAC (0.0001–0.05 mg mL⁻¹).

2.4. Validation of LC-MS and FIA-(ESI)MS Methods

Different parameters were considered in the validation of the previously optimized methods for SAC quantitation. Precision was measured in terms of intra- and inter-day precision by analyzing two aged garlic supplements with different SAC concentrations (AGS2 and AGS22) within the same day ($n = 5$) or in 5 consecutive days, respectively. The linearity of response was assessed in the 0.0001–0.05 mg mL⁻¹ range for both techniques by means of the coefficient of determination (R^2). Limits of detection (LOD) and quantitation (LOQ) were calculated for SAC as three and ten times the signal-to-noise ratio (S/N), respectively. Recovery was calculated after spiking AGS2 and AGS22 extracts with SAC standard solutions at three concentration levels (in the range 0.005–0.01 mg mL⁻¹ for AGS2 and 0.0025–0.0075 mg mL⁻¹ for AGS22). The matrix effect was evaluated by quantifying SAC in AGS2 and AGS22 after dilution with water at different ratios (up to 1:100, v/v).

2.5. LC Coupled to Isotope Ratio Mass Spectrometry (LC-IRMS)

Prior to LC-IRMS analysis of the selected AGS, a clean-up step by solid-phase extraction (SPE) was required. AGS were re-dissolved in 10 mL of Milli-Q water (Millipore), sonicated for 10 min, and centrifuged at 1000× g for 45 min to separate the insoluble fraction. The supernatant was then passed through an SPE column prepared by filling a glass syringe (25 mL) with 5 g of a cation exchange resin (Dowex® 50 WX 8, H⁺ form, 50–100 mesh, Merck KGaA, Darmstadt, Germany) following the procedure described by Kubec et al. [32]. The resin was conditioned with 10 mL of HCl (3%, v/v) and equilibrated with 10 mL of water. 1–2 mL of sample solution were loaded onto the cartridge, and extracts were purified with 10 mL of water. SAC was recovered with 40 mL of NH₄OH (1M). Finally, the purified extracts were freeze-dried and re-dissolved in 1 mL of Milli-Q water (Millipore) for instrumental analysis.

LC-IRMS analysis of SAC in selected AGS, AGRef, and synthetic SAC standards was carried out on a Surveyor HPLC system (Thermo Fisher, Bremen, Germany) using a Primesep A LC column (100 Å, 2.1 × 250 mm, SIELC Co., Wheeling, IL, USA). Separation was carried out using a binary gradient of water (A) and 0.3% H₂SO₄ (B) as follows: 40% B to 100% B for 30 min. The flow rate and temperature were set at 0.3 mL min⁻¹ and 30 °C, respectively. Aliquots of 2–5 µL were injected.

A Delta V Advantage isotope ratio mass spectrometer coupled to an LC-Isolink™ interface (both from Thermo Fisher) was used for isotopic characterization ($\delta^{13}C$) of SAC.

Organic compounds eluted from the LC system were oxidized online using the LC-Isolink™ interface. The effluent was combined with oxidation (50 g L⁻¹ of sodium persulfate) and acid (10% phosphoric acid) reagents and heated at 100 °C. The CO₂ produced in the oxidation reactor was extracted online using a TeflonAF™ membrane (DuPont, Wilmington, DE, USA) and directed into the IRMS system with a stream of He gas. The He flow was dried using Nafion™ (The Chemours Company, Wilmington, DE, USA) membranes before entering the IRMS instrument. Calibration of the IRMS system was performed by applying pulses of CO₂ of a known isotopic composition along the LC run. The standard precision of the $\delta^{13}\text{C}$ data provided by the LC-Isolink-IRMS instrument was 0.7‰.

2.6. Statistical Analysis

Statgraphics Centurion XVI software (Statistical Graphics Corporation) was used for the statistical analysis of data. The agreement between the declared values of SAC and the experimental concentrations determined either by LC-MS or FIA-(ESI)MS analysis was assessed using the *t*-test for independent samples (*p* < 0.05).

3. Results and Discussion

3.1. Development and Validation of LC-MS and FIA-(ESI)MS Methods for SAC Quantitation in AGS

3.1.1. Optimization of (ESI)MS Operating Conditions

Prior to comparing the performance of LC-MS and FIA-(ESI)MS methods for the intended quantitation of bioactive SAC in AGS, the ESI parameters, which are common to both techniques, were optimized.

After preliminary FIA-(ESI)MS experiments in SCAN mode, *m/z* 162 ([M + H]⁺) and *m/z* 160 ([M − H][−]) were shown to be the most abundant adducts under positive and negative polarities, respectively, and were therefore considered for further data acquisition under SIM mode.

The set of 15 experiments provided by the Box–Behnken experimental design described in Section 2.2.2, including different *T*, *FV*, and *P* combinations, was considered for the maximization of the SAC response [*R*_{SAC(+)} and *R*_{SAC(−)}]. The experimental results obtained under positive and negative polarity (peak areas for *m/z* 162 and for *m/z* 160, respectively) are shown in Table S2. The coefficients of the quadratic models proposed for maximization of *R*_{SAC(+)} and *R*_{SAC(−)} and their statistical significance were calculated using a response surface methodology (Figure 1). As for the *R*_{SAC(+)} model, *FV*, *T*·*FV*, and *FV*² were the factors statistically significant at the 95% confidence level. The model equation, the goodness of the fitting and the prediction errors were, respectively, *R*_{SAC(+)} = $-5.2 \times 10^5 + 8.5 \times 10^3 T - 0.8 \times 10^3 FV + 3.5 \times 10^{-10} T^2 - 50.02 T \cdot FV + 24.8 FV^2 + 2.04 \times 10^{-10} FV \cdot P + 1.16 \times 10^{-10} P^2$, *R*² = 89%, *SSE* = 309,417 and *MAE* = 141,152. The maximum response estimated under optimal ESI+ conditions (*T* = 300 °C, *FV* = 40 V, *P* = 60 psi) was *R*_{SAC(+)} = 1.84×10^6 .

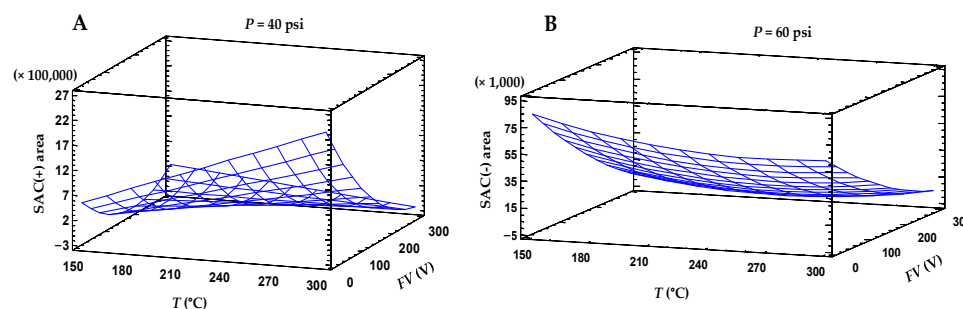


Figure 1. Surface plots of the Box–Behnken experimental design aimed to optimize the ESI parameters under (A) positive and (B) negative polarities for maximization of S-allyl-L-cysteine (SAC) area.

As for the model obtained under negative polarity ($R_{SAC(-)} = 1.2 \times 10^5 - 0.9 \times 10^3 T - 0.3 \times 10^3 FV + 2 \times 10^3 P + 1.64 T^2 + 0.50 T \cdot FV + 0.57 T \cdot P + 0.31 FV^2 - 2.50 FV \cdot P - 17.83 P^2$, $R^2 = 91\%$; $SSE = 9909$; $MAE = 4734$), FV was the only significant ($p < 0.05$) factor. This model provided the maximum response ($R_{SAC(-)} = 70,745$) under optimal ESI conditions ($T = 150\text{ }^\circ\text{C}$, $FV = 40\text{ V}$, $P = 55\text{ psi}$).

From the comparison of both datasets, it was found that although the optimal ESI operating conditions were dependent on the polarity considered, the common setting of $FV = 40\text{ V}$ was required to avoid the easy fragmentation of SAC experimentally observed and give rise to a decrease in response. Under these optimal T , FV , and P conditions, the effect of the capillary voltage (CV) within the range 3000–4000 V was also considered for the optimization of $R_{SAC(+)}$ and $R_{SAC(-)}$. Although CV was not relevant for $R_{SAC(-)}$, the sensitivity of $R_{SAC(+)}$ increased significantly (up to eight times) when CV was set at 3000 V. Finally, when $R_{SAC(+)}$ and $R_{SAC(-)}$ were compared, the response under positive polarity was found to be 26 times higher than that in ESI mode. Therefore, the setting of CV at 3000 V, together with the previously mentioned optimal ESI⁺ conditions, were selected for further validation of a sensitive method for SAC quantitation. Under these conditions, the calculated and experimental responses were very similar ($RSD = 4\%$).

3.1.2. Validation

The analytical parameters considered in the validation of the LC-MS and FIA-(ESI)MS methods previously developed are shown in Table 1. As can be seen, both methods exhibited good linearity ($R^2 > 0.995$) within the SAC concentration range expected for the AGS under study, with LC-MS showing a wider linearity range (0.0001 – 0.05 mg mL^{-1}). In terms of sensitivity, the method by FIA-(ESI)MS outperformed the LC-MS method, as evidenced by a lower LOD (0.84 vs. 1.19 ng mL^{-1} , respectively) and LOQ (2.78 vs. 3.98 ng mL^{-1} , respectively). As compared to the literature, the sensitivity of the LC-MS (SIM) and FIA-(ESI)MS (SIM) methods developed here was significantly higher than that of LC methods with UV or fluorescence detection previously reported (LOD: 29.67 and $6.28\text{ }\mu\text{g mL}^{-1}$, respectively) [24]. However, it was in the order of methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) under the multiple reaction monitoring (MRM) mode described by Woo et al. [17] (LOD: 60 ng mL^{-1}).

Table 1. Validation of LC-MS and FIA-(ESI)MS methods for the analysis of SAC.

LC-MS Validation	AGS2 (1% SAC)	AGS22 (0.1% SAC)
Intra-day precision (RSD, %)	0.99	3.97
Inter-day precision (RSD, %)	3.54	7.36
Calibration curve	$y = 5.43 \times 10^7 x$ ($R^2 = 0.9948$)	
Linear range (mg mL^{-1})	0.0001–0.05	
LOD/LOQ (ng mL^{-1})	1.19 (0.042)/3.98 (0.139) *	
Recovery (%)	112 (10.6)/102 (6.8) *	
FIA-(ESI)MS Validation	AGS2 (1% SAC)	AGS22 (0.1% SAC)
Intra-day precision (RSD, %)	3.57	1.96
Inter-day precision (RSD, %)	5.20	9.25
Calibration curve	$y = 9.41 \times 10^7 x$ ($R^2 = 0.9978$)	
Linear range (mg mL^{-1})	0.0001–0.025	
LOD/LOQ (ng mL^{-1})	0.84 (0.01)/2.78 (0.03) *	
Recovery (%)	105 (4.1)/106 (9.8) *	

* Mean and standard deviation in brackets for $n = 3$.

Regarding the intra- and inter-day precision of these methods, evaluated over two AGS standardized at two different SAC concentrations (0.1 and 1% for AGS2 and AGS22, respectively), the results obtained were similar for both techniques ($RSD < 10\%$). These results are also in good agreement with precision data previously reported for other

LC methodologies based on the use of different detection systems [17,24]. As regards accuracy, recoveries obtained by both techniques by spiking AGS2 and AGS22 with known amounts of SAC were also good (LC-MS: 102–112% and FIA-(ESI)MS: 105–106%). Finally, the potential matrix effect in the analysis of AGS was ruled out for both techniques, as no significant differences at the 95% confidence level were found in the SAC content determined in the original extract and after its dilution in the range 1:20–1:100 for FIA-(ESI)MS and 1:2–1:20 for LC-MS.

3.2. SAC Content of Commercial AGS

The previously optimized and validated LC-MS and FIA-(ESI)MS methods were further applied to the quantitation of SAC in 24 commercial AGS and in AGRef. The experimental results obtained by both techniques, together with the SAC content declared in the labeling of these products, are shown in Table 2.

Table 2. Content (%) of SAC in aged garlic supplements under study (AGS1–AGS24) and in the reference extract (AGRef) experimentally determined by LC-MS and FIA-(ESI)MS and SAC values declared in their corresponding labels.

Code	Experimental SAC (%) *		Declared SAC (%)
	LC-MS	FIA-(ESI)MS	
AGS1	0.106 (0.003) ^a	0.157 (0.008) ^b	0.1 ^a
AGS2	0.89 (0.06) ^a	0.96 (0.03) ^b	1 ^b
AGS3	tr **	tr	0.25
AGS4	0.108 (0.005)	0.162 (0.002)	- ***
AGS5	tr	tr	-
AGS6	0.148 (0.005) ^a	0.082 (0.005) ^b	0.1 ^b
AGS7	0.143 (0.006) ^a	0.092 (0.002) ^b	0.1 ^b
AGS8	0.829 (0.001) ^a	0.68 (0.02) ^b	1 ^c
AGS9	0.083 (0.002)	0.1187 (0.0001)	-
AGS10	tr	tr	-
AGS11	tr	tr	-
AGS12	tr	tr	0.1
AGS13	n.d. **	n.d.	-
AGS14	tr	tr	-
AGS15	0.07 (0.01) ^a	0.09 (0.01) ^b	0.1 ^b
AGS16	0.20 (0.01) ^a	0.125 (0.001) ^b	0.1 ^c
AGS17	0.0169 (0.0002)	0.0084 (0.0001)	-
AGS18	0.073 (0.001) ^a	0.074 (0.003) ^a	>0.1 ^b
AGS19	0.069 (0.003) ^a	0.043 (0.002) ^b	0.013 ^c
AGS20	0.06 (0.01) ^a	0.032 (0.002) ^b	0.013 ^c
AGS21	0.57 (0.03) ^a	0.62 (0.01) ^a	1 ^b
AGS22	0.090 (0.003) ^a	0.062 (0.001) ^b	0.1 ^a
AGS23	1.13 (0.04) ^a	0.75 (0.11) ^b	1 ^{a,b}
AGS24	1.33 (0.02) ^a	1.01 (0.35) ^a	1 ^a
AGRef	0.141 (0.005) ^a	0.1105 (0.0003) ^b	≥0.1 ^b

^{a–c} Significant ($p < 0.05$) differences between experimental (by LC-MS and by FIA-(ESI)MS) and declared SAC content. * Average and standard deviation are in brackets ($n = 3$). ** tr, traces ($<LOQ$) and n.d., non-detected ($<LOD$). *** -, non-declared.

A good match between the results obtained by LC-MS and FIA-(ESI)MS was found for most of the samples under study (e.g., AGS18, AGS21, and AGS24), with 29% of the analyzed supplements (AGS3, AGS5, and AGS10–14) equivalent to 88% of non-SAC-standardized AGS, showing levels of SAC below the LOQ of both methodologies. Although the standardization in SAC was not detailed for most of these supplements, the experimental results obtained here would question the potential health benefits associated with their consumption. Moreover, composition fraud, if other reasons are ruled out, would be responsible for the discrepancies observed regarding the SAC content of AGS3 and AGS12.

As for the SAC-standardized supplements, 50% showed good agreement between the SAC content experimentally determined and that declared in the labeling (e.g., AGS1, AGS6, AGS7, AGS16, etc.), whereas significant ($p < 0.05$) differences were found for the remaining AGS under study. Within this last group, SAC-standardized AGS showing a bioactive content lower than expected (e.g., AGS3, AGS8, AGS12, and AGS21) would represent a potential labeling fraud.

3.3. Characterization of AGS Composition

In order to gain insight into the potential composition or labeling frauds identified in Section 3.2. regarding the SAC content of the commercial AGS under study, the LC-MS profiles of these samples were compared with that of the AGRef extract. As can be seen in Figure 2, supplements were grouped into three different classes according to their similar composition. Supplements of Group 1 (AGS4, AGS6, AGS8, AGS90, AGS19, and AGS20) were considered to be genuine as they were characterized by the presence of a number of organosulfur compounds, including SAC, its diastereoisomer S1PC, as well as their precursors, γ -GSAC, and γ -glutamyl-S-1-propenyl-L-cysteine (γ -GS1PC) [23,33]. Other OSC present in these samples were alliin and its isomer, cycloalliin [12,34], and the dipeptide glutamyl-phenylalanine (Glu-Phe), all of which are typical of black garlic [12,33], and were experimentally detected in the reference sample used here (AGRef).

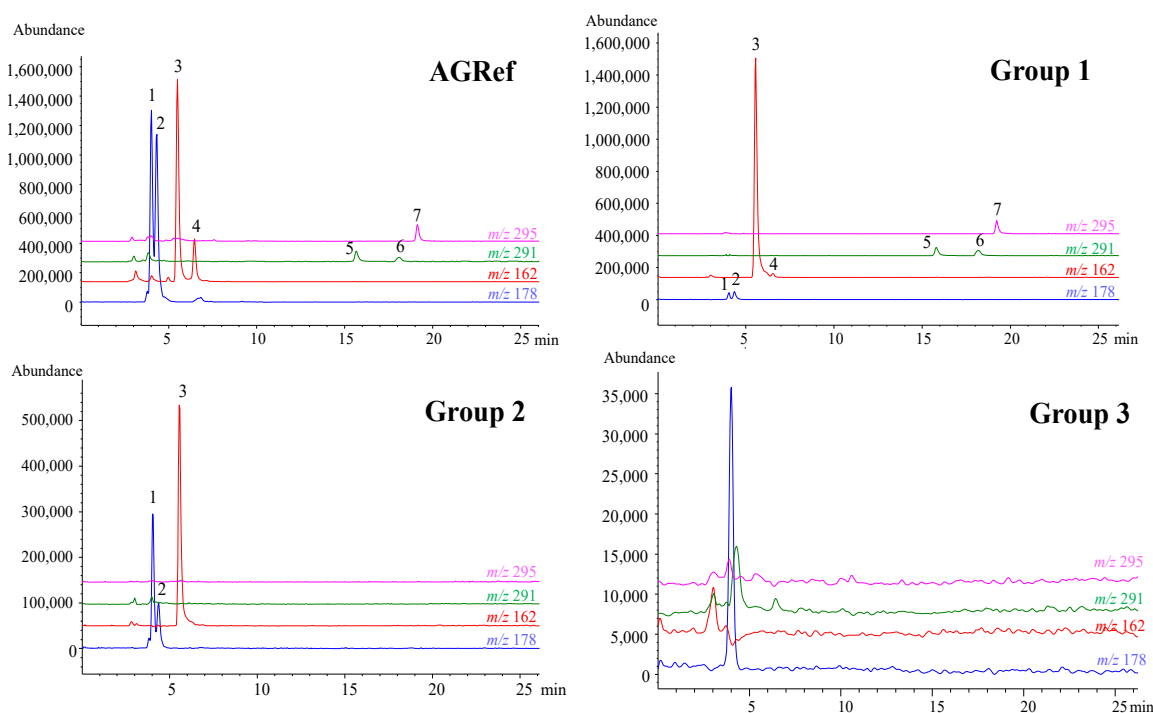


Figure 2. LC-MS profiles of AGRef, AGS4 (Group 1), AGS1 (Group 2) and AGS10 (Group 3). Traces of m/z 178 (blue), 162 (red), 291 (green), and 295 (pink) correspond to $[M + H]^+$ adducts of (1) cycloalliin, (2) alliin, (3) SAC, (4) S1PC, (5) γ -GSAC, (6) γ -GS1PC, and (7) Glu-Phe.

Compared to Group 1, supplements included in Group 2 showed a simpler LC-MS profile, in which either SAC (e.g., AGS1, AGS7, AGS18, and AGS22–24) or both SAC and S1PC in a wide range of concentrations (AGS2, AGS15–17, AGS21) were the most abundant OSC detected. Although the required presence of SAC would support the intended physiological effect associated with their consumption, the genuineness of these supplements would be questionable due to the lack of other typical aged garlic compounds. Therefore, Group 2 samples were presumably considered supplements in which SAC of non-natural origin was used for manufacturing and evaluation of SAC isotopic composition

by LC-IRMS analysis (Section 3.4) was found to be required for further confirmation of this hypothesis.

Lastly, supplements with no SAC content were grouped together in Group 3 (AGS3, AGS5, and AGS10–14). These supplements predominantly contained low molecular weight carbohydrates such as mono-, di-, and trisaccharides, probably arising from the antiaggregants used for their formulation, and compounds derived from sugar degradation such as HMF. In this case, fraud is committed not only because the desired physiological effect associated with bioactive SAC is prevented but also because supplements marketed as standardized in SAC and not complying with the labeling represent composition fraud (e.g., AGS3 and AGS12).

A noticeable advantage of the developed LC-MS method is the ability to differentiate between SAC and its diastereoisomer S1PC, to which a number of bioactive properties (e.g., antioxidant, cardioprotective, etc.) have been ascribed [21,23]. This differentiation was not possible because the FIA-(ESI)MS configuration used here as a single quadrupole mass spectrometer does not discriminate compounds with identical nominal mass. As shown in Table 3, 42% of the AGS under study contained variable amounts of S1PC in addition to SAC. These contents, normalized to the corresponding SAC concentration, were below 3% in most cases and close to that of AGRef (11%). However, for samples AGS4, AGS9, and AGS17, these relative values were noticeably higher (in the range of 72–93%).

Table 3. Concentration of S1PC (in mg g^{-1} and as a percentage of SAC content) determined by LC-MS in the AGS under study.

Code	S1PC (mg g^{-1})	S1PC (% Relative to SAC)
AGS2	0.23 (0.01) *	3
AGS4	0.78 (0.12)	72
AGS6	0.111 (0.004)	7
AGS8	0.228 (0.001)	3
AGS9	0.7 (0.2)	91
AGS15	0.006 (0.001)	1
AGS16	0.011 (0.002)	1
AGS17	0.16 (0.01)	93
AGS19	0.004 (0.001)	1
AGS21	0.17 (0.02)	3
AGRef	0.16 (0.002)	11

* Average and standard deviation are in brackets ($n = 3$).

A detailed evaluation of the available information related to the processing of some of these samples (e.g., AGS4 and AGS9) revealed that they had not been aged by a conventional heating process but by soaking in hydroalcoholic solutions at room temperature [17]. This procedure has been described to give rise to a white-colored product, different from the typical brown-black aged garlic usually appreciated by consumers, with high contents of both SAC and S1PC. Alternatively, it has also been reported that anaerobic fermentation of garlic using different microorganisms (e.g., *S. cerevisiae*, *Bacillus subtilis*, etc.) leads to highly variable proportions of γ -GSAC and γ -GS1PC, which are precursors of SAC and S1PC, respectively [15–18]. Although a greater number of AGS from different garlic varieties, locations, etc., and aged under known conditions should be analyzed to confirm these results, the information provided by LC-MS seems to be useful not only to determine the differential content of these two bioactive compounds in AGS but also to identify those that have presumably undergone a non-conventional aging process.

As regards AGS17, for which information on processing is not available, confirmation of its potential non-genuine origin supported by the results on OSC previously mentioned would require that a non-selective synthetic procedure providing the similar yields of SAC and S1PC experimentally determined is considered (see Section 3.4).

On the other hand, the results shown in Table 3 were also used to evaluate the extent that which S1PC may interfere with the quantitation of SAC by FIA-(ESI)MS, as compared to LC-MS analysis. Thus, considering the similar MS response of both bioactives, an overestimation of SAC by FIA-(ESI)MS would be expected for AGS showing either a high S1PC content and/or a high relative abundance of this bioactive compared to SAC. However, although this effect was evidenced for some of the samples under study (e.g., AGS2, AGS4, and AGS9), it was not clear for others (particularly for those with the lowest content of these two bioactives). Therefore, the usefulness of FIA-(ESI)MS as a rapid (4 min/sample) tool for screening SAC in AGs is demonstrated, and a particularly good match with LC-MS data will be obtained when this approach is applied to AGS processed under conventional aging procedures, usually giving rise to low S1PC contents.

3.4. Non-Natural SAC in Commercial AGS

LC-IRMS has been proven to be useful for a number of applications in food authentication [35–37] and, as regards garlic, has previously been described for the successful classification of samples from different Italian regions [37] or Asian countries [38]. However, no previous reference has addressed its use for the assessment of the natural origin of SAC present in AGS, and therefore, it was considered here for confirmation of the genuineness results previously described (Section 3.3).

Four samples from both Group 1 and Group 2 were selected for these analyses. AGS4 and AGS8 were considered representative supplements classified as genuine based on their OSC-rich chromatographic profiles and similitude to AGRef. In contrast, AGS17 and AGS23 were chosen as presumably non-genuine supplements enriched with non-natural (either synthetic or biotechnologically produced) SAC. The isotopic compositions of these samples determined by LC-IRMS, together with that of a synthetic SAC standard and AGRef (natural reference), are shown in Table 4.

Table 4. Isotopic ratios of $\delta^{13}\text{C}$ in selected AGS.

Sample	$\delta^{13}\text{C}$ of SAC (‰)
Synthetic SAC standard	−22.3
AGRef (natural reference)	−28.0
AGS4	−27.3
AGS8	−27.9
AGS17	−20.4
AGS23	−20.4

The variability observed for the isotopic composition of SAC in the samples under study ($\delta^{13}\text{C}$ data ranging from −28.0 to −20.4‰) allowed their effective classification. Thus, $\delta^{13}\text{C}$ data for AGS8 and AGS4 (−27.9 and −27.3‰, respectively) were close to the $\delta^{13}\text{C}$ value for the natural SAC reference (−28.0‰) and were in line with data previously reported by different authors for *Allium sativum*, the C_3 pathway plant expected to be used for manufacturing AGS (−29.9 to −24.4‰, [38]; −28.5 to −25.6‰, [37]); average $\delta^{13}\text{C}$ = −27‰, [39]). Therefore, in agreement with the conclusions drawn from the LC-MS analysis, the genuineness of the AGS included in Group 1 was assured.

The $\delta^{13}\text{C}$ data for AGS17 and AGS23 (−20.4‰) were far from those of genuine samples and were similar to the $\delta^{13}\text{C}$ value determined for the synthetic SAC standard (−22.3‰). These results were also in agreement with previous studies in which synthetic compounds have been reported to exhibit $\delta^{13}\text{C}$ values out of the range for natural compounds in vegetable matrixes [40–42]. Therefore, LC-IRMS data would support the use of non-natural SAC for the formulation of supplements included in Group 2, in agreement with the poor profiles obtained by LC-MS analysis. The dissimilarity observed with respect to the synthetic SAC could be attributed, among others, to the different carbon sources and procedures followed for the synthesis of this bioactive.

4. Conclusions

Two analytical methods by LC-MS and by FIA-(ESI)MS have been optimized and validated for the first time for the quantitative determination of *S*-allyl-L-cysteine in aged garlic supplements. Although both methodologies have been proven to allow the reliable quantitation of this bioactive, the LC-MS approach enables the discrimination between SAC and its diastereoisomer, S1PC, a bioactive detected in some of the AGS under study, and an additional quality marker whose content depends among others, on the garlic aging process.

Mass fingerprints by FIA-(ESI)MS are proposed as an advantageous alternative to the LC-MS approach when fast (4 min/sample) screening of AGS for their SAC content is intended and when no differentiation of SAC and S1PC is required. This would be the case for applications highly demanded by both industries of the nutraceutical sector or by regulatory bodies, such as the high-throughput quality control of AGS. In this sense, only those AGS samples that do not meet specifications or do not contain SAC would need to undergo in-depth studies using more time-consuming and expensive LC-MS approaches, thus contributing to the rational use of facilities for AGS authentication and quality control.

Finally, the application of both methodologies to 24 commercial AGS has revealed not only the wide variability regarding their SAC content but has also allowed the identification of a variety of potential composition frauds affecting compliance with labeling, presumed functionality, or genuineness. The results of this study contribute to the demand for improved methodologies and scientifically based data to ensure the quality and authenticity of AGS.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/foods13172645/s1>, Table S1: Composition of AGS under study; Table S2: Box–Behnken experimental design for optimization of ESI parameters in the LC-MS and FIA-(ESI)MS analysis of SAC in AGS.

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4. Desarrollo de procesos de extracción y fraccionamiento de compuestos bioactivos eficaces y medioambientalmente respetuosos

4. DESARROLLO DE PROCESOS DE EXTRACCIÓN Y FRACCIONAMIENTO DE COMPUESTOS BIOACTIVOS EFICACES Y MEDIOAMBIENTALMENTE RESPETUOSOS

4.1. Prefacio

4.1.1. Obtención de extractos ricos en compuestos bioactivos del ajo y sus subproductos

Como se ha comentado en la introducción, la obtención de extractos ricos en compuestos bioactivos presenta un gran interés para su empleo como ingredientes alimentarios o complementos alimenticios, siendo la SLE la técnica más empleada para este fin en la industria alimentaria. Así, en el caso de la extracción de compuestos bioactivos del ajo, tanto fresco como negro, suelen emplearse reactores de gran volumen, operando en unas condiciones de temperatura y agitación previamente seleccionadas, para llevar a cabo este proceso de extracción convencional. Sin embargo, dichos procesos son lentos, normalmente implican el uso de grandes volúmenes de disolvente, orgánicos en su mayoría, y están poco automatizados. Por tanto, cada vez está adquiriendo un mayor interés el desarrollo de **nuevos métodos de extracción basados en técnicas avanzadas** como la PLE, MAE y UAE que permitan realizar una extracción más eficaz, rápida y medioambientalmente más sostenible de dichos compuestos.

Si como matriz de partida se consideran además los residuos de la industria agroalimentaria, este interés por la extracción de compuestos bioactivos es aún mayor. Los residuos agrícolas suponen un riesgo ambiental ya que su tratamiento se basa en la quema, enterrado, almacenamiento o abandono de los mismos en el medio natural. En este contexto, ya se está implementando un mejor aprovechamiento, tanto económico como medioambiental, de estos recursos basado en la **economía circular**. Así, se hablaría de subproductos agrícolas en el caso de productos secundarios obtenidos durante el proceso de producción principal de un cultivo o subproductos del procesamiento, en el caso, por ejemplo, de los producidos durante la elaboración de un alimento. En el caso del ajo (Figura 4.1.), al sólo consumirse su raíz bulbosa, son muchos los residuos que se obtienen de la planta (hojas, tallos, pieles...), por lo que su aprovechamiento es crucial para el medioambiente y la industria alimentaria. Hasta el momento los **subproductos del ajo** se destinan a la alimentación del ganado (Europa Press, 2024) o a la producción de materiales derivados de la celulosa (Moreno *et al.*, 2020), etc. Sin embargo, se ha comprobado que las pieles de ajo son una fuente rica en compuestos fenólicos (Kallel *et al.*, 2014), mientras que el tallo y las hojas lo son de compuestos azufrados como la alicina (Arzanlou & Bohlooli, 2010). A pesar de estos estudios, todavía resulta necesario profundizar en el conocimiento sobre la composición los extractos obtenidos a partir de estos subproductos por técnicas avanzadas de extracción, para su mejor aprovechamiento como fuentes alternativas de extractos ricos en bioactivos.

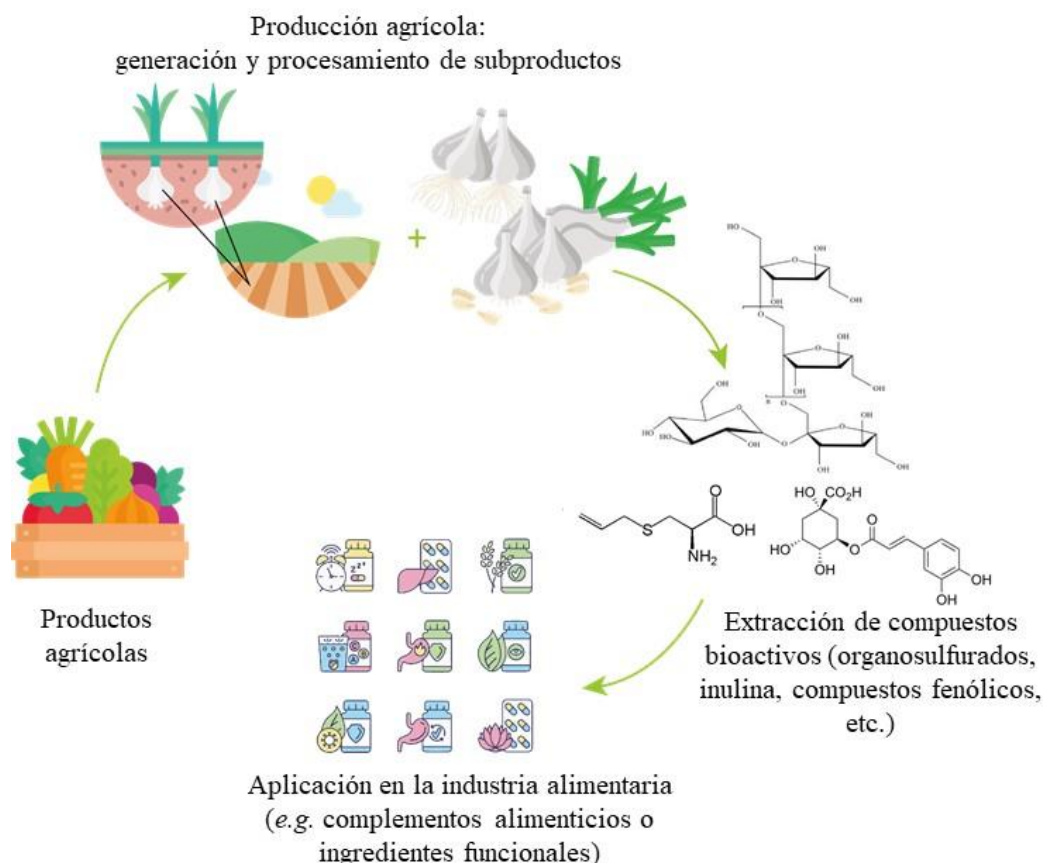


Figura 4.1. Concepto de economía circular aplicada al aprovechamiento de residuos agrícolas del ajo.

En cuanto a la aplicación de la **MAE** para la extracción de compuesto bioactivos del ajo, se ha empleado principalmente para la obtención de extractos enriquecidos en alicina a partir de ajo fresco (Putra *et al.*, 2019; Dhvani *et al.*, 2021), aunque también se ha descrito su uso para la extracción de otros compuestos como taninos (Pardede *et al.*, 2020), pectinas (Şen *et al.*, 2024), etc. a partir de este alimento. En un estudio llevado a cabo por Oke *et al.* (2023) se evaluaron las condiciones MAE óptimas y los aspectos tecno-económicos de la recuperación de compuestos fenólicos (ácido elágico, ferúlico y clorogénico) del ajo. Además, se demostró la viabilidad económica de este proceso a diferentes escalas de producción mediante el empleo de un algoritmo computacional (simulación MonteCarlo). Esta técnica ha sido también empleada para la revalorización de pieles de ajo (Şen *et al.*, 2024), aunque no se ha evaluado su uso para la obtención de extractos enriquecidos en compuestos bioactivos a partir de ajo negro, por lo que sería de interés profundizar en su estudio para la obtención de nuevos ingredientes alimentarios de alto valor añadido.

Respecto a la **PLE**, las aplicaciones para la extracción de compuestos bioactivos del ajo fresco son todavía limitadas. Algunos trabajos previos describen el uso de esta técnica para la extracción de alicina o de compuestos fenólicos del ajo usando agua y mezclas hidroalcohólicas como extractantes (Farías-Campomanes *et al.*, 2014; Krstić *et al.*, 2023); sin embargo, no se ha aplicado

todavía para la obtención de extractos de ajo negro, ni para la revalorización de subproductos de ajo.

Además de la **selección de la técnica de extracción y de la optimización de sus condiciones de operación** para obtener el mayor rendimiento de los compuestos bioactivos de interés, resulta también imprescindible que la matriz vegetal empleada sea una fuente rica en dichos compuestos. En el caso de los alimentos procesados, como el ajo negro, el tratamiento al que se someten afecta a la formación o degradación de los compuestos bioactivos. Como se ha comentado en la Introducción, durante el proceso de envejecimiento del ajo, éste pierde parte de su contenido en alicina que se transforma en SAC, en mayor o menor medida en función del tiempo y la temperatura a la que es sometido. Por tanto, las **condiciones de envejecimiento** han de ser optimizadas para conseguir la mayor concentración de SAC en ajo negro. Sin embargo, esta optimización debe tener también en cuenta las **características organolépticas del producto final**, por las que el ajo negro presenta gran aceptación por parte del consumidor (sabor entre dulce y salado, olor suave, etc.). Es sabido que procesos de envejecimiento más suaves (temperaturas bajas en torno a 40 °C y tiempos reducidos de hasta 15 días) permiten obtener mayores concentraciones de SAC, pero dan lugar a un ajo negro con características organolépticas que podrían no ser las adecuadas (Bae *et al.*, 2014; Putra *et al.*, 2019). En cuanto a la composición volátil del ajo negro, existen algunos estudios sobre la identificación y caracterización de los principales compuestos responsables de su aroma (Abe *et al.*, 2019, 2020; Yang *et al.*, 2019). Además, también hay estudios que analizan el contenido y la evolución los compuestos volátiles del ajo durante el proceso de envejecimiento cuando se modifican las condiciones de procesado, como variaciones en la temperatura, el tiempo de envejecimiento y la humedad relativa (Molina-Calle *et al.*, 2017; Ríos-Ríos *et al.*, 2019). Si bien hay trabajos que evalúan conjuntamente la composición volátil y algunas propiedades físico-químicas (*e.g.* actividad antioxidante, contenido total en polifenoles, etc.) (Ríos-Ríos *et al.*, 2019; Najman *et al.*, 2022; Nam *et al.*, 2022; Yuan *et al.*, 2022), la **evaluación simultánea de los cambios en el contenido en SAC y en la composición aromática del ajo negro**, en función de las condiciones de procesado, es limitada hasta el momento (Cinar *et al.*, 2022), siendo de interés para la selección de la fuente vegetal óptima y la posterior obtención de extractos bioactivos de ajo negro.

4.1.2. Fraccionamiento de compuestos bioactivos

En la mayoría de los casos, los procesos de extracción de compuestos bioactivos llevan implícita la co-extracción de otros compuestos, bien de la misma o de diferente naturaleza, que pueden interferir en sus propiedades bioactivas, siendo por tanto necesaria una etapa de fraccionamiento entre ellos. Uno de estos casos es la obtención de carbohidratos bioactivos, que se encuentran generalmente presentes en forma de mezclas complejas tanto en matrices naturales

como en productos de síntesis. Esta etapa de fraccionamiento es necesaria no sólo para posible uso como **ingredientes bioactivos**, sino también para su **análisis o caracterización**.

Son muchos los procesos de fraccionamiento que se han propuesto para la separación de carbohidratos bioactivos, tales como el empleo de membranas de filtración (ultra o nanofiltración), tratamientos con carbón activo o cromatografía de exclusión molecular (SEC). Sin embargo, aunque estas técnicas de fraccionamiento pueden ser útiles para la separación de carbohidratos de distinto grado de polimerización (DP), no son adecuadas para el fraccionamiento de carbohidratos del mismo DP en base a sus enlaces glicosídicos o composición monomérica. Para este fin, recientemente se ha propuesto el empleo de disolventes respetuosos con el medio ambiente como los líquidos iónicos (ILs) o los **disolventes eutécticos profundos naturales (NADESs)**. Los primeros, son sales con puntos de ebullición inferiores a 100 °C, compuestas por aniones orgánicos e inorgánicos y cationes orgánicos. Los ILs se consideraban respetuosos con el medioambiente, ya que minimizan su difusión en la atmósfera, y se han propuesto como alternativa ecológica a los disolventes orgánicos volátiles tradicionales para la separación selectiva de inositoles y carbohidratos prebióticos (Ruiz-Aceituno *et al.*, 2013, 2019; Carrero-Carralero *et al.*, 2014). Sin embargo, las opiniones sobre su toxicidad son contradictorias y no se puede garantizar que no lleguen al medioambiente y supongan un peligro para diferentes ecosistemas (Choi & Verpoorte, 2019). Debido a esta desventaja, existe un creciente interés por la búsqueda de nuevos disolventes que eviten estos problemas, pero que sean eficaces para el fraccionamiento selectivo de carbohidratos. Este podría ser el caso de los NADESs, mezclas de compuestos en relaciones molares específicas, que presentan un punto de fusión inferior al de cada uno de sus componentes por separado. Las mezclas eutécticas están constituidas por dos o más componentes (al menos uno de ellos sólido), que actúan como donadores de enlace de hidrógeno (HBD) o aceptores de enlace de hidrógeno (HBA). Los HBD son, con frecuencia, sales de amonio cuaternarias como el cloruro de colina (ChCl), mientras que los HBA comprenden aminas, ácidos carboxílicos, alcoholes, polialcoholes, amidas ácidas o carbohidratos, que aparecen como metabolitos primarios que coexisten en la naturaleza. Además de su alta biodegradabilidad, bajo coste y fácil preparación, los NADESs presentan una serie de propiedades fisicoquímicas que los hacen idóneos para su utilización en diferentes aplicaciones y, en este caso, para el fraccionamiento selectivo de carbohidratos. Sin embargo, es un campo escasamente abordado y requiere de una mayor investigación.

En el **Anexo 4** se presenta una revisión bibliográfica titulada “Advances and challenges in the selective extraction of low molecular weight carbohydrates using ionic liquids and deep eutectic solvents” de Jiménez-Amezcuca *et al.*, publicada en *Trends in Analytical Chemistry* 171 (2024) 117507. En esta revisión, que sirve como punto de partida en esta novedosa temática, se recogen

y discuten los fundamentos, ventajas e inconvenientes y aplicaciones de los ILs y los DESs para la extracción y fraccionamiento de carbohidratos de bajo peso molecular.

Considerando estos antecedentes, el objetivo de esta sección de la Tesis Doctoral se centró en el “Desarrollo de procesos de extracción de compuestos bioactivos eficaces y medioambientalmente respetuosos”.

La **Sección 4.2.** incluye el artículo titulado “Changes in the bioactive content and aroma profile of aged garlic with processing conditions” de Jiménez-Amezcu *et al.*, enviado para su publicación a la revista *LWT - Food Science and Technology*. En este trabajo se evalúan y seleccionan las condiciones óptimas de envejecimiento del ajo que simultáneamente maximizan el contenido en SAC, y en su isómero bioactivo, S1PC, y que proporcionan una mejor apreciación sensorial de este producto, al reducir su contenido en compuestos volátiles típicos del ajo. Para la determinación de ambos compuestos bioactivos (SAC y S1PC), se han empleado métodos por LC-MS previamente desarrollados en esta Tesis Doctoral, mientras que se han desarrollado en este trabajo nuevos métodos por SPME GC-MS y por SPME GC-O para la caracterización de la composición volátil de las muestras en estudio.

El artículo titulado “Simultaneous microwave-assisted extraction of bioactive compounds from aged garlic” de Jiménez-Amezcu *et al.* publicado en la revista *Journal of Chromatography A* (2023) 1704-464128 constituye la **Sección 4.3.** En este artículo se evalúa el empleo de diferentes disolventes y técnicas de extracción (MAE, UAE y SLE) para la obtención de extractos de ajo negro ricos en compuestos organosulfurados, aminoácidos y carbohidratos bioactivos. Una vez seleccionada la MAE como técnica de extracción más eficaz, se procedió en este artículo a la optimización de sus correspondientes condiciones de operación y a la aplicación de estas a diversas muestras de ajo negro.

La **Sección 4.4.** incluye el artículo, pendiente de publicación, “Evaluation of advanced extraction techniques to obtain bioactive enriched extracts from garlic (*Allium sativum* L.) by-products” de Jiménez-Amezcu *et al.* enviado para su publicación a la revista *Journal of Agriculture and Food Research*. En este trabajo se evalúa la composición multicomponente (compuestos organosulfurados, carbohidratos prebióticos y amino ácidos) de distintos subproductos del ajo (raíz, pieles, tallo y hojas), y se comparan diversas técnicas de extracción (UAE, PLE, y SLE), para la selección de la combinación subproducto-técnica que contribuya a una mayor revalorización de estos productos mediante la obtención de extractos ricos en compuestos bioactivos.

En relación al fraccionamiento de carbohidratos, el artículo “Natural Deep Eutectic Solvents for Solubility and Selective Fractionation of Bioactive Low Molecular Weight Carbohydrates” de

Jiménez-Amezcuca *et al.* publicado en la revista *Foods* (2023) 12, 4355 constituye la **Sección 4.5**. Dicho artículo aporta nuevos datos sobre la solubilidad de LMWCs en NADESs hidrofílicos basados en ChCl como HBA, con objeto de emplearlos para la separación selectiva de LMWCs bioactivos y no bioactivos. Como ejemplo de aplicación, los NADESs evaluados se utilizaron para el fraccionamiento de estos compuestos en mezclas binarias y de lactulosa con respecto a otros carbohidratos presentes en su mezcla de síntesis como lactosa, epilactosa o glucosa.

Finalmente, la **Sección 4.6** incluye el artículo “Use of choline chloride-based deep eutectic solvents for the selective separation of bioactive polyalcohols” de Jiménez-Amezcuca *et al* enviado para su publicación a la revista *Advances in Sample Preparation*. Este trabajo aporta nueva información sobre los datos de solubilidad a distinta temperatura de diferentes polialcoholes en los NADESs elegidos en la Sección anterior como más prometedores. La utilidad de estos NADESs para el fraccionamiento selectivo de polialcoholes bioactivos y monosacáridos ha sido evaluada en mezclas binarias y en muestras vegetales (apio y algarroba).

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4.2. Cambios en el contenido de bioactivos y en el perfil aromático del ajo envejecido durante las condiciones de envejecimiento

Changes in the bioactive content and aroma profile of aged garlic with processing conditions

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Changes in the bioactive content and aroma profile of aged garlic with processing conditions

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Abstract

Garlic (*Allium sativum* L.) is a popular culinary ingredient but also the natural source of a number of bioactives with a variety of health benefits which support their use as food supplements. However, the widespread use of garlic supplements is sometimes hampered by their strong flavor and aroma. To overcome these limitations, raw garlic is subjected to aging to provide aged garlic bulbs (AGB) with improved bioactivity and better sensory acceptance which can be further used for supplement manufacturing.

Aging of garlic bulbs (either untreated or crushed during their processing under mild conditions (Abg Cool-Tech™)) was optimized in terms of time (0-40 days). To that aim, a multicomponent characterization of AGB samples, including the LC-MS analysis of bioactive S-allyl-L-cysteine and S-1-propenyl-L-cysteine, the volatile profiling by SPME-GC-MS, and the identification of aged garlic key odourants by SPME GC-O was addressed for the first time. For comparative purposes, results from these samples were also compared with those of fresh garlic and of commercial black garlic samples.

Aging of garlic bulbs for 21 days, with no crushing, was proposed as a trade-off to meet the highest content of bioactives in the shortest processing time, together with the intended improvement of the aromatic profile, associated with a lower content of pungent organosulfur compounds and higher levels of volatiles providing fresh, green and sweet notes (hexanal, 2-hexenal and furfural).

These findings are a contribution to satisfy both industry and consumer demand for improved processing methods providing AGB with improved properties and better characterized.

Keywords: aged garlic, aging optimization, bioactives, S-allyl-L-cysteine (SAC), S-1-propenyl-L-cysteine (S1PC), volatiles, key odourants.

1. Introduction

Garlic (*Allium sativum* L.) is widely used, not only as a condiment for culinary applications, but also as the basis for a number of garlic-based products including garlic supplements, due to its wide variety of biological properties (Shang *et al.*, 2019). However, the consumption of supplements is sometimes conditioned by their strong aroma and taste, and its tendency to cause stomach upset in sensitive individuals (Rais *et al.*, 2023). To overcome these limitations, supplements based on deodorized garlic or the use of alternative sources such as aged garlic (AG) are increasingly being considered for the development of garlic supplements with improved organoleptic properties and better consumer acceptance.

Although fermentation of raw garlic with microorganisms (*Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Monascus pilosus*, etc.) (Kim *et al.*, 2011; Ríos-Ríos *et al.*, 2019; Nam *et al.*, 2022; Rais *et al.*, 2023) or by soaking in hydroalcoholic mixtures for an extended period of time (e.g. 15–20 % ethanol for at least 18 months) (Chen *et al.*, 2020) have been described for garlic aging, heating under controlled temperature (60–90 °C) and humidity conditions (70–90 %) for 1–3 months is by far the most common aging procedure implemented in industry (Kodera *et al.*, 2002; Xu *et al.*, 2015; Lee *et al.*, 2016; Yang *et al.*, 2019). As a result of non-enzymatic browning reactions (e.g. Maillard reaction), caramelization, and chemical oxidation taking place during aging of garlic bulbs, AG with a brown or even black colour (depending on processing conditions), sticky jelly-like texture, sweet taste, reduced pungency and a more pleasant aroma is obtained. Moreover, as compared to fresh garlic, AG provides enhanced antioxidant activity, cardiovascular benefits (e.g. reduction of high blood pressure and cholesterol levels), immune system reinforcement, anti-cancer effects, etc. (Ahmed & Wang, 2021), all of them supporting its preferred use for manufacturing of food supplements.

Among the most significant changes associated with garlic aging, the increase of bioactive molecules such as S-allyl-L-cysteine (SAC) and its stereoisomer S-1-propenyl-cysteine (S1PC) (Ahmed & Wang, 2021; Jiménez-Amezcuca *et al.*, 2023), or the reduction of other sulfur volatiles (e.g. allyl methyl sulfide, diallyl disulfide, etc.) responsible for the pungent garlic aroma have been reported (Molina-Calle *et al.*, 2017; Abe *et al.*, 2019). These changes have been described to be greatly affected by the pre-treatment (freezing, high-pressure, pulsed electric field, ultrasound (US), etc.) of garlic bulbs (Xu *et al.*, 2015; Chen *et al.*, 2020), as well as by the procedure and operating conditions followed for garlic aging (Bae *et al.*, 2014; Kang *et al.*, 2022).

Regarding bioactive content, SAC is one of the major sulfur-containing amino acid present in AG and is thought to be the main responsible for its beneficial health effects (antioxidant, anticancer, antihepatopathic and neurotrophic activities, among others) (Kodera *et al.*, 2002). The amount of this bioactive, noticeably higher than that of fresh garlic (Kodera *et al.*, 2002; Bae *et*

al., 2012; Rais *et al.*, 2023), is usually considered in the standardization of AG supplements and, therefore, a number of studies have aimed to select the processing conditions providing SAC-enriched AG. Thus, in a study by Chen *et al.*, (2020), US and freezing pre-treatments prior to heat processing were evaluated to obtain AG with high levels of this bioactive compound. Although a shorter US-treatment (2 vs 30 h) was shown to maintain the garlic whole appearance and original colour, the SAC content of garlic undergoing freezing prior to aging was significantly higher (5.46 vs 3.22 mg g⁻¹ dry weight) and, therefore, preferred.

On the other hand, SAC is formed by the enzymatic hydrolysis of γ -glutamyl-S-allyl-cysteine (γ -GSAC) by γ -glutamyl transpeptidase (γ -GTP), whose activity is affected by temperature (Kodera *et al.*, 2002). In agreement with this, a higher SAC content has been reported to be promoted by mild (below 60 °C) thermal processing of garlic bulbs, as higher temperatures give rise to an increased water loss leading to the reduction of γ -GTP activity (Bae *et al.*, 2014; Manoonphol *et al.*, 2023). Additional studies on γ -GTP activation by soaking garlic in CaCl₂ solutions prior to thermal treatment have also been reported to enhance SAC content of AG samples (Xu *et al.*, 2015).

A number of biological activities including immunomodulatory and antihypertensive effects have recently been ascribed to S1PC (Suzuki *et al.*, 2016; Matsutomo *et al.*, 2017). These findings together with its safety data have contributed to propose S1PC as a bioactive, additional to SAC, worthy of consideration in the standardization of AG preparations (Kodera *et al.*, 2017). However, this determination is not still widespread considered. As SAC, S1PC is only present in trace amounts in raw garlic, but its concentration may increase almost up to SAC levels depending on the AG processing conditions and procedure considered (Woo *et al.*, 2022).

Changes in the volatile composition of aged garlic with the pre-treatment of raw garlic bulbs prior to aging or with the aging conditions have been previously evaluated (Molina-Calle *et al.*, 2017; Abe *et al.*, 2019; Varga-Visi *et al.*, 2019; Manoonphol *et al.*, 2023). Thus, it has been reported that crushing of garlic followed by thermal processing give rise to a decrease in the content of organosulfur compounds (OSC) responsible for the pungent and unpleasant aroma of garlic (Tocmo *et al.*, 2017; Varga-Visi *et al.*, 2019). Similarly, aging at temperatures above 60 °C can lead to the volatilization or the formation to a higher extent of garlic key odourants, including those providing appealing notes (Varga-Visi *et al.*, 2019), but may also reduce AG bioactive content (Xu *et al.*, 2015; Ríos-Ríos *et al.*, 2019). Despite this background, no previous study has addressed the changes in AG aroma when garlic bulbs are subjected to crushing once the thermal processing has been initiated, in an attempt to provide a trade-off between AG functionality and organoleptic acceptance.

A number of methodologies based on solid phase microextraction (SPME) coupled to/ followed by gas chromatography-mass spectrometry (GC-MS) or GC-Olfactometry (GC-O) have been reported for the green and rapid isolation of garlic volatiles, and for the high resolution separation and identification of garlic odour-active compounds, respectively (Molina-Calle *et al.*, 2017; Abe *et al.*, 2019, 2020; Yang *et al.*, 2019; Hwang & Kim, 2022). Despite these methodologies have also been reported for the evaluation of the impact of processing conditions on AG aroma, no previous reference has considered volatile data in combination with bioactive content for the improved processing of aged garlic bulbs (AGB).

At the sight of these antecedents, aging of garlic bulbs (either untreated or crushed during their processing) under mild temperature conditions (Abg Cool-Tech™) was optimized in terms of aging time to provide AGB with improved bioactivity and sensory profile, by means of a multianalytical approach. To that aim, AG bioactives (SAC and S1PC) were determined by liquid chromatography-mass spectrometry (LC-MS), whereas SPME-GC-MS and SPME GC-O methods were optimized for volatile profiling and identification of aged garlic key odourants, respectively. Fresh garlic and commercial black garlic samples were also analysed for comparative purposes.

2. Material and methods

2.1. Standards and samples

Analytical standards (purity > 99%) of S-1-propenyl-L-cysteine (S1PC) and 1,2-dichlorobenzene were obtained from Sigma-Aldrich (St. Louis, MO, USA), and of S-allyl-L-cysteine (SAC) from Tokyo Chemical Industry (Zwiindrecht, Belgium).

Garlic samples under study included: sun dried fresh garlic (FG) cultivated in Las Pedroñeras (Castilla-La Mancha, Spain), and aged garlic samples obtained from FG using an innovative 100% natural aging process (Abg Cool-Tech™) under controlled temperature (< 65 °C) and humidity (60-90 %) conditions. In addition to aged garlic samples from untreated garlic bulbs (AGB), garlic bulbs subjected to crushing after 11 aging days (aged garlic crushed bulbs, AGCB) were also considered for aging. In both cases, sampling was done after 0, 14, 21, 28 and 40 days of thermal processing. Commercial black garlic samples (CBG1 and CBG2) were purchased online (details related to the aging processing were not specified). Before analysis, all samples were frozen, freeze-dried, ground to fine particles using an IKA A10 basic mill (IKA-Werke, Germany), sieved through a 500 µm mesh and stored in dry and dark conditions at room temperature.

2.2. Bioactive content

2.2.1. Sample preparation

Garlic samples (0.1 g) were subjected to extraction with 1 mL of Milli-Q water (Millipore, Bedford, MA, USA) using an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) operating at 35 °C for 25 min, 80 Hz of frequency and 30 % of power. The extracts were centrifuged (MiniSpin®, Eppendorf, Hamburg, Germany) at 4401 *g* for 10 min, diluted 10 times and filtered (0.2 µm x 13 mm, UptiDisc PTFE filters, Montluçon, France) prior to their LC-MS analyses. All experiments were carried out in triplicate.

2.2.2. LC-MS analysis

LC-MS analysis of garlic bioactives and other typical garlic polar compounds was carried out following the method previously described by Jiménez-Amezcu et al. (2023). An Agilent 1100 series system equipped with an autosampler, a quaternary pump, a temperature-controlled column compartment and a diode array detector coupled to a 6195 mass spectrometry detector provided with an electrospray ionization (ESI) source (Agilent Technologies, Santa Clara, CA, US) was used. Chromatographic separation was done using an InfinityLab Poroshell 120 Bonus-RP column (150 mm × 4.6 mm, 2.7 µm; Agilent Technologies) operating at 0.4 mL min⁻¹ and 30 °C. A binary gradient consisting of water (A) and acetonitrile (B), both with 0.1% formic acid, was performed as follows: 0–7 min, 7% B; 21 min, 60 % B; 22–32 min, 95 % B; 33–48 min, 7 % B. The injection volume was 5 µL.

ESI parameters were set as indicated below: capillary voltage, 3 kV; drying gas temperature (N₂, 99.5 % purity), 300 °C; drying gas flow, 12 L min⁻¹; nebulization pressure, 276 kPa and fragmentor voltage, 40 V. Mass spectra were simultaneously acquired in SCAN (90–2000 *m/z* range), and in SIM mode for the quasimolecular ion corresponding to the [M+H]⁺ adduct of both SAC and S1PC (*m/z* 162).

Compound identification was based on chromatographic retention and MS data and was confirmed, when possible, by co-injection of the corresponding commercial standards and by comparison with literature data (Woo *et al.*, 2022; Jiménez-Amezcu *et al.*, 2023, 2024). Quantitative analysis of bioactives was performed in triplicate using an external standard calibration curve of SAC (0.0005–0.05 mg mL⁻¹) and S1PC (0.0001–0.05 mg mL⁻¹). Data acquisition and processing were carried out using HP Chemstation Rev. B.04.02 software (Agilent Technologies).

2.3. Volatile profiling and key odourant analysis

2.3.1. Solid phase microextraction coupled to gas chromatography-mass spectrometry (SPME-GC-MS) analysis

Garlic volatiles were sampled in headspace (HS) mode by SPME using two different fibres (both from Supelco, Bellefonte, Palo Alto, USA): (i) a 75 μm carboxen/polydimethylsiloxane (C/PDMS) fiber and (ii) a 50/30 μm divinylbenzene/carboxen on polydimethylsiloxane (DVB/CAR/PDMS) fibre. First, the sample amount (20–200 mg) to be optimized, together with 0.5 mL of salty (NaCl) (Sigma Aldrich, St. Louis, MO, US) water and 0.1 mL of internal standard (2 $\mu\text{g mL}^{-1}$, 1,2-dichlorobenzene, Sigma-Aldrich), were placed into a 20 mL vial fitted with a screw cap and a predrilled PTFE-lined septum (Thermo Scientific™, MA, USA) and vortexed for 2 min. Other operating conditions such as extraction temperature (40–55 °C) and extraction (5–30 min) times were further optimized. Constant stirring (220 rpm) was set during the whole procedure.

Volatiles isolated by SPME ($n = 3$) were analysed on an Agilent 7890-5977A GC-MS system equipped with an CTC120 autosampler (both from Agilent Technologies, Stock-port, UK). The SPME fibre was desorbed into the injection port, after evaluation of different temperatures (200–250 °C) and splitless times (0.3, 0.75, 1.5 and 3 min), using a SPME liner (0.75 mm, Supelco Co.). Chromatographic separations were performed on a Zebron ZB-5MS 5% phenyl methyl polysiloxane capillary column (30 m x 0.25mm i.d., 0.25 μm film thickness; Phenomenex, CA, USA), using helium as carrier gas (0.9 mL min⁻¹). The oven temperature was 40 °C, rising by 4 °C min⁻¹ to 250 °C, and held for 8 min.

Mass spectra were recorded in electron impact (EI) mode at 70 eV, scanning the 40–350 m/z range. Quadrupole, source and interface temperatures were set at 150, 230 and 280 °C, respectively. For data acquisition and analysis, MSD ChemStation software (Agilent Technologies) was used.

Volatile compounds were tentatively identified by comparison of their experimental mass spectra with data from mass spectral libraries (Wiley 6.0, NIST 2020). Identifications were further confirmed by using linear retention indices (LRI) calculated from retention data of suitable n -alkanes (from C₆ to C₃₀) analysed under identical conditions. For all compounds, concentrations recovered by SPME (expressed as $\mu\text{g g}^{-1}$ dry sample) was calculated by comparing the peak area against that of the internal standard, using a response factor of 1.

2.3.2. SPME followed by GC-olfactometry (SPME GC-O) analysis

Analysis of garlic key odourants was carried out following the procedure previously optimized in section 2.3.1. (except for the sample amount, 20 mg), using a manual SPME holder from

Supelco rather than an automated device. For SPME GC-O analyses, an Agilent HP5890 Series II ODO 2 (SGE) GC-O system equipped with the same column used for SPME-GC-MS analysis was used. The outlet of this column was split between a flame ionisation detector (FID) and a sniffing port. After SPME isolation, volatiles were desorbed at 250 °C for 3 min in splitless mode onto five small loops of the column in a coil, which were cooled by using the solid carbon dioxide placed in a 250 mL beaker. Once desorption was complete, the solid carbon dioxide was removed. The oven temperature was held at 40 °C during desorption and for a further 1 min and then the temperature was raised at 4 °C min⁻¹ to 250 °C, and held for 5 min. Helium at 2.0 mL min⁻¹ was used as carrier gas and the detector temperature was set at 280 °C. *n*-Alkanes (C₇–C₄₀) were analysed under these operating conditions to obtain LRI for comparison with the GC-MS data.

Three assessors were involved in the detection and verbal description of the odour active components of the extracts. The sniffing time was approximately 20 min. Each odour was scored on a nine-point line scale (1-9) where 1-3 = weak, 4-6 = medium and 7-9 = strong.

2.4. Statistical analysis

Statistica 7.0 software (StatSoft, Inc., Tulsa, OK, USA) was used for the statistical analysis of data. Significance ($p < 0.05$) of differences was determined by the analysis of variance (ANOVA, Tukey test).

3. Results and Discussion

3.1. Evolution of the bioactive content with garlic aging

Taking into account the intended selection of the processing conditions giving rise to aged garlic with the highest content of bioactives (SAC and S1PC) and improved organoleptic properties, the quantitative determination by LC-MS of these two bioactives, together with the qualitative profiling of related compounds, in aged garlic samples subjected to different processing conditions was first addressed. For comparison purposes, these analyses were also carried out on the FG and CBG samples previously described in section 2.1.

As shown in **Figure 1**, the LC-MS profiles of both FG and AGB samples were characterized by a number of compounds typical of garlic, including isoalliin, alliin, SAC, S1PC, γ -GSAC and γ -glutamyl-S-1-propenyl-L-cysteine (γ -GS1PC), whose concentration were dependent on the sample considered. SAC and S1PC were present in all the samples under study (**Table 1**), except for commercial black garlic samples (CBG1 and CBG2), where only traces of these bioactives were detected. In both FG and garlic bulbs subjected to aging for 14-40 days, S1PC concentration was lower than that of SAC. In a study on raw garlic by Koderá *et al.* (2017), it was stated that γ -GS1PC, precursor of S1PC, is found at higher concentration than γ -GSAC, precursor of SAC.

However, as a consequence of heat treatment, γ -GS1PC is affected to a greater extent by S-oxidation and deglutamylation, giving rise to isoalliin or cycloalliin (Lee *et al.*, 2016; Kodera *et al.*, 2017), whereas the competitive hydrolysis of γ -GS1PC to S1PC is favoured with increasing processing times. This explanation would agree with the experimental results here reported.

In general, SAC and S1PC contents of AGB increased with aging time (**Table 1**), reaching the maximum values of 1.55 and 0.13 mg g⁻¹ dry sample after 21 and 28 aging days, respectively. From day 21 onwards, SAC values remained stable, whereas S1PC content significantly decreased after 40 aging days (0.047 mg g⁻¹ dry sample), according to the lower stability of this compound previously reported (Woo *et al.*, 2022).

As compared to FG (0.18 mg g⁻¹ dry sample), the aging process increased by nine the SAC concentration. These results are in good agreement with previous studies where aging was reported to increase the SAC content of fresh garlic in a ratio of 3-10 (Xu *et al.*, 2015: 98-198 μ g g⁻¹; Bae *et al.*, 2014: 19.61-124.67 μ g g⁻¹; Jiménez-Amezcuca *et al.*, 2023: 0.32-2.32 mg g⁻¹), depending on the garlic variety and harvesting conditions, as well as the aging process considered. S1PC levels of aged garlic samples were close to that of the FG sample here analysed (0.0995 μ g g⁻¹ dry weight), and similar to data previously reported by other authors such as Woo *et al.* (2022) for a number of aged garlic products (in the range 120-530 μ g g⁻¹ dry weight).

Regarding the processing of crushed garlic cloves, aging for 14 days was shown to provide a significantly ($p < 0.05$) higher content of SAC over that of the corresponding non-treated sample (AGB14). However, a sharp decrease from then onwards was observed, ending in a 2-fold lower level of this bioactive at the end of the process. Moreover, SAC content of AGCB at 14 days of aging was lower than that of AGB samples processed for intermediate times (21-28 days). Similarly, levels of S1PC in AGCB drastically decreased (45 fold) during processing and were always significantly lower than those of AGB, irrespective of the aging period considered.

3.2. Characterization of the aromatic composition of garlic samples

As previously mentioned, SPME has shown to be advantageous for the clean, fast and sensitive isolation of volatiles in a number of food matrices with different purposes (Merkle *et al.*, 2015; Xu *et al.*, 2016). Therefore, in this study the optimization of a SPME procedure for garlic volatile isolation and preconcentration, and the selection of other optimal chromatographic conditions, was considered before the application of SPME-GC-MS and SPME GC-O approaches for, respectively, garlic volatile fingerprinting and identification of key odourants in the aged garlic samples under study.

3.2.1. Optimization of the SME-GC-MS procedure

As selectivity of SPME greatly depends on the fiber coating considered, two fibers (C/PDMS and DVB/CAR/PDMS) with different characteristics were evaluated under identical experimental conditions (200 mg aged garlic, $T = 40\text{ }^{\circ}\text{C}$, $t_{eq} = t_{ext} = 15\text{ min}$) for sampling of garlic volatiles. Despite C/PDMS is recommended for isolation of high volatility compounds such as organosulfur compounds (*e.g.* allyl methyl sulfide, diallyl disulfide, 3-Vinyl-1,2-dithiacyclohex-5-ene), no noticeable differences in the recovery of these compounds were observed over the DVB/CAR/PDMS fiber. Moreover, this last fiber provided a richer GC-MS profile in the low-medium volatility range and, therefore, it was selected for further analyses.

The development of a miniaturized method for garlic volatile profiling was then addressed, not only to fulfil the requirements of green chemistry, but also to minimize the unpleasant effect associated with the handling of high amounts of garlic samples for analysis. To that aim, sample amount in the range 100-200 mg was evaluated. As garlic samples are rich in volatiles, 100 mg were shown to be an enough sample amount to provide a sensitive characterization of minor volatiles, at the same time preventing the unwanted detection of major compounds as saturated peaks in GC-MS chromatograms.

Considering 20 min as equilibrium time, the extraction temperature and time were further evaluated in the ranges previously described in section 2.3.1. ($40\text{--}55\text{ }^{\circ}\text{C}$ and $5\text{--}30\text{ min}$, respectively). Temperature has been usually described as one of the operating factors having a more significant effect on recovery of volatile recovery by SPME (Soria *et al.*, 2017). In this study, GC-MS profiles gathered at different temperatures were shown to be qualitatively similar in the whole elution range. However, the overloaded signals for some major garlic volatiles at the highest extraction temperatures evaluated, together with the intended preservation of the original volatile composition of garlic samples under study (with no thermal degradation artifacts), pointed at the selection of $40\text{ }^{\circ}\text{C}$ as optimal SPME extraction temperature. On the other hand, although longer extraction times (30 min) were shown to positively affect the recovery of several volatiles (*e.g.* 5-methyl-1,2,3,4-tetrathiane), this beneficial effect was not found to justify an extended analysis time and, therefore, 20 min were considered as a trade-off value for the intended characterization of the garlic samples under study.

Once optimal SPME operating conditions were selected (100 mg sample, $T = 40\text{ }^{\circ}\text{C}$, $t_{eq} = t_{ext} = 20\text{ min}$), the amount of volatiles thermally desorbed from the SPME fiber and further introduced in the GC column was evaluated by means of the optimization of the injector temperature and splitless time. Again, the combination of a high injector temperature and a short splitless time ($250\text{ }^{\circ}\text{C}$ for 45 s) provided a better detection as non-saturated peaks of some major organosulfur volatiles, and a better focusing of high volatility garlic compounds.

3.2.2. Identification and quantitation of aged garlic volatiles

The optimized SPME-GC-MS procedure was further applied to the characterization of the volatile composition of the different garlic samples under study. A total of 62 compounds with different functionality (organosulfur, alcohols, esters, aldehydes, ketones, furan derivatives and pyrazines) were identified in the garlic samples (FG, AGB, AGCB and CBG) here analysed. **Table S1** shows a compilation of these compounds together with their experimental and literature LRI. Moreover, **Figure 2** shows, for comparative purposes, the volatile profiles gathered by SPME-GC-MS for two of these samples: FG and aged garlic processed for 40 days (AGB40).

OSC were the predominant class of volatiles, with 34 compounds identified in the different garlic samples under study. Among them, 11 OSC were selected for their quantitative monitoring because of their significant role in garlic aroma and/or their higher variability throughout the aging process (**Table 2**). Most of OSC here detected were derivatives of alliin, an odourless sulfoxide generated from γ -glutamyl-S-allyl-L-cysteine by a series of reactions, including oxidation (Rais *et al.*, 2023). It has been described that when fresh garlic is cut, crushed, etc., alliinase enzyme is released and activated, converting alliin and the remaining S-alk(en)-yl-L-cysteine-S-oxides into sulfenic acid intermediates (Molina-Calle *et al.*, 2017; Abe *et al.*, 2020). In the cellular environment, these intermediates are quickly condensed into thiosulfonates such as allicin, which is the unstable precursor of a number of volatile OSC such as diallyl sulfide, diallyl disulfide, allyl methyl disulfide, diallyl trisulfide, among others (Kim *et al.*, 2011; Abe *et al.*, 2019). These compounds, experimentally detected at different concentrations in the garlic samples here analysed, have been reported to be responsible for the pungent aroma of FG, their concentration decreasing with aging (Colín-González *et al.*, 2012; Kang *et al.*, 2022), as experimentally evidenced in this study. Among major OSC, concentrations of diallyl sulfide and diallyl disulfide were the most variable within the garlic samples evaluated, with FG and AGB samples showing significantly higher contents of both compounds (in the ranges 1-5.4 and 10-38 $\mu\text{g g}^{-1}$ dry sample, respectively) than those of commercial black garlic samples (in the ranges 0.02-0.24 and 0.2-3 $\mu\text{g g}^{-1}$ dry sample, respectively).

Taking into account the intended application of the aged garlic samples here processed as the vegetable source used for manufacturing of supplements with higher bioactive content, but also with improved organoleptic properties (lesser content of pungent volatiles), the effect of crushing on the 11th day of processing (AGCB samples) was considered. As for AGB samples, most of OSC with unpleasant aroma showed a decrease with processing time, their contents being significantly lower than those of FG (*e.g.* 1-3.7 vs 5.4 μg diallyl sulfide g^{-1} dry sample for AGCB and FG, respectively), and higher than those of CBG samples. The mild conditions here employed

for garlic aging (Abg Cool-Tech™ technology), involving low processing temperatures, could justify these results.

In addition to unpleasant OSC, a number of volatiles from different chemical families and showing positive aromatic attributes (sweet, fruity, etc. notes) were found to increase their concentration during garlic aging. Thus, aldehydes such as furfural and benzaldehyde, present in low concentrations in FG, were detected at higher levels in garlic samples thermally processed (up to 0.22 µg furfural g⁻¹ dry sample in AGCB28 and up to 1.4 µg benzaldehyde g⁻¹ dry sample in AGB28), in agreement with previous references (Molina-Calle *et al.*, 2017; Bae *et al.*, 2020). As compared to CBG samples, benzaldehyde content was higher in the AGB21/AGB28 samples here processed, whereas furfural was significantly lower. Furfural, an aroma compound commonly formed in foods during the Maillard reaction (Gong *et al.*, 2021), together with other products of this non-enzymatic browning reaction such as 2-furanmethanol, have been previously detected in aged garlic (Ríos-Ríos *et al.*, 2019; Nam *et al.*, 2022), as a result of the thermal degradation of sugars, their content being dependent on the aging conditions employed (Molina-Calle *et al.*, 2017). In agreement with this, the mild conditions here employed for garlic aging (Abg Cool-Tech™ technology) would be responsible for the lower levels of these Maillard derivatives detected in untreated garlic bulbs, which were approximately ten times lower than those of CBG samples.

Regarding the alcohols showing a greater variability, 2-propen-1-ol (allyl alcohol) content of FG (0.78 µg g⁻¹ dry sample) was found to significantly increase during aging, particularly for garlic processed for up to 14-21 days (9.5 µg g⁻¹ dry sample for AGB21). As compared to crushed bulbs, untreated garlic provided values of this compound significantly higher irrespective of the aging period considered (*e.g.* 9.5 vs 4 µg g⁻¹ dry sample for AGB21 and AGCB21, respectively). These results match well with those published by Martínez-Casas *et al.* (2017), who detected that 2-propen-1-ol was not detected in purple garlic but it was abundant in black garlic, and with those of Kim *et al.* (2011), who reported a 2-fold increase of 2-propen-1-ol when raw crushed garlic was subjected to a high temperature treatment (72 °C for 14 days) to obtain black crushed garlic.

3.2.3. Analysis of aged garlic key odourants

As previously mentioned, optimal processing conditions should be chosen as a trade-off to provide aged garlic samples that meet the highest content of bioactives (SAC and S1PC), the lowest level of unpleasant volatiles (*e.g.* OSC) and a processing time as short as possible. Taking into account these considerations, the intended balance between bioactivity, organoleptic properties and processing efficiency was found for garlic samples subjected to aging for 21 days (AGB21 and AGCB21 samples) and, therefore, these samples were chosen for further analyses.

The GC-O technique was applied to these samples, and for comparison to FG and CBG2, for evaluation of their key odourants. Identification of the most active odour compounds was done in combination with SPME-GC-MS data (**Table 1S**). As shown in **Table 3**, GC-O analyses yielded a total of 13 odour-active compounds which were mainly detected in the FG and aged garlic sample analysed. OSC (dimethyl sulfide, diallyl sulfide, diallyl disulfide, etc), which are responsible for the sulfurous odour of FG, have been described to undergo decomposition or transformation into other substances during the heat processing of black garlic. As a result of the reduction of these compounds, the pungent smell of black garlic is significantly diminished compared to its white counterpart (Kilic-Buyukkurt *et al.*, 2023). In this study, odour scores for several OSC such as allyl mercaptan and, particularly for diallyl sulfide, in AGB21 were found to be noticeably lower than those of FG. Notably, OSC were less strongly associated with the overall odour profile of AGCB, and dimethyl sulfide was the sulfur volatile whose odour score was the highest irrespective of the sample considered.

On the other hand, fresh, green and sweet were the characteristic notes of aged garlic which were mainly associated to compounds such as hexanal, 2-hexenal and furfural. As shown in **Table 3**, the scores for these odour active compounds were found to be affected by the garlic bulb treatment and processing. In agreement with these experimental results, it has been described the important role of processing conditions on the sensory properties of black garlic (Najman *et al.*, 2022; Ríos-Ríos *et al.*, 2019), as well as the influence of furan derivatives (furfural, 2-furanmethanol, etc.) and aldehydes (*e.g.* hexanal) on appealing flavour notes of aged garlic (Abe *et al.*, 2019; Ríos-Ríos *et al.*, 2019).

4. Conclusion

A multianalytical approach based on LC-MS, SPME-GC-MS and SPME GC-O analyses has been used to evaluate the differences in the bioactive content and aroma (volatile composition and odour active compounds) of aged garlic samples that can be attributed to either the processing period or the pre-treatment of garlic bulb, when compared to fresh garlic and commercial black garlic. As for the intended use of aged garlic samples here processed for manufacturing of supplements, samples subjected to aging for 21 days were chosen as a trade-off to meet the highest content of SAC, as supplements are standardized and valued for the content of this bioactive, and a processing time as short as possible. As regards the volatile profile of these samples, playing a significant role on consumer's acceptance, crushing pre-treatment was found to slightly increase the loss of pungent OSC and to increase the levels of other compounds with desirable odours such as furfural, all of them having an important role on the sensory profile of aged garlic samples, as evidenced by their corresponding odour scores. However, and taking into account the significant detriment of aged garlic bioactives associated with crushing, this pre-treatment was ruled out and,

therefore, AGB21 aging conditions were selected as optimal. This decision was also supported by the easier processing usually preferred by industries when this does not have a significant effect for the intended application. To conclude, the findings here presented are a valuable contribution in the food science and technology field as they have addressed for the first time the optimization of aged garlic processing with regard both its bioactive composition and aromatic profile. In addition to shed light on the changes in volatile composition during the transition from fresh garlic to black garlic and the possible reactions taking place during this process, the results from this study deep insight into of the key aromatic components of black garlic.

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Table 1. Evolution of SAC and S1PC content (mg g⁻¹ dry sample) of fresh garlic (FG), aged garlic bulb (AGB) and aged garlic crushed bulb (AGCB) samples with aging time (up to 40 days).

Aging time (days)	[SAC] (mg g ⁻¹ dry sample)		[S1PC] (mg g ⁻¹ dry sample)	
0 (FG)	0.18 ^{*a} (0.01)		0.099 ^a (0.006)	
	AGB	AGCB	AGB	AGCB
14	1.24 ^{b,1} (0.02)	1.46 ^{b,2} (0.02)	0.121 ^{b,c,1} (0.003)	0.089 ^{b,2} (0.001)
21	1.55 ^{c,1} (0.06)	1.12 ^{c,2} (0.01)	0.111 ^{a,b,1} (0.006)	0.037 ^{c,2} (0.001)
28	1.5 ^{c,1} (0.1)	1.01 ^{d,2} (0.04)	0.13 ^{c,1} (0.01)	0.023 ^{d,2} (0.001)
40	1.48 ^{c,1} (0.05)	0.74 ^{e,2} (0.03)	0.047 ^{d,1} (0.002)	0.002 ^{e,2} (0.001)

* Average ($n = 3$) and standard deviation in brackets.

^{a-e} Different letters within the same column indicate significant ($p < 0.05$) differences for SAC and S1PC content associated with aging time.

^{1,2} Different numbers within the same row indicate significant differences ($p < 0.05$) for SAC and S1PC content associated with the crushing treatment of aged garlic bulbs.

Table 2. Concentrations ($\mu\text{g g}^{-1}$ dry sample) for selected volatiles determined by SPME-GC-MS in fresh garlic (FG), aged garlic samples processed for different times (14, 21, 28 and 40 days) from bulbs (AGCB) and crushed bulbs (AGC) and commercial black garlic samples (CBG).

LRI ¹	Compounds ²	FG	14 days		21 days		28 days		40 days		CBG1	CBG2
			AGB	AGCB	AGB	AGCB	AGB	AGCB	AGB	AGCB		
Thiols												
598	Allyl mercaptan	0.87 (0.04)	0.88 ^a (0.05)	0.86 ^e (0.03)	0.54 ^{b,1} (0.03)	0.372 ^{f,2} (0.03)	0.50 ^b (0.02)	0.33 ^f (0.05)	0.23 ^c (0.02)	0.22 ^g (0.03)	-	0.08 (0.04)
	Monosulfides											
698	Allyl methyl sulfide	1.2 (0.6)	0.28 ^a (0.06)	0.2 ^e (0.1)	0.24 ^{a,1} (0.06)	0.17 ^{e,1} (0.06)	0.2 ^a (0.1)	0.16 ^e (0.04)	0.10 ^a (0.04)	0.3 ^e (0.1)	0.09 (0.03)	0.142 (0.001)
862	Diallyl sulfide	5.4 (0.4)	3.5 ^a (0.3)	3.7 ^e (0.9)	2.5 ^{a,b,1} (0.8)	2 ^{f,1} (1)	2.0 ^{b,c} (0.8)	1.3 ^f (0.5)	1.0 ^c (0.4)	1.0 ^f (0.3)	0.02 (0.02)	0.24 (0.07)
Disulfides												
746	Dimethyl disulfide	0.47 (0.01)	0.06 ^a (0.01)	0.04 ^e (0.01)	0.054 ^{a,b,1} (0.004)	0.03 ^{e,2} (0.01)	0.06 ^a (0.02)	0.03 ^e (0.01)	0.03 ^b (0.01)	0.08 ^f (0.02)	0.4 (0.1)	0.22 (0.02)
922	Allyl methyl disulfide	15.1 (0.5)	3.1 ^a (0.1)	2 ^e (0.2)	2.7 ^{a,1} (0.1)	1.4 ^{f,2} (0.3)	2.5 ^a (0.5)	1.1 ^f (0.1)	1.3 ^b (0.1)	1.8 ^e (0.2)	0.5 (0.2)	1.4 (0.5)
1092	Diallyl disulfide	38 (7)	24 ^a (5)	23 ^e (3)	19 ^{a,1} (3)	14 ^{f,2} (1)	18 ^a (1)	11 ^{f,g} (1)	10 ^b (1)	7 ^g (1)	0.2 (0.1)	3 (2)
Trisulfides												
978	Dimethyl trisulfide	3.31 (0.03)	0.35 ^a (0.03)	0.17 ^e (0.01)	0.25 ^{b,1} (0.01)	0.09 ^{f,2} (0.02)	0.22 ^b (0.01)	0.09 ^f (0.01)	0.1 ^c (0.01)	0.20 ^g (0.05)	0.2 (0.1)	0.4 (0.1)
1153	Allyl methyl trisulfide	18 (4)	9 ^a (2)	6 ^e (1)	6 ^{b,1} (2)	1.7 ^{f,2} (0.2)	4.7 ^b (0.4)	1.5 ^f (0.3)	1.7 ^c (0.2)	1.4 ^f (0.3)	0.12 (0.08)	2.4 (0.02)
1318	Diallyl trisulfide	22 (3)	30 ^a (2)	26 ^e (2)	21 ^{b,1} (1)	11 ^{f,2} (1)	18 ^b (0.5)	9 ^f (1)	7.2 ^c (0.3)	3.1 ^g (0.2)	-	2 (0.5)
Cyclic sulfides												
1171	4-Methyl-1,2,3-trithiolane	2.4 (0.4)	6 ^a (1)	4.6 ^e (0.4)	3.6 ^{a,1} (0.3)	1.8 ^{f,2} (0.2)	3.3 ^a (0.2)	1.6 ^{f,g} (0.2)	1.4 ^b (0.1)	0.7 ^g (0.1)	0.01 (0.008)	0.98 (0.01)

Table 2. Cont.

1229	3-Vinyl-1,2-dithiacyclohex-4-ene	3.7 (0.5)	1.6 ^a (0.6)	0.9 ^e (0.3)	1 ^{ab,1} (0.3)	0.4 ^{f,2} (0.1)	0.8 ^{ab} (0.2)	0.3 ^f (0.1)	0.3 ^b (0.1)	0.1 ^f (0.03)	-	0.07 (0.04)
Alcohols												
566	2-propen-1-ol	0.78 (0.01)	2.9 ^a (0.1)	2.4 ^e (0.2)	7.3 ^{b,1} (0.2)	4.6 ^{f,2} (1.0)	9.5 ^c (0.7)	4 ^f (0.5)	8 ^b (1)	2.1 ^e (0.4)	0.16 (0.01)	1.2 (0.03)
776	2,3-butanediol	-	0.09 ^a (0.05)	0.13 ^e (0.06)	0.1 ^{ab,1} (0.01)	0.3 ^{e,f,1} (0.2)	0.12 ^a (0.05)	0.18 ^{e,f} (0.05)	0.15 ^a (0.03)	0.4 ^f (0.1)	0.09 (0.03)	0.01 (0.001)
Aldehydes												
541	2-propenal	0.4 (0.1)	0.13 ^a (0.02)	0.15 ^e (0.01)	0.2 ^{b,1} (0.01)	0.09 ^{f,2} (0.01)	0.18 ^b (0.01)	0.08 ^f (0.01)	0.12 ^a (0.01)	0.04 ^e (0.008)	0.01 (0.001)	0.02 (0.001)
965	Benzaldehyde	0.39 (0.04)	0.5 ^a (0.2)	1.2 ^e (0.3)	1.2 ^{b,1} (0.3)	1.2 ^{e,1} (0.1)	1.4 ^b (0.2)	0.8 ^e (0.1)	0.6 ^a (0.08)	0.18 ^f (0.04)	0.05 (0.02)	0.08 (0.03)
Pyrazines and furan derivatives												
823	Methyl pyrazine	-	0.015 ^a (0.003)	0.012 ^e (0.001)	0.06 ^{b,1} (0.01)	0.12 ^{f,2} (0.01)	0.10 ^c (0.01)	0.16 ^g (0.01)	0.12 ^d (0.003)	0.1 ^f (0.002)	0.007 (0.001)	0.27 (0.05)
833	Furfural	0.005 (0.001)	0.01 ^a (0.003)	0.10 ^e (0.01)	0.07 ^{b,1} (0.01)	0.19 ^{f,2} (0.01)	0.15 ^c (0.01)	0.22 ^f (0.03)	0.14 ^c (0.01)	0.21 ^f (0.02)	1.5 (0.3)	2.2 (0.6)
854	2-Furanmethanol	-	-	0.01 ^e (0.002)	0.004 ^{a,1} (0.001)	0.15 ^{f,2} (0.004)	0.02 ^b (0.0003)	0.26 ^g (0.04)	0.03 ^c (0.003)	0.32 ^g (0.05)	0.015 (0.002)	0.015 (0.005)

¹ Experimental linear retention index (LRI) on DB-5 column, calculated from retention data of *n*-alkanes (C₆-C₃₀) analysed under identical experimental conditions.

² Compounds identified by comparing the LRI value and mass spectral data with mass spectral libraries (Wiley, NIST).

* Average ($n = 3$) and standard deviation in brackets.

^{a-d} Different letters within the same row indicate significant ($p < 0.05$) differences for AGB samples associated with aging time.

^{e-h} Different letters within the same row indicate significant ($p < 0.05$) differences for AGCB samples associated with aging time.

^{1,2} Different numbers within the same row indicate significant differences ($p < 0.05$) for AGB and AGCB samples processed for the same aging time.

-: Non detected

Table 3. Aroma descriptors and GC-O odour scores for volatiles identified in selected garlic samples.

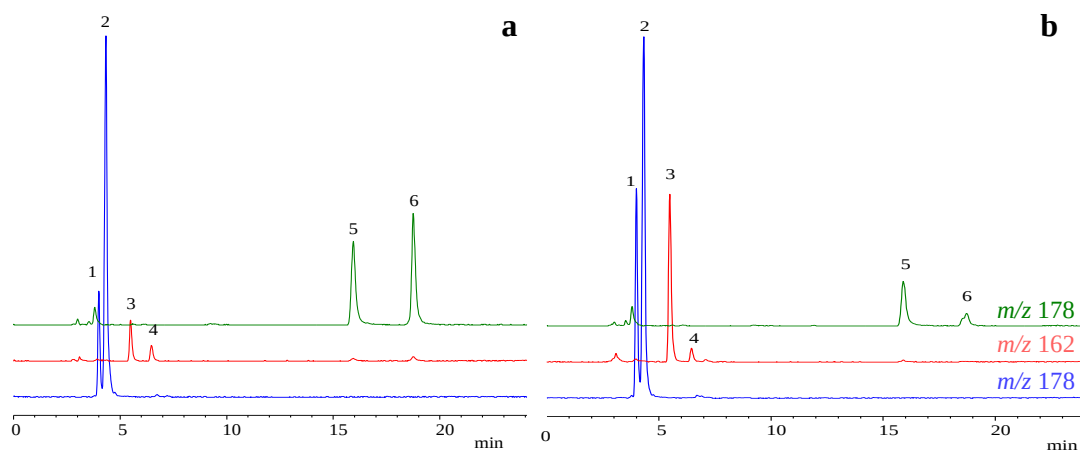
Experimental LRI ^a		Aroma descriptors	Identity	GC-O Score ^{b,c}			
GC-O	GC-MS			FG	AGB21	AGCB21	CBG2
<600	556	Sulfurous Onion Green	Dimethyl sulfide	6	9	6	3
<600	566	Pungent Mustard	2-Propen-1-ol	-	4	4	1
601	598	Sulfurous Garlic	Allyl mercaptan	5	-	-	-
612	616	Garlic cooked	Methyl-thiirane	-	3	4	-
703	698	Garlic	Allyl methyl sulfide	1	3	4	2
799	800	Fresh Green Fatty	Hexanal	4	1	6	-
829	833	Almond-like Sweet	Furfural	-	-	2	4
847	853	Fresh Green	2-Hexenal	3	2	6	-
853	854	Musty Sweet	2- Furanmethanol	3	3	5	-
865	862	Garlic Metallic Meaty	Diallyl sulfide	5	1	-	2
874	874	Herbaceous, sweet	5-Methyl-3(2H)- furanone	3	-	3	7
920	922	Sulfurous Garlic	Methyl allyl sulfide	5	4	4	-
972	978	Sulfurous Meaty	Dimethyl trisulfide	5	4	5	-
1089	1092	Strong garlic	Diallyl disulfide	5	5	5	4

^a Experimental linear retention index (LRI) on DB-5 column for volatiles detected by SPME GC-O analysis and for volatiles separated and identified by SPME-GC-MS analysis.

^b GC-O score for fresh garlic (FG), aged garlic bulbs and aged garlic crushed bulbs processed for 21 days (AGB21 and AGCB21, respectively) and for a commercial black garlic sample (CBG2).

^c Odour score: weak (1-3), medium (4-6) and strong (7-9).

581



582

583 **Figure 1.** LC-MS profile of garlic **(a)** before aging (FG) and **(b)** after 21 days of processing (AGB
584 21). Labeled peaks are as follows: (1) Isoalliin, (2) Alliin, (3) *S*-allyl-L-cysteine (SAC), (4) *S*-1-
585 propenyl-L-cysteine (S1PC), (5) γ -glutamyl-*S*-allyl-L-cysteine (γ -GSAC) and (6) γ -
586 glutamyl-*S*-1-propenyl-L-cysteine (γ -GS1PC).

587

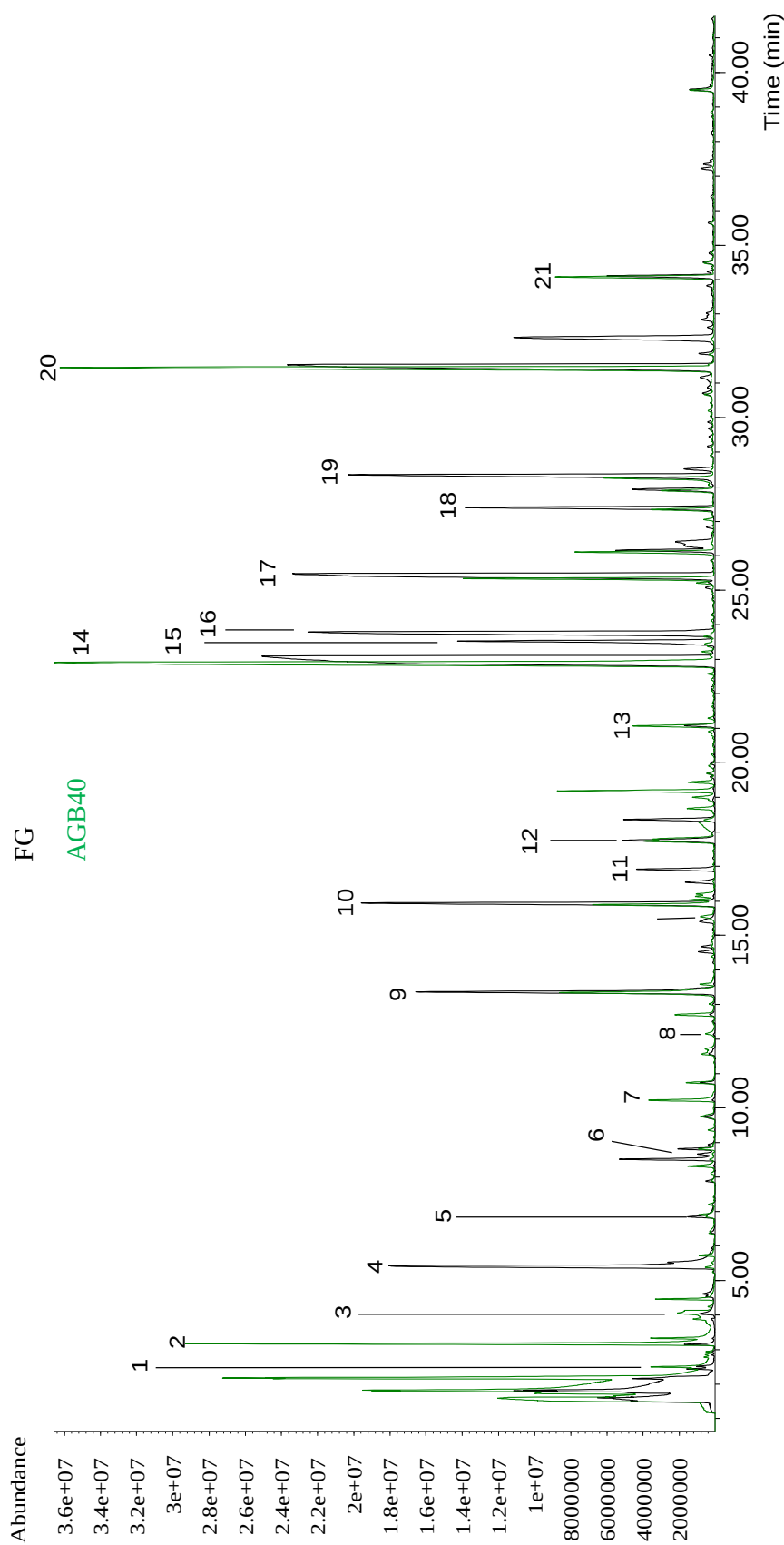


Figure 2. Chromatographic profiles of FG (black) and AGB40 (green) samples. 1. 2-Propenal, 2. 2-Propen-1-ol, 3. Allyl mercaptan, 4. 2-Butenal, 5. Allyl methyl sulfide, 6. Dimethyl disulfide, 7. 5-Hexenal, 8. Furfural, 9. Diallyl sulfide, 10. Allyl methyl sulfide, 11. Benzaldehyde, 12. 3-H-1,2-Dithiole, 13. Internal Standard, 14. Diallyl disulfide, 15. (Z)-1-propenyl propyl disulfide, 16. (E)-1-propenyl-2-propen-1-yl disulfide, 17. Allyl methyl trisulfide, 18. 4-Methyl-1,2,3-trithiolane, 19. 3-Vinyl-1,2-dithiacyclohex-5-ene, 20. Diallyl trisulfide, 21. 5-Methyl-1,2,3,4-tetrathiane.

4.3. Extracción asistida por microondas de compuestos bioactivos de ajo envejecido

Simultaneous microwave-assisted extraction of bioactive compounds from aged garlic

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Simultaneous microwave-assisted extraction of bioactive compounds from aged garlic

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ABSTRACT

In this work, the simultaneous extraction of bioactives (organosulfur compounds, such as S-allyl-L-cysteine (SAC), carbohydrates, such as neokestose and neonytose, and total phenolic compounds) from aged garlic has been optimized for the first time to obtain multifunctional extracts for further application as food ingredients. Analytical methods using liquid chromatography coupled to mass spectrometry (HPLC-MS) and by hydrophilic interaction liquid chromatography with evaporative light scattering detection (HILIC-ELSD) were also previously optimized. High sensitivity (limits of detection between 0.013 and 0.77 $\mu\text{g mL}^{-1}$) and appropriate repeatability ($< 12\%$) and accuracy ($> 92\%$) for the analysis of bioactives were achieved. After selecting water as the extraction solvent and microwave-assisted extraction (MAE) as the most efficient technique, operation conditions were optimized using a Box-Behnken experimental design (60 min; 120 °C; 0.05 g mL^{-1} ; 1 cycle) to maximize the content of bioactives from different aged garlic samples. Regarding organosulfur compounds, only SAC (traces–2.32 mg g^{-1} dry sample) and cycloalliin (1.23–3.01 mg g^{-1} dry sample) were detected in all samples, while amino acids such as arginine (0.24–3.45 mg g^{-1} dry sample) and proline (0.43–3.91 mg g^{-1} dry sample) were, in general, the most abundant. Bioactive carbohydrates (from trisaccharides to nonasaccharides) were only detected in fresh garlic and aged garlic processed under mild conditions, whereas all garlic extracts showed antioxidant activity. The developed MAE methodology is shown as a successful alternative to other procedures for the simultaneous extraction of aged garlic bioactives intended by the food and nutraceutical industries, among others.

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1. Introduction

Garlic (*Allium sativum* L.) is highly consumed all over the world as a food, condiment and dietary supplement, because of both its organoleptic characteristics and numerous health benefits. Among them, the antioxidant, antimicrobial, anticancer, anti-inflammatory, antihyperglycemic and antihypertensive activities deserve to be highlighted [1,2]. Despite the many beneficial properties of garlic, its consumption is sometimes conditioned by its slightly unpleasant flavor and aroma, and its tendency to cause upset stomachs in sensitive individuals. To overcome these limitations, and to provide an alternative that is richer in bioactives, either for direct consumption or for further application as a dietary supplement, raw

garlic is subjected to an aging process to produce aged garlic (also known as black garlic).

Although a wide variety of processing methods, including heat treatment, fermentation, soaking in hydroalcoholic mixtures, etc., have traditionally been used or reported in the literature for garlic aging [1,3,4], garlic heating for a preset time under controlled temperature and humidity conditions is usually the method of choice by the food and nutraceutical industries. However, the wide variety of processing conditions (ranging from 13 to 69 days of incubation at 60–85 °C and 60–90% humidity) that can be applied greatly condition the composition of the final product and, therefore, the quality, organoleptic properties and bioactivity of commercial aged garlic [5].

The aging process changes the chemical composition of the garlic [6,7], for instance, producing the bioactive S-allyl-L-cysteine (SAC) and other organosulfur compounds (OSC) including allicin, diallyl sulfide, diallyl disulphide, ajoene, etc. [5]; degrading

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polysaccharides to monosaccharides or oligosaccharides [8], whose degree of polymerization (DP), and therefore prebiotic properties [9,10], depends on the thermal treatment applied; and producing 5-hydroxymethylfurfural (HMF) and other Maillard reaction products [4,6]; etc. Although these compounds have been reported to provide various health benefits to aged garlic (antioxidant, prebiotic, anti-inflammation, etc.), increasing its therapeutic potential for various human diseases [2,4,11–14], the overall benefit of black garlic should always be considered as the result of the synergistic effect of all them [15]. However, food supplements based on black garlic only standardize their SAC content and no data is generally provided on additional bioactive compounds. It is therefore important, particularly when multifunctional extracts are intended for their use as food supplements, to maximize the extraction not only of SAC, but also of other garlic bioactives.

Although conventional solid-liquid extraction (SLE) methods are most commonly used to obtain SAC-enriched extracts from garlic [16], other techniques such as ultrasound-assisted extraction (UAE) [17] or microwave-assisted extraction (MAE) have been proposed to overcome the limitations of conventional extraction regarding efficiency, extraction time and solvent consumption [18,19]. However, so far the application of these techniques for the extraction of bioactive compounds from aged garlic is limited and mainly refers to the extraction of phenolic compounds [20] and allicin [21]. Further studies are therefore required to evaluate the full potential of these advanced extraction techniques for the multicomponent extraction of aged garlic bioactives.

It is known that extraction conditions (i.e., temperature, pressure, type of solvent, technique, etc.) significantly influences the amount and type of bioactive compounds extracted and thus the biological activity of the extract [19]. Multivariate experimental design approaches, in which the effect of different operating parameters and their interactions are considered for selection of optimal operating conditions, have been described to be good alternatives to univariate procedures to optimize the extraction [22,23]. This sample preparation step is also crucial for the analytical determination of garlic bioactives, as it conditions not only the required isolation or preconcentration of target analytes, the removal of matrix interferences, etc., but also the technique(s) to be used for analysis [24].

Therefore, this work evaluates the efficiency of SLE, UAE and MAE for the simultaneous extraction of OSC, bioactive carbohydrates and total phenolics from aged garlic for the first time. MAE conditions were carefully optimized using an experimental design and then applied to the extraction of these bioactives from different commercial aged garlic samples. A comparative analysis with fresh garlic was also carried out. Moreover, analytical methods using liquid chromatography coupled to mass spectrometry (HPLC-MS) and by hydrophilic interaction liquid chromatography (HILIC) with evaporative light scattering detector (ELSD) were also previously optimized and validated for the required characterization of the different garlic extracts under study.

2. Material and methods

2.1. Standards

Analytical standards of fructose, glucose, sucrose, gallic acid, HMF, amino acid standard mixture (AAS18) and γ -glutamyl-cysteine were obtained from Sigma-Aldrich (St. Louis, MO, US). S-allyl-L-cysteine (SAC) was acquired from TCI (Tokyo Chemical Industry, Zwiindrecht, Belgium), while 1-kestose [Fru-(2-1)-Fru-(2-1)-Glc] and nystose [Fru-(2-1)-Fru-(2-1)-Fru-(2-1)-Glc] were purchased from TargetMol (Wellesley Hills, MA, US).

Neokestose [Fru-(2-6)-Glc-(1-2)-Fru] and neonytose [Fru-(2-6)-Glc-(1-2)-Fru-(1-2)-Fru] were synthesized following the pro-

cedure described by Gimeno-Perez et al. [9] using a β -fructofuranosidase of *Xanthophyllomyces dendrorhous*, generously provided by Dr. F. Plou (IC, CSIC, Spain) and Dr. M. Fernández-Lobato (CBM, CSIC, Spain). The synthesis was followed by gas chromatography coupled to mass spectrometry (GC-MS) and neokestose and neonytose structures were confirmed as indicated by Ruiz-Matute et al. [25].

2.2. Samples

Fresh garlic (FG), cultivated in Las Pedroñeras (Castilla-La Mancha, Spain) and brown aged garlic (AG1) produced from FG using an innovative 100% natural process (ABG Cool-Tech™) which employs controlled temperature (<65 °C) and humidity conditions (60–90%) for an aging period of 30–90 days, were provided by Pharmactive Biotech Products S.L.U. Commercial samples of black aged garlic (AG2-AG6) were purchased online; details on processing conditions were not specified in their labeling. All samples were analyzed prior to their expiration date.

As sample pretreatment before extraction, fresh garlic cloves from four garlic heads were peeled, cut, frozen and freeze-dried. FG and AG1 samples were ground to fine particles using an IKA A10 basic mill (IKA-Werke, Germany), sieved through a 500 μ m mesh and stored in dry, hermetically sealed containers protected from light at ambient temperature until extraction. Although AG2-AG6 samples were also subjected to freeze-drying, moisture could not be totally removed and dry matter was gravimetrically measured according to the AOAC method [26]. This determination was made in triplicate.

2.3. Solid-liquid extraction (SLE)

Prior to the evaluation of the effect of different extraction conditions on the composition of FG and AG extracts, the most appropriate solvent was selected. Three milliliters of different extractants [Milli-Q water, ethanol, methanol, ethanol:water (1:1, v:v) and methanol:water (1:1, v:v)] were added to 300 mg of AG1 powder and were stirred (700 rpm) at 70 °C for 30 min in a heating plate (IKA-Werke, Staufen, Germany). The extracts obtained were immediately centrifuged at 4401 g for 10 min. Supernatants were collected and diluted (1:10, v/v) for further chromatographic analyses. All experiments were carried out in triplicate.

2.4. Ultrasound-assisted extraction (UAE)

AG1 sample (300 mg) was subjected to extraction with 3 mL of Milli-Q water using an ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) operating at 70 °C for 30 min, 80 Hz of frequency and 30% of power. UAE extracts were centrifuged and diluted as previously described in Section 2.2. All experiments were carried out in triplicate.

2.5. Microwave-assisted extraction (MAE)

MAE extractions were carried out using MARS6 equipment (CEM, Matthews, NC, USA) provided with X-Press 1500 vessels (CEM) and an optic fiber (MTS-300, CEM, Matthews, NC, USA) for temperature control. Microwave power was set at 1200 W and solvent volume to 10 mL for all experiments. After completion of the extraction time, MAE vessels were immersed in an ice-water bath to stop extraction. MAE extracts were subsequently centrifuged and diluted as described in Section 2.3.

2.5.1. Experimental design

MAE conditions were optimized following a Box-Behnken experimental design using an AG1 sample. The effect of three independent variables [sample amount (s); temperature (T) and time

(*t*) on extraction of SAC, bioactive carbohydrates and total phenolic content (TPC) was evaluated. A total of 15 experiments were carried out in random order and three central points were considered for error estimation. Experimental ranges for factors evaluated were: *T* = 40–120 °C, *t* = 5–60 min, and *s* = 0.5–1.5 g. Each experiment was carried out in triplicate.

The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_3 s + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{3,3} s^2 + \beta_{1,2} Tt + \beta_{1,3} Ts + \beta_{2,3} ts + \varepsilon \quad (1)$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,j}$ the quadratic coefficients for i^{th} factor, $\beta_{i,j}$ the coefficients for interaction between the factors *i* and *j* and ε is the error. Response surface methodology (RSM) was used to calculate the coefficients of R_1 (concentration of extracted SAC, mg g⁻¹ dry sample), R_2 (content of 1-kestose, neokestose and neonytose, mg g⁻¹ dry sample) and R_3 (TPC, mg of gallic acid equivalents (GAE) g⁻¹ dry sample), and to estimate the statistical significance of the regression coefficients. The experimental conditions individually maximizing R_1 , R_2 and R_3 were estimated by multiple regression using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA). A multiple response (R_D) analysis simultaneously maximizing R_{1-3} was also considered to select the optimal MAE operating conditions; this R_D function takes values between 0 (completely undesirable value) and 1 (completely desirable or ideal response). Under optimized conditions, the effect of three consecutive extraction cycles was also evaluated to maximize the recovery of AG1 bioactive compounds. After extraction, samples were centrifuged at 4401 g for 10 min and the supernatant was collected, diluted (1:10 v/v) and frozen until analysis.

2.5.2. Optimal conditions

FG and AG1-AG6 garlic samples (0.5 g) were subjected to an MAE process at 120 °C and 60 min with 10 mL of Milli-Q water. All experiments were carried out in triplicate.

2.6. Analytical methods

2.6.1. HILIC-ELSD

Analysis of carbohydrates was performed by HILIC-ELSD using an Agilent 1200 Series system (Agilent Technologies, Palo Alto, CA, USA), equipped with an autosampler, a binary pump and a thermostatted column compartment, and coupled to a 1260 Infinity Evaporative Light Scattering Detector (ELSD). Chromatographic separation was carried out using an XBridge BEH-amide column (150 mm × 4.6 mm, 3.5 μm, 135 Å; Waters, Hertfordshire, UK), operating at 1 mL min⁻¹. Injection volume was set as 10 μL. The separation of carbohydrates was optimized by considering different binary gradients of acetonitrile (AcN, eluent A): water (eluent B), both with and without 0.1% NH₄OH as mobile phase modifier. The elution temperature was set at 30 °C.

The following ELSD parameters were also varied for optimization: nebulization temperature (45–75 °C), gas flow (1–1.60 mL min⁻¹) and signal smoothing (1–3); evaporation temperature was always set 10 °C above the nebulization temperature. Data acquisition and processing were performed using HP Chemstation Rev. A.07.01 software. Carbohydrate identification was done by co-injection of the corresponding commercial (when available) or synthesized (neokestose and neonytose) standards.

Quantitative analysis was performed in triplicate using the external standard calibration curves of carbohydrates such as fructose, glucose, sucrose (0.05–0.5 mg mL⁻¹); 1-kestose and nystose (0.05–0.25 mg mL⁻¹). Carbohydrates with DP > 4 were quantified using a nystose calibration curve. Goodness of fit was evaluated from the correlation coefficients of these calibration curves.

Repeatability of the method was estimated in terms of the intra-day and inter-day precision evaluated within the same day (*n* = 5) or in 5 consecutive days, respectively. Recovery was calculated in triplicate after spiking AG1 sample with a known amount of fructose, sucrose, 1-kestose and nystose standards. Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as three and ten times, respectively, the signal to noise ratio (*S/N*), where *N* is five times the standard deviation of the noise [27].

2.6.2. HPLC-Q MS

Qualitative and quantitative analysis of OSC, HMF and amino acids was performed on a 1260 Infinity II Prime LC System, including an autosampler, a quaternary pump, a thermostatted column compartment and a diode array detector, coupled to a 6125 single quadrupole mass detector (Q MS, Agilent Technologies) provided with an electrospray ionization (ESI) source. Chromatographic separation was carried out by testing two columns: C1, an Infinity-Lab Poroshell 120 Bonus-RP column (150 mm × 4.6 mm, 2.7 μm; Agilent Technologies) and C2, a Kinetex F5 (100 mm × 2.1 mm, 2.6 μm, 100 Å; Phenomenex, CA, USA) operating at 0.4 mL min⁻¹ and thermostatted at 30 °C. Different binary gradients consisting of water (eluent A) and acetonitrile or methanol (eluent B), both with 0.1% formic acid, were assayed for optimization of the LC-MS method. The injection volume was set as 5 μL. ESI parameters were set as indicated below: spray voltage, 4 kV; drying gas temperature (N₂, 99.5% purity), 300 °C; drying gas flow, 12 L min⁻¹; nebulization pressure, 276 kPa and fragmentator voltage, 80–100 V. Analyses were carried out both in SCAN mode (90–2000 *m/z* range) and in SIM mode, for the *m/z* 162 ion corresponding to the [M + H]⁺ adduct of SAC. Data acquisition and processing were performed using HP Chemstation Rev. A.07.01 software. Compound identification was based on chromatographic retention and MS data and confirmed, when possible, by co-injection of the corresponding commercial standards.

Quantitative analysis was performed in triplicate using an external standard calibration curve of SAC (0.0004–0.1 mg mL⁻¹), HMF (0.025–0.1 mg mL⁻¹), γ-glutamyl-cysteine (0.1–0.3 mg mL⁻¹) and amino acid standard mixture (0.0005–0.01 mg mL⁻¹). The quantitation of SAC derivatives (e.g. alliin, cycloalliin, etc.) and γ-glutamyl-compounds (e.g. γ-glutamyl-phenylalanine) was carried out using SAC and γ-glutamyl-cysteine calibration curves, respectively. Validation of the optimized HPLC-Q MS method was done for SAC as described in Section 2.5.2. Matrix effect was also determined by comparing the slope of SAC calibration curve with that obtained after spiking the AG1 extract with different known concentrations of this analyte (0.002 – 0.007 mg mL⁻¹).

2.6.3. HPLC-QToF MS

Identification of OSC present in garlic samples was carried out using an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 quadrupole time of flight (QToF) mass spectrometer (Agilent Technologies) provided with an ESI interface working in positive-ion mode. Chromatographic column and conditions were those selected as optimum for HPLC-Q MS (Section 2.5.2). The electrospray voltage was set at 4.5 kV and the fragmentation voltage at 150 V. Nitrogen (99.5% purity) was used as nebulizer (270 kPa) and drying gas (300 °C, 6 L min⁻¹), while nitrogen of higher purity (99.999%) was used as the collision gas. Tandem mass spectra were obtained by collision-induced dissociation (CID), applying collision energies from 10 to 30 eV to selected precursors ([M + H]⁺). Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition Rev. B.02.00 software.

2.6.4. TPC assay

TPC was determined by the Folin-Ciocalteu method ($n = 3$). First, 100 μL of aged garlic extracts diluted 1:10 (v/v) were mixed with 100 μL of methanol used as extractant and with 100 μL of Folin-Ciocalteu reagent (Sigma-Aldrich). The mixture was stirred (1200 rpm) in a vortex (Velp Scientifica, Usmate, Italy) and left to rest 5 min in the dark. Then, 700 μL of a 75 g L^{-1} solution of Na_2CO_3 (Sigma-Aldrich) in Milli-Q water were added, mixed gently and incubated in the dark for 20 min. Finally, samples were centrifuged at 13,000 rpm for 3 min and transferred to a 96-well plate for absorbance measurement at 750 nm. For quantitation, calibration curves of gallic acid in Milli-Q water (in the range 0.01–0.1 mg mL^{-1}) were used. Results were expressed as mg of gallic acid equivalents (GAE) g^{-1} dry sample.

2.7. Analysis of antioxidant activity

2.7.1. DPPH assay

The antioxidant activity of garlic extracts was measured ($n = 3$) in terms of hydrogen donating or free radical scavenging ability using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical method [28]. Aliquots (50 μL) of extracts diluted in the 1:2–1:10 (v/v) range were mixed with 45 μL of a 0.001 M methanolic solution of DPPH (Sigma-Aldrich). After 30 min of incubation in the dark at 40 °C, the decrease in absorbance was read at 540 nm. The antioxidant reagent Trolox (Sigma-Aldrich) was used as a positive control (0.005–0.07 mg mL^{-1}) and results were expressed as mg of Trolox equivalents (TE) g^{-1} dry sample.

2.7.2. ABTS assay

A 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich) and 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ (Sigma-Aldrich) aqueous solution was prepared and allowed to stand at 4 °C in the absence of light for 16 h. After this time, the ABTS^+ was equilibrated at 30 °C and diluted with ethanol (1:30, v/v) until reaching an absorbance of 0.80 (± 0.02) at 750 nm. Garlic extracts under study were then diluted (1:15–1:50, v/v) with Milli-Q water. For colorimetric measurements, in a 96 well-plate, 50 μL of the diluted extracts and 150 μL of the diluted ABTS^+ solution were added to each well. The same procedure was carried out for the antioxidant reagent Trolox (0.005–0.07 mg mL^{-1}) in ethanol (positive control). After stirring the mixtures, absorbance ($n = 3$) at 750 nm was determined. The results of this assay were expressed as mg of Trolox equivalents (TE) g^{-1} dry sample.

2.8. Statistical analysis

Statistica 7.0 software (StatSoft, Inc. Tulsa, OK, USA) was used for statistical analysis. Significance ($p < 0.05$) of differences in the studies aimed at the selection of the optimal solvent and extraction technique was determined by the analysis of variance (ANOVA, Tukey test).

3. Results and discussion

3.1. Optimization of analytical methods

Optimization of both HILIC-ELSD and HPLC-Q MS methods was required prior to the evaluation of the different techniques considered in this study for the extraction of garlic bioactives.

First, neutral ($\text{AcN:H}_2\text{O}$) and basic ($\text{AcN:H}_2\text{O}$ both with 0.1% NH_4OH) mobile phases were tested for carbohydrate analysis by HILIC-ELSD. At neutral pH, reducing carbohydrates showed double peaks corresponding to α and β isomers, while a single peak was obtained under basic conditions, as previously observed by Brokl

et al. [29]. Although the presence of several non-reducing carbohydrates has been reported in garlic extracts [30], more complex chromatographic profiles were obtained under neutral conditions due to the contribution of reducing carbohydrates. Different binary gradients of $\text{AcN:H}_2\text{O}$, both with 0.1% NH_4OH , were assayed. The optimal conditions for the separation of carbohydrates were as follows: 0 min, 20% B; 11 min 38% B; 25 min, 50% B; 35 min, 65% B; 40 min, 80%; B 45 min, 80% B; 46 min, 20% B; 50 min, 20% B.

Operating conditions for the ELSD detector were also optimized. As shown in Fig. S1, a higher chromatographic response was observed when the evaporator temperature was kept at 85 °C, the nebulizer temperature at 75 °C and the gas flow at 1 mL min^{-1} . As for signal smoothing, no noticeable differences were observed between the different conditions evaluated, so it was kept at 1.

Under these conditions, monosaccharides (glucose and fructose), polyalcohols (*myo*-inositol), disaccharides (sucrose), trisaccharides (1-kestose and neokestose) and tetrasaccharides (nystose and neonytose) were detected. Moreover, oligosaccharides with DP 5–9 were tentatively identified as shown in Fig. 1A. SAC was also identified by this technique, but it coeluted with other non-identified compounds at the beginning of the chromatogram (t_R : 2.0 min) and, therefore, it was not analyzed using this technique.

Regarding HPLC-Q MS, the efficiency of two C18 columns (C1 and C2) on the retention of SAC was evaluated. Although shorter retention times result in high throughput analysis, coelution of SAC with other barely retained AG compounds (mainly carbohydrates) occur on C18 columns. Therefore, a trade-off between the retention of such compounds and analysis time must be considered. Isocratic conditions using 93:7 (v/v) $\text{H}_2\text{O:AcN}$ (both with 0.1% formic acid) at 0.4 mL min^{-1} were first assayed for both columns. While scarce retention of SAC in column C2 was observed under these conditions ($t_R = 1.25$ min), longer retention was achieved with column C1 ($t_R = 5.6$ min). Changes in the composition of the mobile phase used for column C2 [lower percentage of AcN (3–5%)] and a slower flow (0.2 mL min^{-1}) resulted in an SAC peak with wider width at half height ($w_h > 0.8$) and only a slight increase in retention (1.5–1.8 min); therefore, column C1 was selected for the following studies. The use of AcN and MeOH in the mobile phase was also evaluated. It is known that acetonitrile exhibits a higher elution strength than methanol for reversed-phase chromatography; therefore, SAC t_R was slightly shorter for AcN as compared to MeOH (t_R 4.7 min vs 5.6 min). However, considering the minimal differences in SAC retention provided by these two organic solvents and the advantages of methanol in terms of availability and lower cost, water:methanol (both with 0.1% formic acid) was selected for further studies. Different gradients were then assayed; the best conditions for the separation were as follows: 0–7 min, 7% B; 21 min, 60% B; 22–32 min, 95% B; 33 min, 7% B; 48 min, 7% B.

Once chromatographic conditions were optimized, OSC was identified by HPLC-QToF MS. Retention, MS and MS/MS data of the different compounds are shown in Table S1 of Supplementary Material. SAC and other OSC, such as alliin, cycloalliin, S-1-propenyl-L-cysteine (S1PC) and S-allylmercaptocysteine (SAMC), and their precursors [γ -glutamyl-S-methylcysteine (GSMC), γ -glutamyl-S-allyl-L-cysteine (GSAC), γ -glutamyl-S-1-propenyl-L-cysteine (GS1PC) and γ -glutamyl-S-allylmercaptocysteine (GSAMC)] were identified, as shown in Fig. 1B. Furthermore, several amino acids (e.g. tyrosine, phenylalanine, tryptophane) were also detected.

3.2. Validation of analytical methods

Table 1 shows the analytical parameters considered for the validation of the HILIC-ELSD method here developed. Fructose, sucrose, 1-kestose and nystose were selected as target carbohydrates. Although a linear response was found for all these standards in their expected concentration range in the AG samples under study

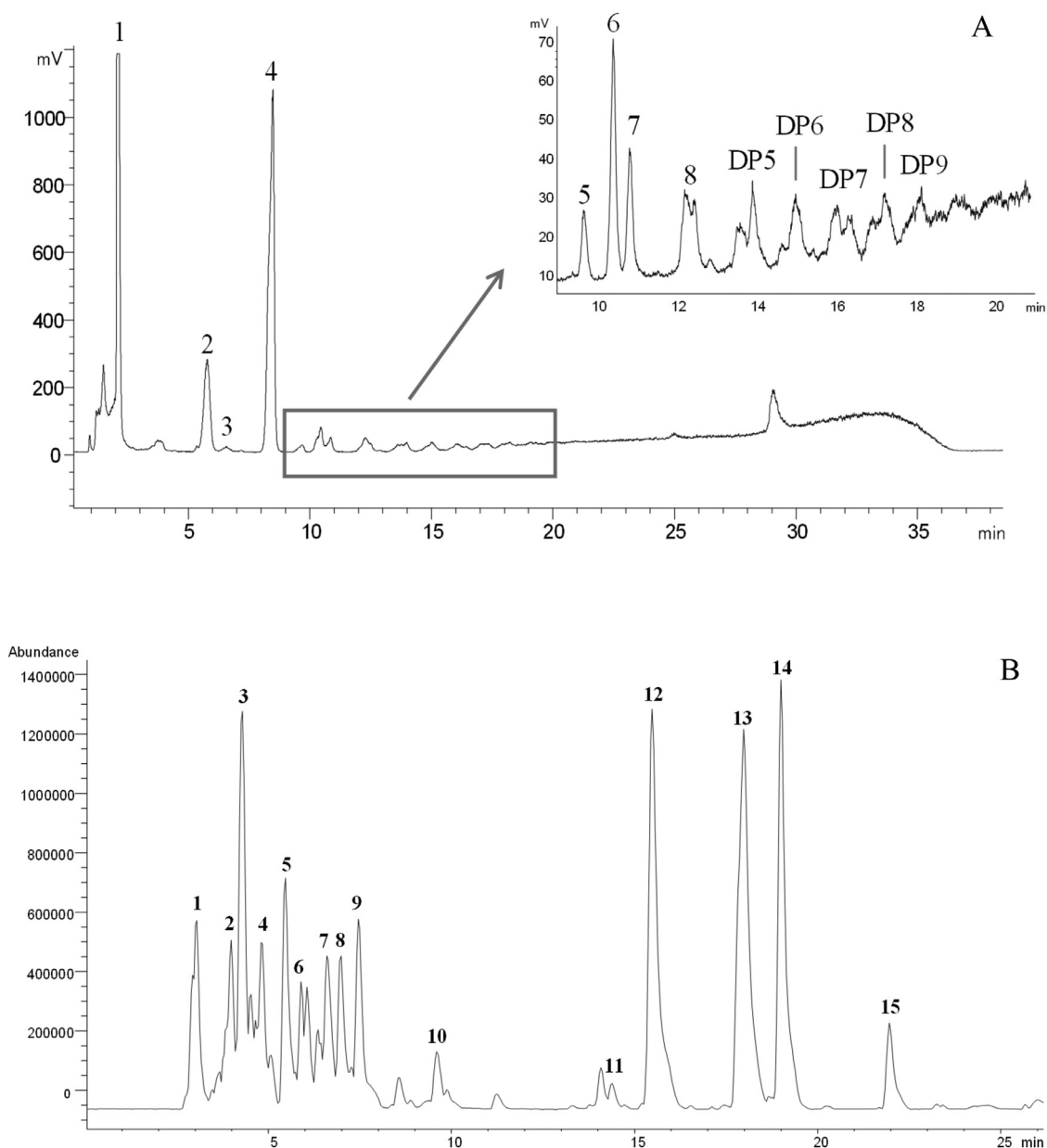


Fig. 1. AG1 extract chromatographic profiles by HPLC-ELSD (A): 1.- SAC, 2.- Fructose, 3.- Glucose, 4.- Sucrose, 5.- *myo*-inositol, 6.- Neokestose, 7.- 1-Kestose and 8.- Nystose + neonytose and HPLC-Q MS (B) 1.- Proline, 2.- Glutamic acid, 3.- Cycloalliin, 4.- Alliin, 5.- SAC, 6.- Tyrosine, 7.- S1PC, 8.- GSMC, 9.- Phenylalanine, 10.- SAMC, 11.- Tryptophane, 12.- GSAC, 13.- GS1PC, 14.- γ -glutamyl-phenylalanine and 15.- GSAMC. *DP: degree of polymerization.

($R^2 = 0.991$ – 0.993), the linearity range was short (0.05 – 0.5 mg mL $^{-1}$ for glucose, fructose and sucrose; 0.05 – 0.25 mg mL $^{-1}$ for 1-kestose and nystose). Intra-day and inter-day precision values at the two concentrations assayed were lower than 10% in all cases except for 1-kestose (11%), while recovery ranged from 92.1% to 99.2%, and the sensitivity of the method (LOD and LOQ) was in the μ g mL $^{-1}$ order (< 3 μ g mL $^{-1}$ for carbohydrates and < 0.05 μ g mL $^{-1}$ for SAC). Although lack of linearity and high LOD and LOQ values are sometimes associated to ELSD detectors, satisfactory data were achieved in this study for the analysis of aged garlic carbohydrates, allowing the detection of oligosaccharides up to DP9.

The analytical parameters considered to validate the HPLC-Q MS method developed to analyze SAC are shown in Table 1. Due to the lack of commercial standards for OSC other than SAC, this bioactive was the only one considered for validation. A linear response on the expected concentration range of SAC in AG samples (0.0004 – 0.1 mg mL $^{-1}$) was obtained ($R^2 = 0.993$). The relationship between the slopes of spiked and non-spiked calibration

curves was 97%, indicating no matrix effect in its determination. This was also statistically evidenced by the t -test (t experimental $< t$ tabulated) at the 95% confidence level. Good values for intra- and inter-day precision ($< 8\%$) and recovery (93.2%) were also achieved. Sensitivity of the method was in the ng mL $^{-1}$ order (LOD : 13.3 ng mL $^{-1}$; LOQ : 44.3 ng mL $^{-1}$), in close agreement with the values reported by Lee et al. [31] for the HPLC-MS analysis of SAC in heated garlic ($20/70$ ng mL $^{-1}$), and better than that of HPLC methods for the analysis of SAC in black garlic using UV detectors (e.g. $0.25/1$ μ g mL $^{-1}$ [32]; $29.67/89.91$ μ g mL $^{-1}$ [33]) or fluorescence detectors (e.g. $6.18/19.02$ μ g mL $^{-1}$ [33]).

3.3. Selection of extraction solvent and technique

To optimize the simultaneous SLE of bioactive compounds from the AG1 sample, representative garlic bioactive compounds (i.e. SAC, 1-kestose, neokestose and nystose+neonytose) and TPC were considered.

Table 1

Validation of methods for quantitation of carbohydrates (fructose, sucrose, 1-kestose y nystose) by HILIC-ELSD and of S-allyl-L-cysteine (SAC) by HPLC-Q MS.

	Fructose	Sucrose	1-Kestose	Nystose	SAC
Calibration curve	$y = 12383x - 672.38$ $R^2 = 0.9911$	$y = 16451x - 769.77$ $R^2 = 0.9925$	$y = 10365x - 346.34$ $R^2 = 0.991$	$y = 8226.6x - 245.4$ $R^2 = 0.991$	$y = 6.4 \cdot 10^8 x + 1.5 \cdot 10^5$ $R^2 = 0.993$
Linearity range (mg mL ⁻¹)	0.05–0.5	0.05–0.5	0.05–0.25	0.05–0.25	0.0004–0.1
Intra-day precision (RSD%)					
0.05 mg mL ⁻¹	5.64	3.64			3.52
0.10 mg mL ⁻¹			9.18	4.10	4.52
0.15 mg mL ⁻¹	2.37	2.81	11.33	2.55	
Inter-day precision (RSD%)					
0.05 mg mL ⁻¹	4.24	3.34			8.12
0.10 mg mL ⁻¹			11.33	4.10	5.75
0.15 mg mL ⁻¹	2.31	2.56	4.34	3.21	
LOD (µg mL ⁻¹)	0.77 (0.04)*	0.32 (0.03)	0.74 (0.04)	0.72 (0.05)	0.013 (0.001)
LOQ (µg mL ⁻¹)	2.59 (0.15)	1.09 (0.09)	2.47 (0.14)	2.39 (0.16)	0.044 (0.004)
Recovery (%)	96.7 (4.1)	99.2 (3.9)	92.1 (4.7)	95.5 (1.2)	93.2 (4.0)

* Average ($n = 3$) value and standard deviation in brackets.**Table 2**Concentration of SAC and bioactive carbohydrates (mg g⁻¹ sample) and TPC [mg of gallic acid equivalents (GAE) g⁻¹ of sample] of AG1 extracts obtained by SLE at 70 °C for 30 min using different solvents.

	H ₂ O	MeOH	EtOH	EtOH:H ₂ O	MeOH:H ₂ O
SAC	1.31 (0.01) ^{a,*}	0.98 (0.01) ^b	0.63 (0.14) ^c	1.34 (0.12) ^a	1.33 (0.00) ^a
1-Kestose	8.94 (0.30) ^a	4.50 (0.01) ^c	1.78 (0.06) ^d	6.24 (0.23) ^b	6.02 (0.08) ^b
Neokestose	14.31 (1.26) ^a	5.58 (0.09) ^c	1.82 (0.07) ^d	8.25 (0.37) ^b	8.42 (0.27) ^b
Nystose + Neonystose	14.70 (1.55) ^a	4.92 (0.09) ^c	- ** ^d	7.67 (0.22) ^b	8.40 (0.48) ^b
TPC	5.49 (0.17) ^a	4.26 (0.13) ^c	1.38 (0.10) ^d	4.44 (0.39) ^b	4.66 (0.44) ^b

* Average and standard deviation ($n = 3$) in brackets;

** Lower value than LOD;

^{a,b,c} Different letters within the same row indicate significant ($p < 0.05$) differences for the solvents assayed.**Table 3**SAC and bioactive carbohydrates concentrations (mg g⁻¹ sample) and TPC [mg of gallic acid equivalents (GAE) g⁻¹ of sample] of AG1 extracts obtained by SLE, UAE and MAE at 70 °C for 30 min.

	SLE	UAE	MAE
SAC	1.50 (0.17) ^{b,*}	1.39 (0.12) ^b	1.99 (0.26) ^a
1-Kestose	8.48 (0.88) ^a	8.31 (0.49) ^a	7.41 (0.07) ^a
Neokestose	12.71 (2.19) ^a	11.85 (1.05) ^a	10.48 (0.18) ^a
Nystose + Neonystose	13.53 (2.14) ^a	12.23 (1.27) ^a	10.21 (0.08) ^a
TPC	5.74 (0.50) ^a	6.44 (0.43) ^a	6.53 (0.20) ^a

* Average and standard deviation ($n = 3$) in brackets;^{a,b} Different letters within the same row indicate significant ($p < 0.05$) differences for the extraction technique assayed.

Firstly, five different solvents (water, methanol, ethanol, 50:50 (v/v) methanol:water and 50:50 (v/v) ethanol:water) were evaluated at 70 °C for 30 min under constant stirring conditions. Whereas no significant differences for SAC concentration were obtained using water and hydroalcoholic mixtures as extractants, ethanol and methanol provided the lowest recovery of this compound (Table 2). Regarding bioactive carbohydrates and TPC, the highest yields were obtained using water; values significantly lower were found for the remaining solvents. Taking these results into account and considering the advantages of using green solvents for the extraction of bioactives, water was selected as the best solvent.

The efficiency in terms of extraction yield of bioactives was further evaluated for the three techniques (SLE, UAE and MAE) compared in this study; in all cases extraction conditions were 70 °C, 30 min and a sample amount (g)/solvent volume (mL) ratio of 1/10. Results are shown in Table 3. No significant differences in TPC and bioactive carbohydrate concentrations extracted by the three tech-

niques were observed. However, the highest extraction of SAC using MAE justified its selection for further studies.

3.4. Optimization of an MAE method

Experimental design and RSM have been shown to be useful for developing, improving and optimizing processes [23]. Thus, the influence of three independent variables (T , t and s) on the efficiency of the MAE of SAC (R_1), 1-kestose, neokestose and nystose+neonystose (R_2) and TPC (R_3) of aged garlic was evaluated using a Box-Benhen experimental design. Table 4 shows the results for the 15 experiments performed. Total concentrations varied between 1.17 and 2.32 mg g⁻¹ dry sample for SAC, 1.97–6.85 mg g⁻¹ dry sample for 1-kestose, 2.91–9.41 mg g⁻¹ dry sample for neokestose, 2.80–13.00 mg g⁻¹ dry sample for nystose+neonystose and 4.87–10.63 GAE g⁻¹ dry sample for TPC. In general, higher concentrations of SAC were extracted at high temperatures for long/medium times (120 °C/80 °C and 60/32.5 min, experiments 2 and 4), while higher yields of bioactive carbohydrates were obtained at 120 °C and 60 min (experiment 6). The highest TPC was also achieved at 120 °C for 32.5 min (experiment 4). It has been reported that high temperatures usually provide a higher extraction efficiency, as a result of enhancing solvent diffusivity into the matrix, and of improved analyte desorption from the active sites of the matrix [34,35]. As for the extraction time, its increase (up to 60 min) improved the intended extraction of target compounds. Regarding sample amount, the highest SAC and TPC values were obtained with the lowest sample amount (0.5 g), whereas the highest yields of bioactive carbohydrates were obtained with higher amounts (1.0 g) of aged garlic.

RSM was followed to calculate the regression coefficients of the R_1 , R_2 and R_3 quadratic models proposed and to estimate their

Tabla 4

SAC, 1-kestose, neokestose, nystose+neonystose concentrations (mg g⁻¹ dry sample) and TPC [mg of gallic acid equivalents (GAE) g⁻¹ sample] in MAE AG1 extracts obtained following a Box–Behnken experimental design. T: temperature, t: time, s: sample amount.

Experiment	T (°C)	t (min)	s (g)	SAC	1-Kestose	Neokestose	Nystose+ Neonystose	TPC
1	80	32.5	1.0	1.48 (0.10)*	6.42 (0.26)	8.74 (0.23)	8.75 (0.14)	6.02 (0.46)
2	80	60	1.5	2.29 (0.21)	6.11 (0.30)	9.09 (0.43)	9.14 (0.73)	4.87 (0.19)
3	80	5	0.5	1.58 (0.11)	5.39 (0.87)	8.25 (0.92)	7.78 (1.74)	9.68 (0.24)
4	120	32.5	0.5	2.32 (0.33)	6.85 (1.08)	8.41 (0.88)	11.43 (2.84)	10.63 (0.68)
5	40	5	1.0	1.90 (0.33)	5.55 (0.16)	7.71 (0.1)	7.13 (0.22)	6.63 (0.16)
6	120	60	1.0	2.04 (0.05)	8.21 (0.50)	9.41 (0.23)	13.00 (1.86)	7.89 (0.93)
7	120	32.5	1.5	1.76 (0.08)	6.33 (0.37)	8.15 (0.29)	10.65 (1.27)	5.90 (0.42)
8	80	60	0.5	1.84 (0.11)	4.89 (0.53)	7.80 (0.25)	7.28 (0.99)	9.44 (0.53)
9	80	32.5	1.0	1.50 (0.19)	5.80 (0.52)	7.57 (0.86)	6.80 (0.76)	6.01 (0.28)
10	80	32.5	1.0	1.41 (0.01)	6.25 (0.19)	8.16 (0.24)	7.64 (0.22)	5.88 (0.08)
11	120	5	1.0	1.53 (0.09)	1.97 (0.10)	2.91 (0.12)	2.80 (0.13)	6.40 (0.07)
12	80	5	1.5	1.20 (0.04)	6.12 (0.17)	8.81 (0.21)	8.32 (0.31)	5.06 (0.20)
13	40	32.5	1.5	1.17 (0.03)	6.45 (1.13)	9.05 (1.72)	8.97 (1.77)	4.80 (0.56)
14	40	60	1.0	1.48 (0.10)	6.42 (0.26)	8.74 (0.23)	8.75 (0.14)	6.02 (0.46)
15	40	32.5	0.5	2.19 (0.21)	6.11 (0.30)	9.09 (0.43)	9.14 (0.73)	4.87 (0.19)

* Standard deviation (n = 3).

statistical significance, as well as the prediction errors (estimation standard error and mean absolute error) (Table S2). In the case of SAC, T, t and t-s were the most significant coefficients ($R_1 = 1.64077 + 0.00174148 T - 0.0214545 t - 4.90455 s + 0.000170455 T \cdot t + 0.150909 t \cdot s$; $R^2 = 0.75$, $p < 0.1$) and the optimal conditions to maximize R_1 were 120 °C, 60 min and 1.5 g of black garlic. T and T^2 were the most significant variables to maximize the concentration of bioactive carbohydrates ($R_2 = 28.2384 - 0.227978 T + 0.0645 t + 0.00166217 T^2$; $R^2 = 0.73$, $p < 0.05$) while the optimal conditions to maximize R_3 were 119 °C, 60 min and 0.8 g of black garlic. Regarding the R_3 model, the most significant coefficients ($p < 0.05$) to maximize TPC were s, T^2 and s^2 ($R_3 = 18.8691 - 0.0802699 T - 0.0278182 t - 140.775s + 0.000479687 T^2 + 0.000347727 Ts + 461.0 s^2$; $R^2 = 0.98$); according to this model, the optimal conditions were 120 °C, 32.5 min and 0.5 g of sample. Response surface plots (Fig. S2) and ANOVA results (Table S2) have been included as Supplementary Material.

A multiple response that simultaneously maximized R_1 , R_2 and R_3 was further considered. Optimal ($R_D = 0.86$) operating parameters were T: 120 °C; t: 60 min and s: 0.5 g. A good agreement between theoretical and experimental concentrations of bioactives under optimal conditions was observed in all cases (RSD < 10%).

Once optimal conditions were established, the use of different cycles for the exhaustive extraction of SAC, bioactive carbohydrates and TPC was evaluated. An extraction efficiency of 88% of SAC and 92–95% of bioactive carbohydrates was achieved in the first cycle, and the remaining 5–12% of target compounds were extracted in the second cycle, whereas no compounds were detected in the third one. Consequently, considering the low amounts recovered in the second cycle and that a new extraction cycle would require longer times, more energy consumption, higher solvent volume, etc., only one cycle was selected for the subsequent experiments.

3.5. Application of MAE to garlic samples

Optimum MAE conditions were applied to the simultaneous extraction of OSC, carbohydrates and TPC from fresh garlic (FG) and different black garlic (AG1–6) samples. HMF and amino acid concentrations and antioxidant activity (measured by DPPH and ABTS methods) were also determined; results are shown in Table 5.

Regarding OSC, SAC was detected in all samples studied, although quantifiable amounts were only found in FG, AG1 and AG6. SAC concentration of AG1 (2.32 mg g⁻¹) was noticeably higher than that of FG (0.32 mg g⁻¹), while that of AG6 (0.78 mg g⁻¹) was twice that of FG. As previously reported, SAC content of aged garlic is usually 3–6 times higher than that of FG [5]; in this work,

the ratio for AG1 was even higher (SAC_{AG1/FG} 7.4). Concentration of AG1 extract was also higher than those described in the literature for different black garlics (0.095–0.427 mg g⁻¹ [36] 0.085–0.194 mg g⁻¹, [32]; 0.114 mg g⁻¹, [33]). However, AG2–AG5 samples showed extremely low SAC contents (lower than LOQ values). These variances may be due to different temperature, time and humidity conditions used during garlic aging. Previous studies have shown that SAC production is greatly favored by using mild temperatures (40–60 °C) and high humidity (90%) conditions [1]. The results obtained in this work seem to indicate that the commercial samples analyzed had been subjected to high temperature or low humidity treatments that negatively affected the formation of SAC.

Regarding other OSC, the SAC isomer S1PC could only be quantified in AG1 (0.91 mg g⁻¹), while only traces of this compound were detected in FG and AG6. Precursors of SAC and S1PC were also found in these extracts; AG1 showed the highest concentrations of both GSAC and GS1PC (10.26 and 10.86 mg g⁻¹, respectively).

Alliin was only detected in FG and AG1, with the highest concentration in FG (5.39 mg g⁻¹). This compound is a sulfoxide derived from cysteine, which is transformed into allicin (the main component responsible for garlic aroma) when garlic is cut or crushed, due to a reaction catalyzed by the enzyme alliinase. Alliin is also degraded when heat treatments are applied, giving rise to other organosulfur compounds, which explains its lower content in AG1. Alliin concentrations found in both AG1 and FG were higher than those detected by other authors in raw garlic (0.300 mg g⁻¹) [6]. On the contrary, alliin was not detected in AG2–6, probably as a result of its degradation during their production process; however, its isomer, cycloalliin, was found in all samples. The formation of this compound during thermal processing by the action of enzymes with γ-glutamyl transpeptidase and oxidase activities has been previously described [37], which explains its presence in AG samples. Other compounds such as GSAMC could be quantified in samples other than AG4 and AG5, while SAMC was only present in AG1 and FG, and GSMC in AG1.

Essential amino acids such as leucine + isoleucine, lysine, phenylalanine, tryptophan and threonine were detected in most of the samples; in general, the highest concentrations were found in FG. These results were in line with the results reported by Liang et al. [37] who found an increase in amino acid content during the first 5 days of thermal processing and then a marked decrease. Some amino acids such as aspartic acid, glutamic acid and serine were found in AG samples. Furthermore, the dipeptide γ-glutamyl-phenylalanine (Glu-Phe) was successfully identified and quantified in all garlic samples analyzed. As previously reported by Bedrníček

Table 5

Organosulfur compounds, 5-hydroxymethylfurfural (HMF), amino acids and carbohydrate concentrations (mg g^{-1} dry sample), total phenolic content [mg of gallic acid equivalents (GAE) g^{-1} of sample] and antioxidant activity (determined by DPPH and ABTS assays, mg TE g^{-1} dry sample) of fresh garlic (FG) and aged garlic (AG1–6) MAE extracts. DP: degree of polymerization.

		FG	AG1	AG2	AG3	AG4	AG5	AG6
OSC	Alliin	5.39 (1.23)*	1.08 (0.20)	---	—	—	—	—
	Cycloalliin	1.95 (0.11)	1.23 (0.08)	1.29 (0.27)	1.72 (0.56)	3.00 (0.16)	3.01 (0.14)	2.07 (0.540)
	GSAC	4.73 (1.9)	10.26 (0.59)	—	—	—	—	1.59 (0.02)
	GSAMC	1.16 (0.10)	1.81 (0.26)	1.17 (0.10)	1.14 (0.03)	—	—	1.52 (0.13)
	GSMC	—	3.50 (0.68)	—	—	—	—	—
	GS1PC	8.06 (1.4)	10.86 (0.58)	—	—	—	—	1.04 (0.02)
	SAC	0.32 (0.14)	2.32 (0.16)	tr**	tr	tr	tr	0.78 (0.15)
	SAMC	1.75 (0.13)	0.43 (0.15)	—	—	—	—	—
	S1PC	tr	0.91 (0.03)	—	—	—	—	tr
	HMF	—	—	9.0 (1.9)	5.4 (1.4)	7.7 (0.1)	4.3 (0.8)	—
	Arginine	3.45 (0.02)	1.99 (0.04)	1.03 (0.09)	0.98 (0.06)	0.24 (0.08)	0.37 (0.06)	1.88 (0.29)
Amino acid compounds	Aspartic acid	—	0.13 (0.03)	2.68 (0.24)	1.29 (0.25)	1.62 (0.14)	1.51 (0.35)	0.65 (0.01)
	Glutamic acid	—	0.72 (0.08)	0.42 (0.05)	0.25 (0.03)	0.38 (0.05)	—	0.29 (0.09)
	Isoleucine + leucine	0.14 (0.01)	0.50 (0.04)	0.27 (0.02)	0.25 (0.01)	0.23 (0.02)	0.23 (0.04)	0.53 (0.02)
	Lysine	0.78 (0.08)	0.55 (0.03)	0.16 (0.01)	0.15 (0.01)	0.05 (0.01)	—	0.3 (0.004)
	Phenylalanine	1.10 (0.1)	0.83 (0.04)	0.73 (0.02)	0.59 (0.02)	0.27 (0.02)	0.24 (0.08)	0.70 (0.06)
	Proline	3.91 (0.03)	0.50 (0.03)	0.17 (0.02)	0.88 (0.04)	0.65 (0.05)	0.97 (0.04)	0.43 (0.01)
	Serine	—	0.82 (0.04)	0.65 (0.07)	0.17 (0.02)	0.17 (0.10)	0.07 (0.01)	0.87 (0.01)
	Threonine	0.69 (0.03)	0.53 (0.11)	0.36 (0.02)	0.19 (0.02)	0.24 (0.01)	0.28 (0.06)	0.27 (0.05)
	Tryptophane	1.27 (0.09)	0.14 (0.02)	—	0.16 (0.01)	0.16 (0.01)	0.13 (0.05)	0.15 (0.02)
	Tyrosine	1.65 (0.14)	0.67 (0.06)	0.27 (0.04)	0.38 (0.08)	0.28 (0.05)	0.52 (0.08)	0.56 (0.02)
	Glu-Phe	8.5 (0.39)	9.95 (1.83)	3.11 (0.69)	3.41 (0.40)	1.46 (0.20)	1.40 (0.16)	5.44 (0.77)
Carbohydrates	Fructose	12.9 (1.7)	64.6 (3.8)	466.7 (16.1)	759.3 (47.9)	627.9 (43.1)	734.8 (32.2)	513.4 (17.6)
	Glucose	27.3 (0.8)	11.6 (0.5)	58.9 (1.3)	51.1 (2.5)	45.2 (1.39)	39.97 (0.94)	30.8 (1.2)
	Sucrose	42.3 (1.5)	54.1 (2.8)	—	—	—	—	20.3 (1.8)
	DP3	6.9 (0.1)	21.0 (0.8)	—	—	—	—	—
	DP4	6.5 (0.1)	17.7 (1.2)	—	—	—	—	—
	DP5–9	35.7 (1.6)	77.4 (2.7)	—	—	—	—	—
	TPC	8.2 (1.1)	9.2 (0.6)	27.5 (1.7)	24.7 (3.9)	26.2 (0.1)	25.4 (2.3)	30.4 (1.3)
Bioactivity assays	DPPH	5.6 (0.1)	4.1 (0.1)	26.4 (0.1)	34.1 (0.8)	24.3 (1.4)	32.7 (0.1)	23.9 (0.8)
	ABTS	1.13 (0.04)	1.2 (0.1)	6.15 (0.9)	10.9 (1.7)	9.5 (0.8)	11.0 (0.6)	7.4 (0.7)

* Average and standard deviation ($n = 3$) in brackets;

** tr: traces;

*** -: non-detected.

et al. [38], this compound has beneficial effects on human tissues (e.g., antioxidant, anticancer activity, etc.) and its concentration is lower in black garlic than in fresh garlic. This behavior was observed in the FG and AG2–6 samples here evaluated; however, AG1 showed higher concentrations.

HMF was detected in most of the commercial AG samples (AG2–AG5) with values ranging from 4.3 to 9 mg g^{-1} dry sample. However, it was not found in FG, AG1 and AG6. This compound is produced due to the thermal degradation of carbohydrates during food processing and its concentration in aged garlic depends on the thermal treatment applied. Pakakaew et al. [39] found that low temperatures (around 60 °C) resulted in lower HMF content in the final product (up to 0.9 mg g^{-1} dry weight), while treatments at 70–80 °C produced higher HMF concentrations (up to 7.24 mg g^{-1} dry weight). Similar results were also reported by Yuan et al. [40] who found 8 mg g^{-1} of HMF after 12 days of garlic treatment at 75 °C and 85% relative humidity. Therefore, in view of the experimental results obtained, it is probable that samples AG2–5 had been subjected to temperatures higher than 70 °C for their aging processing. Moreover, as reported by Ahmed and Wang [5], concentrations of HMF higher than 4 mg g^{-1} were found in AG2–5 samples, indicating that the products have undergone complete aging, in agreement with the black color of the samples.

Regarding carbohydrates, AG2–6 samples showed different profiles than AG1 and FG extracts. While only large amounts of fructose (> 450 mg g^{-1}) and glucose (30.8–58.9 mg g^{-1}) were found in the AG2–6 extracts, with the exception of sucrose in AG6 (20.3 mg g^{-1}), the AG1 and FG extracts presented a more complex carbohydrate profile, with quantifiable amounts of up to DP9 oligosaccharides. Similar results to those obtained for commercial samples were described by Martínez-Casas et al. [8], who found

concentrations of 447.3 mg g^{-1} of fructose and 55.1 mg g^{-1} of glucose in black garlic samples. The high concentrations of these sugars are related to the effect of heat treatment on higher molecular weight carbohydrates (FOS and fructans), which degrade into monosaccharides (mainly fructose and lower amounts of glucose) and disaccharides (sucrose), giving a sweeter flavor to black garlic [8]. The lower amounts of glucose detected may be justified not only by its lower presence in FOS structures, but also by its faster involvement in the Maillard reaction [40]. Although to a lesser extent, this behavior was also observed in the AG1 sample, which had a higher concentration of carbohydrates up to DP9 than the FG sample, except for glucose. Lu et al. [41] observed that the production of black garlic at high temperatures for long times favors the conversion of oligosaccharides into monosaccharides. These results seem to support the hypothesis that commercial AG2–6 samples were obtained using more extreme temperature and time conditions than AG1.

As for TPC values, the highest contents were found in AG2–6 samples (24.7–30.4 mg GAE g^{-1}), while values in FG and AG1 extracts were lower (8.2 and 9.2 mg GAE g^{-1} , respectively). Similar results (14.03–82 mg GAE g^{-1} , [34]; 8.20 mg GAE g^{-1} , [8]) or even lower values (0.412–0.982 mg GAE g^{-1} , [42]) have been reported in the literature for different black garlic samples. Although TPC values were high in AG extracts, phenolic compounds could not be detected in these samples neither by HPLC–Q MS nor by HPLC–QToF MS. This suggests that the values obtained could mainly be due to other compounds different to polyphenols, i.e., organosulfur compounds (SAC, S1PC...), HMF, amino acids or dipeptides (γ -glutamyl-phenylalanine), etc., which can interfere with the Folin–Ciocalteu reagent [8,43]. The same conclusions were reached by Bedrníček et al. [38] and Molina-Calle et al. [44] who also failed

to detect polyphenols in garlic samples (fresh and black) using QToF MS detectors.

Accordingly, antioxidant activity determined by both DPPH and ABTS methods was noticeably higher for AG samples (24.3–34.1 mg TE g⁻¹ and 6.2–11.0 mg TE g⁻¹ for DPPH and ABTS methods, respectively) than for AG1 and FG which showed similar values (4.1 and 1.2 mg TE g⁻¹ for AG1; 5.6 and 1.13 mg TE g⁻¹ for FG, respectively). These results were in line with those published by Bedrníček et al. [38] for black garlic (12.93 mg TE g⁻¹) and fresh garlic (1.9 mg TE g⁻¹) samples.

In general, higher concentrations of bioactive compounds were detected in the AG1 extract compared to the other aged garlic samples (AG2–6), which were probably subjected to more intense aging conditions (thermal treatment, humidity and time).

4. Conclusions

This comparative study, which focused for the first time on different extraction techniques, showed MAE as the most promising procedure to obtain multifunctional AG extracts rich in SAC. The combination of the analytical data provided by the HILIC-ELSD and HPLC-Q MS methods developed in this work was shown to improve the multicomponent characterization of aged garlic MAE extracts. The application of the previously optimized MAE procedure (0.5 g of sample in 10 mL of water at 120 °C for 60 min) to different commercial samples revealed a wide variability in the AG extracts, both in composition and concentration, probably associated with the garlic processing conditions. In conclusion, the new MAE method developed here can be proposed as an environmentally friendly and efficient procedure for the simultaneous extraction of AG bioactives, which can be employed for different applications (e.g., food ingredients, dietary supplements, etc.).

Declaration of Competing Interest

All authors declare no conflict of interest. I.J.-A., which is granted with an Industrial Doctorate from the Comunidad de Madrid, carried out all the investigations in the CSIC facilities under the supervision of CSIC academic researchers, assuring the integrity and objectivity of the results obtained.

CRediT authorship contribution statement

I. Jiménez-Amezcu: Formal analysis, Methodology, Validation, Investigation, Writing – original draft. **A. González-Prada:** Methodology, Validation, Writing – original draft. **M. Díez-Municio:** Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing. **A.C. Soria:** Data curation, Investigation, Project administration, Resources, Validation, Visualization, Writing – original draft, Writing – review & editing. **A.I. Ruiz-Matute:** Conceptualization, Data curation, Investigation, Supervision, Writing – original draft, Writing – review & editing. **M.L. Sanz:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2023.464128.

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4.4. Evaluación de técnicas de extracción avanzadas para la obtención de extractos enriquecidos en bioactivos procedentes de subproductos de ajo (*Allium sativum* L.)

Evaluation of advanced extraction techniques to obtain bioactive enriched extracts from garlic (*Allium sativum* L.) by-products

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Evaluation of advanced extraction techniques to obtain bioactive enriched extracts from garlic (*Allium sativum* L.) by-products

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Abstract

Garlic (*Allium sativum* L.) residues generated during its production are very abundant and their disposal can have a negative impact on the environment, in addition to entailing high management costs for producers. On the other hand, the numerous health benefits of garlic associated to its composition in organosulfur compounds (OSC), bioactive carbohydrates (BCH) and free amino acids (aa), among others, could be also attributed to these discarded residues. Therefore, there is great interest in the development of new, efficient, fast and environmentally friendly extraction methodologies of bioactive compounds for the revalorization of these by-products.

Thus, in this work, new ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) methodologies were optimized using experimental designs to efficiently obtain multifunctional extracts from different garlic by-products. Stems were selected as the most promising bioactive source for their highest content of bioactive compounds. Concentration of bioactives extracted by these techniques were compared with those achieved by conventional solid liquid extraction (SLE). Among them, the optimized UAE methodology (40 °C, 60 min and 77 mg mL⁻¹) was selected as optimal and applied to garlic stems of different ecotypes and stages of plant growth, obtaining high concentrations of OSC (e.g. *S*-allyl-L-cysteine, *S*-1-propenyl-L-cysteine and *S*-allyl-mercaptopcysteine), BCH (*myo*-inositol, neokestose, 1-kestose, nystose and neonystose) and free aa (glutamine, lysine, phenylalanine, leucine, isoleucine, methionine, valine and threonine). Thus, UAE extracts of garlic stems are therefore proposed as a very interesting source of bioactive compounds to be used as food ingredients and as a way for revalorization of garlic by-products.

Keywords: Garlic by-products, organosulfur compounds, bioactive carbohydrates, amino acids, ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE)

1. Introduction

Nowadays, there is a growing trend towards the revalorization of agricultural by-products as a suitable and economical source of bioactive compounds. Garlic (*Allium sativum* L.) is one of the most valuable crops throughout the world, with a total harvested area of 1,659,236 ha and a production of 28,204,854 tonnes in 2021. Spain is the first garlic producer of the European Union, with an average of 212,160 tonnes per year [1].

It is worth noting that although almost the entire garlic plant is edible, only the bulbs, mainly the cloves, are globally commercialized [2], and the resultant residues, such as leaves, stems, roots and skins are frequently discarded. Hence, the residues generated from garlic production are very abundant and, therefore, they have a negative impact on the environment that implies significant mitigation costs for producers [3]. In this context, different strategies based on the concept of circular economy are employed to harness the residues generated during garlic production, which include composting of agricultural residues (e.g. organic fertilizers), biogas production, or extraction of bioactive compounds. In the last case, the term ‘residue’ is replaced by ‘by-product’, as some discarded parts of the plant have potential value in the food or pharmaceutical industry and could be used for the production of dietary supplements, cosmetic products, etc [4,5].

Garlic is valued for its organoleptic and bioactive properties, including its antioxidant effects, lipid-lowering activity, antimicrobial action, cardioprotective and antihypertensive properties [1,6]. These numerous health benefits are related to a high variety of phytochemical compounds such as organosulfur compounds [OSC, e.g. alliin, S-allyl-L-cysteine (SAC) and S-1-propenyl-L-cysteine (S1PC)], bioactive carbohydrates [BCH, e.g. inulin-type fructans and fructooligosaccharides (FOS)], free amino acids [aa, e.g. glutamine (Gln), tyrosine (Tyr) and phenylalanine (Phe)] or phenolic compounds (e.g. flavonoids) which are found in garlic bulbs [7,8], but also in the discarded by-products [9–11].

The development of greener non-conventional extraction techniques such as ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE) are usually considered as faster and more efficient alternatives to conventional solid-liquid extraction (SLE) for obtaining extracts enriched in bioactive compounds from agro-food by-products [12]. While these techniques have been previously applied to the extraction of OSC [13,14], BCH [15,16] and specially, phenolic compounds [13,17–19] from garlic bulbs, only few studies related to garlic by-products are reported [20,21]. In this sense, Shekhar *et al.* [21] optimized an UAE method for the extraction of OSC (alliin, SAC and allicin) and phenolic compounds (gallic acid, p-coumaric acid, benzoic acid, among others) from garlic leaves, while Ferioli *et al.* (2020) evaluated the efficiency of high pressure extraction against conventional extraction of the main OSC from garlic cloves and stems

[11]. The limited information available in the literature related to the use of assisted extraction techniques to obtain extracts enriched in bioactive compounds from garlic by-products highlights the need for further research to fully explore the potential of these promising and innovative, but also underutilized, techniques for the revalorization of these samples. These studies would contribute to improve the sustainability and to provide value-added products.

Then, this work was focused on the development and comparison of different methodologies using advanced extraction techniques (UAE and PLE) and SLE to efficiently obtain multifunctional extracts from different garlic by-products (roots, skins, stems and leaves). The most effective methodology was applied to the extraction of these bioactives from the most promising garlic by-product.

2. Material and methods

2.1. Reagents

Analytical standards of S1PC, carbohydrates (fructose, glucose and sucrose) and of the amino acid standard mixture (AAS18) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while SAC was obtained from TCI (Tokyo Chemical Industry, Zwiindrecht, Belgium). 1-Kestose and nystose standards were acquired from Target Mol (Wellesley Hills, MA, US). The analytical grade solvents used were purchased from Scharlau (Barcelona, Spain). Ultrapure water was obtained using a Milli-Q system (Millipore Advantage A10 system, Massachussets, USA).

2.2. Plant material

By-products from different garlic plants (G1-G8) at different growth stages of different wild ecotypes (purple and white garlic) and from different geographical origin [Colmenar de Oreja (Madrid, Spain), Talavera de la Reina (Castilla La-Mancha, Spain) and the geographical area of ‘Las Pedroñeras’ (Castilla La-Mancha, Spain)] were obtained from different local producers or provided by Pharmactive Biotech S.L.U. (**Table S1** of Supplementary Material). As sample pretreatment, all the by-products, including roots, skins, stems and leaves, were cut, frozen and freeze-dried. Finally, samples were ground to fine particles in an IKA A10 basic mill (IKA-Werke, Germany), sieved through a 500 μ m mesh and stored in closed containers at room temperature and protected from the light until extraction.

2.3. Extraction methods

2.3.1. By-product selection

Prior to the optimization of the extraction procedures, the different parts of garlic plant G5, including skins (Sk5), roots (R5), stem (S5) and leaves (L5), were evaluated to select the by-

product with the highest content in bioactives. To this end, 100 mg of each sample were extracted by UAE using an Elmasonic P ultrasonic bath (Elma Schidbauer GmbH, Singen, Germany) equipped with temperature control and operating at 37 Hz of frequency and 30% power in 1 mL of MilliQ-water (Millipore) at 40 °C for 25 min. Extracts obtained were centrifuged at 4401 *g* for 10 min. Supernatants were collected, filtered with 0.2 µm teflon filters (Interchim, Montluçon, France) and diluted (1:10, v/v). All extracts were frozen and protected from the light until their analysis. Experiments were carried out in triplicate.

2.3.2. Optimization of UAE method

UAE conditions were optimized following a Box-Behnken experimental design using the stem sample S5. Three independent variables were included: temperature (*T*, °C), time (*t*, min) and sample amount/solvent volume ratio (*s/v*, mg mL⁻¹). The concentrations of bioactive compounds (OSC, BCH and aa) were considered as dependent variables. Fifteen experiments were conducted in random order, including three central points for error estimation. Experimental ranges for the factors evaluated were: *T* = 40-80 °C, *t* = 10-60 min, and *s/v* = 50-150 mg mL⁻¹. Each experiment was carried out in triplicate.

The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_3 s/v + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{3,3} s/v^2 + \beta_{1,2} Tt + \beta_{1,3} T s/v + \beta_{2,3} t s/v + \varepsilon \text{ (Ec. 1)}$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for *i*th factors, $\beta_{i,j}$ the coefficients for interaction between the factors *i* and *j* and ε is the error. Response surface methodology was used to calculate the coefficients of *R*₁ [concentration of OSC (including SAC, S1PC and S-allylmercaptocysteine (SAMC))], *R*₂ [concentration of BCH, including *myo*-inositol, 1-kestose, neokestose, nystose, neonystose and oligosaccharides with a degree of polymerization (DP) of 5] and *R*₃ [content of amino acids, including Gln, Tyr and Phe], and to estimate the statistical significance of the regression coefficients. The experimental conditions individually maximizing *R*₁, *R*₂ and *R*₃ were estimated by multiple linear regression (MLR) using StatGraphics Centurion 19 software (Statistical Graphics Corporation, Rockville, MD, USA). A multiple response analysis that simultaneously maximized *R*₁, *R*₂ and *R*₃ was also considered to select the optimal UAE operating conditions; this response function considers values between 0 (completely undesirable value) and 1 (completely desirable or ideal response). After extraction, samples were processed as described in section 2.3.1. Results were expressed as mg g⁻¹ dry weight (dw).

2.3.3. Optimization of PLE method

PLE was carried out in a PSE one instrument (Applied separations, PA, USA) using a 11 mL stainless steel extraction cell. A cellulose filter was placed at the bottom of the extraction cell and

a mixture of 100-500 mg of S5 with 7 g of sea sand (Scharlab S.L., Barcelona, Spain) was introduced. Additionally, a top layer of 4 g of sand was also added. Water was used as extraction solvent, and a single extraction cycle was conducted in static mode at 100 bar pressure. Nitrogen was used as purge gas for 1 minute at the end of the cycle.

Considering the higher volumes used in this technique, and therefore, the larger sample amounts needed, prior to the experimental design, the sample amount was optimized for a total volume of 10 mL and 100, 300 and 500 mg were considered using PLE conditions of 85 °C and 15 min.

A 3-level factorial design was used for optimization of the extraction conditions, in which two factors were considered: time ($t = 5-30$ min) and temperature ($T = 50-120$ °C). In this case, extraction times were shorter than those of UAE, as the PLE instrumentation allows the use of higher temperatures (120 °C). In total, 12 experiments with 4 replicates as central points were conducted. The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{1,2} Tt + \varepsilon \quad (\text{Ec. 2})$$

where the nomenclature is the same as those indicated in section 2.3.2. For comparative purposes, the same three response variables, R_{1-3} (OSC, BCH and aa) considered for UAE were selected and a multiple response analysis that simultaneously maximized R_{1-3} was considered to select the optimal operating conditions.

The volume of extracts obtained were measured, filtered and further processed as described in section 2.3.1. before analysis.

2.3.4. SLE method

Once the most appropriate advanced technique was selected, the same optimal extraction conditions were assayed by SLE for comparative purposes. Sample S5 (77mg) was treated with 1 mL of Milli-Q water and stirred (700 rpm) at 40 °C for 60 min in a heating plate (IKA-Werke, Staufen, Germany). The obtained extracts were centrifuged at 4401 g for 10 minutes. The supernatants were then collected and diluted at a ratio of 1:10 (v/v) for subsequent chromatographic analysis. All experiments were carried out in triplicate.

2.4. Analytical methods

2.4.1. LC-MS analysis

Qualitative and quantitative analysis of OSC, carbohydrates (bioactive and non bioactive) and aa were performed on an Agilent 1100 series system, including an autosampler, a quaternary pump, a thermostated column compartment and a diode array detector, coupled to a 6125 single

quadrupole mass detector (Agilent Technologies, Santa Clara, CA, US) provided with an electrospray ionization (ESI) source. The LC-MS analyses of OSC and aa were carried out following the methodology of Jiménez-Amezcuca *et al.* [6]. In brief, chromatographic separation was carried out by an InfinityLab Poroshell 120 Bonus-RP column (150 mm × 4.6 mm, 2.7 µm; Agilent) operating at 0.4 mL min⁻¹ and thermostated at 30 °C. A binary gradient consisting of water (eluent A) and methanol (eluent B), both with 0.1% formic acid, was used as follows: 0–7 min, 7% B; 21 min, 60% B; 22–32 min, 95% B; 33 min, 7% B; 48 min, 7% B.

Attending CHs, the analyses were performed as indicated by Jiménez-Amezcuca *et al.* [22], using a BEH X-Bridge column (150 mm × 4.6 mm; 3.5 µm particle size; 135 Å pore size; Waters, Hertfordshire, UK). A gradient of acetonitrile (A) / water (B), both with 0.1% of NH₄OH, at a flow rate of 0.4 mL min⁻¹ was used as follows: 0 min, 20% B; 20 min, 40% B; 30 min, 40% B; 31 min, 20% B, 40 min, 20% B. Injection volume was set as 5 µL.

Mass spectra were acquired using ESI spray voltage at 3 kV, 300 °C of drying N₂ temperature (99.5% purity), 12 L min⁻¹ of drying gas flow, 276 kPa of nebulization pressure, and a fragmentor voltage of 100 V. Analyses were carried out both in SCAN mode (90–2000 *m/z* range) and in SIM mode, registering [M+H]⁺ adducts of the different OSC and aa. In the case of CHs, ammonium adducts, [M+NH₄]⁺, were the most abundant for monosaccharides and disaccharides while sodiated adducts, [M+Na]⁺, were the predominant for 1-kestose, nystose and oligosaccharides with DP5. Thus, they were selected for SIM mode analysis.

Data acquisition and processing were performed using OpenLAB CDS Software (v.2.19.20, Agilent Technologies). Identification of compounds was carried out based on chromatographic retention and MS data and confirmed, when possible, by co-injection of the corresponding commercial standards.

Quantitative analysis was performed in triplicate in SIM mode using external standard calibration curves of SAC (0.0006–0.075 mg mL⁻¹), the aa standard mixture (0.0005–0.01 mg mL⁻¹), glucose, fructose and sucrose (0.005–0.1 mg mL⁻¹), 1-kestose (0.005–0.07 mg mL⁻¹) and nystose (0.005–0.07 mg mL⁻¹). Other compounds such as S1PC and SAMC were quantified using the SAC calibration curve, while *myo*-inositol and DP5 oligosaccharides were quantified with glucose and nystose calibration curves, respectively.

2.5. Statistical analysis

Statgraphics Centurion 19 software (Statistical Graphics Corporation, Rockville, MD, USA) was used for statistical analysis of data. Significance (*p* < 0.05) of differences for results obtained was determined by the analysis of variance (ANOVA, Tukey test).

3. Results and discussion

3.1. Selection of garlic by-products

Prior to the optimization of the extraction techniques, composition of garlic stems, leaves, roots and skins was evaluated in order to select the garlic by-product with the highest content of bioactive compounds. For this purpose, UAE extracts from the different by-products of garlic sample 5 (Sk5, R5, S5 and L5, **Table S1**) were obtained at 40 °C for 25 min and analyzed by LC-MS. Water was selected as extractant based on previous results regarding the extraction of OSC and BCH in aged garlic samples [6]. Moreover, its GRAS (Generally Recognized As Safe) nature and low cost, facilitate the possibility of further application of the obtained extracts in the food industry [13,14].

Different compounds were identified in G5 garlic by-products (**Figure 1**), including: (i) organosulfur compounds (such as SAC, S1PC and SAMC), (ii) carbohydrates of different DP such as monosaccharides (e.g. fructose and glucose), disaccharides (sucrose), and those considered bioactives [polyols (*myo*-inositol), trisaccharides (such as neokestose and 1-kestose), and tetrasaccharides (such as nystose and neonystose)], and (iii) free amino acids (such as Gln, Tyr and Phe). As it can be seen in **Table 1**, the stem, followed by the leaves, were the by-products with the highest content of OSC, while the roots showed the lowest contents (0.78 and 0.62 vs 0.12 mg g⁻¹ dw). Regarding BCH, values ranged from 2.7 mg g⁻¹ dw in the root extract to 19.2 mg g⁻¹ dw in the stem extract and aa were also more abundant in the stem extract (14.6 mg g⁻¹ dw). Therefore, this by-product was selected for further studies.

3.2. Optimization of UAE method

Optimization of the extraction conditions is critical, as they affect the efficiency of the process as well as the quality and bioactivity of the extracts obtained. Thus, a total of 15 experiments were carried out according to a Box-Behnken experimental design to optimize the UAE parameters that maximize the extraction of bioactive compounds from garlic stem samples. The effect of three independent variables (*t*, *T* and *s/v*) on the yields obtained for OSC (*R*₁, mg g⁻¹ dw), BCH (*R*₂, mg g⁻¹ dw) and aa (*R*₃, mg g⁻¹ dw) by UAE was evaluated. As it can be seen in **Table 2**, total concentrations varied between 0.70-1.5 mg g⁻¹ dw for OSC, 9.9-24.9 mg g⁻¹ dw for BCH and 4.4-9.5 for aa. In general, for a fixed *t* and *s/v* values, the increase of temperature favored the extraction of OSC (e.g. 80 °C/10 min/100 mg mL⁻¹ vs. 40 °C/10 min/100 mg mL⁻¹, experiments 1 and 5 and 80 °C/35min/50 mg mL⁻¹ vs. 40 °C/35min/50 mg mL⁻¹, experiments 12 and 2), while higher yields of aa were achieved at lower temperatures and *s/v* ratios (40 °C/35min/50 mg mL⁻¹, experiment 2). Regarding BCH, low *s/v* ratios also promoted their extraction at high/medium temperatures and times (experiment 12 and 13).

A response surface methodology was used to calculate the regression coefficients of R_1 , R_2 and R_3 quadratic models proposed and to estimate their statistical significance. The regression equations of corresponding models and response surface plots are shown in **Table 3** and **Figure S1A** respectively. In the case of OSC (R_1), the most significant parameter at a 95% confidence level ($p < 0.05$) was s/v^2 and the optimal conditions to maximize R_1 were 80 °C, 19 min and 95 mg mL⁻¹, while the most significant variables ($p < 0.05$) to maximize the concentration of BCH (R_2) were s/v and t s/v and the optimal conditions were 40 °C, 59 min and 50 mg mL⁻¹. Regarding R_3 , the most significant coefficients ($p < 0.05$) to maximize the aa concentration were T and s/v ; according to this model, the optimal conditions were 40 °C, 60 min and 51 mg mL⁻¹. These quadratic models appropriately described the variability for R_1 ($R^2 = 0.72$), R_2 ($R^2 = 0.79$) and R_3 ($R^2 = 0.88$).

Finally, a multiple response that simultaneously maximize R_{1-3} was also evaluated and the optimal operating parameters ($R_D = 0.74$) were 40 °C, 60 min and 77 mg mL⁻¹.

3.3. Optimization of PLE method

Prior to the optimization of PLE conditions, three experiments were carried out for the selection of the optimum s/v ratio at 100 bar, 85°C and 15 minutes of extraction. For a total solvent volume of 10 mL, different sample amounts (100, 300 and 500 mg) were evaluated to determine the optimum sample/solvent volume ratio. Although no significant differences were found between the obtained concentrations of bioactive compounds (18.3 ± 2.2 mg g⁻¹ dw, 16.6 ± 3.8 and 18.0 ± 2.0 mg g⁻¹ for 100, 300 and 500 mg, respectively), in order to minimize the sample amount, 100 mg was selected for further experiments.

A 3-level factorial design was then carried out to maximize the extraction of bioactives of stem garlic by-product (R_{1-3}) by PLE considering two independent variables, the extraction temperature and time. The results obtained under the different conditions assayed are shown in **Table 4**. As it can be observed, temperatures equal or higher than 85°C enhanced the extraction of OSC and BCH, while a lower concentration of aa was observed at 120°C, probably due to their thermal degradation [23,24]. Time had a minor impact on the extraction of OSC, with not major differences observed between extractions at the same temperature and different times (0.99 mg g⁻¹ dw at 50°C/17.5 min and 0.98 mg g⁻¹ dw at 50°C/30 min, experiments 1 and 2; 1.27 mg g⁻¹ dw at 85°C/30 min and 1.30 mg g⁻¹ dw at 85°C/17.5 min, experiments 8 and 10). **Figure S1B** shows the response surface plots obtained for the evaluated variables while the R_{1-3} quadratic equations and R^2 are presented in **Table 3**. In the case of OSC (R_1), T^2 and T coefficients were the most significant ($p < 0.05$) variables, being the optimal extraction conditions 89 °C and 17 min. Attending BCH, T , t and t^2 were the most significant factors ($p < 0.05$) that affected their

extraction (optimal conditions: 120 °C and 22 min) while the coefficients T^2 , T and t were the most significant to maximize aa (R_3) extraction (optimal conditions: 77 °C and 5 min).

In order to maximize all the responses, a multiple response was also considered and the optimal conditions were 91 °C and 18 min ($R_D=0.64$).

3.4. Comparison of extraction techniques

Figure 2 shows the concentrations of OSC, BCH and aa in the extracts of sample S5, obtained by UAE and PLE under the optimal conditions above described. Extraction conditions were selected according to those described in a previous work [6] for the extraction of OSC and CHs from aged garlic.

As it can be observed, similar results were obtained for UAE and PLE and both techniques would be suitable for the revalorization of garlic stem samples. Although the PLE methodology is faster than the one developed by UAE (extractions of 18 min vs 60 min, respectively), the latter requires lower extraction temperatures than those required for PLE (40 °C vs 89 °C). In addition, the UAE instrumentation allows multiple extractions to be performed simultaneously, which is an advantage over PLE.

Thus, finally, the results obtained by UAE were also compared with those achieved by conventional SLE, using the same extraction conditions (77 mg mL⁻¹, 60 min and 40 °C). In this case, significantly lower yields of all the bioactives were obtained by SLE (i.e., 0.97 ± 0.01 mg OSC g⁻¹ dw, 18.5 ± 0.6 mg BCH g⁻¹ dw and 7.3 ± 0.03 mg aa g⁻¹ dw). It has been described that the disruptive effect of ultrasounds on cell wall structures accelerates the diffusion of the solvent through the membranes and improves the extraction yield of bioactive compounds over conventional extraction approaches [16].

Therefore, based of the above results, the optimized UAE methodology was selected for further applications.

3.5. Application of UAE methodology to garlic stem samples

Stem garlic samples from different ecotypes of raw garlic (including white and purple garlic) were collected at different stages of growth and subjected to UAE under optimized operating conditions. As it is shown in **Table 5**, variable concentrations of OSC, BCH and aa were detected depending on the sample considered. These differences could be due to a number of variables, such as garlic ecotype, geographical location, growing conditions, harvesting time, etc. [25]. It has been described that during whole garlic vegetation period, nutrients are redistributed along the plant, so that organosulfur compounds and other bioactives are transported from the senescent leaves to the newly formed sink organs. Therefore, the content of bioactive compounds of garlic

bulbs can vary considerably depending on the harvesting time [25]. Thus, in this study, stems of garlic plants with different growth stage (with developed and non-developed bulb, samples S1-4 and S5-8, respectively, **Figure S2A**) were considered. Moreover, different parts of a stem (the closest part and the more distal part to the bulb, samples S8a and S8b, respectively) were also included (**Figure S2B**).

Table 5 shows the different OSC detected in the UAE stem extracts, where total concentrations varied from 0.35 to 14.87 mg g⁻¹ dw. S-alk(en)yl-cysteine sulfoxides such as alliin (S-allylcysteine sulfoxide) and isoalliin (S-1-propenylcysteine sulfoxide) were detected. Both have been described as primary sources of the compounds responsible for the garlic bioactivities and its characteristic flavor [26]. Alliin was the OSC with the highest concentration, with sample S3 showing the highest value (10.4 mg g⁻¹ dw), followed by S4 (4.8 mg g⁻¹ dw). This compound has also been described to be the main compound in the flowering stems of Sulmona Red Garlic, amounting to over 99% of all hydrophilic organosulfur compounds quantified [11].

Regarding growth stage, in general, alliin was present in stem samples with less maturation (S1-4). It has been described that this compound is biosynthesized in garlic leaves during the initial stages of bulb development and when the leaves began to wither, alliin is transported from the green foliage leaves to the developing bulb for storage [26]. This behavior was observed in garlic stems of different growth stages by Liu *et al.* [25], where alliin decreased from 4.54 mg g⁻¹ in the first week to 0.22 mg g⁻¹ in the week 6 of cloves development.

Variable amounts of allicin (allyl disulfide oxide) were also detected in the stem extracts, reaching up to 3.51 mg g⁻¹ dw in sample S3. This compound, a garlic defense component due to its antibacterial and antifungal effects, is the main responsible for the characteristic aroma of freshly cut garlic. It is synthesized from alliin by the action of the vacuolar enzyme alliinase when garlic is cut or crushed [11,26,27] and is not present in the intact plant. Allicin is highly reactive and unstable and decompose into other volatile OSC characteristic of the garlic aroma, such as diallyl sulfide, diallyl disulfide, diallyl trisulfide, *E*-, and *Z*-ajoene, etc. Very low amounts of this compound (0.05% of all hydrophilic organosulfur compounds) were found by Ferioli *et al.* [11] in the flowering stems of Sulmona Garlic.

S-alk(en)yl-L-cysteines, such as SAC and S1PC, and SAMC, were also detected in most of the stem samples, except for samples S8a-8b, where neither SAC nor SAMC could be detected. These compounds, reported to have cardioprotective activity and antioxidant, anti-inflammatory, immunomodulatory and hepatoprotective effects [28–30], were also found in garlic stem by Liu *et al.* [25] and the concentration values were in the same range as those shown in **Table 5**.

Regarding carbohydrates, fructose, glucose and sucrose were detected in all stem samples, their total contents ranging from 90 to 231 mg g⁻¹ dw. In general, the content of total low molecular

weight carbohydrates (LMWCs) was higher in those plants with formed cloves, reaching values up to 247 mg g⁻¹ dw in S8b. FOS consist of a sucrose (α -D-glucopyranosyl-(1 $\rightarrow$ 2)- β -D-fructofuranoside) molecule that is elongated by fructosyl units linked through β -(2 $\rightarrow$ 1) bonds [16]. Thus, it was not surprising to find in stem samples high levels of fructose (40-170 mg g⁻¹ dw), followed by sucrose (32-60 mg g⁻¹ dw) and, to a lesser extent, of glucose.

Bioactive carbohydrates were also found in all these samples, with concentrations varying between 7 to 21 mg g⁻¹ dw. Garlic carbohydrates, mainly polysaccharides and oligosaccharides, are important storage compounds present in garlic bulbs. Non-digestible oligosaccharides such as neokestose, 1-kestose, nystose and neonystose are natural prebiotics, that have also been described to have other beneficial properties such as antimicrobial agents, antioxidants, and hepatoprotectives compounds, among others [16]. *myo*-Inositol, a bioactive polyol present in plasma membranes and in other structures of fruits and vegetables [31] was also detected in all the stems analyzed (0.32 and 3.1 mg g⁻¹ dw).

Amino acids varied between 2 and 15.7 mg g⁻¹ dry sample and, in general, a higher content was observed for those samples at the initial growth stages. These results match those previously reported by Liu *et al.* [25], who described that the content of 20 amino acids in five garlic tissues (including stems) was higher in young garlic plants as compared to more advanced growth stages, with values varying in the range of 27.46-74.96 mg g⁻¹ dw. The role of amino acids in garlic plant is essential, as they function as building blocks for proteins and enzymes and they are the main precursors for the formation of cysteine sulfoxides and glutamyl peptides. Moreover, they also act as antioxidants, especially sulfur-containing amino acids, such as methionine [32]. Among the amino acids quantified, Gln, was the most abundant (0.6-8.5) in stem extracts, which is in line with data reported in the literature (3.65-10.1 mg g⁻¹ dw [25]). Essential amino acids such as lysine (Lys), phenylalanine (Phe), leucine (Leu), isoleucine (Ile), methionine (Met), valine (Val) and threonine (Thr) were also detected in all stem samples.

In general, although samples of garlic stem from early growth stages were found to have a higher content of bioactive compounds, stems from plants with fully developed bulbs, which currently are discarded, have also proven to be a potential source of functional ingredients. Thus, they would be a very interesting by-product to be revalorized.

4. Conclusions

Advanced extraction techniques, such as UAE and PLE have proven to be useful to obtain multifunctional extracts from garlic stems, with higher yields of bioactive compounds (OSC, BCH and aa) over classical SLE methodologies. Although PLE methodology was faster, UAE method required lower temperatures and allowed multiple extractions to be performed

simultaneously. The application of the optimized UAE procedure (40 °C, 60 min and 77 mg mL⁻¹) to different stem garlic by-products revealed a wide variability in the composition of extracts, that could be due to the growing stage and the ecotype of the plant, but also to other external factors (environmental conditions, available nutrients, soil quality, etc).

In the context of circular economy, the new developed methodologies, specially UAE, could be considered as valuable and promising tools for the revalorization not only of garlic stems but also of other garlic by-products (such as leaves, peels or roots). These approaches would also be a contribution to minimize garlic residues and to reduce the environmental impact associated with garlic production.

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511 **Table 1.** Garlic by-products composition (mg g⁻¹ dw)

		Stem	Leaves	Roots	Skins
Organosulfur compounds		0.78 (0.06) ^{*a}	0.62 (0.01) ^b	0.12 (0.01) ^c	0.40 (0.02) ^d
Carbohydrates	DP1	128 (5) ^a	75 (4) ^b	32 (2) ^c	44 (2) ^c
	<i>myo</i> -Inositol [#]	1.4 (0.1) ^a	1.4 (0.1) ^a	2.7 (0.4) ^b	0.82 (0.05) ^a
	DP2	42 (3) ^a	0.84 (0.01) ^b	<LOQ	25.3 (3) ^c
	DP3 [#]	5.0 (0.2) ^a	0.44 (0.09) ^b	<LOQ	4.1 (0.3) ^c
	DP4 [#]	6.8 (0.1) ^a	1.5 (0.1) ^b	<LOQ	3.8 (0.2) ^c
	DP5 [#]	6.4 (0.2) ^a	1.5 (0.5) ^b	<LOQ	1.10 (0.05) ^b
Amino acids		14.6 (0.3) ^a	14.2 (0.2) ^a	10.9 (0.5) ^b	10.7 (0.5) ^b

512 ^{*} Standard deviation ($n = 3$) in brackets513 ^{a-c} Different letters indicate significant ($p < 0.05$) differences among the different byproducts for
514 each group of compounds considered515 [#] Bioactive carbohydrates

516

Table 2. Concentration (mg g^{-1} dw) of target bioactive compounds in garlic stems obtained during UAE optimization. OSC: organosulfur compounds; BCH: bioactive carbohydrates; aa: free amino acids.

Exp.	T ($^{\circ}\text{C}$)	t (min)	s/v (mg mL^{-1})	OSC	BCH	aa
1	80	10	100	1.4 (0.1)*	20.8 (0.2)	7.82 (0.05)
2	40	35	50	0.84 (0.06)	22.8 (0.8)	9.5 (0.4)
3	60	60	50	0.70 (0.06)	24.9 (0.1)	8.3 (0.2)
4	80	35	150	0.89 (0.01)	15.4 (0.2)	4.4 (0.2)
5	40	10	100	0.78 (0.05)	12.5 (0.5)	6.0 (0.1)
6	60	35	100	1.4 (0.1)	16.8 (0.7)	8.1 (0.6)
7	40	60	100	1.31 (0.02)	17 (2)	7.85 (0.05)
8	60	35	100	1.4 (0.2)	16.2 (0.8)	7.2 (0.7)
9	40	35	150	1.19 (0.01)	14.5 (0.2)	5.97 (0.09)
10	60	10	50	0.99 (0.09)	9.9 (0.1)	9.0 (0.2)
11	60	10	150	1.07 (0.05)	13.9 (0.5)	5.6 (0.4)
12	80	35	50	1.07 (0.06)	24 (3)	8.0 (0.3)
13	60	35	100	1.34 (0.02)	16.2 (1.3)	7.6 (0.1)
14	60	60	150	1.03 (0.03)	13.4 (0.8)	4.97 (0.03)
15	80	60	100	1.5 (0.1)	16 (1)	6.7 (0.4)

*Standard deviation ($n = 3$) in brackets

Table 3. Regression equations to maximize the extraction of organosulfur compounds (R_1), bioactive carbohydrates (R_2) and amino acids (R_3) from garlic stems by UAE and PLE.

	Model Equation	R ²
UAE	$R_1 = -1.78 + 0.023 t + 0.035 s/v + 0.025 T - 0.00014 t^2 - 0.00022 t T - 0.00013 s/v^2 - 0.00013 s/v T$	0.72
	$R_2 = 13.00 + 0.87 t + 0.049 s/v - 0.40 T - 0.0029 t^2 - 0.0031 t s/v^2 - 0.0046 t T + 0.0047 T^2$	0.79
	$R_3 = 8.36 + 0.089 t - 0.034 s/v + 0.037 T - 0.0015 t T$	0.88
PLE	$R_1 = -0.234 + 0.034 T - 0.00019 T^2$	0.88
	$R_2 = 8.16 + 0.087 T + 0.77 t - 0.017 t^2$	0.84
	$R_3 = 2.40 + 0.16 T - 0.042 t - 0.001 T^2$	0.84

Table 4. Concentration (mg g⁻¹ dw) of target bioactive compounds in garlic stems obtained during PLE optimization. OSC: organosulfur compounds; BCH: bioactive carbohydrates; aa: free amino acids.

Exp.	T (°C)	t (min)	OSC	BCH	aa
1	50	17.5	0.99 (0.06)*	21.1 (0.7)	6.7 (0.1)
2	50	30	0.98 (0.09)	20 (1)	6.0 (0.5)
3	85	17.5	1.3 (0.1)	24 (2)	7.9 (0.2)
4	85	17.5	1.24 (0.06)	24.8 (0.6)	7.4 (0.2)
5	120	5	1.05 (0.2)	24.1 (0.6)	6.1 (0.3)
6	85	5	1.19 (0.08)	17 (1)	7.7 (0.6)
7	120	30	1.1 (0.2)	24 (4)	5.4 (1.2)
8	85	30	1.27 (0.01)	25 (1)	7.6 (0.1)
9	120	17.5	1.16 (0.05)	27 (3)	5.9 (0.2)
10	85	17.5	1.30 (0.06)	22 (2)	7.7 (0.2)
11	85	17.5	1.00 (0.01)	16 (2)	8.35 (0.07)
12	50	5	1.37 (0.06)	22.9 (0.3)	7.7 (0.2)

*Standard deviation ($n = 3$) in brackets

Table 5. Concentration (mg g⁻¹ dw) of bioactive compounds in garlic stem by-products extracts obtained by UAE under optimal conditions (77 mg mL⁻¹, 40 °C, 60 min). OSC: organosulfur compounds; LMWC: low molecular weight carbohydrates; BCH: bioactive carbohydrates; aa: free amino acids.

	By-product	S1	S2	S3	S4	S5	S6	S7	S8a	S8b
OSC	Isoalliin	0.108 (0.007) ^a	0.054 (0.003) ^b	0.32 (0.01) ^c	1.90 (0.01) ^d	0.187 (0.0004) ^e	tr	tr	1.08 (0.04) ^f	0.30 (0.02) ^c
	Alliin	2.5 (0.2) ^a	2.71 (0.05) ^a	10.43 (0.09) ^b	4.8 (0.01) ^c	4.18 (0.07) ^d	-	-	-	-
	SAC	0.87 (0.02) ^a	0.54 (0.01) ^b	0.0129 (0.0001) ^c	tr	0.78 (0.08) ^d	0.3 (0.02) ^e	0.11 (0.01) ^c	-	-
	S1PC	0.217 (0.004) ^a	0.161 (0.001) ^b	0.05 (0.0005) ^c	0.087 (0.001) ^d	0.06 (0.005) ^c	0.097 (0.002) ^e	0.092 (0.002) ^{d,e}	0.051 (0.001) ^c	0.0512 (0.0003) ^c
	SAMC	0.26 (0.01)	0.24 (0.01)	0.539 (0.003)	0.54 (0.003)	0.47 (0.07)	0.09 (0.01)	0.037 (0.003)	-	-
	Allicin	2.8 (0.2) ^a	1.36 (0.07) ^b	3.51 (0.03) ^c	-	0.87 (0.05) ^d	1.81 (0.03) ^e	1.90 (0.005) ^f	-	-
LMWC	Total	6.78 (0.02)^a	5.1 (0.1)^b	14.87 (0.04)^c	6.8 (0.1)^a	6.78 (0.08)^a	2.2 (0.1)^d	2.13 (0.01)^d	1.13 (0.04)^e	0.35 (0.02)^f
	Fructose	86 (3) ^a	84 (8) ^a	44 (2) ^b	54 (6) ^b	108 (4) ^a	52 (3) ^b	106 (8) ^a	138 (13) ^c	170 (13) ^d
	Glucose	38 (2) ^a	34 (3) ^a	14.6 (0.4) ^c	3 (1) ^d	48 (0.2) ^e	22 (1) ^f	27 (2) ^f	26 (3) ^f	-
	Sucrose	58 (6) ^{a,b}	38 (3) ^{b,c}	32 (3) ^c	36 (1) ^{b,c}	54.6 (0.6) ^{a,b}	46 (2) ^{b,c,d}	52 (3) ^{a,b,d}	35 (5) ^c	60 (3) ^{a,b}
	Total	183 (10)^{a,b}	157 (13)^b	90 (6)^e	92 (6)^e	211 (5)^{a,c}	120 (7)^e	186 (12)^{a,b}	198 (13)^{a,c}	231 (16)^c

Table 5. Cont.

	myo -Inositol	3.1 (0.2) ^a	1.3 (0.2) ^{b,c}	1.5 (0.1) ^b	0.6 (0.2) ^d	2.6 (0.1) ^a	0.32 (0.04) ^d	0.8 (0.1) ^{c,d}	1.0 (0.2) ^{b,d}	3.1 (0.2) ^a
BCH	Neokestose	4 (0.5) ^a	3 (0.5) ^{a,b,c}	2.2 (0.1) ^{b,d}	2.3 (0.4) ^{b,d}	2.1 (0.1) ^{b,d}	3.4 (0.2) ^{a,c}	3.5 (0.4) ^{a,c}	1.8 (0.4) ^d	3 (0.1) ^{b,c}
	1-Kestose	3.7 (0.5) ^a	2.6 (0.5) ^{b,c}	1.7 (0.05) ^{b,d,e}	2.6 (0.4) ^{b,c}	1.39 (0.01) ^{e,f}	3 (0.1) ^{a,c}	2.6 (0.4) ^{b,c,d}	0.6 (0.2) ^f	1.1 (0.1) ^{e,f}
	Nystose	3.7 (0.4) ^{a,b}	2.2 (0.1) ^{a,c}	2.9 (0.2) ^{a,b}	3.5 (1.6) ^{a,b}	2.21 (0.05) ^{a,b,c}	4.1 (0.3) ^b	3.4 (0.2) ^a	0.8 (0.1) ^c	2.63 (0.08) ^{a,b}
	Neonystose	1.6 (0.2) ^a	1.2 (0.3) ^a	0.54 (0.02) ^b	1.5 (0.1) ^a	4.18 (0.03) ^d	1.6 (0.2) ^a	2.5 (0.2) ^c	1.1 (0.1) ^{a,b}	2.31 (0.04) ^c
	DP5	4.9 (0.5) ^{a,b}	3.9 (0.5) ^{a,c}	2.7 (0.1) ^{c,d}	5.5 (0.2) ^b	8.0 (0.01) ^e	4.9 (0.7) ^{a,b}	5.6 (0.4) ^b	1.9 (0.2) ^d	4.21 (0.07) ^a
	Total	21 (2)^a	14 (2)^{b,c}	11.5 (0.3)^{b,d}	16 (3)^{b,c,e}	21.14 (0.01)^{a,e}	17 (1)^{a,c,e}	18 (1)^{a,c,e}	7 (1)^d	16 (1)^{a,b,c,e}
aa	Gln	4.2 (0.1) ^a	2.21 (0.04) ^b	8.5 (0.1) ^c	8.48 (0.01) ^c	8.5 (1.1) ^c	1.35 (0.07) ^{b,d}	0.59 (0.02) ^d	0.8 (0.04) ^d	0.6 (0.2) ^d
	Tyr	0.25 (0.05) ^{a,b}	0.37 (0.09) ^{b,d}	0.8 (0.3) ^c	0.24 (0.05) ^{a,b}	0.6 (0.07) ^{c,d}	0.09 (0.01) ^a	0.07 (0.01) ^a	0.06 (0.01) ^a	0.05 (0.02) ^a
	Phe	0.320 (0.001) ^a	0.43 (0.01) ^b	0.66 (0.01) ^c	0.35 (0.004) ^a	1.0 (0.1) ^d	0.14 (0.01) ^e	0.14 (0.01) ^e	0.09 (0.005) ^e	0.11 (0.2) ^e
	Leu+Ile	0.7 (0.02) ^a	1.03 (0.02) ^b	1.69 (0.024) ^c	0.7 (0.01) ^{a,c}	0.67 (0.004) ^c	0.3 (0.02) ^d	0.26 (0.01) ^d	0.25 (0.01) ^d	0.229 (0.001) ^d
	Lys	0.13 (0.01) ^a	0.14 (0.001) ^a	0.14 (0.003) ^b	0.14 (0.003) ^a	0.32 (0.01) ^c	0.044 (0.002) ^d	0.05 (0.01) ^d	0.059 (0.003) ^{d,e}	0.07 (0.01) ^e
	Pro	0.30 (0.01) ^a	0.26 (0.002) ^b	0.36 (0.001) ^c	0.3 (0.005) ^c	0.28 (0.01) ^b	0.1 (0.001) ^d	0.1 (0.01) ^e	0.06 (0.003) ^d	0.1 (0.01) ^e
	Glu	0.60 (0.01) ^a	0.59 (0.02) ^a	1 (0.003) ^{b,c}	0.6 (0.02) ^{a,b}	0.9 (0.01) ^c	0.30 (0.02) ^d	0.35 (0.01) ^e	0.41 (0.02) ^f	0.27 (0.05) ^{d,e}
	Arg	-	-	-	-	-	-	-	-	0.15 (0.01)
	Asp	0.20 (0.01) ^a	0.13 (0.02) ^a	0.32 (0.02) ^c	0.43 (0.01) ^d	0.22 (0.004) ^e	0.05 (0.01) ^f	0.053 (0.005) ^f	0.3 (0.06) ^{c,e}	0.12 (0.05) ^a

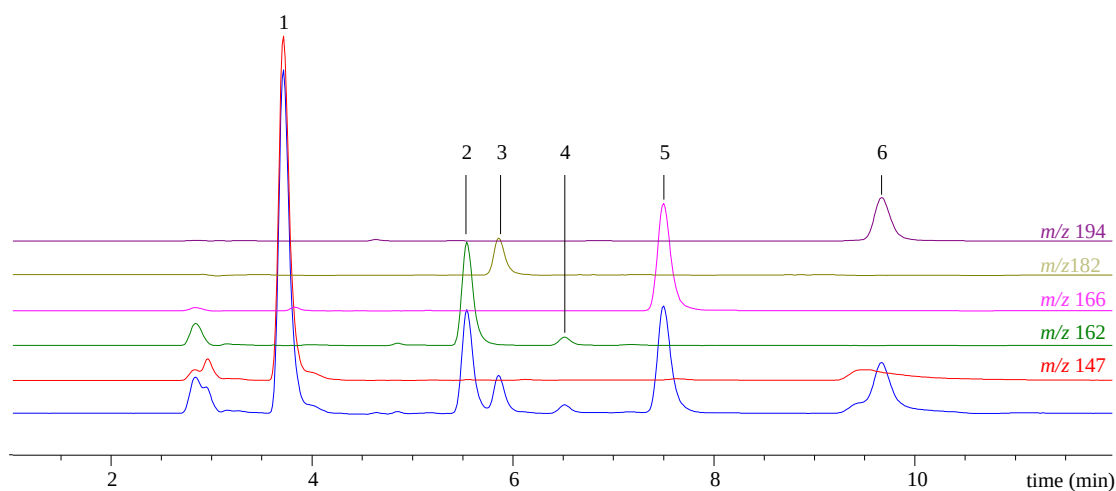
*Standard deviation ($n = 3$) in brackets;a-f Different letters within the same row mean significant ($p < 0.05$) differences for each compound.

Table 5. Cont.

ee	Ser	0.3 (0.02) ^a	0.44 (0.01) ^b	0.62 (0.001) ^c	0.3 (0.01) ^a	0.37 (0.01) ^a	0.2 (0.01) ^d	0.11 (0.01) ^e	0.22 (0.01) ^d	0.14 (0.04) ^f
	Thr	0.2 (0.004) ^a	0.32 (0.01) ^b	0.3 (0.01) ^c	0.1 (0.01) ^d	0.30 (0.01) ^b	0.1 (0.01) ^d	0.1 (0.003) ^d	0.1 (0.001) ^d	0.09 (0.01) ^d
	Met	0.1 (0.01) ^a	0.06 (0.01) ^a	0.14 (0.003) ^b	0.02 (0.001) ^c	0.03 (0.003) ^c	0.02 (0.002) ^c	0.027 (0.003) ^c	0.013 (0.002) ^c	0.02 (0.01) ^c
	Val	0.3 (0.01) ^{a,c,d}	0.26 (0.002) ^a	0.36 (0.001) ^b	0.3 (0.005) ^a	0.28 (0.01) ^{a,c,d,e}	0.1 (0.001) ^{d,e}	0.1 (0.01) ^{d,e}	0.06 (0.003) ^e	0.1 (0.01) ^{d,e}
	Total	7.7 (0.1)^a	6.6 (0.2)^b	15.7 (0.2)^c	12.3 (0.03)^d	13.6 (1.0)^e	2.9 (0.2)^f	2.0 (0.1)^f	2.5 (0.2)^f	2.31 (0.01)^f

*Standard deviation ($n = 3$) in brackets;^{a-f} Different letters within the same row mean significant ($p < 0.05$) differences for each compound.

A



B

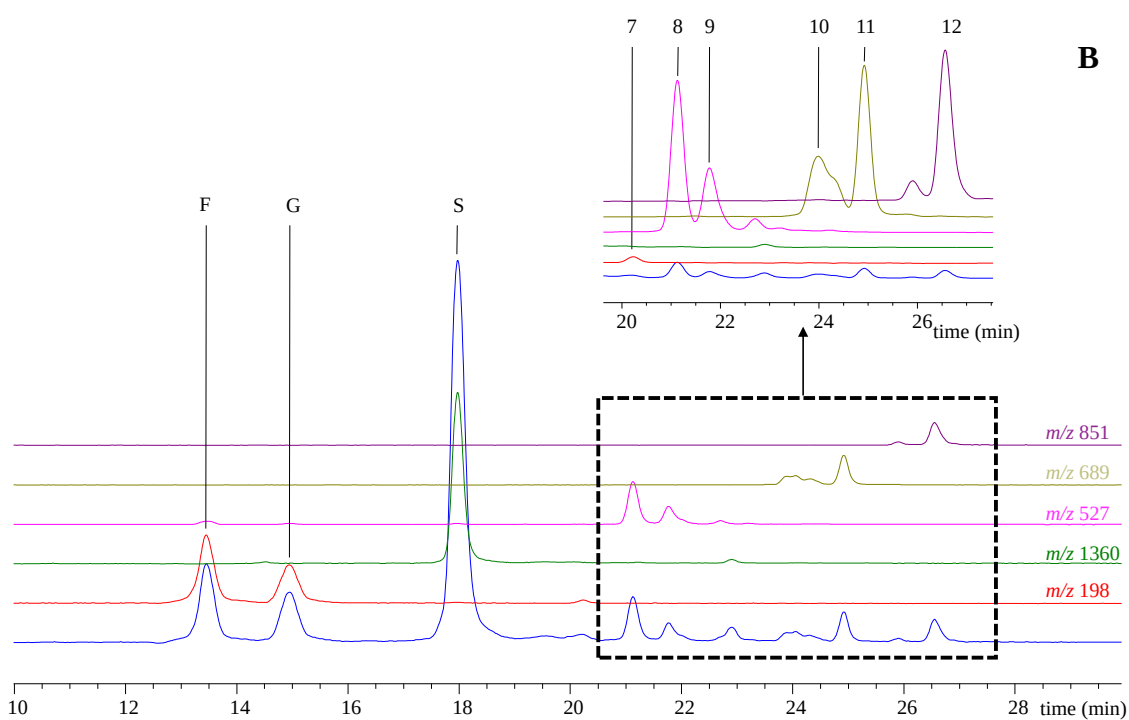
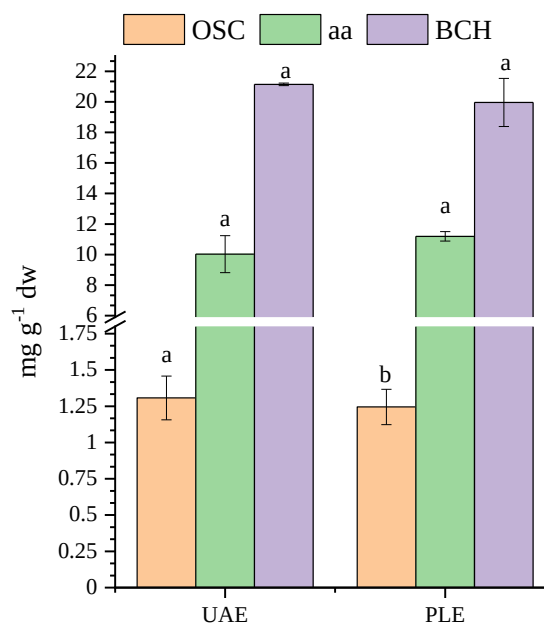


Figure 1. LC-MS profile (SIM mode) of aqueous stem extracts obtained by UAE. Organosulfur compounds and free amino acids (A), and carbohydrates (B) considered in the experimental design: 1. Glutamine; 2. *S*-allyl-L-cysteine; 3. Tyrosine; 4. *S*-1-propenyl-L-cysteine; 5. *S*-allyl-mercaptocysteine; 6. Phenylalanine; 7. *myo*-Inositol; 8. Neokestose; 9. 1-Kestose; 10. Nystose; 11. Neonystose; 12. DP5; F: Fructose; G: Glucose; S: Sucrose.



^{a-b} Different letters indicate significant differences between advanced extraction techniques ($p < 0.05$)

Figure 2. Concentration of organosulfur compounds (OSC), free amino acids (aa) and bioactive carbohydrates (BCH) in garlic stems extracts obtained by UAE and PLE under optimal conditions.

4.5. Disolventes eutécticos profundos naturales para la solubilización y el fraccionamiento selectivo de carbohidratos de bajo peso molecular

Natural Deep Eutectic Solvents for Solubility and Selective Fractionation of Bioactive Low Molecular Weight Carbohydrates

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Article

Natural Deep Eutectic Solvents for Solubility and Selective Fractionation of Bioactive Low Molecular Weight Carbohydrates

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Abstract: Natural deep eutectic solvents (NADESs) have been shown to be selective and environmentally friendly solvents for the extraction of bioactive compounds. However, studies on the solubility of low-molecular-weight carbohydrates (LMWCs) in NADESs are scarce. In this work, new solubility data of LMWCs in NADESs are provided and a new approach based on the use of these solvents for the efficient fractionation of bioactive carbohydrates was explored for the first time. Several mono- and disaccharides and three NADESs based on choline chloride (ChCl) and different donors (2-ethylene glycol (EtG), glycerol (Gly) and ethanedioic acid dihydrate (Eth)) were considered. While the degradation of carbohydrates, mainly ketoses, was detected with ChCl:Eth due to its acidic nature, ChCl:EtG and ChCl:Gly were found to be useful alternatives for selectively separating bioactive ketoses and their corresponding aldoses (e.g., lactulose/lactose and tagatose/galactose) present in equimolar binary mixtures. In addition, the usefulness of ChCl:EtG for the selective enrichment of lactulose to be used as food ingredient or nutraceutical was proven (from a 25% in the reaction mixture to a 56% in the purified sample). NADESs could be used for the selective fractionation of value-added carbohydrates from interfering sugars for several applications, including food science, engineering or pharmaceuticals.

Keywords: natural deep eutectic solvents (NADESs); ketoses; aldoses; fractionation; low-molecular-weight carbohydrates



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1. Introduction

Low molecular weight carbohydrates (LMWCs) are important components of foods as they belong to the group of basic nutrients involved in nutrition and metabolism. They may be naturally present or may be added to improve the sensory, functional and technological properties of food. In addition, certain carbohydrates present functional properties that are beneficial to human health (i.e., prebiotic effect, glycosidase inhibitors, treatment of conditions related to insulin resistance, etc.). However, in natural sources, these LMWCs coexist with other sugars that may interfere with their bioactive or technological properties [1]. The selective fractionation of these bioactive carbohydrates is essential for their analytical characterization, use in the food industry or the evaluation of their bioactivity [2]. However, this is a challenging process due to the similarity and complexity of their structures as well as their varying concentrations in natural samples.

Several techniques based on the use of membranes (such as ultrafiltration, nanofiltration, etc.) have been proposed for the selective separation of carbohydrates [2]; however, their efficiency for LMWCs is limited. Chromatographic techniques (such as ion exchange chromatography, size-exclusion chromatography, simulated moving bead chromatography, etc.) are highly selective for the fractionation of carbohydrate mixtures with different

degrees of polymerization (DP), but the fractionation of LMWCs with the same DP and different monosaccharide compositions or glycosidic linkages remains a challenge [2].

Differences in the solubility of carbohydrates in conventional solvents (ethanol, methanol, 1-propanol, etc.), which cause some of them to precipitate, have been used for their selective separation [3]. Despite the potential utility of these methods, their high solvent consumption and low selectivity have motivated the search for greener and cost-effective alternatives, such as ionic liquids (ILs) or deep eutectic solvents (DESs) [4]. The tunability of these solvents and their unique physicochemical properties (negligible vapor pressure over a wide temperature range, high thermal stability, etc.) open up a range of possibilities for the selective extraction of bioactive compounds such as carbohydrates.

The solubility of LMWCs in ILs has been extensively studied in recent years [5–7]. These solvents have also been proposed for the selective fractionation of bioactive carbohydrates such as bioactive ketoses/aldoses [8] and value-added polyols/sugars [1]. However, some studies suggest that, depending on their chemical structure, ILs may be as toxic as or even more dangerous than organic solvents and are more expensive [9].

DESs, a new generation of green solvents, are obtained by the complexation of a hydrogen acceptor (HBA), often a quaternary ammonium salt such as choline chloride, and a hydrogen bond donor (HBD), which can be carboxylic acids, alcohols, amines, polyols, acid amides or carbohydrates [10]. If the compounds that make up the DESs are primary metabolites, such as organic acids, amino acids, choline derivatives or sugars, they are known as natural deep eutectic solvents (NADESs) [11,12]. NADESs have been shown to be environmentally friendly and selective for the extraction of certain compounds, mainly phenolic compounds [13–15] and polysaccharides [16,17]. However, to the best of our knowledge, there are limited studies related to the solubility of LMWCs in NADESs (glucose, fructose, sucrose and erythritol) [18,19], while these solvents have not yet been used for the selective fractionation of specific LMWCs. Gomez et al. [20] proposed the combined use of NADESs and microwave-assisted extraction to remove soluble sugars from banana puree to obtain an enriched non-starch polysaccharide fraction.

In the present work, the solubility of various LMWCs (including mono- and disaccharides, aldoses and ketoses) in choline chloride-based NADESs and their usefulness for the separation of bioactive ketoses (i.e., tagatose or lactulose) from their corresponding aldoses (i.e., galactose or lactose) was evaluated for the first time. Finally, the potential of NADESs as a new alternative for the selective separation of carbohydrates in real and more complex samples was considered by applying the proposed method to the enrichment of lactulose in a lactose isomerization reaction mixture.

2. Materials and Methods

2.1. Standards and Reagents

The following, along with 5-hydroxymethyl-2-furaldehyde (HMF), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Carbohydrate standards: fructose (Fru), galactose (Gal), glucose (Glc), lactose (La), lactulose (Lu), maltose (Mal), sucrose (Suc), tagatose (Tg) and trehalose (Trh). DES constituents: choline chloride (ChCl), 2-ethylene glycol (EtG), glycerol (Gly) and ethanedioic acid dehydrate (Eth).

2.2. Synthesis and Characterization of NADESs

NADESs were synthesized as indicated by Hakkinen and Abbott [18] with slight modifications. In brief, HBD (EtG, Gly and EthAc) were mixed with ChCl at 2:1 molar ratio for ChCl:EtG (NADES1, ethaline) and ChCl:Gly (NADES2, glyceline) and 1:1 molar ratio for Eth:ChCl (NADES3, oxaline) in a water bath at 50 °C under stirring until a homogeneous liquid was formed. Characteristics of these NADESs are shown in Table 1.

Table 1. Viscosity and water content of synthesized NADESs.

	Hydrogen Bond Acceptor (HBA)	Hydrogen Bond Donnor (HBD)	Molar Ratio HBA:HBD	Viscosity (cP)	Water Content (%)
NADES1	Choline chloride (ChCl)	2-Ethylene glycol (EtG)	1:2	35.6 (0.9) *	0.29
NADES2		Glycerol (Gly)	1:2	309.2 (0.9)	0.23
NADES3		Ethanedioic acid dihydrate (Eth)	1:1	143.8 (0.7)	9.96

* Standard deviation in parentheses ($n = 3$).

Dynamic viscosity (η) of synthesized NADESs was determined following the Poiseuille's law (Equation (1)) as indicated by Geurink et al. [21]. An Agilent 7100 CE instrument with a diode array detector (DAD) and an Agilent bare fused silica (BFS) capillary of 50 μm id, total length 57.5 cm and with acetone as tracer were used. A hydrodynamic pressure (ΔP) of 4 bar was applied on one side of the capillary (length (L) 57.5 cm, inner diameter (d) 50 μm) and homogeneously filled with the BGE solution. The velocity (v_{gem}) of the tracer was calculated by measuring the migration time (t_m) over an effective length (L_{eff}) of 8.5 cm:

$$\eta = (\Delta P \cdot d^2) / (32 \cdot v_{\text{gem}} L) = (\Delta P \cdot d^2) / (32 \cdot L_{\text{eff}} / t_m \cdot L) \quad (1)$$

The water content of the NADESs was measured using a C20 Compact Karl Fischer coulometer (Mettler Toledo; Ohio, OH, USA) with HYDRANAL[®]-Coulomat AG (Sigma Chemical Co., San Luis, CA, USA) as the reagent for the volumetric titration.

Fourier transform infrared spectroscopy (FT-IR) analyses were also carried out to determine the functional groups of the individual components (ChCl, EtG, Gly and Eth) as well as those of the respective NADESs. For this purpose, a Fourier transform infrared spectrophotometer Perkin Elmer Spectrum Two using an attenuated total reflectance device (FTIR-ATR; Perkin Elmer, Waltham, MA, USA) was used. Spectra were recorded between 400 and 4000 cm^{-1} with 10 scans and a resolution of 4 cm^{-1} .

2.3. Synthesis of Lactulose

Lactulose was obtained via the isomerization of lactose according to the method of Montilla et al. [22] with some modifications. Briefly, 2 mL of a solution of lactose (250 mg mL^{-1}) and powdered egg shell (final concentration: 30 mg mL^{-1}) were added to 8 mL of Milli-Q water and kept at 125 °C under reflux for 4 h. The reaction was stopped via immersion in an ice bath, the egg shell was removed via filtration through a 0.4 μm paper filter (Merck KGaA, Darmstadt, Germany) and the sample was lyophilized.

2.4. Solubility of LMWCs in NADESs

One gram of each NADESs was added to an excess amount of the individual carbohydrates. These mixtures were magnetically stirred at 200 rpm at 25 °C (and at 45 °C for NADES2) for 24 h and left to stand for a further 24 h at the same temperature. Preliminary experiments were carried out to confirm that the stirring time was sufficient to achieve reproducibility and accuracy and to determine the amounts of each carbohydrate required (from 20 mg to 1000 mg). Aliquots were collected from the upper layer and diluted between 1:1000 and 1:40,000 (v/v) with Milli-Q water. Samples were analyzed via high-performance liquid chromatography coupled to mass spectrometry (HILIC-MS). All solubility assays were made in triplicate.

2.5. Solubility of Binary Mixtures of LMWCs in NADESs

One gram each of NADES1 and NADES2 were, respectively, added to 1:1 (w/w) binary mixtures containing lactose/lactulose and galactose/tagatose. The samples were stirred at

200 rpm for 24 h and allowed to stand for a further 24 h at 25 °C. Aliquots of each mixture were diluted as required with Milli-Q water and analyzed via HILIC–MS.

2.6. NADESs Treatment of the Lactose Isomerization Mixture

NADES1 (0.5 g) was mixed with 500 mg of the lyophilized isomerization mixture and subjected to the same treatment as described in Section 2.5 for the dilution of binary mixtures. Aliquots of the supernatant were analyzed by HILIC–MS.

2.7. Evaluation of Carbohydrate Degradation

Color development of NADES1 and NADES3 samples was assessed using Spectra Max Plus 384 Microplate Reader (Molecular Devices, San Jose, CA, USA) through an absorbance at 420 nm, according to the method of Meydavi et al. (1977) [23]. Samples were diluted (from 1:1 to 1:10 (*v/v*)) in sodium phosphate buffer (PBS, 1 mM; pH, 7.02; control value).

The formation of HMF was determined through high-performance liquid chromatography using an HPLC-UV 1100 series with a G1314B VWD detector (Agilent Technologies, Santa Clara, CA, USA), an XDB-C18 column (Zorbax®, 5 µm particle size and 80 Å pore size, 150 mm × 4.6 mm i.d., Agilent Technologies) and a binary gradient consisting of methanol/water as indicated by Viñas et al. [24]. An external standard calibration curve (0.025–0.1 mg mL^{−1}) was used for quantitative analysis.

2.8. HILIC–MS Analysis

Hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC–MS) was used for the analysis of LMWCs using a 1260 Infinity II Prime LC System equipped with a diode array detector coupled to a 6125 single quadrupole mass detector (Agilent Technologies, Santa Clara, CA, USA) provided with an electrospray ionization (ESI) source. These analyses were developed by using an ethylene bridge hybrid with trifunctionally bonded amide phase column (150 mm × 4.6 mm; 3.5 µm particle size; 135 Å pore size; BEH X-Bridge, Waters, Hertfordshire, UK). A gradient of acetonitrile/water with an addition of 0.1% of ammonium hydroxide at a flow rate of 0.4 mL min^{−1} was used as follows: from 0 to 30 min, a linear gradient from 20% to 50% aqueous phase, at these conditions, then ramped to the original composition in 1 min, and finally equilibrated for 8 min. The ESI source operated under positive polarity using the following MS parameters: capillary voltage, 4 kV; temperature, 300 °C; nitrogen drying gas flow, 12 L min^{−1}; nebulizer (N2, 99.5% purity) pressure, 276 kPa; and fragmentor voltage, 100 V. Mono-sodiated adducts [M+Na]⁺ were formed for the different carbohydrates and monitored in SIM mode. Data were processed using OpenLAB CDS Software (v.2.19.20, Agilent Technologies). External calibration curves of the different LMWCs (0.0001–0.5 mg mL^{−1}) were performed in triplicate.

2.9. Statistical Analysis

Statistical assays were carried out using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA, Tuckey test) followed to evaluate significance (*p* < 0.05) of differences among solubility values.

3. Results and Discussion

NADES1 (ethaline), NADES2 (glyceline) and NADES3 (oxaline) were chosen in the present work based on results reported by Hakkinen and Abbott [18], who determined the solubility of glucose, sucrose and erythritol in these solvents. In addition to their good efficacy in solubilizing carbohydrates, choline-based NADESs are also valued for their biological activity [25]. Characterization of synthesized NADESs was carried out on the basis of their viscosity and water content, considering the relevance of these properties for their use as solvents. As shown in Table 1, the water content was below 0.3% for NADES1 and NADES2, while NADES3 contained 10% of water.

Regarding viscosity, NADES2 showed the highest values (309.2 cP) while NADES1 was the least viscous (35.6 cP). These values agreed with those described by Hakkinen and Abbot [18], except for NADES3, which showed a markedly lower viscosity (137.4 cP) than that reported (767 cP). However, this value was in good agreement with that described by Skulcová et al. [26]. Viscosity of NADESs is associated to the strong hydrogen bonding interactions produced between HBA and HBD. The high viscosity of NADESs is one of the main disadvantages of these solvents as it can reduce mass transfer during extraction. However, the NADESs used in this work showed adequate viscosity values, which makes them promising solvents for carbohydrate solubility studies.

Moreover, FT-IR analyses were carried out to confirm the formation of the NADESs. As shown in Figures S1–S3 (Supplementary Material), spectra of the pure compounds and their eutectic mixtures were obtained and compared, where conformational changes due to intermolecular interactions between HBA and HBD were observed, according to other authors [27–29].

3.1. Carbohydrate Degradation

Before measuring the solubility of the different LMWCs in the selected NADESs, their potential degradation in these solvents was evaluated. When some carbohydrates were dissolved in NADES3 at 25 °C, a change in color from transparent to brown was observed. On the contrary, color changes were not observed for carbohydrates dissolved in NADES1 and NADES2 at this temperature.

The change in color could be due to the non-enzymatic degradation of the carbohydrates dissolved in NADES3 [18]. To evaluate this reaction, absorbance at 420 nm was measured (Table 2). Before the measurement, 1:2 and 1:6 (*v/v*) dilutions in PBS were mandatory for fructose and tagatose solutions, respectively. Under the same conditions, a control assay was performed with each carbohydrate dissolved in PBS; the absorbance values were in all cases lower than 0.059 units of absorbance (UA). A noticeable increase in the absorbance was mainly found for tagatose, followed by fructose, sucrose and lactulose. Aldose solutions, such as glucose and lactose, also showed a slight increase in the absorbance values but was less than for ketoses. Changes in the color of maltose and galactose solutions were not observed.

Table 2. Measure of absorbance at 420 nm and 5-hydroxymethylfurfural content (% *w/w*) of carbohydrate/NADES3 mixtures at 25 °C. Absorbance data of carbohydrate solutions in phosphate-buffered saline (PBS) are also included as reference.

LMWCs	Absorbance at 420 nm (A.U.)			HMF (% <i>w/w</i>)
	Dilution	PBS	NADES3	
Tagatose	1/6	0.0467 (0.0003) *	0.5837 (0.0065)	6.23 (0.68)
Fructose	1/2	0.0491 (0.00017)	0.4631 (0.0018)	6.32 (0.16)
Glucose	1/1	0.0458 (0.0002)	0.1226 (0.0003)	0.02 (0.01)
Sucrose	1/1	0.0477 (0.0002)	0.4752 (0.0002)	4.00 (0.13)
Lactose	1/1	0.0584 (0.0002)	0.0970 (0.0005)	0.040 (0.001)
Lactulose	1/1	0.0587 (0.0001)	0.3595 (0.0004)	2.63 (0.08)

* Standard deviation in parenthesis (*n* = 3).

The degradation of glucose and cellobiose in oxaline at 25 °C has been previously reported, while erythritol remained stable under the same conditions [18], probably because it does not contain a reducing end. A similar behavior has been observed for some carbohydrates dissolved in acidic ILs; it has been described that fructose dissolved in these solvents is more prone to the formation of dehydration products (e.g., HMF) than glucose [7,30].

In this work, HMF was also determined in carbohydrate solutions that presented higher absorbance values (Table 2). The greatest HMF concentrations (ratios above 6%, *w/w*) were found for tagatose and fructose in NADES3, followed by sucrose (4.0% *w/w*) and lactulose (2.6% *w/w*). On the contrary, the production of HMF via the degradation of the aldoses, glucose and lactose was very low (below 0.05%, *w/w*). Wu et al. (2018) indicated that, in comparison with neutral or basic DESs, acidic DESs can contribute to the equilibration of a higher proportion of furanose forms, which have been described as the major forms responsible for converting ketoses such as fructose and tagatose into 5-HMF [31]. Based on these results, acidic NADESs could be used as catalysts for these dehydration reactions, mainly for ketoses and, to a lesser extent, for some aldoses, and likewise, for some imidazolium-based ILs [7,32].

Considering these results and to provide reliable solubility data, NADES3 was discarded for the following studies.

3.2. Solubility of Carbohydrates at 25 °C

Table 3 shows the solubility data of the different carbohydrates in NADES1 and NADES2 at 25 °C. In general, the solubility of monosaccharides was higher in NADES2, except for glucose, whereas that of disaccharides was higher in NADES1, except for trehalose.

Table 3. Solubility (mg g^{-1}) of monosaccharides and disaccharides in NADESs at 25 °C and 45 °C.

LMWCs	NADES1		NADES2	
	25 °C	25 °C	25 °C	45 °C
Monosaccharides	Glucose	242 (16) ^{*,f}	135.7 (0.2) ^c	364 (1) ^c
	Galactose	31.4 (0.4) ^a	43 (4) ^a	71 (6) ^b
	Fructose	191 (1) ^d	245 (5) ^d	806 (16) ^f
	Tagatose	72 (2) ^b	150.0 (0.4) ^c	214 (7) ^b
Disaccharides	Lactose	147 (5) ^c	26.3 (2.9) ^a	92 (29) ^{a,b}
	Maltose	323 (10) ^g	280 (14) ^e	630 (90) ^e
	Trehalose	183 (3) ^{d,e}	252 (17) ^d	503 (13) ^d
	Sucrose	152 (3) ^{c,e}	82 (11) ^b	368 (15) ^c
	Lactulose	666 (25) ^h	135.1 (0.3) ^c	250 (11) ^b

* Standard deviation in parenthesis ($n = 3$). ^{a–h} Different letters within the same column indicate significant ($p < 0.05$) differences for the CHs assayed.

Regarding monosaccharides, significant differences in the solubility of glucose and fructose were observed in both NADESs. The solubility of fructose was higher than that of glucose in NADES2 (245 vs. 135.7 mg mL^{-1}). This ketohexose has also been found to be more soluble than glucose in different imidazolium-based ILs [6,7,33], considering its more stable structure and its lower melting temperature (378.1 K vs. 423.1 K for Fru and Glc, respectively) and enthalpy [6]. However, in this work, the solubility of fructose in NADES1 (191 mg mL^{-1}) was significantly lower than that of its corresponding aldose (242 mg mL^{-1}). Moreover, great differences were observed between the solubility of glucose and its C-4 epimer (galactose) in both NADESs (Glc/Gal: 7.7 in NADES1 and 3.2 in NADES2); the same behavior was observed for fructose and its C-4 epimer (tagatose), although the differences were smaller (Fru/Tg: 2.6 in NADES1 and 1.6 in NADES2). These results point to these NADESs as suitable solvents for the selective fractionation of these isomeric carbohydrates.

As for the disaccharides, large differences in solubility values were observed for both NADESs. In this sense, lactulose (4-O- β -galactopyranosyl-D-fructofuranose) was the most soluble sugar in NADES1 (666 mg mL^{-1}), while the solubility of lactose (4-O- β -galactopyranosyl-D-glucopyranose) in this solvent was the lowest (147 mg mL^{-1} ; Lu/La: 4.5). Similarly, lactulose (135.1 mg mL^{-1}) was more soluble than lactose (26.3 mg mL^{-1} ; Lu/La: 5.1) in NADES2, although solubility values of both carbohydrates were lower than in NADES1. Hakkinen and Abbot [18] observed a similar behavior between sucrose (α -D-glucopyranosyl- β -D-fructofuranose) and cellobiose in ethaline (the former was fourteen

times more soluble than the latter). They attributed these differences to the more rigid structure of cellobiose (disaccharide with two symmetrical glucose units linked with a 1-4 glycosidic linkage) that restricts bond rotation, while sucrose has a less symmetrical structure with more conformational freedom in solution, resulting in a higher solubility. However, maltose, which is also constituted by two symmetrical glucose units linked with 1-4 linkage, showed higher solubility values in both NADESs than sucrose. Similarly, the solubility of trehalose (1-O- α -D-glucopyranosyl- α -D-glucopyranose) was significantly higher than that of sucrose in NADES2, although differences were lower in NADES1. It is likely that the different factors such as their folded structure in the solution or the greater hydrogen bond capacity [18,34], among others, could contribute to the solubility of these disaccharides in the NADESs.

Considering that the HBA (choline chloride) of studied NADESs is the same in both solvents, differences in solubility values observed for the different carbohydrates could be attributed to the HBD (2-ethylene glycol in NADESs 1 and glycerol in NADES2).

3.3. Solubility of Carbohydrates at 45 °C in NADES2

The effect of the temperature on carbohydrate solubility in NADESs was investigated. While color changes in carbohydrate solutions at 45 °C in NADES2 were not detected, some solutions in NADES1, mainly of ketoses, became darker. Then, NADES2 was selected for the following studies and results are shown in Table 3. As expected, most carbohydrate solubility values were significantly higher with increasing temperature from 25 °C to 45 °C, as previously observed with other solvents [7]. A moderate increase was found for the solubility of tagatose and galactose, which increased by a factor of 1.4 and 1.6, respectively. However, higher differences were observed in the solubility of fructose, lactose and sucrose, which increased by a factor of 3.3, 3.5 and 4.5, respectively. Intermediate increases in solubility values were found for other carbohydrates.

3.4. Fractionation of Bioactive Ketoses from Aldoses in Equimolar Binary Mixtures

Some ketoses such as tagatose and lactulose are used as additives or functional ingredients in food, cosmetic and pharmaceutical formulations due to their texturizing and stabilizing behaviors and to their bioactive properties, such as prebiotic effect [35,36]. These bioactive ketoses are usually synthesized from their corresponding aldoses (galactose and lactose) using different catalysts [22,37], which affect their isomerization yields. The remaining aldoses must be removed from the product, requiring an efficient selective fractionation process. Considering the different solubility of aldoses and ketoses in the studied NADESs, the feasibility of these solvents for the selective fractionation of lactulose and tagatose from their corresponding aldoses (i.e., lactose and galactose) in binary mixtures was evaluated.

According to the solubility data shown in Table 3, NADES2 at 25 °C was chosen for tagatose/galactose fractionation because it provided the highest differences between the individual solubility values of both sugars (Tg/Gal: 3.5), which, in principle, could lead to an effective fractionation. For lactulose/lactose, NADES1 at 25 °C was selected, also taking into account the high solubility of lactulose in this solvent.

Figure 1 shows the solubilized and precipitated percentage of each carbohydrate of the equimolar binary mixtures after NADESs treatment. Regarding the lactulose/lactose mixture (Figure 1a), all lactulose dissolved in NADES1, while only 18.8% of lactose remained in this solvent. Then, lactose could be purely obtained in the precipitate, while lactulose was highly enriched in the solution (77%). Similarly, in the galactose/tagatose binary mixture (Figure 1b), 92.7% of the total tagatose dissolved in NADES2, while only 13.5% of the galactose was found in the solution. High-purity galactose could be separated in the precipitate, while an enriched solution of tagatose (16% galactose; 84% tagatose) was obtained. In both cases, ketoses were more soluble than aldoses, which agreed well with the individual solubility data reported in Table 3. These data point out the feasibility of using these green solvents for ketose/aldose fractionation.

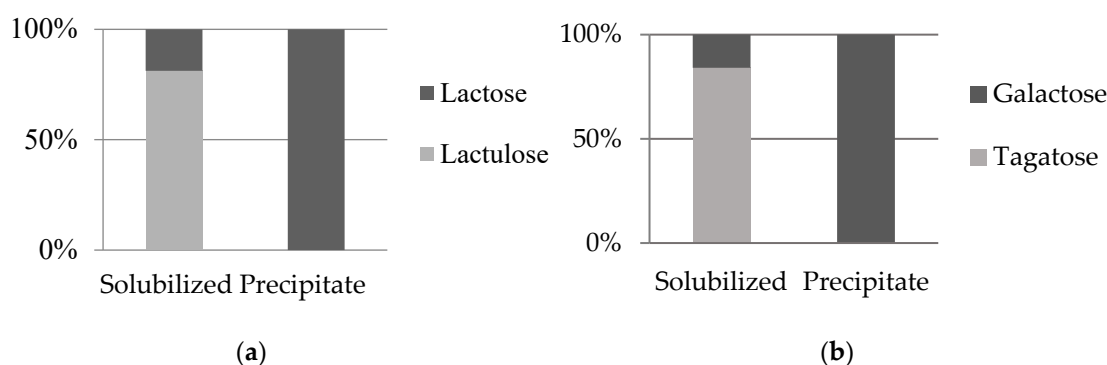


Figure 1. Content (as percentage, %) of lactulose and lactose (a) and tagatose and galactose (b) in 1:1 (*w/w*) binary mixtures in NADES1 and NADES2, respectively, determined as remaining in solution and precipitate after treatment at 25 °C.

3.5. Fractionation of Lactulose from the Isomerization Reaction Mixture

The efficiency of NADESs as solvent candidates for the fractionation of carbohydrates in real mixtures was evaluated. Lactulose was synthesized via the isomerization of lactose using egg shell as catalyzer; Figure 2a shows the HILIC–MS profile of this reaction mixture. The synthesis yielded 25% of lactulose, which is in good agreement with previously published results [8,22], 14% monosaccharides (glucose and galactose) and 3% epilactose; 60% lactose remained as unreacted sugar in the mixture.

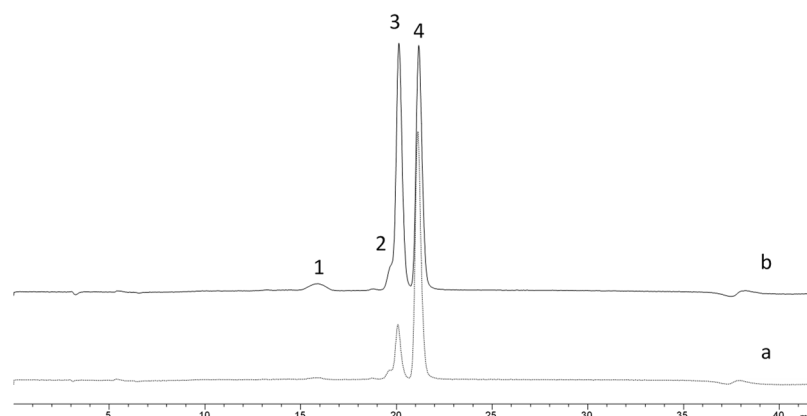


Figure 2. HPLC–MS profile of the isomerization with egg shell of lactose to lactulose before (a) and after (b) treatment with NADES1 at 25 °C. Labeled peaks are as follows: (1) monosaccharides, (2) epilactose, (3) lactulose and (4) lactose.

Then, NADES1 at 25 °C, previously chosen as a selective solvent for the lactose/lactulose binary mixture, was used for the fractionation of lactulose from the other isomerization reaction products. As it is shown in Figure 2b, the HILIC–MS profile of carbohydrates in the isomerization mixture after treating with this NADES changed. Lactulose became an abundant carbohydrate in the mixture, while a noticeable reduction in lactose was observed.

The extraction yields in NADES1 of the different carbohydrates present in the mixture were determined: while 100% of lactulose was solubilized, only 25% of the available lactose was extracted. A high percentage of monosaccharides (55%) was also dissolved, mainly of glucose, in agreement with the individual solubility of this carbohydrate in NADES1 (Table 3), while 10% of epilactose was also solubilized. Percentages of these carbohydrates before and after treatment are shown in Figure 3. Lactulose percentage showed a 2-fold increase, while lactose and epilactose decreased notably and monosaccharides remained constant after this treatment. Then, the final mixture constituted of 50% lactulose, 32% lactose, 17% monosaccharides and 1% epilactose. These results were quite similar to

those achieved by Carrero-Carralero et al. (2015) for lactulose enrichment using ILs (58% lactulose, 31% lactose and 11% monosaccharides), but using a more natural solvent [8].

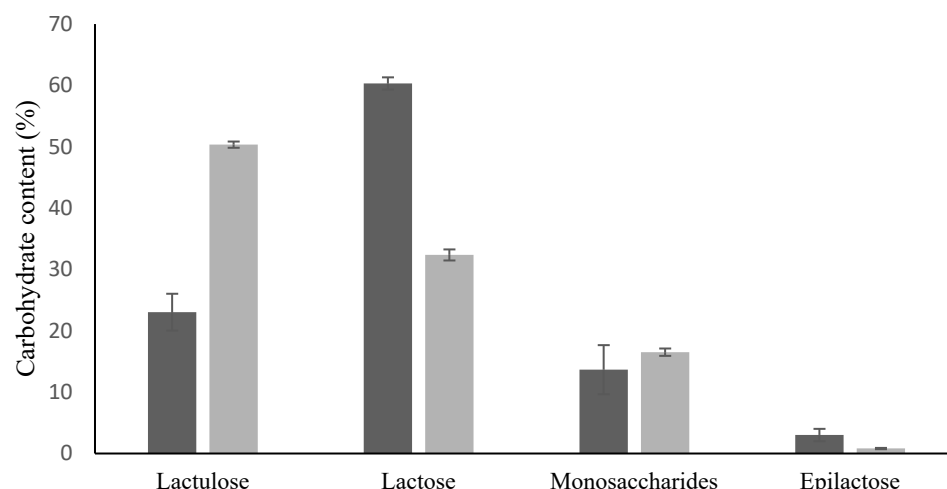


Figure 3. Concentration (%) of lactulose, lactose, epilactose and monosaccharides in the isomerization mixture of lactose to lactulose before (black bars) and after treatment (grey bars) with NADES1.

4. Conclusions

In this work, new solubility data of various mono- and disaccharides in selected NADESs (ChCl:EtG and ChCl:Gly) are presented for the first time. Differences were found between the solubility values of carbohydrates as a function of their structure. In general, the solubilities of the ketoses were higher than those of their corresponding aldoses in both solvents. These NADESs proved to be promising alternatives for the selective fractionation of the binary mixtures galactose/tagatose and lactose/lactulose. Furthermore, the results obtained in this work provide the first evidence of the usefulness of ChCl:EtG (NADESs 1) for the enrichment of lactulose in its synthesis mixture. They may contribute to the development of new applications based on the separation of value-added carbohydrates using this new generation of sustainable bio-solvents.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/foods12234355/s1>, Figure S1. FT-IR spectra of chloride choline (ChCl), ethylene glycol (EtG) and NADES1 (ChCl:EtG), Figure S2. FT-IR spectra of chloride choline (ChCl), glycerol (Gly) and NADES2 (ChCl:Gly), Figure S3. FT-IR spectra of chloride choline (ChCl), oxalic acid dihydrate (Eth) and NADES3 (ChCl:Eth).

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Data Availability Statement: The data used to support the findings of this study can be made available by the corresponding author upon request.

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Conflicts of Interest: Author I. Jiménez-Amezcuca is granted with an Industrial Doctorate from the Comunidad de Madrid to CSIC and Pharmactive Biotech S.L.U. and carries out all the investigations in the CSIC facilities under the supervision of CSIC academic researchers, assuring the integrity and

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4.6. Uso de disolventes eutécticos profundos basados en cloruro de colina para la separación selectiva de polialcoholes bioactivos

Use of choline chloride-based deep eutectic solvents for the selective separation of bioactive polyalcohols

I. Jiménez-Amezcu, M.I. López-Martínez, A.I. Ruiz-Matute, M.L. Sanz

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Use of choline chloride-based deep eutectic solvents for the selective separation of bioactive polyalcohols

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Abstract

Both linear polyalcohols (such as mannitol) and cyclitols (such as inositols) are relevant carbohydrates with technological and bioactive properties. They are chemically synthesised or extracted from natural sources, usually obtained together with other more abundant carbohydrates, in particular mono- and disaccharides, which may interfere with their properties. However, due to the similarity of their chemical structures and the complexity of the mixtures, further fractionation of polyalcohols is a challenge.

In this manuscript, the use of natural deep eutectic solvents (NADESs) for the selective fractionation of polyalcohols from other low molecular weight carbohydrates (LMWCs) has been explored. For this purpose, as a first step, the solubility of a linear sugar alcohol (mannitol), inositols (*myo*- and *chiro*-inositol) and methyl-inositols (pinitol and quebrachitol) in choline chloride (ChCl)-based NADESs, i.e., ChCl: 2-ethyleneglycol (EtG) and ChCl: glycerol (Gly), ethaline and glyceline respectively, has been investigated. The experimental results revealed low solubility values of polyalcohols at 25 °C in these NADESs (under 92 mg g⁻¹) as compared with those of LMWCs previously published (136-245 mg g⁻¹). These NADESs were useful for the selective fractionation of polyalcohols and glucose/fructose in 1:1 (w/w) binary mixture (yields in the 60 – 100 % range). Moreover, advantages of the use of glyceline over only water were achieved for the selective extraction of pinitol from carob and mannitol from celery, although the complete isolation of these polyols could not be achieved. These results show NADESs as promising environmentally friendly solvents for the selective fractionation of polyalcohols and other LMWCs and suggest the interest of further investigation in this particular application field.

Keywords: natural deep eutectic solvents, polyalcohols, inositols, solubility, selective extraction

1. Introduction

Polyalcohols, also called sugar alcohols or polyols, are organic compounds in which the aldo or keto group of a sugar is reduced to a hydroxyl group. They can have both linear or cyclic structures and they are very abundant in the plant kingdom.

Linear polyalcohols (*e.g.* mannitol) are widely used as food additives due to their relatively sweet taste and low caloric contribution. Regarding cyclic polyols, inositols (*e.g.* *myo*- and *chiro*-inositol) are cyclic sugars with a six-carbon ring structure that are chemically very stable to heat, acids and alkalis. Inositols are used for the treatment of several diseases related to insulin resistance, such as polycystic ovary syndrome. *Myo*-inositol is the most abundant isomer in nature and it can be endogenously synthesized from glucose. It is present in several foods at relatively high amounts (*e.g.* citric fruits, legumes, cereals, coffee and honey; 0.9 - 22 mg g⁻¹) [1–4]. D-(+)- and L-(-)-*chiro*-inositols are also relatively abundant in higher plants, mostly as methyl ethers such as L-(-)-quebrachitol and D-(+)-pinitol.

However, polyalcohols are found in nature as part of very complex matrices together with other more abundant low molecular weight carbohydrates (LMWCs), mainly mono- and disaccharides, which can interfere in their technological effects or bioactive properties [1]. The extraction of these added-value carbohydrates currently presents a challenge.

Different techniques have been employed for the selective extraction and purification of these compounds, such as membranes [5], biotechnological methods [3], precipitation with conventional solvents [5–7], among others. However, the selectivity of these methods is inefficient due to the structural similarity between polyols and LMWCs.

Recently, ionic liquids (ILs) have been proposed as green solvents for the selective fractionation of polyols, surpassing the low selectivity of traditional organic solvents [1,5]. However, opinions regarding their toxicity and the environmental concerns associated with these ‘green’ solvents have changed since it is not guaranteed that they cannot reach the environment and, therefore, represent a danger to the ecosystem [8].

Deep eutectic solvents (DESs) are eutectic mixtures formed by combining two or more compounds, where at least one or all are solids, and each component will be either a hydrogen bond donor (HBD) or a hydrogen bond acceptor (HBA). Typically, the HBA is a quaternary ammonium salt, such as choline chloride (ChCl), while the HBD can be alcohols, amines, carboxylic acids, etc. When the components of the DES are primary metabolites like amino acids, organic acids, or choline derivatives, they are considered natural deep eutectic solvents (NADESs) [9,10]. NADESs are tuneable solvents which have demonstrated their versatility in various applications, including analytical chemistry and food sector serving as media for

enzymatic or chemical reactions, preservatives for labile compounds [11], and agents for extracting bioactive compounds from natural sources [12]. Moreover, the use of natural compounds reduces the production costs and become a green alternative to conventional solvents for solubilizing bioactive compounds like polysaccharides [13,14] or polyphenols [15,16]. Recently, the solubility of mono- and disaccharides (aldoses and ketoses) in NADESs has been determined and the usefulness of these solvents for the selective fractionation of bioactive ketoses from their corresponding aldoses has been proven [17]. However, solubility studies of polyalcohols in NADESs have not yet been performed and their potential utility for their selective fractionation from other coextracted LMWCs has not been previously evaluated.

Then, in this work, the solubility of different polyols (both linear and cyclic) in two choline chloride-based NADESs was explored for the first time. The potential use of these solvents in the fractionation of polyols from non-bioactive sugar (glucose and fructose) in binary mixtures was also evaluated. The selected NADES was then applied for the selective extraction of polyols from natural sources.

2. Materials and methods

2.1. Chemical and reagents

All chemicals and reagents were of analytical grade. *chiro*-Inositol, mannitol, *myo*-inositol, pinitol (3-O-methyl-D-*chiro*-inositol), quebrachitol (2-O-methyl-D-*chiro*-inositol) and NADESs constituents [ChCl, 2-ethyleneglycol (EtG), and glycerol (Gly)] were purchased from Sigma Chemical Co. (St. Louis, USA). Milli-Q water was obtained from an Advantage A10 system (Millipore, Bedford, MA, USA), while acetonitrile (AcN) and ammonium hydroxide were acquired at Fisher Chemical (Newington, NH, USA).

2.2. Samples

Celery and carob samples were obtained from local markets. In both cases, the sample was pre-treated before extraction by freeze-drying, grinding to fine particles using an IKA A10 basic mill (IKA-Werke, Germany), sieving through a 500 µm mesh and storage in dry in the absence of light at room temperature until extraction.

2.3. Synthesis of NADESs

NADESs were synthesized following the methodology previously described by Jiménez-Amezcuca *et al.* (2023) [17]. In brief, a mixture of HBD compounds (EtG and Gly) was combined with ChCl at a specific molar ratio (2:1) to create NADES1 (ethaline) and NADES2 (glyceline) under stirring at 50°C until a uniform liquid was achieved. Formation of NADESs was confirmed by Fourier transform infrared spectroscopy (FT-IR) using a spectrophotometer Perkin Elmer

Spectrum Two using an attenuated total reflectance device (FTIR-ATR; Perkin Elmer, Waltham, MA, USA; 400 and 4000 cm^{-1} with 10 scans and a resolution of 4 cm^{-1}).

2.4. Solubility of polyalcohols in the evaluated NADESs

1 g of each NADES was added to an excess amount of the respective polyalcohol. The resulting mixtures were magnetically stirred at 200 rpm at 25 °C for both NADESs and also at 45 °C for NADES2 for 24 h and left to stand for another 24 h at the same temperature. The amounts of each polyalcohol used varied from 20 to 150 mg. Aliquots were taken from the upper layer and diluted with Milli-Q water between 1:2,000 and 1:10,000 (v/v). Finally, samples were analysed by HILIC–MS. All solubility assays were made in triplicate.

2.5. Solubility of binary mixtures of LMWCs in NADESs

Binary mixtures (1:1, w/w) comprising different polyalcohols and LMWCs combinations were analysed: (i) *myo*-inositol with glucose, (ii) pinitol with glucose, (iii) mannitol with fructose and (iv) mannitol with glucose. In each experiment, each mixture was dissolved in 1 g of the specific NADESs (NADES1 for mixtures 1 and 2, and NADES2 for mixtures 3 and 4) with a slight excess (10% above the corresponding solubility limit experimentally determined in this study or previously reported by Jiménez-Amezcu *et al.*, 2023). The mixtures were stirred (200 rpm, 24 h) and allowed to stand for another 24 h at 25 °C. Aliquots of each mixture were diluted as required with Milli-Q water before their analysis via HILIC–MS.

2.6. Extraction of polyols from natural samples: carob and celery

0.1 g of carob and celery powder was subjected to extraction with 1 mL of Milli-Q water and the mixture was stirred (700 rpm) at 25 °C for 30 min in a heating plate (IKA-Werke, Staufen, Germany) and centrifuged at 4,401 g for 10 min. Then, supernatants were collected and diluted (1:10, v/v) before the analysis. Simultaneously, an extraction of carob (0.3 mg) and celery (0.1 mg) powder was performed using NADES2 (1 g) under the same temperature and agitation conditions as previously mentioned. In this case, the extraction time was extended to 24 hours, followed by an additional 24 hours of resting. Finally, the extract was collected, centrifuged, and diluted prior to analysis.

Additionally, 0.3 mg of freeze-dried carob aqueous extract was extracted with 0.5 g of NADES1 and 0.5 g NADES2, respectively, and the mixtures were stirred at 25 °C for 24 hours and left to stand for another 24h at the same temperature. The same treatment was followed for the freeze-dried celery aqueous extracts but using only NADES2 (1 g). Supernatant aliquots (10 μL) of all samples were diluted as required with MilliQ water before their analysis by HILIC–MS. All experiments were carried out in triplicate.

2.7. HILIC-MS analysis

Analysis of polyalcohols was performed using a 1260 Infinite II Prime LC System equipped with a diode array detector coupled to a 6125 single quadrupole mass detector (Agilent Technologies, Santa Clara, CA, USA) provided with an electrospray ionization (ESI) source. The analyses were carried out by using a BEH X-Bridge column (150 mm× 4.6 mm; 3.5 µm particle size; 135 Å pore size; Waters, Hertfordshire, UK). A gradient of acetonitrile (A) / water (B), both with 0.1% of NH₄OH, at a flow rate of 0.4 mL min⁻¹ was used as follows: 0 min, 20% B; 20 min, 40% B; 30 min, 40% B; 31 min, 20% B, 40 min, 20% B. MS operated under positive polarity using 4 kV of capillary voltage, 300 °C of temperature, 2 L min⁻¹ of nitrogen drying gas flow; 276 kPa of nebulizer pressure, (N₂, 99.5% purity); and 100 V of fragmentor voltage. Mono-sodiated adducts ([M+Na]⁺) of polyalcohols were monitored in SIM mode. For data processing OpenLAB CDS Software (v.2.19.20, Agilent Technologies) was used. External calibration curves for the different LMWCs and polyalcohols in the concentration range of 0.0001 - 0.5 mg mL⁻¹ were performed in triplicate.

2.8. Statistical analysis

Differences among solubility values were determined by the analysis of variance (ANOVA, Tuckey test) using the Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Solubility of polyalcohols in NADESs

Table 1 shows the experimental data related to the solubility of the studied polyalcohols in both NADESs. At 25 °C, the highest solubilities were generally found in NADES1, except for *chiro*-inositol, whose solubility value was 4 times higher in NADES2 (14 vs 59 mg g⁻¹). With the exception of mannitol and quebrachitol (92 and 90 mg g⁻¹, respectively), the other polyalcohols showed solubility values below 59 mg g⁻¹ in both NADESs, which are extremely low compared to those of LMWCs (*e.g.* glucose 136-247 mg g⁻¹ and fructose 191-245 mg g⁻¹), as reported in previous works [17]. This behaviour has previously been observed in solubility studies of LMWCs and polyols in other alternative solvents, such as imidazolium ILs, although these solubility differences are greater for NADESs [1,18].

Attending the solubility values of inositols, *myo*-inositol was the less soluble in both NADESs. This behaviour could be justified taking into account the symmetry of these molecules. In this sense, *myo*-inositol has one axial hydroxyl group and one plane of symmetry while *chiro*-inositol has two axial hydroxyl groups and no plane of symmetry, giving the D- and L- forms [19]. It is known that melting properties and solubility of organic compounds is affected by their molecular structure. In general, molecules that are symmetrical in crystalline form have higher melting

temperatures and lower solubilities compared to molecules with a similar structure but less symmetry [20]. In this case, the symmetrical structure of *myo*-inositol limits its ability to form strong interactions with solvent molecules, thus reducing its solubility in comparison with *chiro*-inositol. Both sugar alcohol also exhibited the same behaviour in different imidazolium-ILs [1].

As expected, higher temperatures (45°C) increased the solubility in NADES2 in all cases except for *chiro*-inositol, whose value remained in the same order. It is worth to point out the high increase in the solubility of pinitol as compared with quebrachitol. While this last methyl-inositol increased its solubility with the temperature approximately 1.8 times, the solubility of pinitol was 4.5 times higher at 45°C. Ruiz-Aceituno *et al.*(2019) also observed this trend change with increasing temperature in solubility studies of both methyl-inositols in IL [HMIM][Cl]; pinitol increased its solubility by up to 10-fold at 45°C, while quebrachitol only increased by 6-fold at 45°C [1].

3.2. Fractionation of polyalcohols from monosaccharides in binary mixtures

Considering the solubility values of the different polyalcohols in NADES1 and NADES2 (**Table 1**) and the data of LMWCs previously reported by Jiménez-Amezcuca *et al.*, (2023) using the same NADESs [glucose (242 mg g⁻¹ in NADES1 and 136 mg g⁻¹ in NADES2) and fructose (191 mg g⁻¹ in NADES1 and 245 mg g⁻¹ in NADES2)], solubility ratios (LMWC:polyalcohol) were calculated to evaluate the utility of these solvents for their selective fractionation in equimolar binary mixtures (**Table 2**) [17]. These mixtures were chosen on the basis of a high LMWCs:polyalcohol ratio and the interest in potential future applications in real samples. In general, most of the ratios were similar or lower at 45 °C, then 25 °C was considered for all these studies. In this sense, NADES1 was chosen to selectively fractionate *myo*-inositol and pinitol from glucose (mixtures A and B, respectively), while NADES2 was selected for the separation of mannitol from fructose (mixture C) and glucose (mixture D). **Figure 1** shows the percentages (%) of the solubilized concentrations of LMWC and polyols and their precipitated amounts for these equimolar binary mixtures. As for binary **mixture A**, *myo*-inositol completely precipitated (100%) and only glucose was found in the solvent. Thus, under the proposed conditions, *myo*-inositol could be successfully fractionated (75%) from glucose. In the **mixture B**, almost 60% of glucose remained dissolved in NADES1 with 40% of pinitol. These results yielded a solubility ratio between both compounds of 1.5 in the solution fraction, lower than that expected taking into account the individual solubility results (**Table 2**). A similar behaviour was observed by Ruiz-Aceituno *et al.*(2019) when a binary mixture of pinitol : maltose (disaccharide of two glucose units) was treated with the IL [EMIM][DCA]. In that case, also a 60% of pinitol precipitated, being this enrichment lower than the expected (maltose/pinitol theoretical: 3.3 vs experimental: 2.3) [1].

Regarding the binary mixtures of mannitol and fructose or glucose (**mixture C and D**, respectively), almost pure mannitol (>95%) was precipitated and the monosaccharides were dissolved, achieving in both cases a successful fractionation of the bioactive compound. A selective fractionation between mannitol and glucose was also reported by Ruiz-Aceituno *et al.* (2019) using the IL [EMIM][OAc]. However, in contrast to the results obtained in this work, mannitol was completely solubilized in the IL, which would require an additional recovery step considering the potential toxicity of ILs.

Then, depending on the aim of the study, both NADES1 and NADES2 could be promising solvents for the selective separation of bioactive polyols from LMWCs.

3.3. Selective extraction of polyols from natural samples

Due to the unexpected results obtained for pinitol in NADES1, the potential use of NADES2 for its selective extraction from freeze-dried carob was evaluated. Extracts using Milli-Q water were also prepared for comparative purposes. As shown in **Figure 2A**, similar concentration of pinitol were extracted by both solvents (77 and 78.7 mg g⁻¹ with Milli-Q water and NADES 2, respectively). However, sucrose and fructose concentrations were noticeably lower in the NADES2 extract (163 vs. 262 mg g⁻¹ and 15 vs. 20 mg g⁻¹, NADES2 and water respectively). This means that pinitol extracts were enriched from 21% pinitol to 30% using NADES2 as solvent.

In an attempt to selectively fractionate pinitol from other LMWCs, water extract was freeze-dried and was independently treated with both NADES1 and NADES2. Glucose and fructose were almost completely extracted in both NADESs (81%-100%), while sucrose was only partially dissolved on them (60% and 63%). However, pinitol was also highly dissolved in the NADES (67% and 72%), contrary to what was expected considering its individual solubility data (**Table 2**). **Figure 2B** shows, as an example, the percentages of each carbohydrate in the initial water extract, in the NADES2 and in the obtained precipitate after treating with NADES2. Although the precipitate was mainly constituted by sucrose and pinitol, considering the high amounts of sucrose, selective fractionation of pinitol of carob extracts could not be achieved with these solvents.

Finally, taking into account the good results obtained in the binary mixtures experiments for the selective fractionation of mannitol from monosaccharides using NADES2, this solvent was used for the selective extraction of this bioactive from freeze-dried celery. Extracts using Milli-Q water were also obtained and analysed. Mannitol, glucose, fructose, *myo*-inositol and sucrose were detected in both extracts (**Figure 3A**). Concentrations of *myo*-inositol were very low and similar in both extracts (1 mg g⁻¹), while mannitol was the most abundant carbohydrate. However, as expected, concentrations of mannitol extracted with water (256 mg g⁻¹) were higher than those

obtained with NADES2 (89 mg g⁻¹), taking into account the low solubility of this polyalcohol in the NADES.

Then, aqueous extract was freeze-dried and treated with NADES2 in an attempt to selectively fractionate the detected LMWCs and polyalcohols. As shown in **Figure 3B**, fructose, glucose and sucrose were greatly extracted in this solvent (76-89 %). Regarding mannitol, only 35% was solubilised on NADES2. Then, the obtained precipitate was highly enriched in mannitol (72%) as compared with the initial aqueous extract, proving the usefulness of NADES2 for this purpose.

4. Conclusions

This research provides new solubility data for bioactive polyols in selected choline chloride-based NADESs and new insights into the usefulness of these environmentally friendly solvents for the selective fractionation of these added-value polyols from other sugars. The use of NADESs has also shown advantages over the use of only water for the selective extraction of polyols from natural samples such as carob and celery, although these solvents did not allow complete isolation of these bioactives. In view of these preliminary results, it would be interesting to carry out further studies in this field, exploring alternative NADESs or other biosolvents for the selective extraction of bioactive polyols from other carbohydrate mixtures.

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Table 1. Solubility (mg g^{-1}) of polyalcohols in NADESs at 25 °C and 45 °C. NADES1: ChCl:EtG 1:2 (Ethaline); NADES2: ChCl:Gly 1:2 (Glyceline).

Polyalcohols	NADES1 (25 °C)	NADES2 (25 °C)	NADES2 (45 °C)
<i>myo</i> -Inositol	10 (1)*	6 (1)	20 (1)
<i>chiro</i> -Inositol	14 (1)	59 (1)	56 (3)
Mannitol	92 (6)	20 (4)	45 (2)
Pinitol	49 (15)	26 (2)	109 (13)
Quebrachitol	90 (16)	44 (3)	82 (7)

* Standard deviation in brackets (n=3)

323 **Table 2.** Solubility ratios of glucose and fructose vs. polyalcohols in NADESs. NADES1:
 324 ChCl:EtG 1:2; NADES2: ChCl:Gly 1:2.

Ratios (LMWC:polyalcohol)	NADES1 (25 °C)	NADES2 (25 °C)	NADES2 (45 °C)
Glucose: <i>myo</i> -Inositol	24	23	18
Glucose: <i>chiro</i> -Inositol	17	2	7
Glucose: Mannitol	3	7	8
Glucose: Pinitol	5	5	3
Glucose: Quebrachitol	3	3	4
Fructose: <i>myo</i> -Inositol	19	41	40
Fructose: <i>chiro</i> -Inositol	14	4	14
Fructose: Mannitol	2	12	18
Fructose: Pinitol	4	9	7
Fructose: Quebrachitol	2	6	10

325

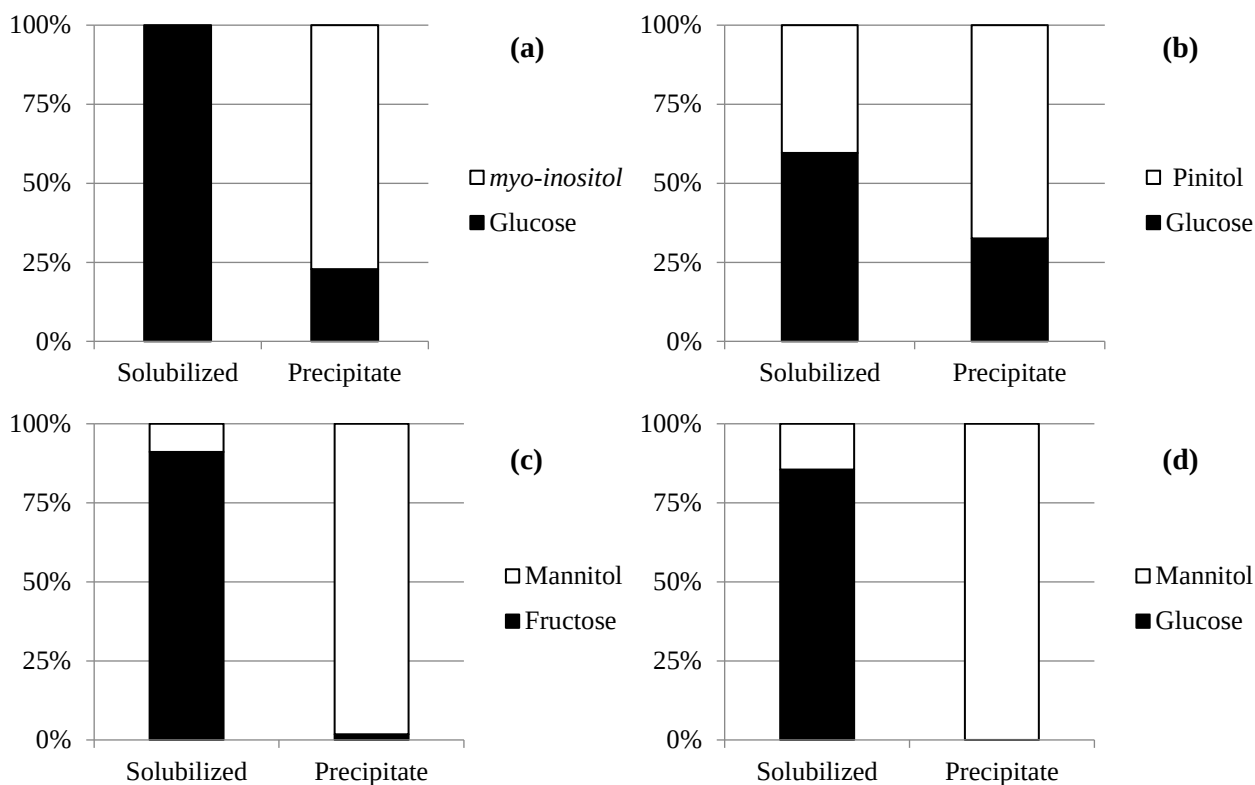


Figure 1. Content (as percentage, %) of LMWCs and polyalcohols (1:1, w/w, binary mixtures) determined as remaining in solution and precipitated after treatment at 25 °C of: (a) *myo*-inositol and glucose (mixture A) and b) pinitol and glucose (mixture B) with NADES 1 and c) mannitol and fructose (mixture C) and d) mannitol and glucose (mixture D) with NADES 2.

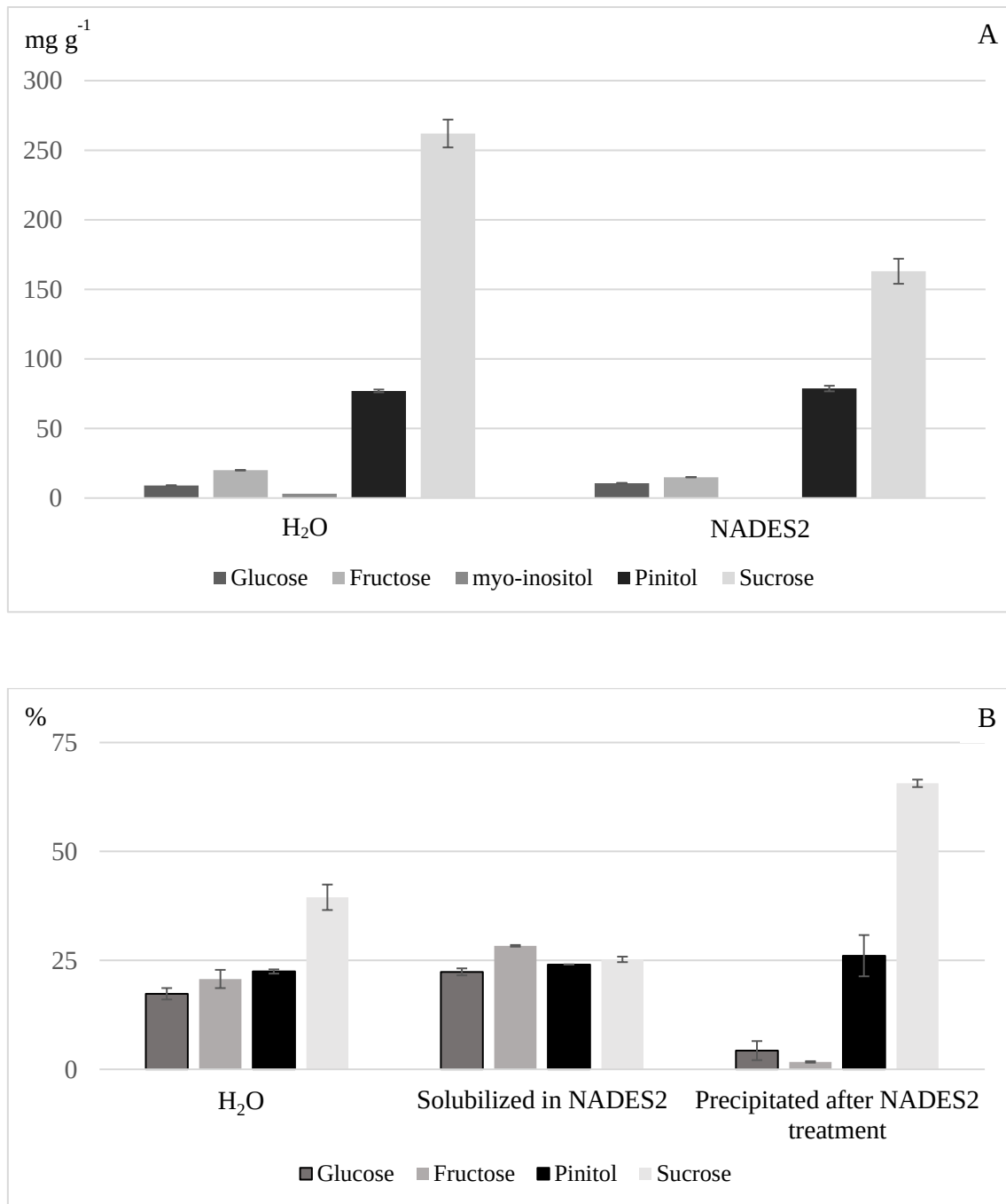


Figure 2. Concentrations (mg g⁻¹) of LMWCs and polyalcohols extracted using Milli-Q water and NADES2 from carob (A) and percentages of these compounds before and after treatment (solubilized and precipitated) of freeze-dried carob water extract with NADES2 (B).

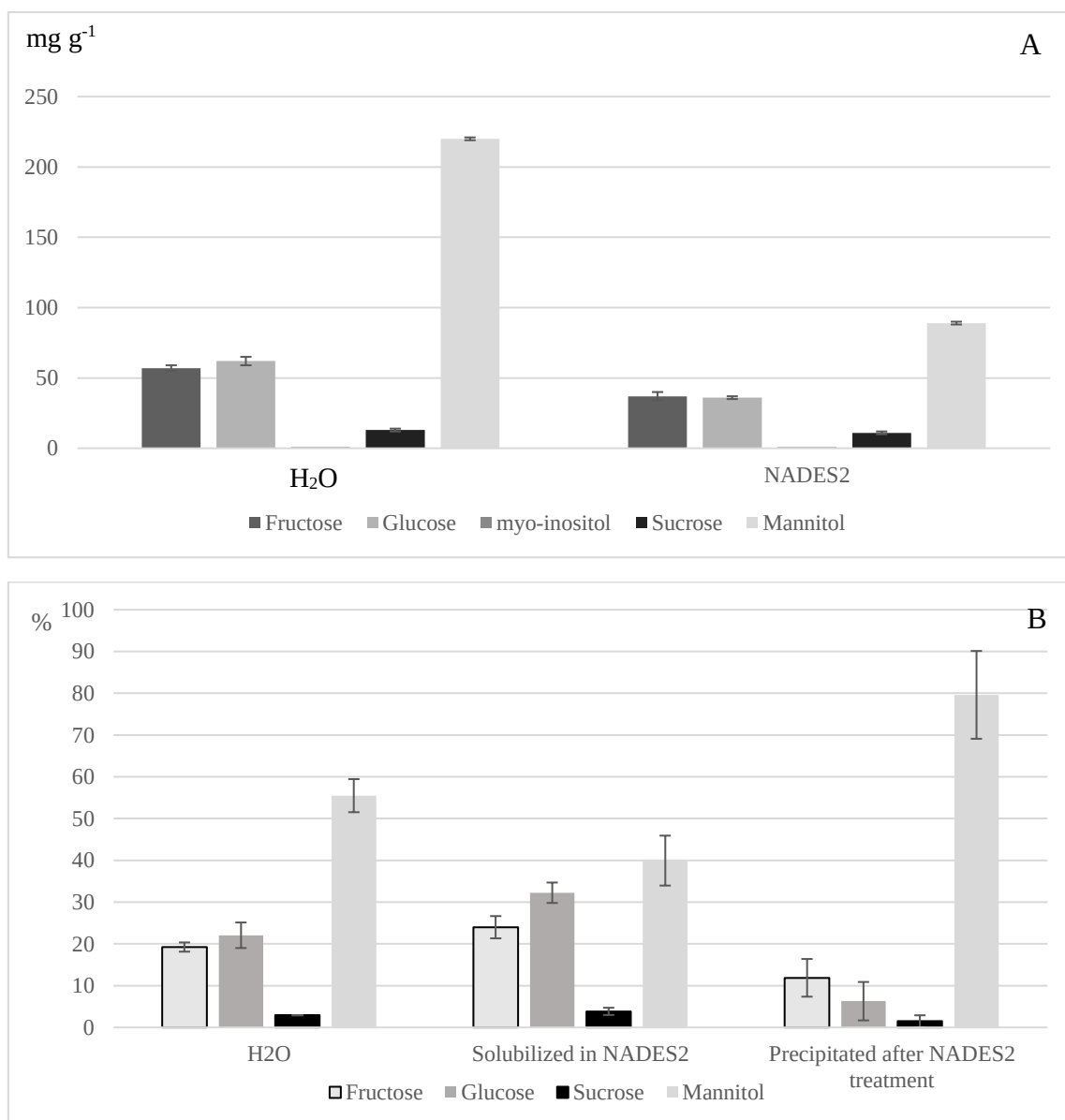


Figure 3. Concentrations (mg g⁻¹) of LMWCs and polyalcohols extracted using Milli-Q water and NADES2 from celery (A) and percentages of these compounds before and after treatment (solubilized and precipitated) of freeze-dried celery water extract with NADES2 (B).



5. Discusión Integradora

5. DISCUSIÓN INTEGRADORA

Los trabajos presentados en esta Tesis Doctoral han pretendido avanzar en el campo de los complementos alimenticios e ingredientes alimentarios, ahondando en el conocimiento de los compuestos bioactivos, con un doble objetivo: por una parte, desarrollar nuevas metodologías analíticas que permitan evaluar la calidad de CAV basados en dichos compuestos y, por otra, proponer nuevos métodos eficaces y medioambientalmente sostenibles de obtención de extractos ricos en compuestos con propiedades bioactivas.

En cuanto a las metodologías de análisis para la detección de fraudes, se han propuesto dos alternativas: (i) por un lado, se han desarrollado estrategias multianalíticas basadas en el empleo de GC-MS y LC-MS o LC-MS y LC-IRMS que han permitido detectar no sólo fraudes asociados al contenido del compuesto bioactivo declarado, sino también los relacionados con la adición de sustancias no declaradas en el etiquetado, la sustitución de la fuente natural por otra de menor valor económico o el empleo de compuestos bioactivos de origen sintético, entre otros; y (ii) por otro lado, se han optimizado metodologías rápidas y fiables mediante FIA-(ESI)MS para la detección de dichos fraudes. En ambos casos se ha puesto de manifiesto el potencial de dichas técnicas y su aplicabilidad diferencial en función del objetivo analítico pretendido. Así, el principal inconveniente que presentan las estrategias multianalíticas es el tiempo de análisis requerido, ya que implican la optimización y validación de métodos por distintas técnicas previo a su aplicación. Como ventaja, este tipo de estrategias proporcionan un conocimiento más detallado de la composición de la muestra, que favorece una mejor evaluación de la autenticidad de complementos, así como la detección de los diversos tipos de fraudes a los que pueden verse sometidos. Por otro lado, las metodologías mediante FIA-(ESI)MS desarrolladas reducen significativamente el tiempo de análisis, por lo que podrían considerarse una alternativa ventajosa para el control de calidad rutinario de complementos alimenticios, basado en el contenido de compuesto bioactivo declarado.

La mejora en la extracción de compuestos bioactivos se ha abordado también desde diferentes puntos de vista. Por un lado, se ha considerado la optimización del procesado de la muestra, con el objetivo de favorecer, tanto la producción de bioactivos, como la mejora de las propiedades organolépticas del producto. Por otra parte, se han desarrollado metodologías de extracción de estos compuestos que contemplan, no sólo el empleo de técnicas avanzadas de extracción (MAE, UAE y PLE) en su aplicación a matrices vegetales y subproductos agroalimentarios para su revalorización, sino el de disolventes naturales medioambientalmente seguros (NADESS) para su extracción y fraccionamiento a partir de mezclas sintéticas o extractos vegetales.

En relación a las muestras seleccionadas en este estudio, se han escogido productos de interés comercial, especialmente para la empresa Pharmactive Biotech Products S.L.U., beneficiaria de

este proyecto de Doctorado Industrial. Dichas muestras, han abarcado tanto alimentos (ajo fresco, ajo negro, algarroba, apio), plantas (raíz de *C. forskohlii*) y subproductos agroalimentarios (tallos, hojas, raíces y pieles de ajo), como CAV y compuestos bioactivos obtenidos mediante síntesis (lactulosa).

El desafío analítico abordado en esta Tesis Doctoral se debe a que las matrices vegetales suelen ser muestras complejas que incluyen una gran variedad de compuestos, siendo muchos de ellos las dianas de interés para su extracción. Además, la diversidad estructural de dichos compuestos (incluyendo isómeros), sus diferentes propiedades físico-químicas y las distintas concentraciones en las que suelen estar presentes, dificultan, en ocasiones, los procesos de extracción y análisis de estos compuestos. Las **metodologías analíticas optimizadas y validadas** en la **Sección 3** han resultado exitosas para la evaluación de la calidad de los complementos alimenticios, tanto de *C. forskohlii* (**Sección 3.2.:** 24 CAV; **Sección 3.3.:** 22 CAV), como de ajo negro (**Sección 3.4:** 24 CAV). Dichas metodologías han permitido comprobar experimentalmente la veracidad del etiquetado de estos productos (cantidad declarada vs cantidad experimentalmente determinada), así como **detectar fraudes** relacionados con la ausencia del compuesto activo declarado, el origen sintético del producto y la sustitución de la fuente natural (**Secciones 3.2.-3.4.**). En cuanto a las técnicas analíticas, la GC-MS ha demostrado ser una herramienta robusta y con alto poder de separación, que ha permitido proponer e identificar **marcadores de autenticidad** de diferente naturaleza para garantizar la genuinidad de CAV de *C. forskohlii* (**Sección 3.2**). La principal desventaja del análisis por GC-MS es la necesidad de derivatizar los compuestos de naturaleza polar, ya que añade una etapa más al proceso de análisis, encareciéndolo e implicando mayores tiempos de preparación de muestra. Sin embargo, la mayor resolución de la GC comparada con otras técnicas, junto con la formación de trimetilsilil oximas, ha permitido la determinación simultánea de carbohidratos y otros compuestos menos polares, como los diterpenoides tipo labdano, en las complejas mezclas que constituyen, por ejemplo, los CAV de *C. forskohlii* analizados en la presente Tesis Doctoral. Por otro lado, la LC-MS, además de no requerir una etapa de preparación previa de la muestra, ha demostrado ser una técnica más sensible que la GC-MS para el análisis de las matrices objeto de estudio, aportando además información complementaria para la confirmación de fraudes. Por su parte, la LC-IRMS ha permitido la identificación de fraudes en complementos de ajo negro comercializados como naturales, evidenciándose la naturaleza sintética u origen biotecnológico del compuesto bioactivo (SAC) declarado en dichos complementos (**Sección 3.4.**). Asimismo, los métodos basados en FIA-(ESI)MS, y desarrollados para el análisis de complementos de *C. forskohlii* y ajo negro (**Secciones 3.3. y 3.4., respectivamente**), han mostrado ser eficaces para la detección rápida (tiempos < 4 min) de fraudes, proporcionando unos resultados muy similares a los obtenidos por LC-MS. Esta reducción del tiempo de análisis permite aumentar la cantidad de muestras a analizar e implica un

menor consumo de disolventes en comparación con LC-MS. Sin embargo, es necesario mencionar que, aunque la aplicación de estas metodologías pudiera presentar en un principio como limitación la cuantificación de compuestos con idéntica masa nominal, tal es el caso de los compuestos bioactivos diana y sus isómeros (**Sección 3.3.:** forskolina e isoforskolina; **Sección 3.4.:** SAC y S1PC), la demostrada bioactividad de estos isómeros hace que su determinación pueda resultar también interesante de cara a la estandarización de CAV, siendo ésta una propuesta de interés a considerar en su implementación por Pharmactive Biotech S.L.U.

Gracias a toda la información aportada por las diferentes técnicas analíticas empleadas, se han encontrado CAV, tanto de *C. forskohlii* como de ajo negro, que presentaban perfiles cromatográficos muy sencillos (con la presencia únicamente del compuesto bioactivo y otros ingredientes declarados, como maltodextrinas, usados como antiagregantes o diluyentes), y otros más complejos (similares a los extractos de la planta genuina de referencia). Atendiendo a los resultados obtenidos sobre los CAV de *C. forskohlii*, sólo se detectó el compuesto bioactivo forskolina en el 50% de los complementos analizados, mientras que un 25% sólo contenía carbohidratos de alto peso molecular (posiblemente empleados como excipientes), un 17% estaba constituido por otra fuente natural distinta a la declarada (té verde, hoja de soja, etc.) y el 8% restante contenía únicamente LMWCs. En el caso de los CAV de ajo negro, los resultados proporcionados por LC-MS y FIA(ESI)MS fueron muy similares, detectándose el compuesto bioactivo, la SAC, en el 71% de los casos y no cumpliendo con lo declarado en el etiquetado en el 50% de ellos. Además, los resultados del análisis por LC-IRMS de algunos complementos identificados como potencialmente adulterados en base a los perfiles cromatográficos obtenidos por LC-MS, permitieron evidenciar el origen sintético de la SAC, a pesar de ser declarado como natural. Estos datos, con una transcendencia no sólo económica, sino con repercusión social al condicionar la confianza del consumidor en estos productos, ponen de manifiesto la necesidad de desarrollar metodologías que permitan un mejor control de la calidad y genuinidad de estos productos, objetivo abordado en la presente Tesis Doctoral. Por otra parte, cabe mencionar también que, además de los organismos reguladores, cada vez son más las empresas que demandan metodologías analíticas innovadoras que permitan mejorar la determinación de los compuestos bioactivos en base a los cuales estandarizan sus productos, y que sean de ayuda para garantizar el cumplimiento del etiquetado de sus productos. En este sentido, las metodologías desarrolladas en este Doctorado Industrial han tenido en cuenta su posible implementación en la empresa Pharmactive Biotech Products S.L.U. con vistas a su posicionamiento preferencial dentro del sector como empresa comercializadora de productos de calidad contrastada.

La **Sección 4** se ha centrado en el **desarrollo de metodologías de obtención de extractos ricos en compuestos bioactivos**. La etapa previa a todo proceso de extracción es la selección de la materia prima a emplear (**Sección 4.2.**). En el caso concreto del ajo envejecido, resulta fundamental seleccionar las condiciones de envejecimiento (tiempo y tratamiento de los bulbos) que, dando lugar a un mayor contenido en los compuestos bioactivos de interés (SAC y S1PC), contribuyan a un perfil organoléptico mejorado (con menor contenido en compuestos volátiles pungentes, entre otros). En este sentido, se ha considerado en este estudio una nueva cámara de fermentación industrial adquirida por la empresa Pharmactive, con capacidad de hasta 400 kg, para el envejecimiento de ajo en distintas condiciones de operación. Por otra parte, la estancia llevada a cabo en la Universidad de Reading (Reino Unido) ha permitido obtener datos sobre la composición aromática de dichas muestras mediante SPME-GC-MS y SPME GC-O. Cabe señalar que las condiciones óptimas de obtención de ajo envejecido (21 días de procesado, sin trituración de los bulbos), están siendo actualmente evaluadas para su implementación por parte de Pharmactive Biotech S.L.U. ya que proporcionan un ajo envejecido con mayor contenido en bioactivos que las muestras de ajo negro comerciales (analizadas con fines comparativos) y con una mayor aceptación sensorial que el ajo fresco de partida.

Por otra parte, las **metodologías optimizadas de extracción** mediante MAE, UAE y PLE, ventajosas en cuanto a eficacia, sostenibilidad y rapidez, han permitido la obtención de extractos ricos en compuestos organosulfurados, carbohidratos bioactivos y aminoácidos a partir del ajo, sus productos derivados (ajo envejecido y negro; **Sección 4.3.**) y sus subproductos (tallos, hojas, pieles y raíces; **Sección 4.4.**). En concreto, la metodología desarrollada por MAE (0.5 g de muestra en 10 mL de agua a 120 °C durante 60 min) ha dado lugar a la obtención de extractos multifuncionales de ajo negro con concentraciones de bioactivos muy por encima de las previamente descritas (*e.g.* concentración de la SAC del orden de 3-7 veces mayor). Esta metodología se ha aplicado para la obtención de extractos multifuncionales a partir de muestras de ajo envejecido aportadas por Pharmactive Biotech Products S.L.U. y, también, de diferentes muestras de ajo negro comerciales. Por otro lado, se ha demostrado que las metodologías desarrolladas por PLE (100 mg mL⁻¹ de muestra a 50 °C durante 18 min) y UAE (84 mg mL⁻¹ de muestra a 40 °C durante 59 min) para la extracción de bioactivos de subproductos agrícolas derivados del cultivo del ajo son más eficaces que la SLE convencional, lo que hace de estas metodologías, y en concreto de la UAE, una alternativa prometedora para la revalorización de estos subproductos. Para evaluar la aplicabilidad industrial de esta técnica, se llevaron a cabo ensayos a mayor escala, optimizándose las condiciones experimentales de extracción (tiempo y temperatura) en un prototipo de UAE (NextTgen Ultrasonic Platform, Synaptec) de hasta 1 L de capacidad, propiedad de la empresa Pharmactive Biotech Products S.L.U. En estos estudios se evaluó el rendimiento de extracción de SAC, obteniéndose los resultados mostrados en el Anexo

9. Como se puede observar, la UAE permitió obtener concentraciones de SAC superiores a las obtenidas por extracción convencional a escala industrial bajo las mismas condiciones (0,39 % vs 0,225 %, respectivamente). Estos prometedores resultados suponen la base para la futura incorporación de esta metodología en los protocolos de producción esta empresa.

Por último, se ha explorado el empleo de los NADESs como disolventes verdes alternativos a los disolventes orgánicos tradicionales para el fraccionamiento selectivo de carbohidratos de bajo peso molecular (**Secciones 4.5. y 4.6.**). A pesar de que, en los últimos años, ha aumentado el número de aplicaciones de los NADESs para la extracción de compuestos de diversa naturaleza (principalmente compuestos fenólicos, flavonoides...), los estudios sobre carbohidratos son limitados, y principalmente orientados a la extracción de polisacáridos. Así, en esta Tesis Doctoral se llevó a cabo, en primer lugar, una revisión bibliográfica que recopiló toda la información disponible en relación a diferentes disolventes verdes [ILs y (NA)DESs] y su empleo como extractantes de carbohidratos (**Anexo 4**). Al observar que los datos disponibles, tanto de solubilidad de LMWCs en NADESs, como de su posible uso para el fraccionamiento selectivo eran muy limitados, se seleccionaron diferentes NADESs hidrofílicos [ChCl:Gly (glicerina) y ChCl:EtG (etanol)], en base a sus aplicaciones para compuestos polares. Las diferencias de solubilidad entre carbohidratos en estos disolventes permitieron evaluar su utilidad, primero en mezclas binarias y, posteriormente, en muestras reales, para el fraccionamiento selectivo de carbohidratos bioactivos frente a no bioactivos, consiguiendo altos porcentajes de enriquecimiento en la mayoría de los casos. Aunque todavía hay aspectos que es necesario abordar para su aplicación industrial, tales como la evaluación de la bioactividad de los extractos obtenidos, la viabilidad de los NADESs para su consumo, etc, estos disolventes resultan una alternativa interesante, novedosa y prometedora a los convencionales para la obtención de extractos enriquecidos en carbohidratos bioactivos. Estos estudios preliminares abren nuevas vías de investigación dentro del campo de la Química Verde y de los carbohidratos.

En base a lo anteriormente expuesto, cabe concluir que el compendio de trabajos incluidos en esta Tesis Doctoral ha contribuido de manera significativa al avance en cuanto al desarrollo de metodologías analíticas innovadoras y mejoradas para el control de calidad/autenticidad de CAV, y sus procesos de obtención, haciendo especial énfasis en el desarrollo de aproximaciones analíticas sostenibles y con potencial de implementación por las industrias del sector de los CAV.



6. Conclusiones / Conclusions

6. CONCLUSIONES

- Las estrategias multianalíticas optimizadas basadas en el empleo de técnicas cromatográficas acopladas a espectrometría de masas (GC-MS, LC-MS, LC-IRMS) han demostrado ser ventajosas sobre las técnicas analíticas individuales para la detección de fraudes en complementos alimenticios de *C. forskohlii* y ajo negro.
- La aplicación de estas metodologías ha permitido identificar un elevado porcentaje de complementos de *C. forskohlii* y ajo negro que no cumplen las declaraciones incluidas en sus etiquetados (*e.g.* por ausencia o discrepancia en el contenido de bioactivo, presencia de otra fuente vegetal, origen no natural del bioactivo, etc.).
- Los métodos optimizados y validados por FIA-(ESI)MS han permitido reducir significativamente el tiempo de análisis requerido para la cuantificación conjunta de forskolina e isoforskolina, así como de SAC y S1PC, en complementos de *C. forskohlii* y ajo negro, respectivamente, siendo una herramienta rápida y eficaz para el control de la calidad de un elevado número de complementos alimenticios de estas fuentes.
- Las metodologías desarrolladas, basadas en LC-MS, SPME-GC-MS y SPME GC-O, y aplicadas a muestras de ajo envejecido sometidas a distintas condiciones de procesado, han permitido seleccionar las condiciones óptimas que aúnan simultáneamente un mayor contenido en compuestos bioactivos (SAC y S1PC) y un perfil aromático mejorado, así como la identificación de los olores clave responsables de sus propiedades organolépticas.
- La MAE ha demostrado ser una técnica más eficaz que la UAE y la SLE para la obtención de extractos de ajo negro enriquecidos en compuestos bioactivos (COS y CH prebióticos). La aplicación esta metodología, previamente optimizada, evidenció la amplia variabilidad en cuanto a la composición cuali- y cuantitativa de los extractos de ajo negro, probablemente asociada a las condiciones de procesado del ajo.
- En el caso de subproductos de ajo, las metodologías por UAE y PLE optimizadas han demostrado ser más eficaces que la SLE para la obtención de extractos multifuncionales ricos en compuestos organosulfurados, carbohidratos bioactivos y aminoácidos, contribuyendo así a la revalorización de estas muestras.
- Se han aportado nuevos datos de solubilidad de mono-/disacáridos y polialcoholes en distintos NADESs hidrofílicos (EtG:ChCl y Gly:ChCl) y se han establecido relaciones entre dichos datos y la estructura química de los analitos investigados.

- Los NADESs seleccionados resultaron ser una alternativa eficaz y sostenible para el fraccionamiento selectivo entre cetosas y sus correspondientes aldosas y entre polialcoholes y monosacáridos en mezclas binarias equimolares, siendo su aplicación de interés para la obtención de extractos enriquecidos en carbohidratos bioactivos a partir de mezclas de síntesis o extractos naturales.
- El conocimiento generado en este Doctorado Industrial, susceptible de ser implementado por empresas del sector de los nutraceúticos, como Pharmactive Biotech Products S.L.U., supone un avance reseñable para el control de la calidad de complementos alimenticios y para la obtención de extractos multifuncionales.

6. CONCLUSIONS

- The optimized multianalytical strategies based on chromatographic techniques coupled to mass spectrometry (GC-MS, LC-MS, LC-IRMS) have proven to be advantageous over individual analytical techniques for the detection of frauds in food supplements of *C. forskohlii* and black garlic.
- The application of these methodologies has enabled the identification of a high percentage of *C. forskohlii* and black garlic supplements which do not comply with the claim declared on their labels (*e.g.*, due to absence or discrepancies in bioactive content, presence of another plant source, non-natural origin of the bioactive compound, etc).
- The optimized and validated methods by FIA-(ESI)MS have significantly reduced the time of the analysis for the simultaneous quantitation of forskolin and isoforskolin, as well as SAC and S1PC, in *C. forskohlii* and black garlic supplements, respectively. These methods are a fast and efficient tool for quality control of a large number of dietary supplements derived from these sources.
- The developed methodologies, based on LC-MS, SPME-GC-MS and SPME GC-O, and applied to aged garlic samples processed under different conditions, have enabled the selection of optimal conditions that simultaneously achieve a higher content of bioactive compounds (SAC and S1PC) and an improved aromatic profile, as well as the identification of key odors responsible for their organoleptic properties.
- MAE has proven to be a more effective technique than UAE and SLE for obtaining black garlic extracts enriched in bioactive compounds (OSC and prebiotic CH). The application of this optimized methodology revealed a wide variability in the qualitative and quantitative composition of black garlic extracts, probably associated with the garlic processing conditions.
- Regarding garlic by-products, the optimized UAE and PLE methodologies have shown greater efficiency than SLE to obtain multifunctional extracts enriched in organosulfur compounds, bioactive carbohydrates and amino acids, thereby contributing to the revalorization of these samples.
- New solubility data for mono-/disaccharides and polyols in different hydrophilic NADESs (EtG:ChCl and Gly:ChCl) have been provided, and the relationship between these new data and the chemical structure of these analytes has been investigated.
- The selected NADESs were an effective and sustainable alternative for the selective fractionation of ketoses and their corresponding aldoses and also between polyols and

monosaccharides in equimolar binary mixtures; its application is of great interest for obtaining extracts enriched in bioactive carbohydrates from synthetic mixtures or natural extracts.

- The knowledge generated in this Industrial PhD, which can potentially be implemented by companies in the nutraceutical sector, such as Pharmactive Biotech Products S.L.U., represents a noteworthy advancement in the quality control of dietary supplements and the production of multifunctional extracts.

Anexo 1: Correspondiente a la sección 3.2.

Development of a multianalytical strategy for detection of frauds in *Coleus forskohlii* supplements

Supplementary material

Table S1. Formulation and composition of FKS under study, as declared in the labelling.

Supplement	Formulation	<i>P. barbatus</i> extract amount/formulation (forskolin content/formulation)	Other declared constituents	Daily dose (forskolin amount)
FKS1	Capsules	500 mg (10%)	Maltodextrin, rice flour, silicon dioxide	2 capsules (100 mg)
FKS2	Capsules	400 mg (20%)	Maltodextrin	1 capsules (80 mg)
FKS3	Capsules	50 mg (20%)	-	1 capsules (10 mg)
FKS4	Capsules	500 mg	-	1 capsules
FKS5	Capsules	50 mg (15 mg)	Maltodextrin, magnesium stearate, silicon dioxide	2 capsules (30 mg)
FKS6	Capsules	500 mg (10%)	Cornstarch	
FKS7	Tablets	750 mg (10%)	Magnesium stearate, silicon dioxide, Cr-enriched yeast, cellulose	1-2 tablets (125-250 mg)
FKS8	Capsules	125 mg (20%)	Magnesium stearate, cellulose	2 capsules (50 mg)
FKS9	Capsules	530 mg	Magnesium stearate, cellulose	2 capsules
FKS10	Capsules	125 mg (10%)	Black pepper fruit extract (bioperine, 2.5 mg), rice flour, maltodextrin, medium chain tryglycerides, vitamin E, rosemary oil	Non declared
FKS11	Capsules	100 mg (10%)	Microcrystalline cellulose, maltodextrin, vegetable stearate	Non declared
FKS12	Capsules	500mg (20%)	Cellulose	2 capsules (200 mg) Non declared
FKS13	Capsules	500 mg (4:1 extract)	Gelatin, cellulose	1 capsules
FKS14	Powder	-	-	
FKS15	Powder	- (20:1 extract)	-	6-10 g
FKS16	Powder	-	-	
FKS17	Capsules	(20:1 extract)	-	3 capsules
FKS18	Powder	-	-	
FKS19	Powder	-	-	
FKS20	Capsules	100 mg (10%)	Rice flour	
FKS21	Capsules	250 mg (10%)	N-Acetyl-L-tyrosine, gelatine, microcrystalline cellulose, silicon dioxide, magnesium stearate	
FKS22	Capsules	250 mg (20%)	Mycrocrystalline cellulose, calcium silicate	
FKS23	Capsules	250 mg	Cellulose	
FKS24	Powder	(20%)	-	

Table S2. LC-MS analyses of *Coleus forskohlii* supplements

Identification	<i>Empirical formula</i>	<i>M_w</i>	<i>t_R</i> (min)	<i>MS</i>			<i>MS/MS</i>
				[M+H] ⁺	[M+NH ₄] ⁺	[M+Na] ⁺	
Forskolin D	C ₂₀ H ₃₂ O ₆	368	16.5		386.2539		351.2165 333.2059 315.1953
9-DE	C ₂₂ H ₃₄ O ₆	394	17.4	395.2428	412.2696	417.2251	377.2322 249.1483
Isoforskolin	C ₂₂ H ₃₄ O ₇	410	18.8		428.2646		393.2270 375.2166 315.1952
1-Acetoxycoleosol	C ₂₂ H ₃₄ O ₆	394	19.7	395.2428			377.2321 249.1434
1,6-diacetyl-7-deacetylforskolin	C ₂₄ H ₃₆ O ₈	452	22.2	453.2481	470.2747		435.2381 375.2164
Forskolin	C ₂₂ H ₃₄ O ₇	410	22.5	411.2371	428.2639		393.2265 375.2157
Deoxyforskolin isomer	C ₂₂ H ₃₄ O ₆	394	24.3	395.2425	412.2691	417.2246	377.2320
1,9-DDF	C ₂₂ H ₃₄ O ₅	378	25.5	379.2478	396.2748	401.2299	283.2579
6-acetylforskolin	C ₂₄ H ₃₆ O ₈	452	25.5	453.2484	470.2750		435.2385

Figure S1. Gas chromatographic separation of: 1,9-DDF and phenyl- β -D-glucoside and forskolin and an unknown compound (m/z ion 545) under different chromatographic conditions: A) 150 °C at 6 °C min⁻¹ to 270 °C, then at 10 °C min⁻¹ to 310 °C (15 min) and B) 150 °C at 8 °C min⁻¹ to 225 °C, then at 10 °C min⁻¹ to 310 °C (10 min).

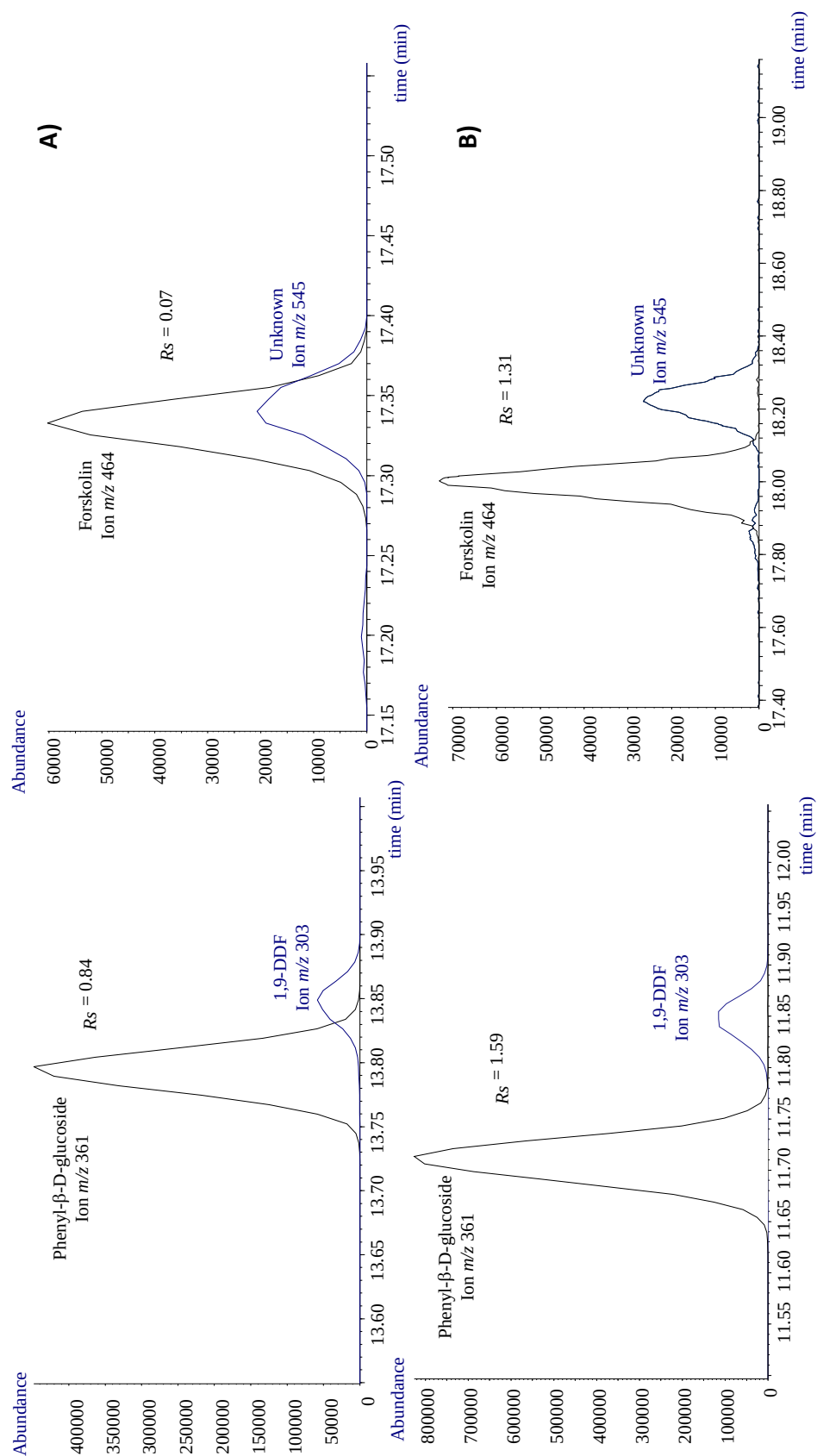


Figure S2. Graphical representation of the analyzed *C. forskohlii* food supplements regarding the type of compounds detected

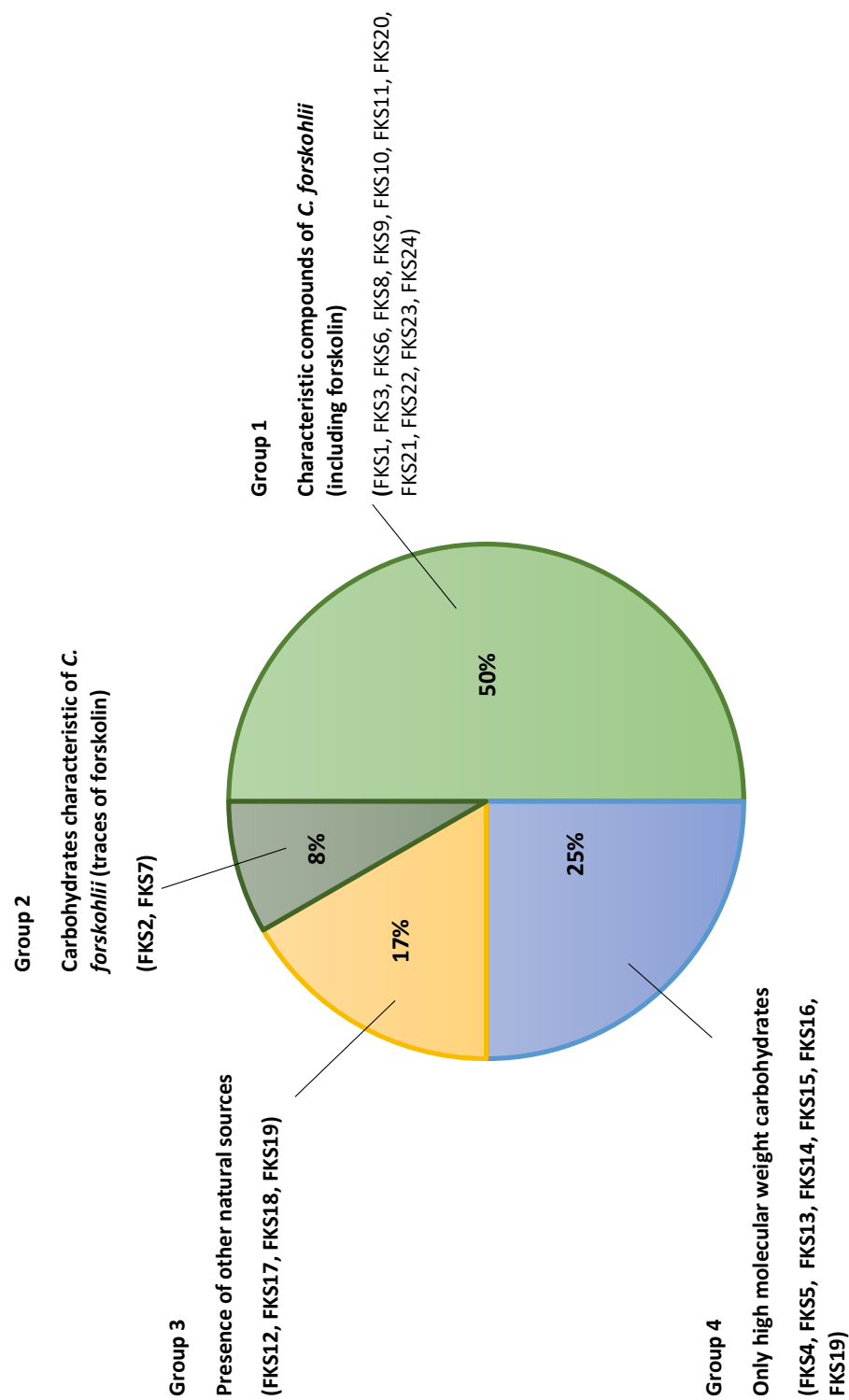
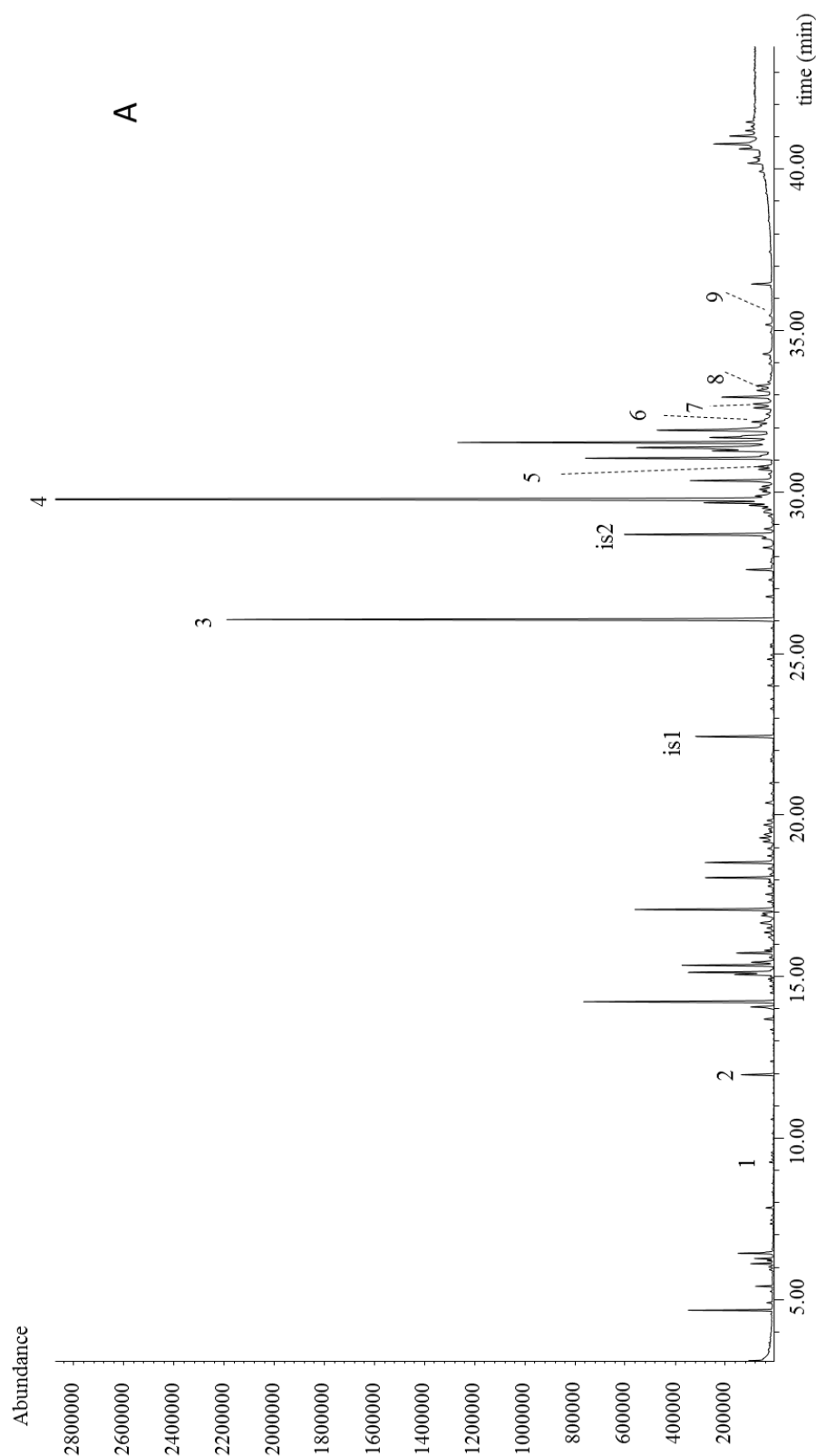
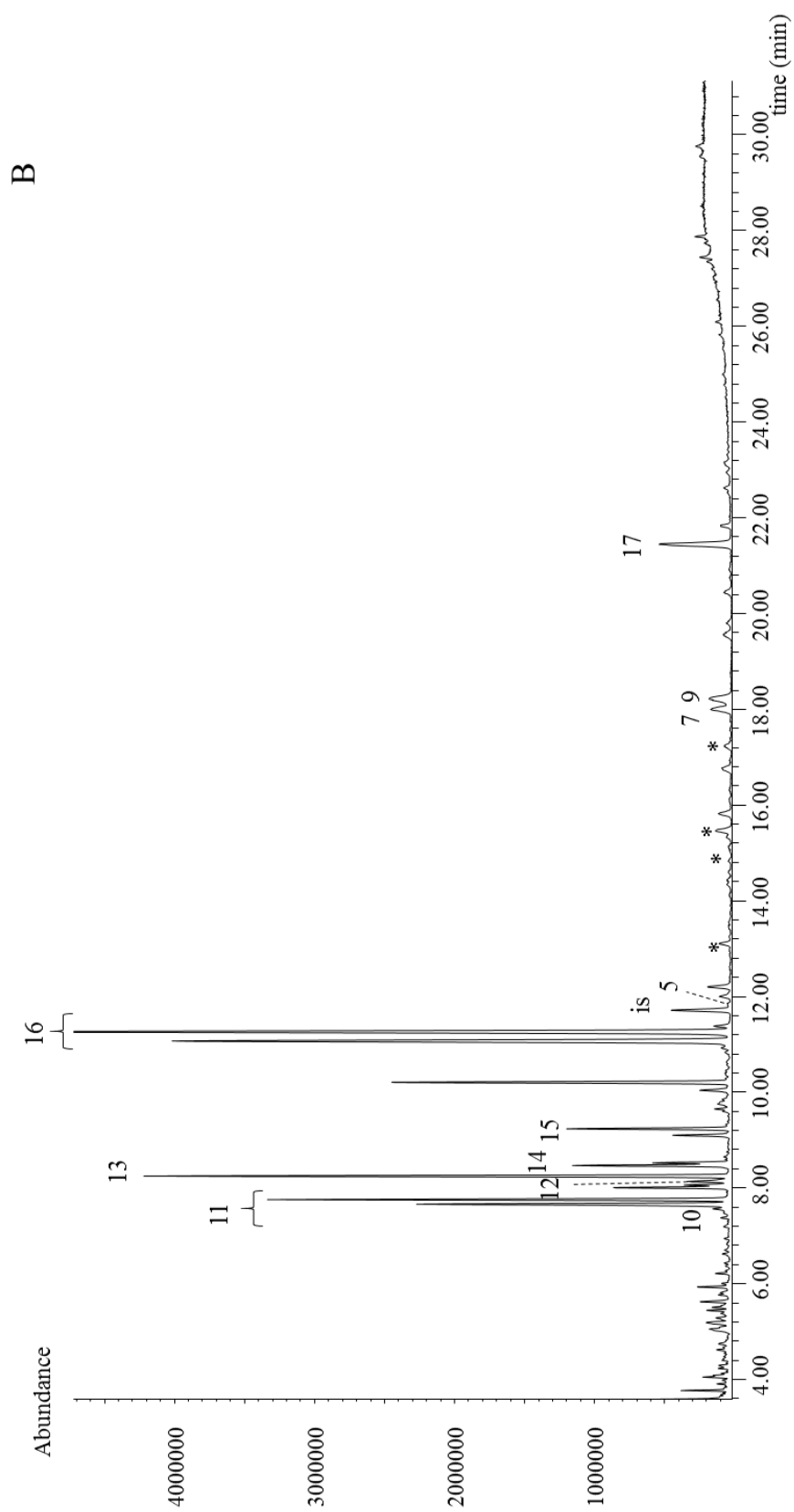


Figure S3. Chromatographic profiles of reference root extract: **A)** GC-MS of heptane extract, **B)** GC-MS of methanolic extract and **C)** LC-MS of methanolic extract. 1. borneol, 2. bornyl acetate, 3. abieta-8,11,13-triene, 4. *trans*-ferruginol, 5. 1,9-DDF, 6. 1,9-DDF related compound, 7. 9-DE, 8. sugiol, 9. forskolin, 10. mannitol, 11. fructose, 12. galactose *E* + mannose *E*, 13. glucose *Z* + galactose *Z* + mannose *Z*, 15. *myo*-inositol, 16. sedoheptulose, 17. sucrose, 18. isoforskolin, 19. 1-acetoxycyclohexanol, 20. 1-acetyl-7-deacetylforskolin + forskolin B, 21. deoxyforskolin isomer, 22. 6-acetylforskolin, is1 and is2: internal standards, * unknowns.





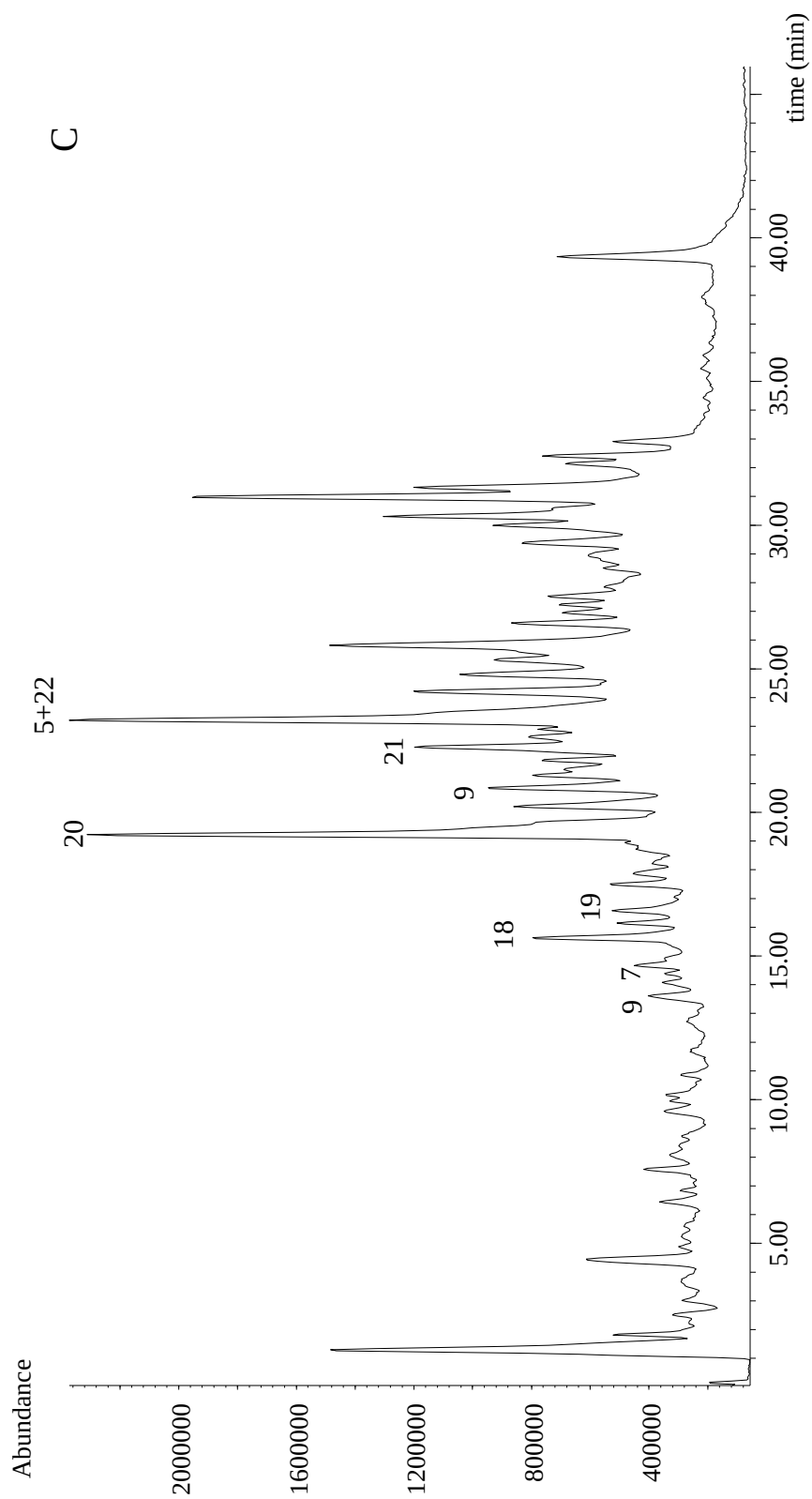
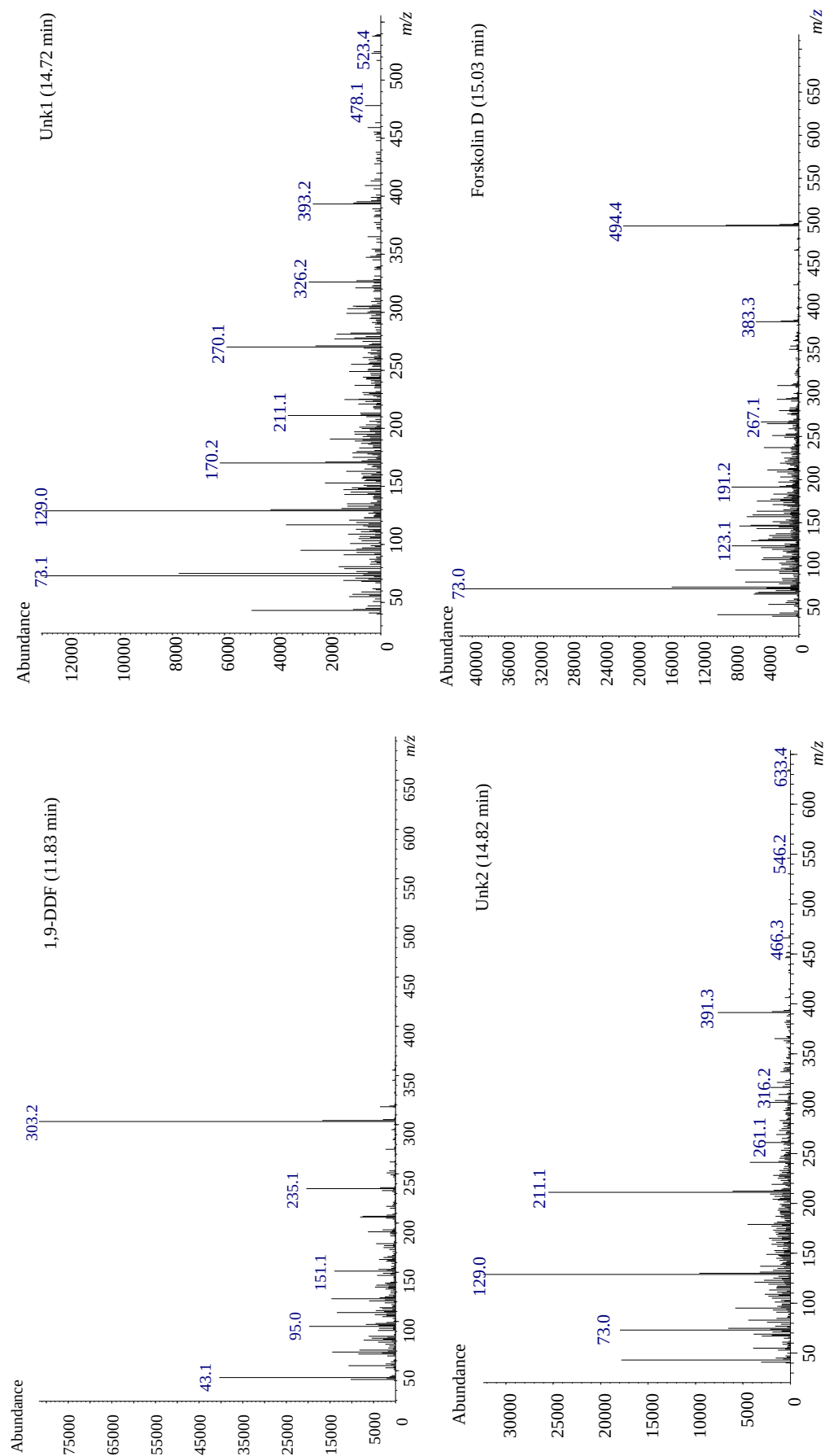
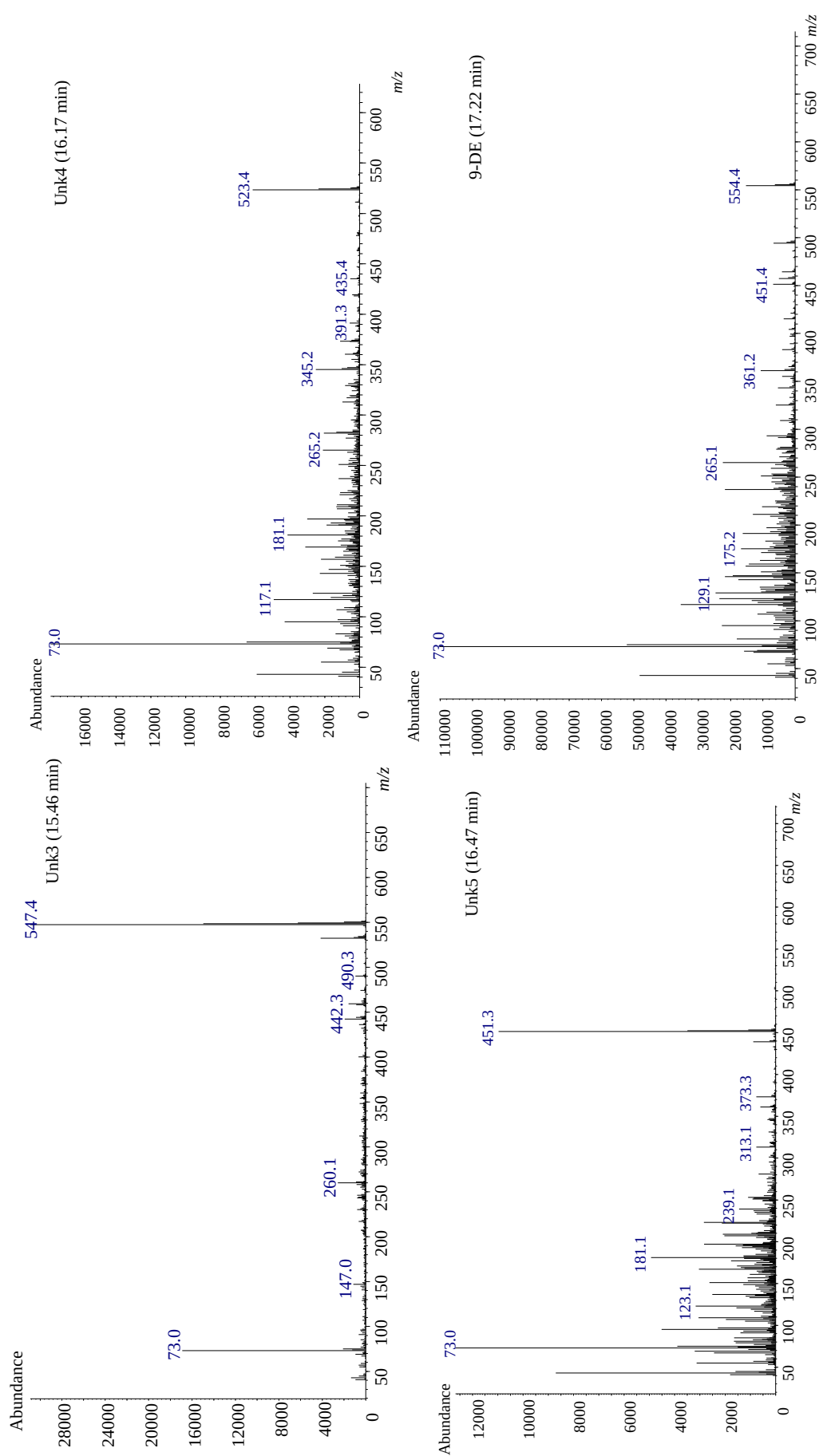


Figure S4. Mass spectra of diterpenoids detected in methanolic extracts of Group 1 supplements. Unk: unknown.





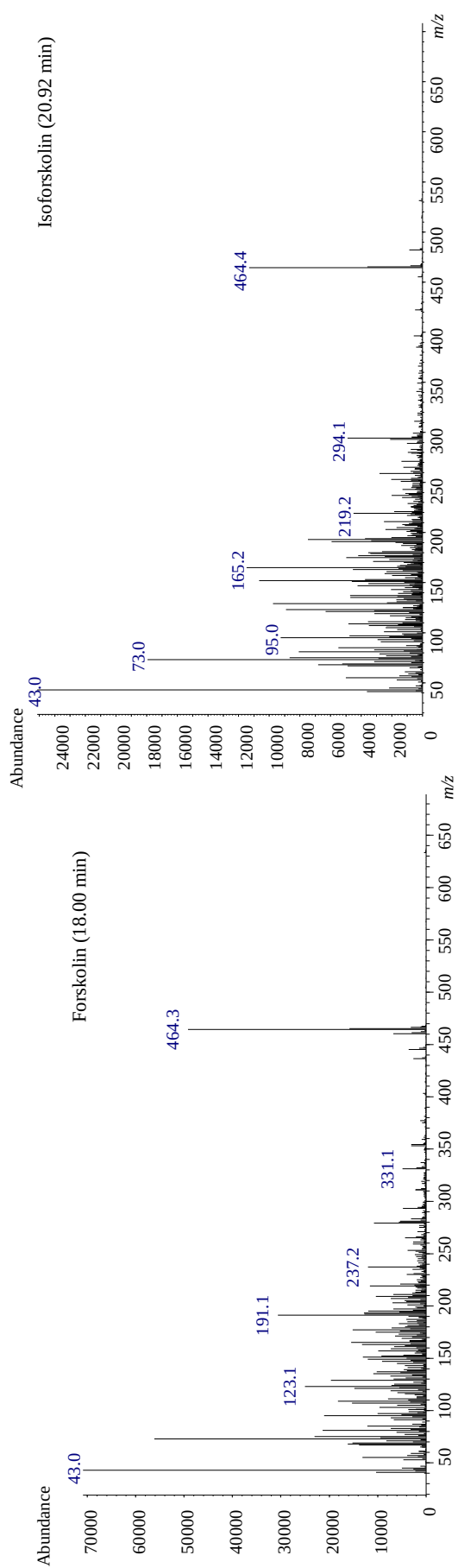
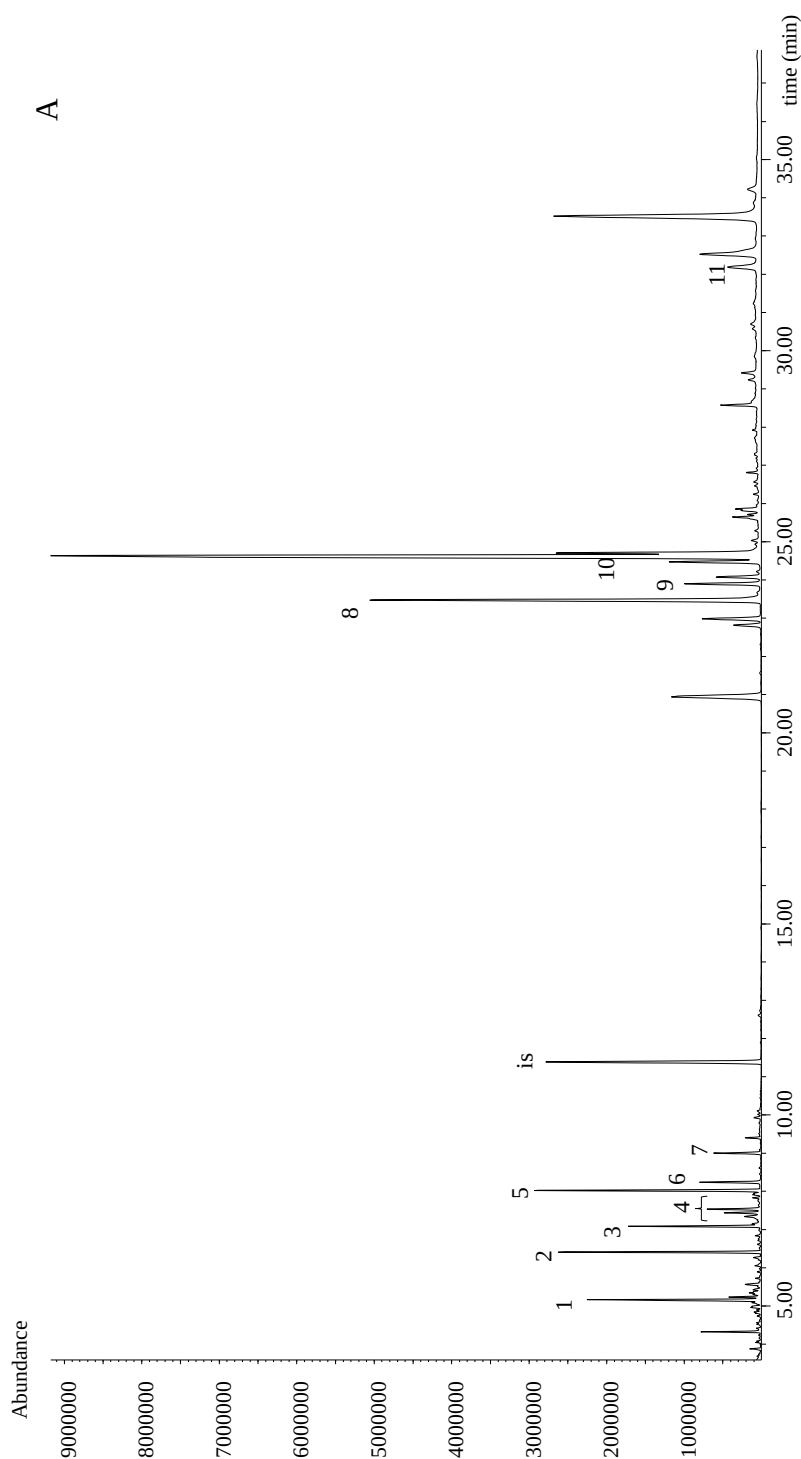


Figure S5. GC-MS profiles of FKS17 (A) and FKS19 (B) methanolic extracts. 1. caffeine, 2. quinic acid, 3. gallic acid, 4. fructose, 5. glucose *E*, 6. glucose *Z*, 7. *myo*-inositol, 8. epicatechin, 9. catechin, 10. maltose, 11. maltotriose, 12. pinitol, 13. ononitol, 14. *chiro*-inositol, 15. sucrose, is.



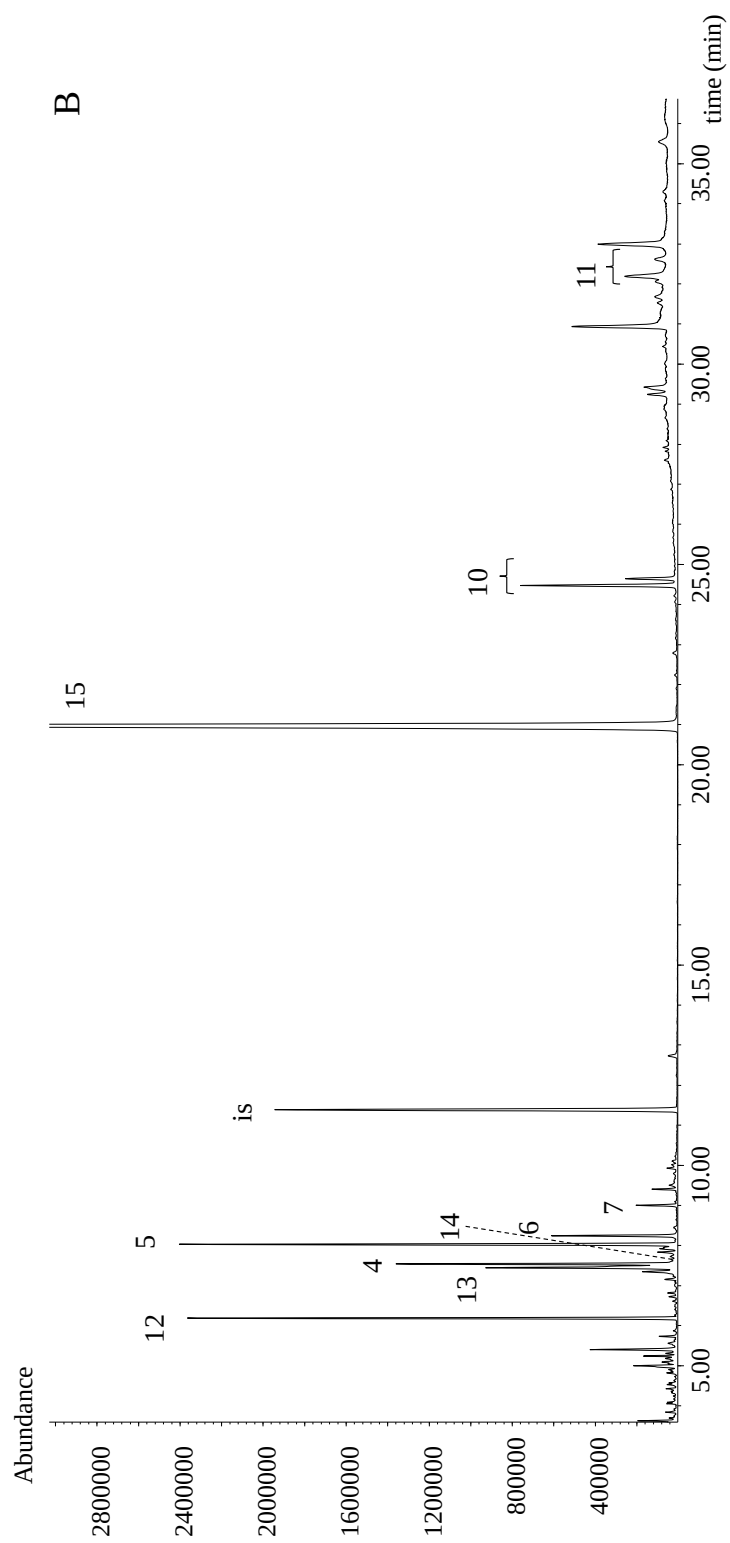
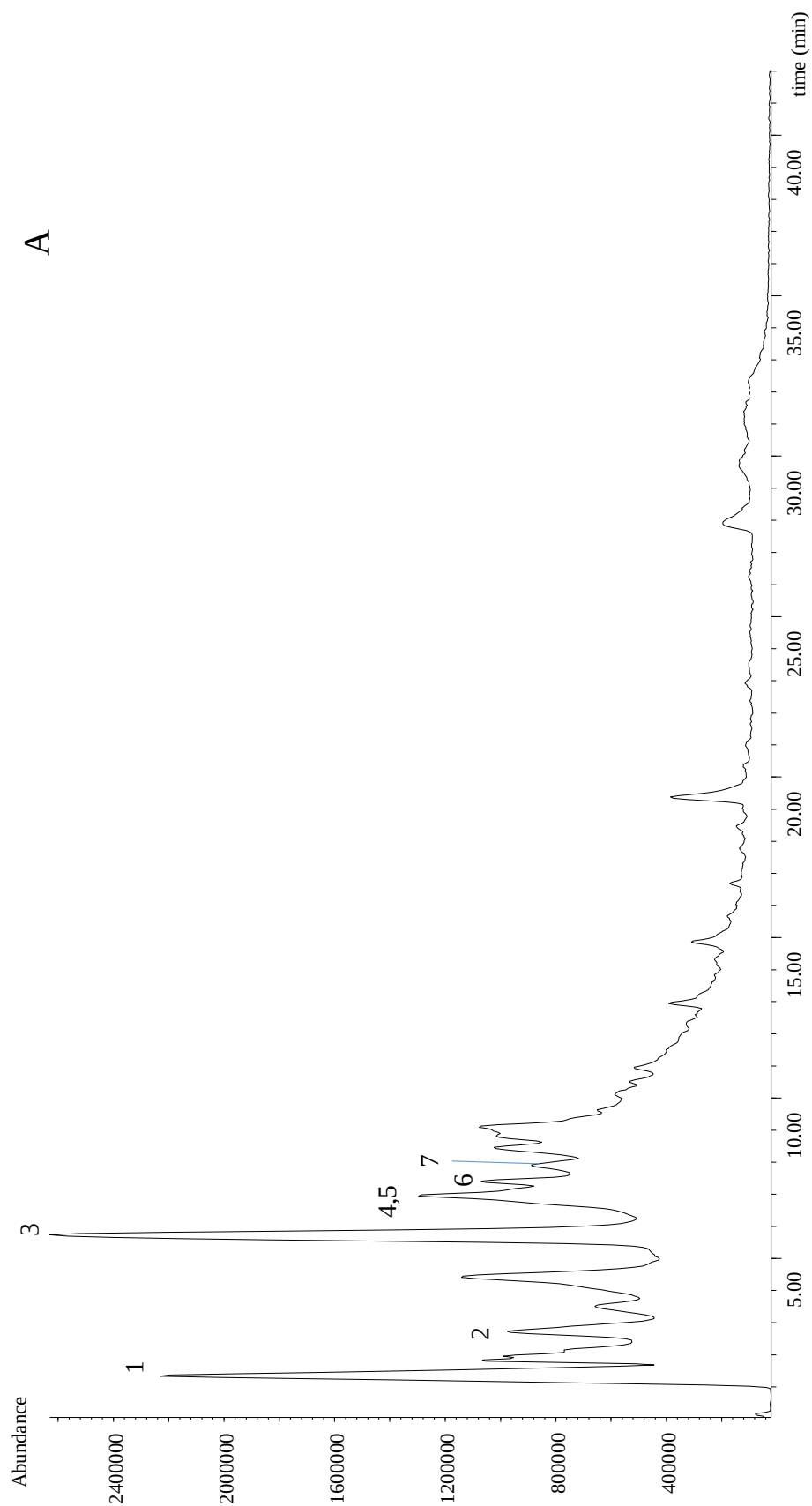


Figure S6. LC-MS profiles of FKS17 (A) and FKS19 (B) methanolic extracts. 1. theanine, 2. theobromine, 3. caffeine, 4. epicatechin, 5. catechin, 6. epigallocatechin gallate, 7. theaflavin, 8. genistin, 9. daidzin, 10. glycitin, 11. daidzein, 12. glycitein.



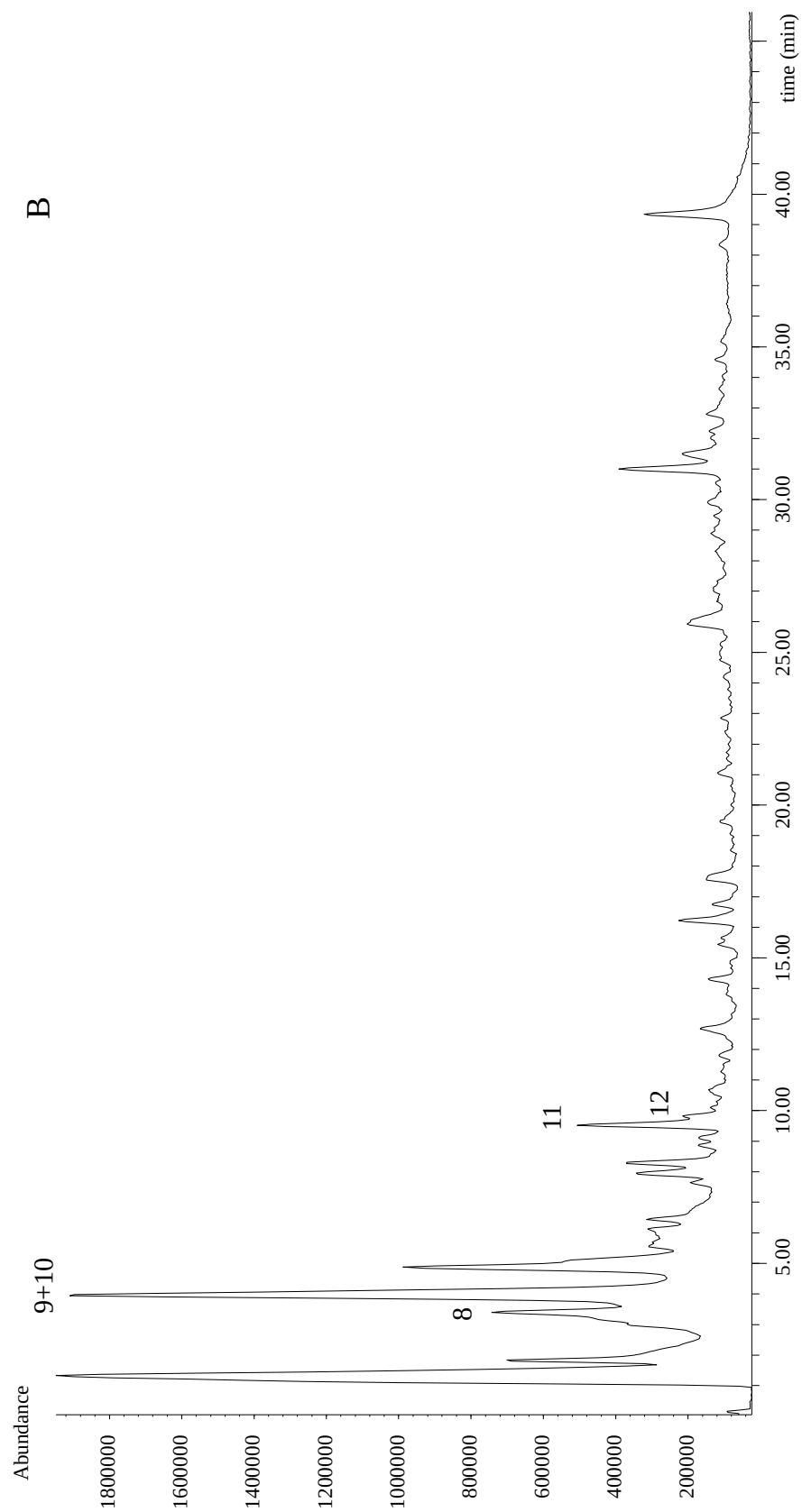
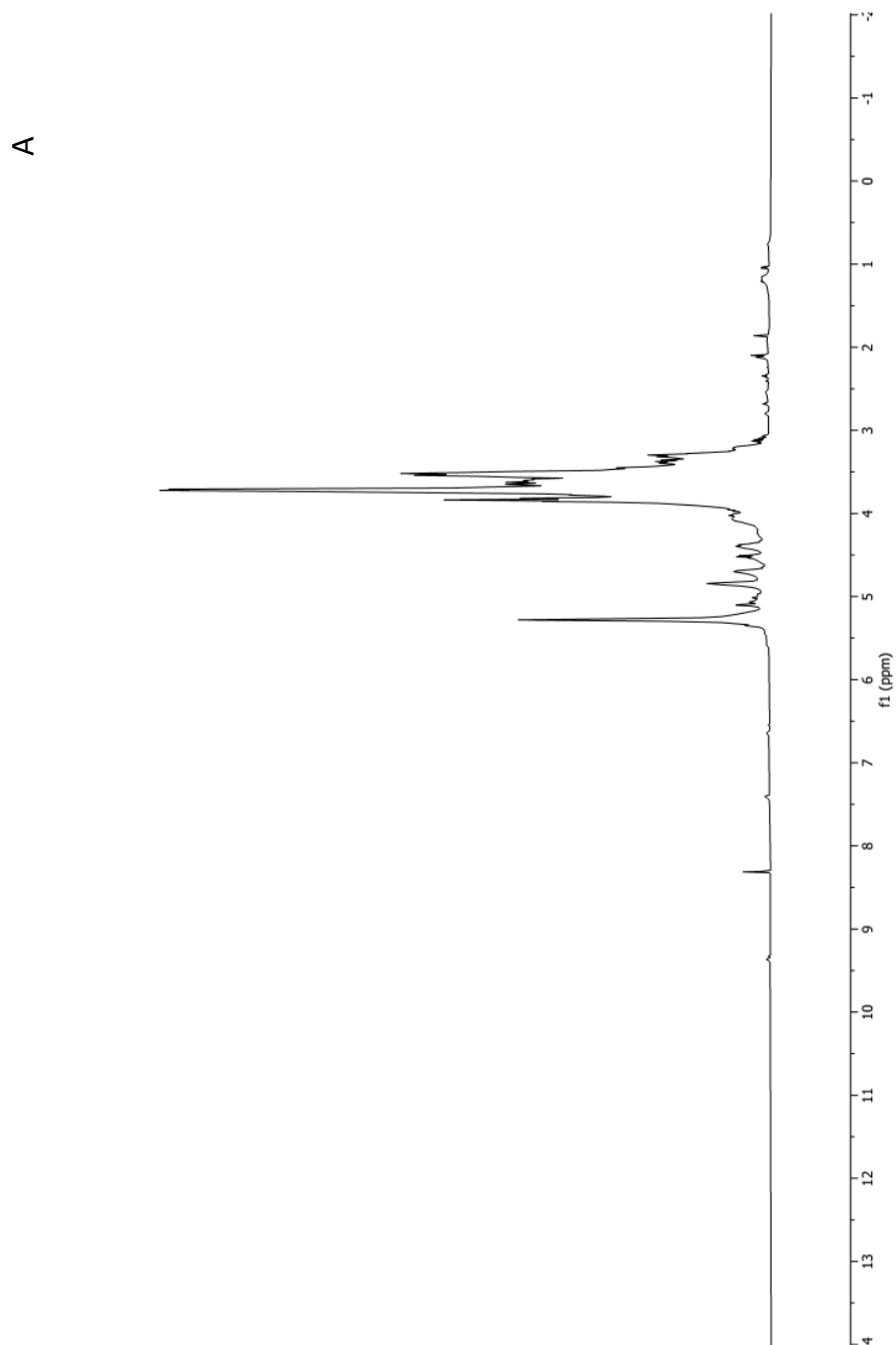
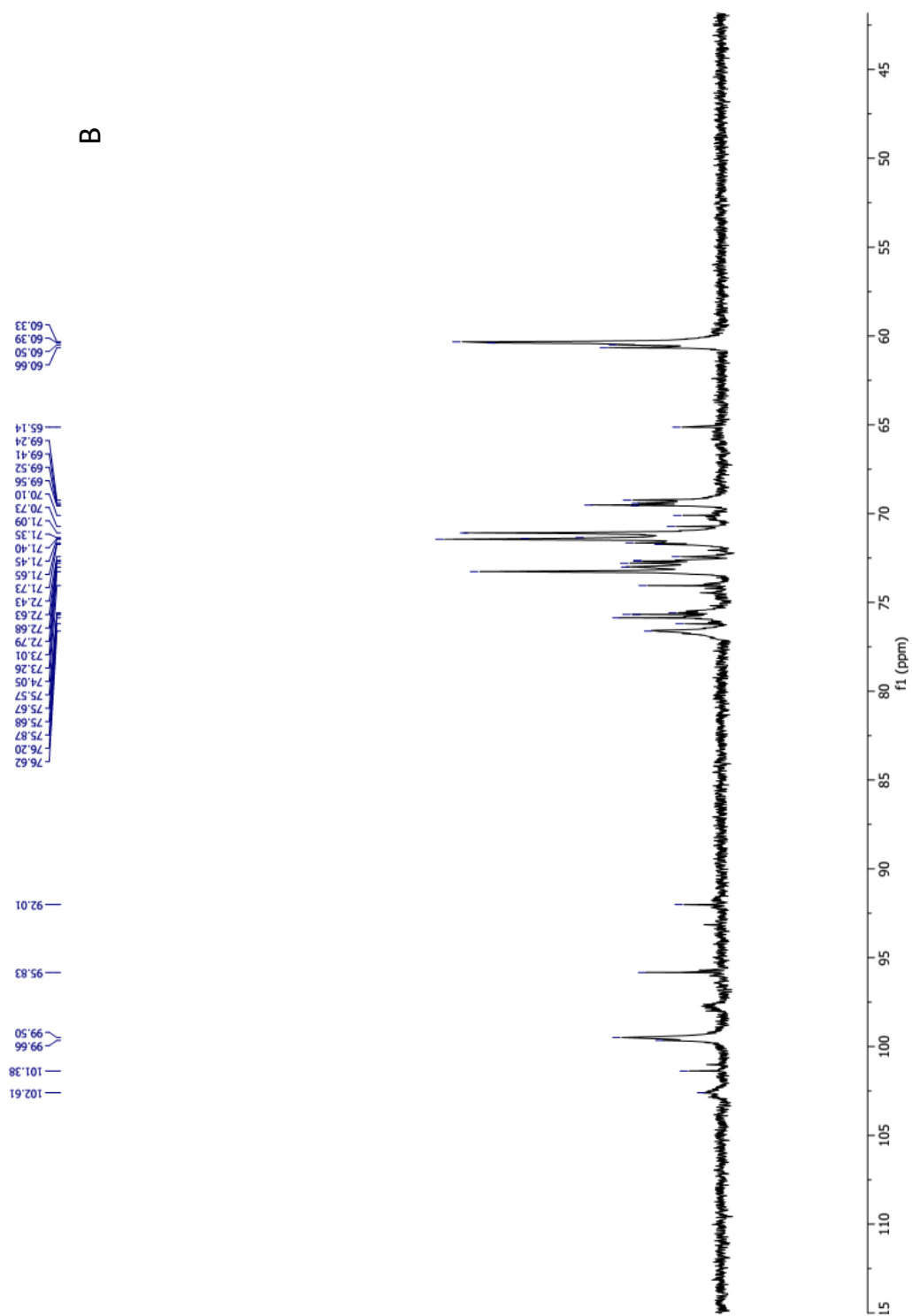


Figure S7. ^1H -NMR(A) and ^{13}C -NMR (B) analysis of FKS14.





Anexo 2: Correspondiente a la sección 3.3.

Flow Injection Analysis–Mass Spectrometry for the fast detection of frauds in *Coleus forskohlii* food supplements

Supplementary material

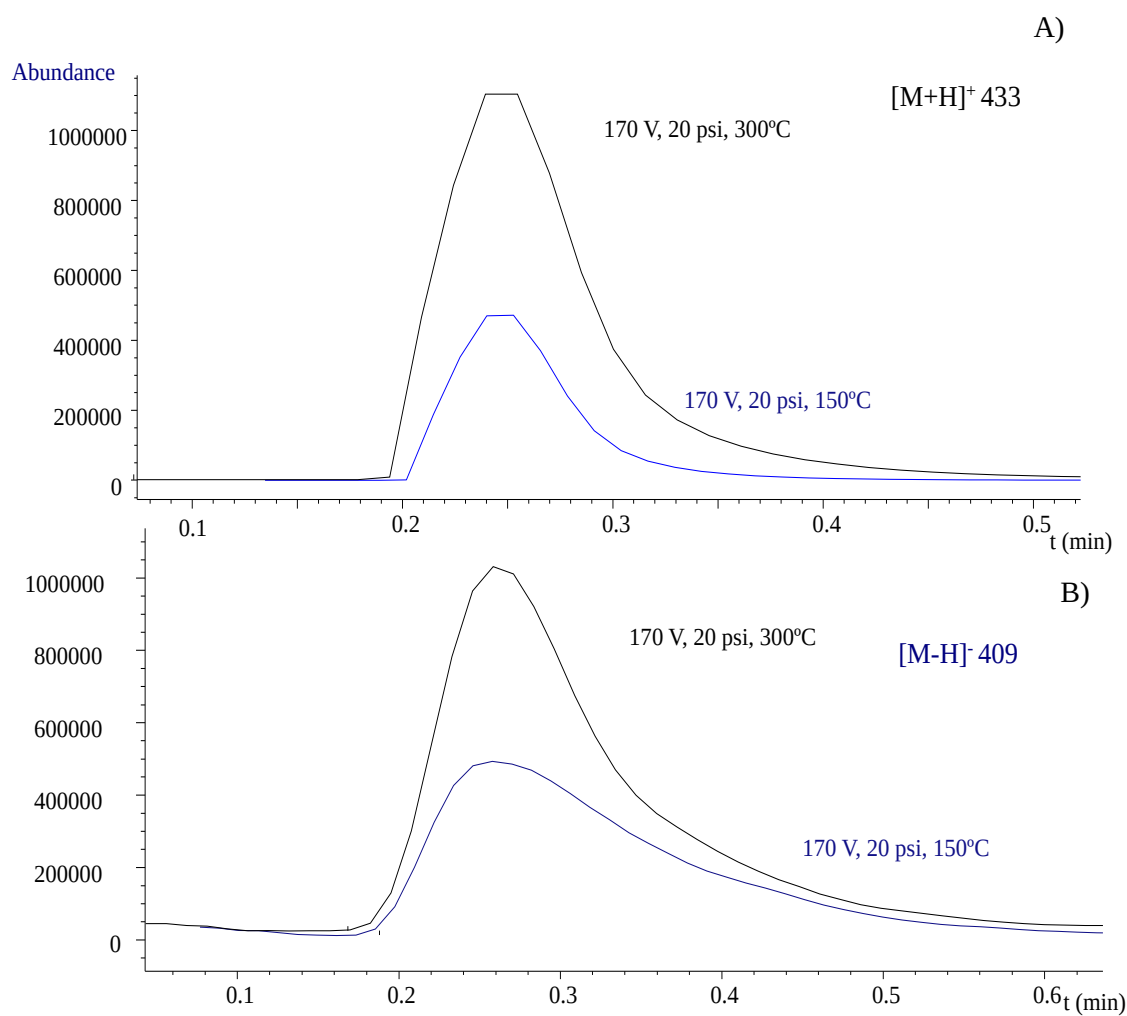


Figure S1. Effect of nebulizing gas temperature on the intensity of forskolin adducts response in A) positive and B) negative ionization modes.

A)

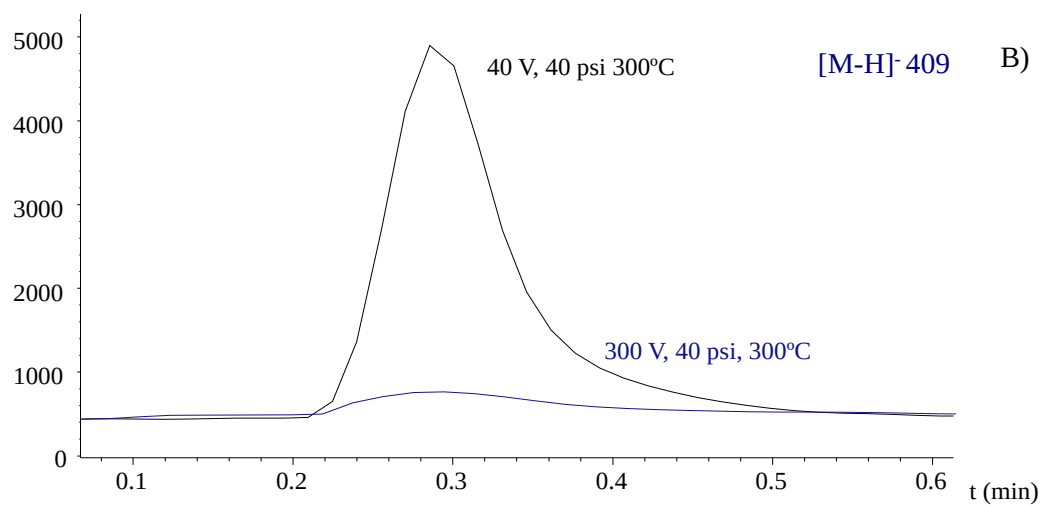
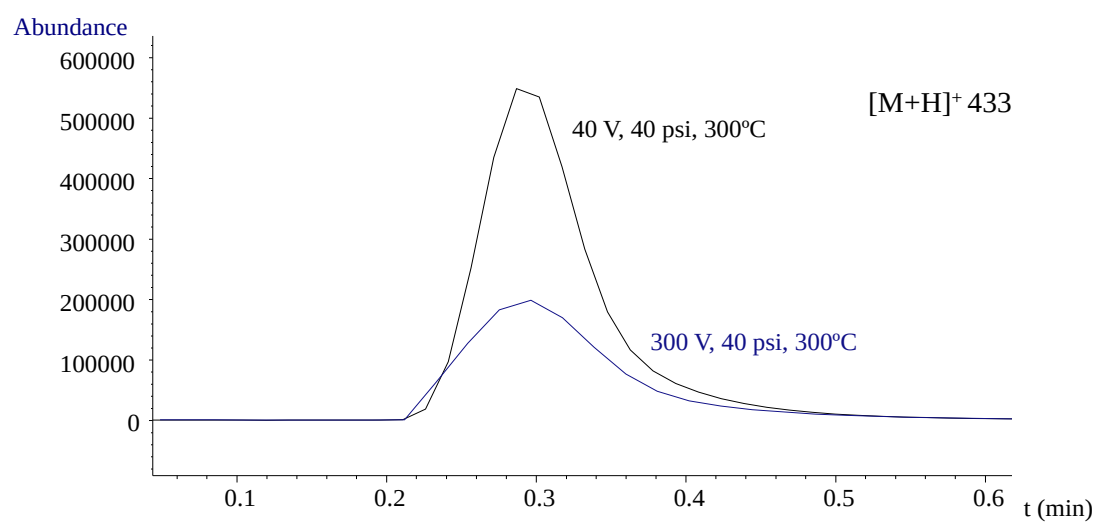


Figure S2. Effect of fragmentor voltages on the intensity of forskolin adducts response in A) positive and B) negative ionization modes.

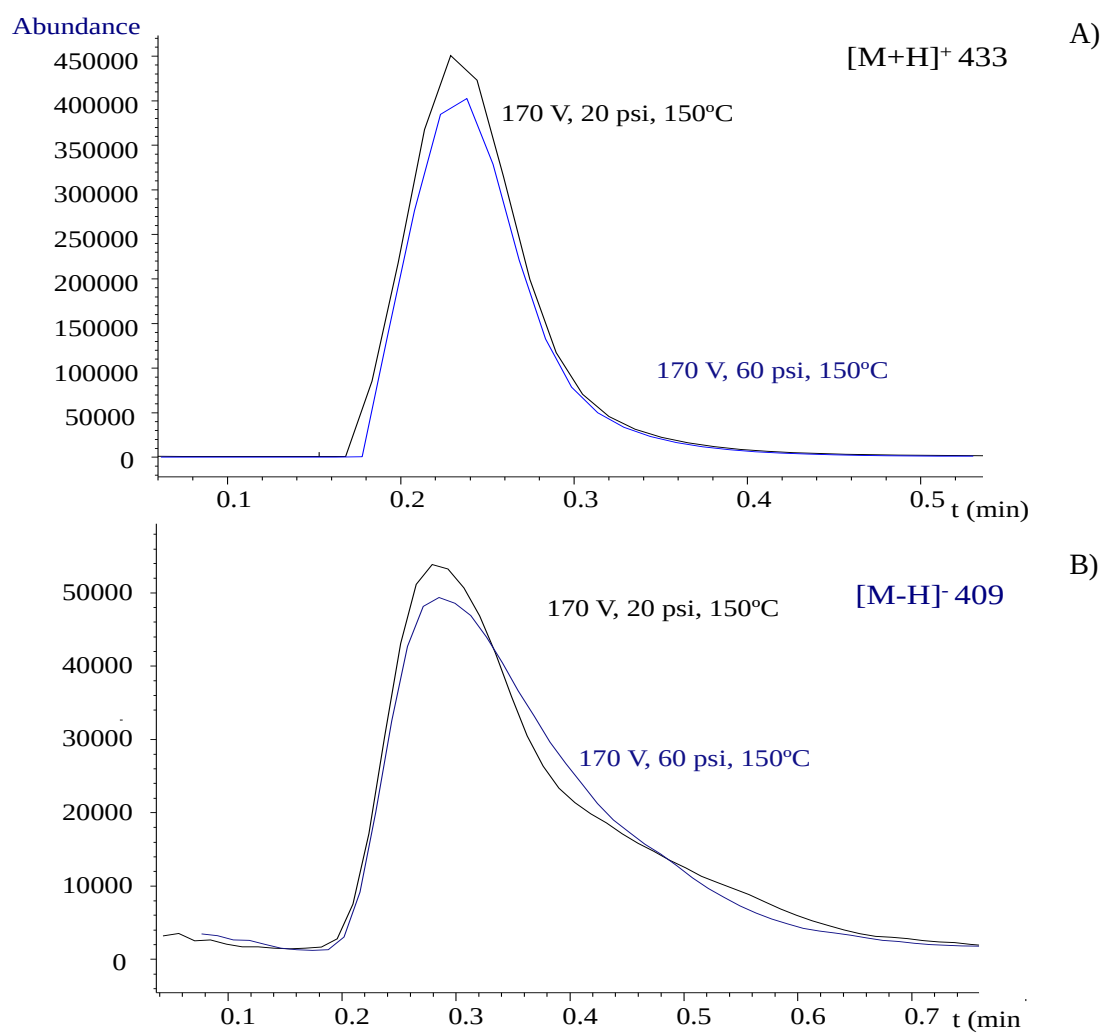


Figure S3. Effect of nebulizing pressure on the intensity of forskolin adducts response in A) positive and B) negative ionization modes.

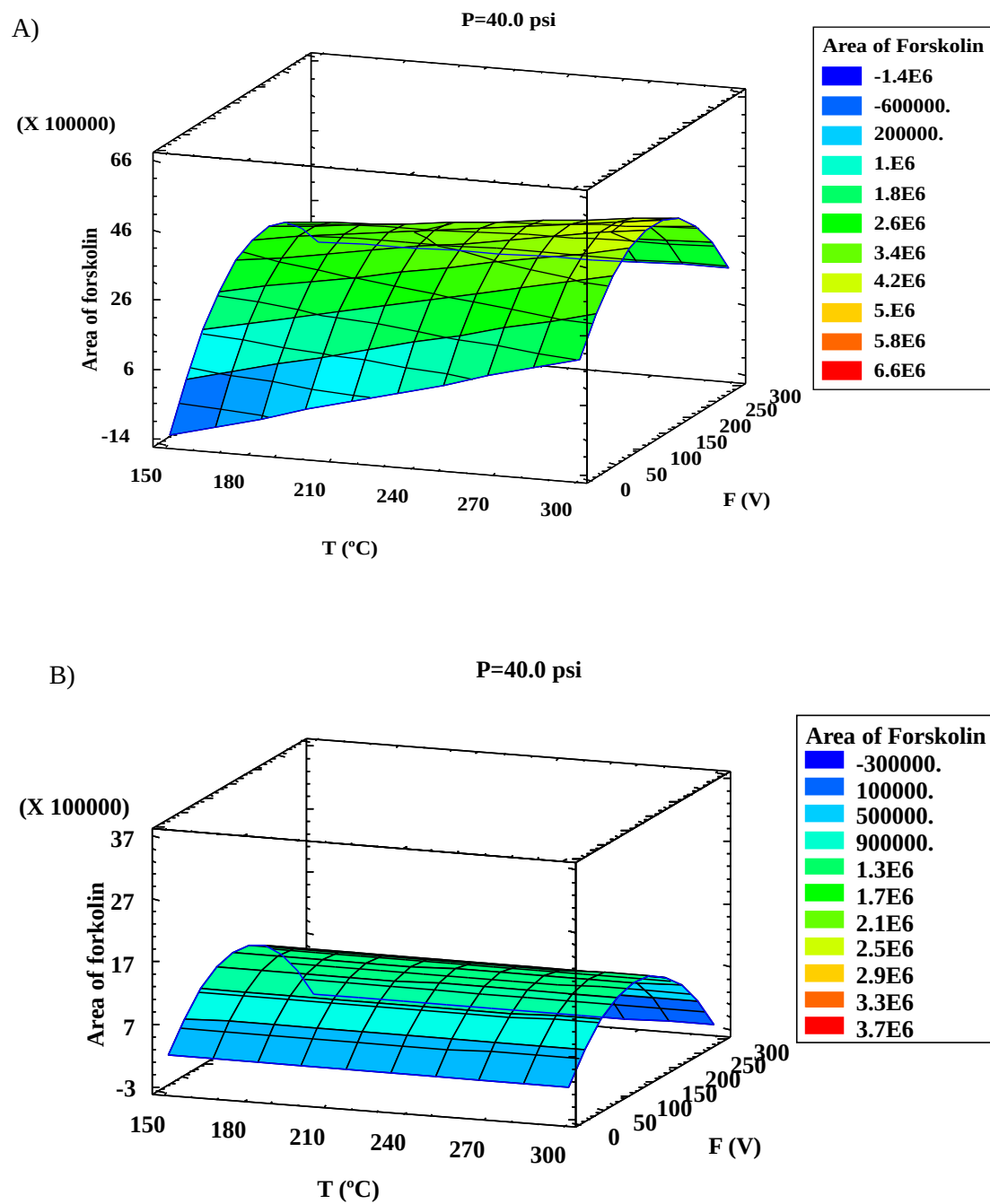


Figure S4. Three-dimensional response surface plot of quadratic regression equation for $R_{f(+)}$ model (A) and $R_{f(-)}$ model (B).

Anexo 3: Correspondiente a la sección 3.4.

A comparative study of LC-MS and FIA-(ESI)MS for quantitation of S-allyl-L-cysteine in aged garlic supplements

Supplementary material

Table S1. Composition of AGS under study.

Code	Formulation	Composition
AGS1	125 mg extract / capsule	Ingredients of ecological agriculture. ABG10+ [®] extract (<i>Allium sativum</i> L.), 0.1% SAC , maltodextrin, acacia fibers.
AGS2	120 mg extract /capsule	Aged black garlic extract (ratio 100:1), 1% SAC . Bulking agent (corn starch and cellulose), anti-caking agent (magnesium stearate, magnesium silicate and silicon dioxide).
AGS3	500 mg extract / capsule	Black garlic extract (82%) including 4% of flavonoids, 0.25% SAC , gelatin, anti-caking agents (silicon dioxide and rice extract), arabic gum and sunflower oil.
AGS4	300 mg extract / capsule	Aged garlic extract powder (bulb), whey, gelatine and magnesium stearate.
AGS5	600 mg extract / capsule	Black garlic extract 15:1 (bulb), rice starch.
AGS6	500 mg extract / capsule	Aged black garlic extract with dextrans (10:1) with 3% polyphenols, 0.1% SAC , anti-caking agent (silicon dioxide).
AGS7	250 mg extract / capsule	ABG10+ [®] extract (<i>Allium sativum</i> L. - maltodextrin), 0.1% SAC , 150 mg citric bioflavonoids (with maltodextrins), hesperidin (60%), 12 mg of vitamin E (100% VRN), 200 µg of folic acid (vit. B9 100%). Anti-caking agents: calcium phosphate, α-tocopherol acetate, etc.
AGS8	120 mg extract / capsule	Dry extract of aged black garlic (<i>Allium sativum</i> L., bulb), 1% SAC . Bulking agents: corn starch and cellulose. Anti-caking agents: magnesium stearate, magnesium silicate and silicon dioxide.
AGS9	600 mg extract / capsule	Aged garlic extract powder (bulb), gelatin, microcrystalline cellulose, magnesium stearate and silica.
AGS10	250 mg extract / capsule	Aqueous extract of fermented black garlic bulbs, gelatine 75 mg. Anti-caking agents: magnesium stearate 30 mg, talc 20 mg.
AGS11	250 mg black garlic / capsule	Powder of biological black garlic bulbs (50%), maltodextrin 100 mg. Anti-caking agents: talc 20 mg.
AGS12	450 mg black garlic / capsule	Fermented black garlic (<i>Allium sativum</i>), hydroxypropylmethylcellulose. 0.45 mg SAC (0.1%) , 10.75 mg polyphenols / capsule.
AGS13	187.5 mg extract /capsule	Black garlic extract 4:1 (<i>Allium sativum</i>), microcrystalline cellulose, silica and magnesium stearate.
AGS14	650 mg black garlic / capsule	Aged black garlic (<i>Allium sativum</i>) by enzymatic fermentation. Other ingredients: gelatin, rice bran, calcium palmitate, silica.

Table S1. Cont.

AGS15	250 mg extract / capsule	Black garlic extract (<i>Allium sativum</i>) ABG10+®, 0.1% SAC , maltodextrin, microcrystalline cellulose, silicon dioxide, magnesium stearate.
AGS16	250 mg extract / capsule	Dry extract of black garlic (ratio 10:1, 0.1% SAC , 3% polyphenols). Bulking agent: microcrystalline cellulose, magnesium stearate. Anti-caking agent: silicon dioxide.
AGS17	200 mg extract / capsule	Black garlic extract (<i>Allium sativum</i>) (ratio 10:1), rice powder.
AGS18	500 mg extract / tablet	Black garlic extract (50.2%) ABG10+®, >0.1% SAC . Bulking agents: arabic gum, rice flour. Anti-caking agent: lecithin, starch. Gelling agent: guar gum. Emulgent: glycerol.
AGS19	245 mg black garlic extract / capsule	Powder and extract of black garlic (<i>Allium sativum</i>) bulb, SAC (130 µg). Others: BIO apple (<i>Malus domestica</i>) fibers.
AGS20	245 mg black garlic extract / capsule	Powder (totum integral) and extract of black garlic (<i>Allium sativum</i>) bulb, SAC (130 µg). Others: BIO apple (<i>Malus domestica</i>) fibers.
AGS21	120 mg extract / capsule	Dry aged black garlic extract (<i>Allium sativum</i> L.) (ratio 100:1) and corn starch, 1% SAC . Anti-caking agents: magnesium salts of fatty acids, magnesium silicate and silicon dioxide.
AGS22	200 mg extract / capsule	Dry black garlic extract (<i>Allium sativum</i>) (ratio 10:1, 0.1% SAC) and maltodextrin.
AGS23	200 mg FBG22 TM / capsule	Fermented black garlic bulb (FBG22 TM , <i>Allium sativum</i>) (200 mg extract: 2 mg SAC; 1% SAC), ginger rhizome/root (fresh freeze dried) (100 mg <i>Zingiber officinale</i>) and vegetable capsule shell (hydroxypropyl methylcellulose).
AGS24	200 mg FBG22 TM / capsule	Fermented black garlic bulb (FBG22 TM , <i>Allium sativum</i>) (200 mg extract: 2 mg SAC; 1% SAC), ginger rhizome/root (fresh freeze dried) (100 mg <i>Zingiber officinale</i>) and vegetable capsule shell (hydroxypropyl methylcellulose).

Table S2. Box-Behnken experimental design for optimization of ESI parameters in the LC-MS and FIA-(ESI)MS analysis of SAC in AGS.

<i>T</i> (°C)	<i>FV</i> (V)	<i>P</i> (psi)	SAC Area	
			ESI ⁺	ESI ⁻
150	170	20	8825	26218
225	170	40	93448	21026
300	300	40	864	5728
150	40	40	52548	74059
225	170	40	81232	21094
300	170	20	48283	13411
150	300	40	267	6501
300	170	60	53645	20790
225	40	60	1108185	46440
225	300	20	745	3842
150	170	60	19631	30164
225	40	20	639815	18682
225	170	40	87881	19515
300	40	40	2003949	53716
225	300	60	889	5594

Anexo 4: Correspondiente a la sección 4.1.

Advances and challenges in the selective extraction of low molecular weight carbohydrates using ionic liquids and deep eutectic solvents

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Advances and challenges in the selective extraction of low molecular weight carbohydrates using ionic liquids and deep eutectic solvents

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ABSTRACT

Low molecular weight carbohydrates (LMWCs) are usually found in Nature as part of complex mixtures with proteins, lipids, amino acids, etc. or other carbohydrates with different degrees of polymerization, monomeric units or glycosidic bonds. Selective extraction or fractionation processes are then required to enable their analytical characterization and the measurement of their bioactivity. In these processes, the use of the extraction solvent plays a key role and although water is the most commonly used, poor selectivity is achieved. Recently, the use of novel, environmentally friendly and tunable solvents, such as ionic liquids (ILs) and deep eutectic solvents (DESs), has been explored for the selective solubilization and extraction of LMWCs. In this review, properties and parameters of ILs and DESs affecting LMWC solvation have been discussed, while recent applications of these solvents for the selective extraction of LMWC has been reviewed. Novel approaches based on the use of ILs as solution-out agents or on the combination of DESs with advanced extraction techniques, such as microwave-assisted extraction, to obtain faster and more efficient carbohydrate extraction processes are also reported.

1. Introduction

Carbohydrates are one of the most important molecules in Nature and play a key role in living organisms. They can present different degrees of polymerization (DP), i.e. monosaccharides (DP1), disaccharides (DP2), oligosaccharides (DP3–9) and polysaccharides (DP≥10), glycosidic linkages and monomeric composition [1]. Although the energetic and structural function are the main physiological properties of carbohydrates, some of these compounds also provide functional properties that promote the human health [2]. In this sense, some polysaccharides could act as dietary fibres, which have some beneficial activities related to the reduction of blood cholesterol levels, the prevention of gastrointestinal disorders, etc [3]. Also, some oligosaccharides have prebiotic properties and are selectively utilized by host microorganisms conferring a health benefit [4].

Low molecular weight carbohydrates (LMWCs) such as mono- and disaccharides, polyalcohols or iminosugars, also possess several bioactivities. For instance, lactulose promotes intestinal transit and modulates gut microbiota [5], and inositols are used to treat several pathologies such as polycystic ovary syndrome, metabolic syndrome and gestational diabetes [6]. Specifically, *myo*-inositol and *D-chiro*-inositol

present insulin mimetic-activity and are effective in lowering blood glucose levels [7]. Linear polyols such as mannitol can be used as a substitute for blood plasma in cerebral haemorrhage and as an osmotic diuretic in pathologies such as nephrotic syndrome or intracranial hypertension [8], while iminosugars have α -glucosidase inhibitory activity and they contribute to the regulation of body weight and the prevention of obesity [9].

LMWCs can be found directly in Nature or can be synthesized both chemically and enzymatically [10]. In both cases, they are usually part of complex mixtures with other carbohydrates of different structures or with other compounds such as lipids, proteins, etc. [11]. Therefore, a fractionation or selective extraction step is essential for their analytical characterization and also for their production as functional ingredients or for the evaluation of their activity. For this purpose, several methods have been proposed such as those based on chromatography, the use of membranes, solvents or microbiology. However, this step is not a straightforward task, mainly due to the similarity of carbohydrate structures and the relatively high abundances of the interferents in the complex mixtures.

The solvent is one of the most influential variables in the efficiency and selectivity of an extraction process. The selection of economical and

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environmentally friendly solvents is one of the main concerns of both researchers and food companies. Water is generally the best solvent for solubilising LMWCs, whereas, high molecular weight carbohydrates are insoluble in it. Although water is the most environmentally friendly and economical solvent, its low selectivity and the high energy treatments required to dry aqueous solutions make it ineffective for the selective extraction of carbohydrates [12].

On the other hand, carbohydrates are poorly soluble in organic solvents; some exceptions such as dimethylformamide and dimethyl sulfoxide have many undesirable properties [13] and they are not applicable in the food industry. Considering the different solubilities of carbohydrates in alcohols or hydroalcoholic mixtures, which lead to the selective precipitation of some of them, several applications have been developed for their fractionation based on the use of these solvents. For example, tagatose and lactulose, prebiotic disaccharides obtained by isomerization of the corresponding aldoses (galactose and lactose, respectively), can be separated from these non-bioactive aldoses using hydroalcoholic mixtures [14]. However, these methods often require large volumes of organic solvents and their applications are limited.

New alternatives for carbohydrate extraction based on the use of novel, more ecological and tunable solvents such as ionic liquids (ILs) and deep eutectic solvents (DESs) have recently been proposed. Recent studies based on the use of ILs and DESs for the extraction of polysaccharides such as cellulose [15–17], starch [18,19], agarose [20,21], chitin [22,23], etc., and for the valorisation of biomass have been recently reviewed [24]. In contrast, studies on the use of these solvents for the selective extraction of LMWC have been scarcely considered. Therefore, in this work, the properties of ILs and DESs affecting the extraction of these carbohydrates and their recent applications are described and discussed.

2. Ionic liquids

Room temperature ionic liquids (RTILs or ILs) are salts with melting

points below 100 °C, some even below 0°C, composed of organic and inorganic anions (chloride, bromide, trifluoroacetate, hexafluorophosphate, etc.) and organic cations (imidazolium, pyridinium, trimethylammonium, etc.) (Fig. 1). Taking into account their low volatility, ILs have been considered as environmentally friendly solvents and have been widely proposed as a green alternative to traditional volatile organic solvents to minimize their diffusion into the atmosphere [25]. Currently, the opinion on the toxicity of ILs is changing, as it is not possible to guarantee that they will never reach the environment and could pose a threat to aquatic and terrestrial ecosystems. However, given their tunable nature, they could be designed to be non-toxic, leading to increasingly environmentally friendly and more efficient ILs [2].

Many studies have been carried out on the use of ILs for the extraction of bioactive components from natural products, mainly volatile compounds, antioxidants, etc. [26–28]. Although the applications for the extraction of LMWCs are more limited, in recent years numerous studies have been developed to determine the solubility of these compounds in ILs, and to evaluate their usefulness for their selective extraction [29–31].

2.1. Properties of ILs affecting carbohydrate extraction

The unique physicochemical properties of ILs (Table 1) make them an advantageous alternative to conventional solvents. Apart from their negligible vapour pressure, one of the most advantageous characteristics of ILs is their highly tunable properties; ILs can be tailored to meet specific requirements. The possibility of combining different cations and anions increases the chances of achieving a selective extraction of carbohydrates. In this sense, some carbohydrates that are poorly soluble in certain ILs, may be highly soluble in others. As an example, *myo*-inositol shows high solubility values at 25°C (41.9 % w/w) in 1,3-dimethylimidazolium dimethyl phosphate ([MMIM][Me₂PO₄]), while its solubility in 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]) is

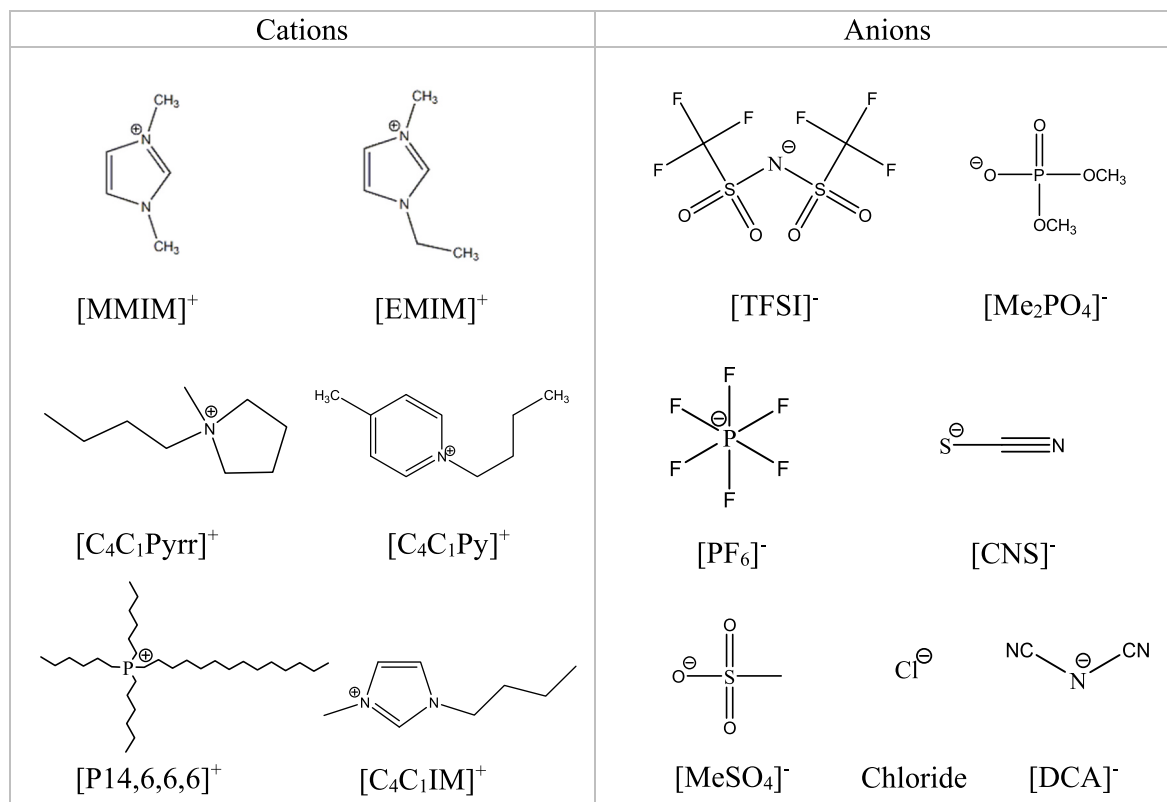


Fig. 1. Most common anions and cations of ILs used in LMWCs extraction.

Table 1

Differences and similarities between physicochemical properties of ILs and DESs.

		Ionic Liquids (ILs)	Deep Eutectic Solvent (DES)
Differences	Composition	An anion and a cation (salts)	HBA and HBD (salts, sugars, amino acids, etc.)
	Molecular bonds	Ionic bonds	Hydrogen bonds
	Conductivity	Moderate to poor	Lower than ILs
	Cytotoxicity	Positive for many	Toxicity profile accepted
	Preparation and cost of production	DES are easier to prepare and cheaper than ILs	
	Thermal stability	High	Relatively high
	Renewable	Non-biodegradable	Most are eco-friendly and biodegradable
Similarities	Melting point	Below arbitrary temperature, some are liquid at or below room temperature	
	Volatility	Low	
	Solvating ability	Both organic and inorganic compounds	
	Viscosity	Both polar and non-polar compounds	
	Tune	Linear correlation with temperature	

notably lower (1.7 % w/w) [31]. On the contrary, the solubility of mono- and disaccharides in the latter solvent is high (in the range of 28.1–55.0 % w/w), which could allow the selective fractionation of *myo*-inositol from mixtures containing different mono- and disaccharides.

Another important feature of ILs is their high thermal stability. It has been proven that most of the ILs do not decompose up to extremely high temperatures (300–400°C), however, this not imply long-term thermal stability at relatively high temperatures (e.g. an appreciable mass loss of 1-alkyl-3-methylimidazolium hexafluorophosphates is observed after 10 h at 200°C). Thermal stability has been associated to the nature of the anion and the cation that make up the IL. ILs with less nucleophilic or coordinating anions such as trifluoromethanesulfonic acid ([CF₃SO₃]) and bis(trifluoromethanesulfonyl)imide ([TFSI]) usually require high temperatures to decompose, whereas ILs with anions such as halides are stable up to lower temperatures. For ILs with the same anion, e.g. [TFSI], the thermal stability was different depending on the cation considered (e.g. [C₄C₁Pyrr] > [C₄C₁IM] > [C₄C₁Py]) [32].

The high viscosity of ILs is one of their main drawbacks for carbohydrate extraction, as it hinders sample preparation (filtration, decantation, etc.) and reduces the solubility rate [33]. The viscosity of ILs can range from 10 to 500 mPa s, which is notably higher than that of organic solvents (i.e. 0.3 and 0.6 mPa s for hexane and benzene, respectively) and of water (0.9 mPa s) at room temperature [29]. High carbohydrate solubilities have been found in low viscosity polar ILs [34], noting that viscosity is mainly influenced by the nature and size of the ILs cation, although temperature can also affect. Abbasi et al. [35] studied the viscosity changes of sorbitol dissolved in [MMIM][MeO₂PO₂] with temperature and at different concentrations of this polyalcohol. As shown in Fig. 2, the viscosity of the solution decreased with increasing temperature, although the rate of change was smoother at higher values, and increased with increasing sorbitol concentration.

One way of reducing the viscosity of the resulting carbohydrate-IL solution is the assistance of microwave irradiation [36,37], which increases the efficiency of dissolution compared to thermal heating. However, special care should be taken as decomposition and incomplete dissolution may occur [29]. Another possibility to overcome the negative effect of their high viscosity is to add suitable cosolvents to the IL (e.g. DMSO), which can reduce the viscosity and enhance the solubility of the carbohydrates at lower temperatures [38].

In terms of surface tension, it is generally higher for ILs than for conventional solvents (e.g. octane, 21.1 mJm⁻²), although not as high as for water (72 mJm⁻²) [39]. It is strongly influenced by the IL anion, as it

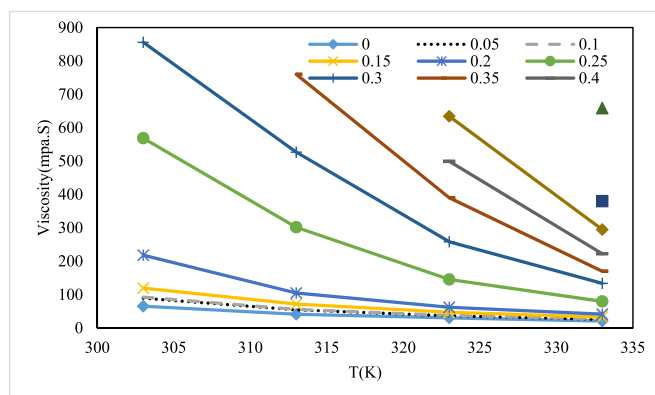


Fig. 2. Viscosity changes in carbohydrate-IL solution based on temperature in various solute concentration. (Reprinted with permission of Abbasi et al. [34]).

decreases with increasing anion size. Although the influence of the cation is small, it has been observed that the lengthening of the alkyl substituent in the cation decreases the surface tension [40]. The surface tension of ILs increase in the presence of carbohydrates [41].

ILs are miscible with other solvents. As an example, the miscibility of ILs with water has been found to be crucial to be used as separation media in extraction processes and it depends mainly on the anion of the IL, while the role of the cation is secondary [42].

The polarity of the ILs is another relevant feature, as it affects the solubility of the carbohydrates. It is higher for primary, secondary, and tertiary alkylammonium salts, and the addition of hydrogen-bond donor functional groups such as hydroxyl significantly increases it. On the contrary, when the alkyl-chain length or the number of alkyl chains of the cations is higher, a decrease in the polarity of ILs is observed [43], reducing the solubility of carbohydrates.

Other property of ILs is their recyclability; these solvents can be recovered after their use by different procedures (activated-charcoal treatments, antisolvent effect ...), which can contribute to promote their applicability [10,44]. The antisolvent method was proposed by Mohan et al. [45] for the separation of sugars (fructose and sucrose) and ILs ([Emim][EtSO₄]). Both experimental and theoretical studies (using the Conductor-like Screening Model for Real Solvents (COSMO-RS)) revealed that dichloromethane was the best antisolvent, obtaining 90–99 % removal of sugars and 80–98 % recovery of ILs.

2.2. Solubility of LMWC in ILs

Solubility studies of LMWCs (mainly mono- and disaccharides) in ILs have been extensive in recent years and have focused on improving knowledge of their solvating capacity and understanding the mechanisms of dissolution. Table 2 shows the solubility data of some LMWCs in different ILs. Among monosaccharides, the solubility of glucose and fructose has been the most studied, while for disaccharides it has been for sucrose and lactose [29,46–48].

ILs containing 1-methylimidazolium as cation are mainly used and are, in general, effective for the solubilization of sugars [12]. Good solubility of various sugars has also been observed in novel protic ILs containing imidazolium chloride as the cation [48]. However, some studies revealed that the cation interacts only weakly with carbohydrates and their solvation is the result of the established interactions between the sugar and the IL anion (e.g. glucose and chloride; [52]). On the contrary, Teles et al. [49] found that both, the IL anion and cation, play a significant role in the solubility of sugars; for example, glucose was more soluble in 1-butyl-3-methylimidazolium ([C₄C₁im])[DCA] (mole fraction solubility (x): 0.229 ± 0.0010) than in [C₄C₁im]dimethylphosphate ([C(CH₃)₂PO₄] (x: 0.132 ± 0.005), but also the size of the cation was inversely related to the solubility of the sugar; i.e. the solubility of glucose was higher in [C₄C₁im][DCA] than in

Table 2
Solubility data of some LMWCs in ILs.

Ionic Liquid	Target carbohydrate	Yield	Conditions	References
[MMIM] [Me ₂ PO ₄]	myo-inositol	419 mg	T (°C): 25	[31]
	chiro-inositol	g ⁻¹	Time (h): 24	
	Pinitol	566		
	Quebrachitol	639		
	Xylitol	181		
	Mannitol	482		
	Galactinol	494		
[EMIM][DCA]	Fructose	550 mg	T (°C): 25	[31]
	Glucose	g ⁻¹	Time (h): 24	
[BMIM][DCA]	Glucose	145 g L ⁻¹	T (°C): 25	[12,13]
	Sucrose	195	T (°C): 25	
	Lactose	282	T (°C): 60	
		225	T (°C): 75	
[MOEMIM][DCA]	Glucose	91 g L ⁻¹	T (°C): 25	[12]
	Sucrose	220	T (°C): 25	
[MOMMIM] [DCA]	Sucrose	249 g L ⁻¹	T (°C): 25	[12]
		352	T (°C): 60	
[C ₄ C ₁ im][DCA]	Glucose	0.229 (x ^a)	T (°C): 45	[49]
	Mannose	0.261	Time (h): 72	
	Galactose	0.221		
	Xylose	0.280		
	Arabinose	0.221		
	Fructose	0.201		
		0.132 (x)		
[(COCH ₃) ₂ PO ₄]	Glucose	0.121	T (°C): 45	[49]
	Mannose	0.220	Time (h): 72	
	Galactose	0.302		
	Xylose	0.182		
	Arabinose	0.056		
	Fructose	72 mg g ⁻¹		
	Sucrose			
[EMIM][SCN]	Sucrose	52 mg g ⁻¹	T (°C): 50	[38]
			Time (h): 48	
[BMIM][SCN]	Sucrose		T (°C): 50	[38]
			Time (h): 48	
[MMIM] [(MeO) ₂ PO ₂]	Sorbitol	305-570	T (°C): 30-	[35]
		mg g ⁻¹	60	
[(di-h) ₂ DMG] [DCA]	Glucose	2.88 (wt %)	T (°C): 35	[50]
			Time (h): 192	
[MMIM] [Me ₂ PO ₄]	Fructose	0.2-1 g g ⁻¹	-	[51]
[EMIM][EtSO ₄]	Glucose	0.90 g g ⁻¹	-	[51]
	Fructose	1.47		
[HMIM][Cl]	Fructose	20.2 w/w	T (°C): 26	[30]
	Glucose	9.3	Time (h): 48	
	Tagatose	29.5		
	Galactose	8.1		
	Lactulose	13		
	Lactose	3.3		
	Maltose	14.1		
	Maltotriose	32.1		
	Fructose	55 w/w	T (°C): 26	
	Glucose	28.1	Time (h): 48	
[EMIM][DCA]	Tagatose	38		[30]
	Galactose	8.5		
	Lactulose	44.9		
	Lactose	7.9		
	Maltose	39.4		
	Maltotriose	94.8		
[BMIM][MeSO ₄]	Fructose	13.2 w/w	T (°C): 26	[30]
	Glucose	10.3	Time (h): 48	
	Tagatose	8		
	Galactose	1.1		
	Lactulose	4.4		
	Lactose	8.2		
	Maltose	23.3		
	Maltotriose	92.3		

^a x mole fraction solubility.

trihexyltetradecylphosphonium ([P_{6,6,6,14}])[DCA] (x: 0.013 ± 0.001) and, therefore, the greater the bulkiness of the cation, the lower the solubility. Similarly, an increase in the solubility of sucrose was also observed when the chain length of the cation decreased (e.g. dissolution of sucrose was 7.2 wt % in [EMIM] thiocyanate ([SCN]) and 5.2 wt % in 1-butyl-3-methylimidazolium ([BMIM][SCN]) at 323.15 K) [38].

Among the different anions, ILs containing DCA, dissolved higher concentrations of mono-, di- and trisaccharides than other ILs (e.g. glucose >100 g L⁻¹, [33]; sucrose >200 g L⁻¹, [13]), due to its hydrogen bond acceptor properties. In fact, ILs containing this anion may be better solvents for LMWC than water [47,48,53]. Furthermore, some thermophysical properties of ILs, such as density and viscosity, could be influenced by the nature of the anions. In this regard, Mohan et al. [38] found that a decrease in the molecular weight of the anions was associated with a decrease in density and viscosity, which would also induce a higher mass transfer between LMWCs and ILs, with a consequent increase in solubility. On the other hand, some anions that could give good results in sugar solubility, such as chloride anion, might be considered inefficient and expensive due to the high viscosity of the resulting IL [29]. ILs containing saccharin, acetate and thiocyanate anions have also been proposed as alternatives to DCA for dissolving large amounts of carbohydrates [50].

In order to establish a criteria for the selection of the solvent, Mohan et al. [38] screened several ILs with different anions and cations, and concluded that those with a not too large cation and an anion that could act as a good hydrogen bond acceptor could improve carbohydrate solubility.

Both hydrophobic and hydrophilic ILs have been proposed to solubilize carbohydrates. Although the solubility of carbohydrate is lower in the hydrophobic ones [54,55], Rosatella et al. [50] found that these ILs were able to extract large amounts of glucose from an aqueous solution (e.g. tetrahexyldimethylguanidinium [(di-h)₂DMG][DCA] 2.70 (wt. %), [P_{6,6,6,14}][Cl] 3.64 (wt.%) ...) and, remarkably, some ILs such as [P_{6,6,6,14}][DCA] were able to extract larger amounts of glucose than the IL could dissolve (i.e. 4.23 % vs. 1.14 %). These authors attribute this behaviour to the possible creation of aqueous microenvironments formed by the water-solubilized glucose inside the IL. Teles et al. [49] compared the solubility of different monosaccharides in ILs composed of dialkylimidazolium (hydrophilic ILs) or tetra-alkylphosphonium (hydrophobic ILs) cations combined with DCA, dimethylphosphate or chloride anions. In general, xylose was the most soluble sugar and fructose the least soluble. However, discrepancies in the solubility data of some specific carbohydrates can be found in the literature, especially at high temperatures, probably due to the degradation of carbohydrates under these conditions [49,56].

Regarding the solutes, the chemical structure of LMWCs could have a direct influence on their solvation in ILs. The solubility of LMWCs is generally related to their melting properties, independent of the IL considered. In this sense, the lower the values of melting temperature and enthalpy of fusion are, the higher the solubility and *vice versa* [57, 58]. However, Teles et al. [49] found that the solubility of monosaccharides in an IL is not directly related to their melting properties, but is dominated by the interaction between the cohesive energy of the monosaccharides and their strong interactions (dispersive and hydrogen bonding) with the IL. Carrero-Carralero et al. [30], analysed the effect of the chemical structure of different types of sugars in ILs and they concluded that ketoses were more soluble, in general, than their corresponding aldoses, and trisaccharides were more soluble than mono- and disaccharides, although some exceptions were observed depending on the IL tested. Moreover, the solubility behavior of sugars in ILs was different from that of other organic solvents, since an increase in the solubility values of LMWCs was detected with increasing their molecular weight, contrary to what happens with some solvents, such as methanol [30]. The larger the sugar, the greater the number of -OH that could interact with hydrogen bonds, and thus, the greater the solubility [59]. However, other authors have observed contradictory results with

monosaccharides showing a higher solvation capacity than disaccharides [38]. In this sense, Teles et al. [49] found that the solubility of monosaccharides in ILs does not strictly increase with the number of hydroxyl groups in equatorial position, in contrast to what occurs for the solvation of monosaccharides in water.

In all cases, a direct relationship between temperature and solubility was found [57,60,61], i.e. an increase in the solubility of sugars in ILs was observed as temperature increased [49]. Several factors are related to this effect: (i) the viscosity of the ILs decreases with temperature, enhancing the diffusion of the ILs on the sugar and resulting into greater solvation [35]; (ii) most of the biphasic systems generated by LMWC and IL are endothermic, non-spontaneous and have positive entropy, which implies that energy input is required for solvation to occur. Despite this increase in solubility, LMWC, especially monosaccharides, can become susceptible to degradation in ILs at high temperatures [49]. It is therefore necessary to control the temperature limit of the assays to avoid the production of undesirable compounds [62]. However, this property has been deliberately considering for certain applications. In this sense, the catalyst power of some ILs (e.g. 1-butyl-3-methylimidazolium methyl sulfate ([BMIM][MeSO₄]), 1-hexyl-3-methyl-imidazolium chloride ([HMIM][Cl]), tri-cationic ILs, Brønsted–Lewis acidic ILs) can be used for the degradation of carbohydrates to produce 5-hydroxymethylfurfural (HMF), which it is a building block for a wide range of useful biochemical products [63]. This reaction usually occurs at high temperatures (318–373 K), and occasionally in the presence of other co-solvents such as DMSO, methanol, metal halides, etc. [30,64–66]. In this sense, ketoses (e.g. fructose, tagatose) are more prone than aldoses (e.g. glucose, galactose) to participate in non-enzymatic browning reactions, producing dehydration products such as HMF.

2.3. Selective extraction of LMWC using ILs

Although solubility studies of LMWCs in ILs have increased in recent

years, the use of these solvents for extraction of LMWCs from natural sources is still scarce. However, some studies have shown the potential of ILs compared to conventional solvents (e.g. pyridine and acetone) for the selective fractionation of carbohydrates. Al-Nashef et al. [51] patented the use of two ILs ([MMIM][Me₂PO₄] and [EMIM] ethyl sulphate ([EtSO₄])) for the efficient selective separation of fructose and glucose. While glucose was 2–6 times more soluble than fructose in [MMIM][Me₂PO₄], fructose was almost 1.5 times more soluble in [EMIM][EtSO₄]. The sequential use of these ILs allowed the successful extraction of both monosaccharides with a purity of 99 %.

Carrero-Carralero et al. [44] explored the usefulness of [HMIM][Cl], [EMIM][DCA] and [BMIM][MeSO₄] for the selective fractionation of bioactive ketoses (fructose, lactulose and tagatose) from their corresponding aldoses (glucose, lactose and galactose) at 299 K. Considering the promising results obtained with binary mixtures of standard sugars, [EMIM][DCA] was chosen for the selective extraction of lactulose from its synthesis product, which also contains lactose and monosaccharides. This treatment resulted in a remarkable enrichment of this ketose (from 24 % lactulose, 48 % lactose and 28 % monosaccharides in the reaction mixture to 58 % lactulose, 31 % lactose and 11 % monosaccharides after ILs treatment). Furthermore, different approaches to recover the carbohydrates dissolved in ILs were evaluated, such as cooling, solvent/antisolvent method and charcoal-based treatment, the last one showing the best results (only 10 % of the lost).

More recently, studies based on the selective separation of polyols from other LMWC using different ILs ([HMIM][Cl], [EMIM][DCA], [MMIM][Me₂PO₄], [EMIM][OAc]) have been carried out (Fig. 3) [31]. Different binary mixtures consisting of fructose:myo-inositol, maltose:pinitol, maltose:mannitol, glucose:mannitol were prepared and treated with ILs. In general, good results were obtained in all cases, highlighting the efficiency of [EMIM][OAc] and [MMIM][Me₂PO₄], which provided the highest solubility values of the polyols and also the highest capacity to achieve their selective separation from the corresponding sugars

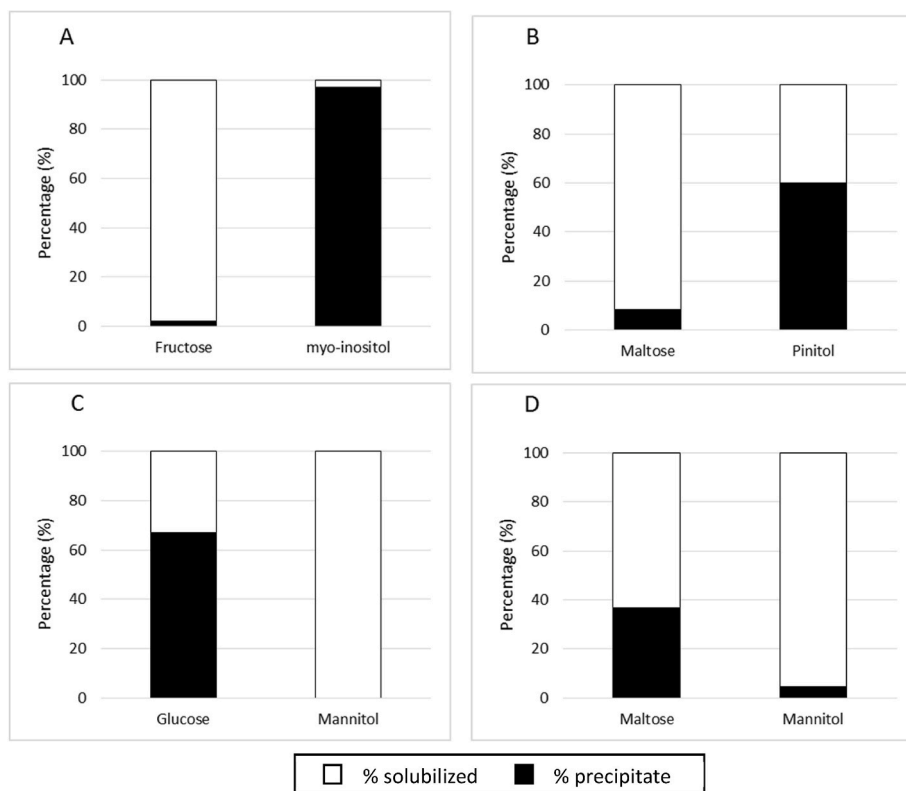


Fig. 3. Selective separation of inositols and sugars in binary mixtures (1:1, w/w) after treatment with ILs: [EMIM][DCA] for (A) and (B); [EMIM][OAc] for (C); and [MMIM][Me₂PO₄] for (D) at 25°C (Reprinted with permission of Ruiz-Aceituno et al. [30]).

(yields with a range of 60–98 %).

ILs have also been used as phase segregation agents in aqueous biphasic systems (ABSs) for the separation or purification of bio-products. In this sense, two ILs with different hydrophilicity (i.e. [BMIM][Br] and [BMIM] hydrogen sulphate ([HSO₄])) have recently been proposed as soluting-out agents for the precipitation of sugars (L-arabinose, D-xylose, D-glucose, D-fructose, maltose, sucrose and maltitol) from their aqueous solutions [67]. [BMIM][HSO₄] was the most hydrophilic IL and showed the strongest soluting-out effect, especially for sucrose, which could be useful for the purification and separation of these carbohydrates for biotechnological applications.

Similarly, ABSs composed of cholinium-derived bistriflimide ILs and carbohydrates (D-glucose or D-sucrose) allowed the solubilization and the simultaneous separation of carbohydrates and antioxidants from a commercial vanillin-rich pudding in one step [68]. Carbohydrates were mainly solubilized in the carbohydrate-rich upper phase of the ABS systems studied, while antioxidants were mainly enriched in the IL-rich phase, with extraction efficiencies of carbohydrates ranging between 89 and 92 % and antioxidant activities ranging between 65 and 75 %.

ILs have also been proposed as alternative solvents to more toxic ones (e.g. pyridine) for derivatization reactions of carbohydrates prior to their GC-MS analysis [69] and as background electrolyte additives for the separation of sugars by capillary electrophoresis [70].

3. Deep eutectic solvents

In general terms, an eutectic system represents a mixture of components whose specific proportions have the lowest melting point [71].

DESs are mixtures constituted by two or more compounds (all solids or at least one of them) acting as either hydrogen bond donors (HBD) or hydrogen bond acceptors (HBA) [72]. The HBA is often a quaternary ammonium salt such as choline chloride (ChCl), while the HBD comprises amines, carboxylic acids, alcohols, polyols, acid amides or carbohydrates [73]. Some examples of HBA and HBD used as components of DES used in carbohydrate extraction are shown in Fig. 4. When the compounds that constitute the DES are primary metabolites, such as organic acids, sugars, amino acids or choline derivatives, the DES are so called natural deep eutectic solvents (NADESs) [74–76].

NADESs have proven to be versatile solvents with many applications in analytical chemistry, in both, sample preparation and analytical techniques [77], and food and pharmaceutical fields, such as media for enzymatic or chemical reactions, preservatives of labile compounds, extraction of bioactive compounds from natural sources, etc [78]. Moreover, the use of compounds from natural sources reduce the cost of the production. Numerous reviews address the applicability of NADESs as a green alternative to conventional solvents for the solubilization of bioactive compounds, such as polyphenols and polysaccharides [79–81], however, the use of these solvents for LMWC extraction, although promising, so far has been less discussed [12].

3.1. Properties of DES affecting carbohydrate extraction

DESs share some of the suitable physicochemical properties of ILs relevant for carbohydrate extraction, such as low volatility, low melting point, high thermal stability, recyclability or high chemical diversity [82,83]. Table 1 summarises the differences and similarities between the

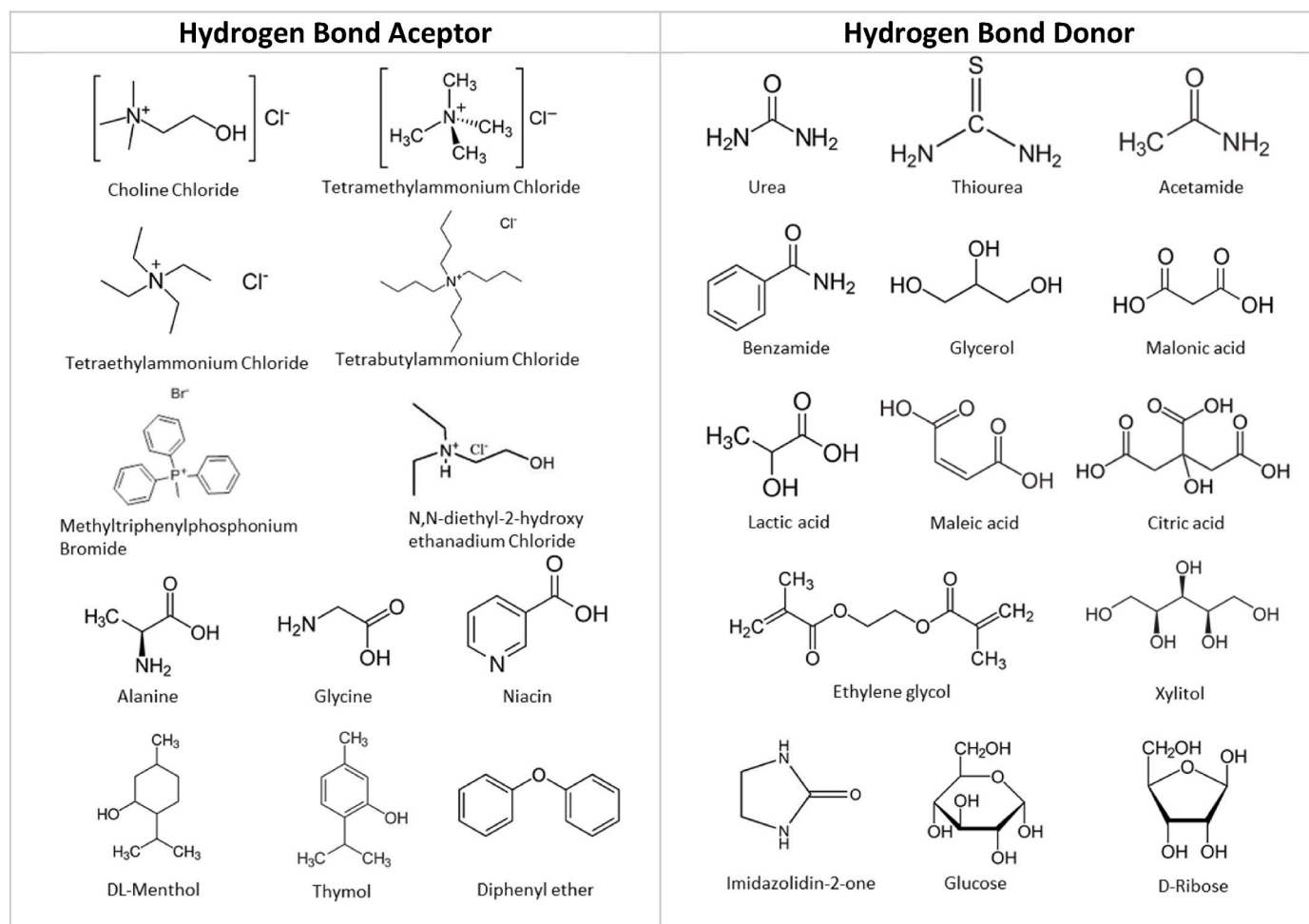


Fig. 4. Most common HBAs and HBDs used in DES preparation for LMWC extraction.

physicochemical properties of ILs and DESs. In general, NADESs are advantageous solvents due to their non-toxicity, biodegradability and lower price. In addition, they are readily metabolised by organisms and their associated environmental damage is reduced compared to organic solvents, and are therefore considered green solvents [84].

In general, the synthesis of DESs is usually simple and their purity is high. It consists of mixing HBD and HBA in a specific molar ratio and at a suitable temperature [85] using different methods, mainly based on stirring and heating with or without additional energy such as micro-waves or ultrasound, and by grinding or extrusion of the components [86] (Fig. 5). Small additions of water can help the synthesis, but it plays an important role in modifying different properties such as density, viscosity or melting point (decreasing all of them) with the consequent disorganisation of the structure and stability of the DESs.

An inherent property of these solvents is that the melting point of the mixture is lower than that of the individual components due to charge delocalisation caused by hydrogen bonding [87]. The melting point of DESs is also influenced by the molar ratio of HBA and HBD components [83]. As the DES components are linked by strong hydrogen bonds, solutes that can form strong molecular interactions with them, such as carbohydrates, are expected to have good solubility in such DESs [88].

DESs can also be either hydrophilic or hydrophobic depending on the nature of their constituents. Although most studies have focused on hydrophilic DESs and are the most suitable solvents for carbohydrate extraction, hydrophobic DESs (HDESs) have recently been shown to have good solvation capabilities for organic and inorganic compounds and have been used for the purification of sugars [89].

The density of the DESs is another property that plays an important role in the solubility of carbohydrates, which is directly related to the nature of the DESs and affects the extraction efficiency. Compared to water, the densities of hydrophilic DESs are higher, while those of

hydrophobic DESs are similar or lower [90–92]. In addition, temperature is inversely related to density; an increase in temperature weakens the component interactions, increasing the matrix hole size and consequently, decreasing the density of DESs.

The viscosity of DESs is an important parameter related to solubility and associated to the strong hydrogen bonding interactions produced that reduce the mobility of free species in the solvent [86]. High viscosities can decrease the solubility of sugars due to a reduction of solvent diffusion [93]. Shekaari et al. [63] evaluated the viscosity of aqueous DES solutions of glucose. As expected, the experimental viscosity of these solutions increased with increasing DES ratios, but also with the number of functional groups and the length of alkyl groups in the DES components, leading to stronger interactions between the compounds, probably due to the establishment of higher hydrogen bonds. DESs exhibit similar viscosities to ILs, and higher than most molecular solvents, which limit their application [92]. Solubility of different sugars (glucose, sucrose, cellobiose and erythritol) in a low viscous DES (i.e. [ChCl][ethylene glycol]) was higher in molar terms than in water [94]. In addition, the lower solubility of glucose observed in some ILs (i.e. [TMA][MeSO₄], [P_{6,6,6,14}][DCA], and [P_{6,6,6,14}][Cl]) than in DES (i.e. tetrabutylammonium bromide ([TBAB])[imidazolium], [TBAB][ethylene glycol] and [TBAB][glycerol]) (Fig. 6) was attributed to the higher viscosity of ILs [95]. As expected, the viscosity of the DESs is inversely related to temperature, so by varying the temperature the viscosity of the DESs can be modulated [87] and the carbohydrate solubility can be modified [95].

Ionic conductivity is another property of DESs that is related to viscosity and is controlled by ion mobility and the availability of voids of suitable dimensions [85]. Moreover, conductivity is also influenced by temperature (main factor of conductivity in an ionic solution) and water content. The low percentages of water content, which is involved in the

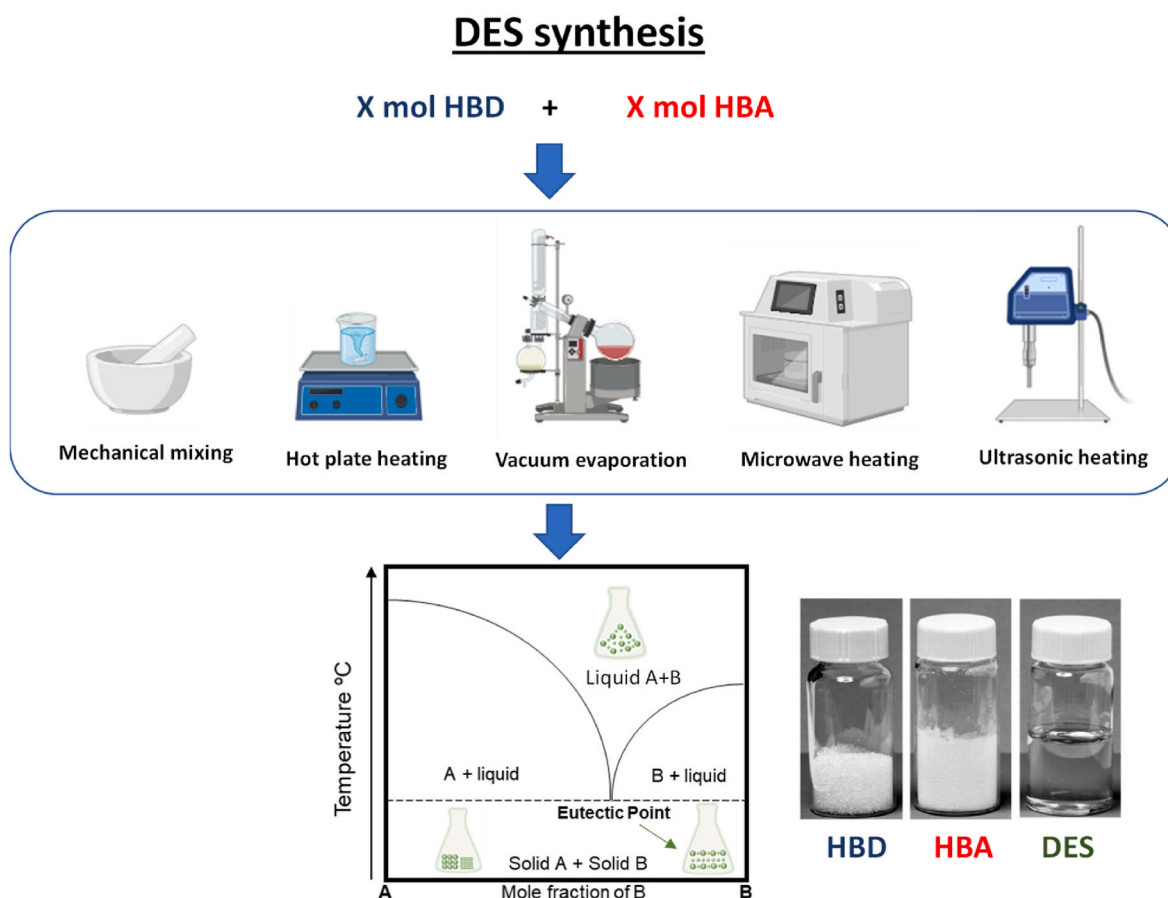


Fig. 5. Schematic representation of DES synthesis and the phase diagram of a two-component DES.

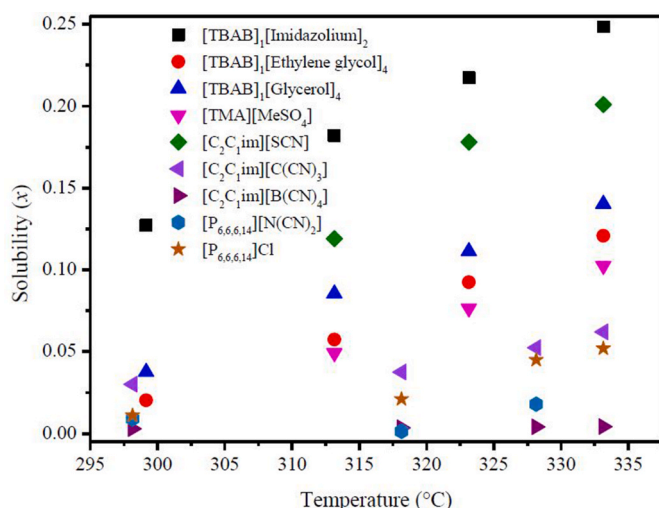


Fig. 6. Comparison between the experimental solubility (x) of glucose in some DESs ([TBAB][imidazolium], [TBAB][ethylene glycol] and [TBAB][glycerol]) and ILs ([TMA][MeSO₄], [C₂C₁im][SCN], [C₂C₁im][C(CN)₃], [C₂C₁im][B(CN)₄], [P_{6,6,6,14}][DCA], [P_{6,6,6,14}][Cl]) and effect of temperature (Reprinted with permission of Mohan et al. [95]).

formation of the DESs matrix, have important effects in reducing the viscosity and increasing the conductivity of the solvent. However, as the water content increases, so does the resistivity. Thus, the correlation between water and conductivity follows a nearly Gaussian shape [75].

In general, the polarity of DESs is similar to or higher than that of conventional organic solvents and is directly dependent on the DES components, in particular the HBD. This parameter is affected by temperature and the percentage of water in the solvent structure, decreasing with increasing temperature and decreasing percentage of water [76].

The thermal stability of DESs is a critical parameter which limits their applications. Although in general, high thermal stability has been found for these solvents (around 150–180°C), some DESs can become unstable at certain temperatures and separate into their HBD and HBA (e.g. [TBAB]-ethylene glycol decomposes at $T > 50^\circ\text{C}$) [95]. The thermal stability of DESs is highly dependent on the stability of their constituents (e.g. the stability of ChCl: ethylene glycol and ChCl: malonic acid is lower than 115°C due to the high volatility of ethylene glycol and the thermal decomposition of malonic acid) [96]. Moreover, the thermal stability of DESs was found to decrease when sugars were added [97].

Similar to ILs, recyclability is another interesting property of DES. Different strategies such as membrane filtration, solid-liquid and liquid-liquid extraction, anti-solvent, supercritical fluid extraction, etc. can be followed; the advantages and disadvantages of these processes have recently been revised by Isci and Kaltschmitt [98].

3.2. Solubility of LMWC in DES

Most recent studies on carbohydrate solubility in DES have focused on polysaccharides, demonstrating the higher extraction capacity of these solvents compared to water [99–104]. In the last decade, however, the behaviour of LMWCs in these solvents has also attracted considerable interest and solubility data of different carbohydrates in DESs has been reported (Table 3).

As expected, hydrophilic DES were found to be the most efficient in the solvation of sugars, with those based on ChCl being the most widely used, mainly due to their low cost, non-toxicity and biodegradability [110]. Five ChCl-based DES with urea (reline), oxalic acid (oxaline), ethylene glycol (ethaline), glycerol (glyceline), and 1,5-pentanediol (pentaline) as HBDs, were evaluated to dissolve different LMWC (glucose, sucrose, erythritol, cellobiose), starch and cellulose [94]. Good solubilities of these LMWC in DESs were reported, mainly in reline and

ethaline (up to 275 mg mL⁻¹), although values were lower than in water (up to 2000 mg mL⁻¹) but comparable, and in some cases, higher than those reported in ILs. Shekaari et al. [63] found that glucose-DES interactions were stronger in choline-based DESs than in the dimethylammonium (Dmac)-based DES. This effect could be due to the hydroxyl group of the choline cation, which could interact with LMWC via hydrogen bonding. Another possible reason for the stronger interaction is the presence of methyl groups in choline chloride, which can cause steric hindrance and interactions.

Mohan et al. [95] compared the solubility of glucose in three [TBAB]-based DES and in different ILs and organic solvents. Although glucose was highly soluble in [C₂C₁im][SCN], in general, the solubility in DES (mainly in [TBAB]-imidazolium) was higher (Fig. 6). These authors found, through molecular dynamic simulations, that the anion of HBA and the HBD structure are the parameters that most influence glucose solubility. In this sense, the lower solubility of this sugar in [TBAB]-ethylene glycol and [TBAB]-glycerol could be due to the strong interaction between HBA and HBD. For these two DES, the higher solubility of glucose in [TBAB]-glycerol could be attributed to the additional hydroxyl group of this HBD, as it allows more hydrogen bonds to be formed with the sugar.

The structure of the solute also influences its solubility. In general, the solubility of sugars decreases with increasing carbon number (polysaccharides are less soluble than disaccharides and disaccharides are less soluble than monosaccharides [95]). However, the glycosidic linkages or the monosaccharide units also have a relevant effect, as sugars with the same number of carbons and hydroxyl groups have different solubilities. In this sense, sucrose was 10 times more soluble than cellobiose in the different ChCl based-DES. This behavior was attributed to the more rigid structure of cellobiose, which restricts bond rotation and limits its solubility. The solubilities of several mono- and disaccharides in ChCl-based DES (ethaline and glyceline) were also been determined, generally showing higher solubilities of ketoses than those of their corresponding aldoses [109].

A good correlation was found between the carbohydrate solubility and the enthalpy of hydrogen bond formation. DES with the highest enthalpy give rise to the best solubility of sugars (e.g. oxaline highly dissolved erythritol as compared to reline, glyceline, pentaline and ethaline [94]).

As expected, the solubility of sugars in DESs generally increases with temperature [97]. However, it should be noted that some DESs can be separated into their respective HBD and HBA at medium/high temperatures, precluding their use as solvents under such conditions (e.g. the HBD in [TBAB]-ethylene glycol became unstable at temperatures above 50°C and were separated out from the DES phase; Mohan et al. [95]).

Moreover, similar to ILs, some DESs catalyse carbohydrate degradation reactions producing HMF (e.g. browning reactions were observed in solutions of glucose and sucrose using acidic DESs such as oxaline), which are favoured at high temperatures (e.g. a browning effect of carbohydrates dissolved in reline was observed at 50°C but not at 25°C, Hakkinen and Abbot [94]). This effect was more drastic for ketoses than for aldoses [109]. Additionally, an isomerization effect of sugars dissolved in DESs has been observed. Mohan et al. [95] detected the isomerization of glucose to fructose at temperatures higher than 50°C in [TBAB]-imidazole without any additional catalyst. The solubility of glucose in this DES was higher probably due to the high interactions between glucose molecule and the bromide anion of HBA, allowing its isomerization to fructose.

3.3. Selective extraction of LMWC using DESs

Although DES tunability is one of the most relevant advantages of these solvents, the infinite number of combinations of their constituents and compositions make the selection of the most appropriate DES to solubilize a specific sugar a non-affordable experimental approach. Then, molecular based modelling and simulation tools such as COSMO-

Table 3

Solubility data of some LMWCs in DESs.

Deep Eutectic Solvent	Natural source	Target carbohydrate	Yield	Conditions	References
Urea: ChCl 2:1 +CuCl ₂	Oil palm fronds	Xylose	14.76 g L ⁻¹	T (°C): 120 Time (h): 4.30	[105]
ChCl: Glycolic acid: oxalic acid 1:1.7:0.3	Olive alperujo	Uronic acids	27 mg g ⁻¹	T(°C): 120	[106]
ChCl: Glycolic acid: Oxalic acid 1:1.7:0.3	Red berries extrudates	Sugars	130	Time (h): 2	[107]
ChCl: Glycolic acid: Oxalic acid 1:1.7:0.3	Red berries extrudates	Acid sugars	1.88 mg g ⁻¹	T(°C): 25	[107]
ChCl: Oxalic acid 1:1	Poplar wood	Sugars (Glucose eq)	82.23	Time (min): 15	[108]
ChCl: Oxalic acid 1:1	Poplar wood	Glucose	22.3 (wt %)	T(°C): 80-100	[108]
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Xylose	34.8	Time (h): 9	[94]
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Glucose	162 mg mL ⁻¹	T(°C): 25	[94]
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Erythritol	230	Time (d): 7	[94]
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Sucrose	299		
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Cellobiose	19		
Oxalic acid dihydrate: ChCl (Oxaline) 1:1	-	Starch	0.3		
Urea: ChCl (Reline) 2:1	-	Glucose	143 mg mL ⁻¹	T(°C): 25	[94]
Urea: ChCl (Reline) 2:1	-	Erythritol	208	Time (d): 7	[94]
Urea: ChCl (Reline) 2:1	-	Sucrose	182		
Urea: ChCl (Reline) 2:1	-	Cellobiose	17		
Urea: ChCl (Reline) 2:1	-	Starch	0.1		
Glycerol: ChCl (Glyceline) 2:1	-	Glucose	240 mg mL ⁻¹	T(°C): 25	[94]
Glycerol: ChCl (Glyceline) 2:1	-	Erythritol	96	Time (d): 7	[94]
Glycerol: ChCl (Glyceline) 2:1	-	Sucrose	142		
Glycerol: ChCl (Glyceline) 2:1	-	Cellobiose	10		
Glycerol: ChCl (Glyceline) 2:1	-	Starch	0.1		
Ethylene Glycol: ChCl (Ethaline) 2:1	-	Glucose	249 mg mL ⁻¹	T(°C): 25	[94]
Ethylene Glycol: ChCl (Ethaline) 2:1	-	Erythritol	113	Time (h): 48	[94]
Ethylene Glycol: ChCl (Ethaline) 2:1	-	Sucrose	275		
Ethylene Glycol: ChCl (Ethaline) 2:1	-	Cellobiose	19		
Ethylene Glycol: ChCl (Ethaline) 2:1	-	Starch	0.04		
1,5-pentanediol: ChCl (Pentaline) 3.5:1	-	Glucose	68 mg mL ⁻¹	T(°C): 25	[94]
1,5-pentanediol: ChCl (Pentaline) 3.5:1	-	Erythritol	28	Time (h): 48	[94]
1,5-pentanediol: ChCl (Pentaline) 3.5:1	-	Sucrose	41		
1,5-pentanediol: ChCl (Pentaline) 3.5:1	-	Cellobiose	3.1		
1,5-pentanediol: ChCl (Pentaline) 3.5:1	-	Starch	0.1		
Ethylene Glycol: ChCl 2:1	-	Glucose	242 mg g ⁻¹	T(°C): 25	[109]
Ethylene Glycol: ChCl 2:1	-	Galactose	31.4	Time (h): 48	[109]
Ethylene Glycol: ChCl 2:1	-	Fructose	191		
Ethylene Glycol: ChCl 2:1	-	Tagatose	72		
Ethylene Glycol: ChCl 2:1	-	Lactose	147		
Ethylene Glycol: ChCl 2:1	-	Maltose	323		
Ethylene Glycol: ChCl 2:1	-	Trehalose	183		
Ethylene Glycol: ChCl 2:1	-	Sucrose	152		
Ethylene Glycol: ChCl 2:1	-	Lactulose	666		
Glycerol: ChCl 2:1	-	Glucose	136/364 mg g ⁻¹	T(°C): 25/45	[109]
Glycerol: ChCl 2:1	-	Galactose	43/71	Time (h): 48	[109]
Glycerol: ChCl 2:1	-	Fructose	245/806		
Glycerol: ChCl 2:1	-	Tagatose	150/214		
Glycerol: ChCl 2:1	-	Lactose	26.3/92		
Glycerol: ChCl 2:1	-	Maltose	280/630		
Glycerol: ChCl 2:1	-	Trehalose	252/503		
Glycerol: ChCl 2:1	-	Sucrose	82/368		
Glycerol: ChCl 2:1	-	Lactulose	135/250		

RS are currently used to theoretically select the DES constituents that are effective to each application. This software has been recently applied to the extraction of glucose and fructose [111]. Among the 212 DESs evaluated, those constituted by carnitine, betaine, and choline chloride as HBA and oxalic acid, ethanolamine, and citric acid as HBD were the most promising.

Hydrophilic DESs have been proposed as efficient solvents for the extraction of different sugars from plants and plant by-products. In this context, Loow et al. [105] successfully extracted xylose (14.76 g xylose L⁻¹) from oil palm fronds by combining the use of a DES (ChCl:urea 1:2) and a CuCl₂ treatment. Fernández-Prior et al. [106] achieved good yields of sugars (130 mg g⁻¹) and uronic acids (27 mg g⁻¹) from olive alperujo using a DES composed of ChCl:glycolic acid:oxalic acid 1:1.7:0.3. The same DES was also used by Vazquez-Gonzalez et al. [107] to extract bioactive compounds from red berries extrudates at room temperature (25°C). Higher extraction yields of free sugars (82.23 mg g⁻¹ fresh weight) and uronic acids (1.88 mg g⁻¹ fresh weight) from strawberries were obtained with this DES than with acetone (41.85 mg sugars g⁻¹ fresh weight and 0.67 mg uronic acids g⁻¹ fresh weight).

Overall, DESs proved to be promising solvents for the extraction of LMCW from plant by-products, contributing to their revalorization.

In order to achieve faster and more efficient carbohydrate extraction processes, the combination of DESs with advanced extraction techniques, such as microwave-assisted extraction (MAE), has recently been explored. Liu et al. [108] investigated the extraction of lignin from poplar wood using ChCl:oxalic acid 1:1 in combination with MAE. In this study, they found that this DES not only removed lignin from the matrix, but was also efficient in saccharide extraction, producing an extract with an interesting glucose (22.3 % wt) and xylose (34.8 % wt) content. However, they observed that under MAE conditions (80 °C; 800 W microwave irradiation; 3 min), some degradation of glucose to HMF occurred. In a more recent study, Gómez et al. [112] evaluated the extraction of soluble sugars from banana puree, selecting four DES (citric acid (CA): beta-alanine (BA), CA:ChCl, malic acid (MA):BA, MA: ChCl) as the most suitable solvents in combination with MAE. Under optimal conditions of temperature, time, and water content, the four DES were more effective than conventional solvents (i.e. water (71.5 g 100 g⁻¹) and ethanol (79.7 g 100 g⁻¹)) in the extraction of simple

sugars; the best results were obtained with MA:BA at 25°C, 30 min, with 30 g 100 g⁻¹ of water (106.4 g sugars 100 g⁻¹).

DESs, mainly HDESs, are useful alternatives to conventional organic solvents for the separation of carbohydrates from other matrix components. HDESs have shown interesting solubility properties for the selective separation of HMF and furfural (FF) from glucose and fructose in aqueous solutions [97]. The monosaccharides showed the lowest solubilities in decanoic acid:tetraoctylammonium bromide (2:1), while their dehydration products were highly soluble. Thus, this HDES is a promising extractant that could be successfully used in industrial applications such as biorefineries.

Gonzalez et al. [89] evaluated the solubility of phenol, guaiacol, syringol and glucose in HDESs based on menthol and three organic acids (octanoic acid, decanoic acid and dodecanoic acid) with the aim of testing the ability of these solvents to remove the phenolic compounds from a glucose solution that could be subsequently subjected to a fermentation process. After optimizing the solubilization conditions (stirring and centrifugation times, solvent/feed ratio, initial concentration of phenolic compounds ...), a selective separation of phenolics from the sugar mixture solution was successfully achieved using menthol:dodecanoic acid (2:1).

The solubilization of mannose and manno-oligosaccharides from supercritical CO₂ defatted date seeds in ChCl: formic acid (1:2 molar ratio) using a MAE method optimized for polyphenol extraction has been evaluated [113]. It was observed that, during extraction, the total concentration of these carbohydrates released into the solvent increased linearly with respect to the microwave energy applied per gram of date seeds.

Recently the combination of DES and ultrasound assisted extraction (UAE) has been proposed to extract glucose, fructose and sucrose from dates [111]. After using COSMO-RS, 5 NADES based in ChCl as HBA and citric acid, oxalic acid, gallic acid.H₂O, ethanolamine and triethylene glycol as HBD were selected. Among the synthesized NADES, the mixture of ChCl, citric acid (CA) and water (1:1:1 with 20 wt% water) resulted in the highest sugar yield (78.30 ± 3.91 g/100 g), which was even higher than that obtained with only water (29.92 ± 1.50 g/100 g). Response surface methodology and artificial neural network were also used to select optimal conditions (30 °C, 45 min, and 40 mL solvent/g dried sample), improving sugar recovery up to 87.81 ± 2.61 g/100 g. This method also allowed the preservation of the heat-sensitive bioactive compounds in dates.

4. Conclusions and future trends

The growing awareness of the environmental impact of conventional extraction processes used in both analytical chemistry and industrial applications has prompted the development of more sustainable and safer alternatives for humans using more efficient and environmentally friendly solvents, such as ILs and DESs, especially when natural sources are incorporated into their design (as for NADESs). These solvents have proven to be promising substitutes of conventional volatile organic solvents such as alcohols, dimethylformamide, dimethyl sulfoxide, etc. for the efficient solubilization of LMWCs, with a higher selectivity capacity than water. However, their overall ecological impact should be considered with caution, as it depends on several factors, including how they are produced, their specific components, their disposal methods, the volume used, etc. Their ecological benefits do not always outweigh those of conventional, and further studies are needed to better understand their safety.

One of the most significant advantages of ILs and DES is that their tunable properties can allow the search for new combinations, essential to increase the selectivity required in analytical methods. Such processes may require the design and development of purification and separation procedures based on these new solvents to achieve high enrichment levels, allowing the use of a specific technique and avoiding unwanted coelution with non-target compounds. However, although several

studies have been developed on the solubilization of LMWCs in ILs and DESs, most of them have been carried out with commercial standards or purified target compounds and those based on real matrices are scarce. Determining the actual usefulness of these solvents remains a challenging field to address and much research remains to be done.

In recent years, investigation has focused on the use of hydrophilic ILs, such as those based on cholinium cations and anions derived from carboxylic acids or amino acids, as fewer toxic alternatives to those based on imidazolium cations and fluorinated anions. Their improved biocompatibility and their high chemical structural diversity, which allows them to be more specifically targeted, make them very attractive for greener applications. In addition, the use of aqueous IL solutions has proven to be a particularly good strategy for reducing viscosity while increasing solvent biocompatibility. Their higher water content results in a lower solvent cost and therefore, a reduction in the cost of the overall extraction process. Therefore, further investigations to extend their application to the extraction of LMWCs from different types of matrices are of increasing interest.

On the other hand, the potential of using DESs and especially NADESs in the field of LMWCs extraction is also gaining more attention due to their high biodegradability, low cost and easy preparation. However, a better understanding of the relationship between their physicochemical properties and their extraction behaviour is crucial to improve the design of NADESs and their applications in complex systems, which has hardly been addressed and needs further investigation. The development of new NADESs composed not only of binary systems but also of three or more components could be a potential solution for more selective and efficient fractionation or extraction of LMWCs from more complex systems.

The great potential of individual, combined or sequential use of enhanced extraction techniques (such as MAE, pressurized liquid extraction, ultrasound-assisted extraction, enzymatic-assisted extraction, etc.) with ILs or DESs to achieve more efficient extractions of LMWCs, clearly emerges as a future line of development. However, special attention should be paid to the high temperatures required in some of these techniques to enhance the extraction of LMWCs, which may lead to undesirable side reactions and degradation of the DES. Improving the enhancement of the temperature stability of these solvents is another factor to be considered.

Moreover, further research is also needed to improve the existing methodologies for the efficient separation of target LMWCs and the recycling of the solvents so that IL and DES-based separation processes can be implemented in industrial applications, as current methodologies are complex and often require the use of multiple cycles, which implies a decrease in the overall efficiency of the process.

Due to the many possible combinations to design ILs and DESs, *in silico* methods (such as those based on the COSMO-RS) which are capable of predicting thermodynamic fluid properties, have emerged as powerful tools for selecting the most suitable solvents for a given application. This avoids the extensive use of experimental data needed to screen new solvents and applications where limited information is available, such as in the case of LMWCs, saving time and resources.

Finally, the use of these more sustainable solvents is only the beginning of an overall green extraction process. Solvent volumes, energy requirements, miniaturization, automation, etc., are some of the numerous criteria that should be also considered. To this end, several metric systems that are now available (e.g. AGREEprep) would be very useful to improve LMWC green extraction.

CRedit authorship contribution statement

I. Jiménez-Amezcu: Investigation, Writing – original draft, Writing – review & editing. **M.I. López-Martínez:** Investigation, Writing – original draft. **A.I. Ruiz-Matute:** Conceptualization, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing. **M.L. Sanz:** Conceptualization, Funding

acquisition, Investigation, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: I. Jimenez reports financial support was provided by Comunidad de Madrid.

Data availability

No data was used for the research described in the article.

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Anexo 5: Correspondiente a la sección 4.2.

Changes in the bioactive content and aroma profile of aged garlic with processing conditions

Supplementary material

Table S1. Volatiles identified by the optimized SPME-GC-MS approach in the garlic samples under study.

COMPOUNDS ^a	Ret. time (min)	Experimental LRI	Literature LRI ^{b,c,d}	Mass (Da)	Formula
Organosulfur compounds (OSC)					
Methanethiol	2.010	525	473	48.107	CH ₄ S
Dimethyl sulfide	2.841	556	550	62.134	C ₂ H ₆ S
Allyl mercaptan	4.033	598	600	74.145	C ₃ H ₆ S
Methyl-thiirane	4.549	616	619	74.145	C ₃ H ₆ S
Allyl methyl sulfide	6.851	698	698	88.171	C ₄ H ₈ S
Thiazole	7.878	726	728	85.128	C ₃ H ₃ NS
Dimethyl disulfide	8.660	746	752	94.199	C ₂ H ₆ S ₂
Diallyl sulfide	13.374	862	860	114.209	C ₆ H ₁₀ S
2,4-Dimethyl-thiophene	14.300	883	885	112.193	C ₆ H ₈ S
(Z)-1-prop-2-enylsulfanylprop-1-ene	14.522	888	888	114.209	C ₆ H ₁₀ S
(E)-1-prop-2-enylsulfanylprop-1-ene	14.677	892	890	114.209	C ₆ H ₁₀ S
2,5-Dimethyl-thiophene	15.404	909	905	112.193	C ₆ H ₈ S
Allyl methyl disulfide	15.941	922	919	120.236	C ₄ H ₈ S ₂
Methyl (Z)-1-propenyl disulfide	16.545	936	937	120.236	C ₄ H ₈ S ₂
Methyl (E)-1-propenyl disulfide	16.918	944	950	120.236	C ₄ H ₈ S ₂
3,4-Dimethylisothiazole	17.235	952	-	113.029	C ₅ H ₇ NS
3H-1,2-Dithiole	17.753	964	958	104.194	C ₃ H ₄ S ₂
Dimethyl trisulfide	18.363	978	975	126.264	C ₂ H ₆ S ₃
Diallyl disulfide	23.100	1092	1094	146.274	C ₆ H ₁₀ S ₂
(Z)-1-propenyl propyl disulfide	23.530	1103	1104	148.289	C ₆ H ₁₂ S ₂
(E)-1-propenyl propyl disulfide	23.777	1109	1114	148.289	C ₆ H ₁₂ S ₂
Allyl methyl trisulfide	25.453	1153	1148	152.301	C ₄ H ₈ S ₃
Methyl propyl trisulfide	25.867	1163	1168	154.317	C ₄ H ₁₀ S ₃
4-Methyl-1,2,3-trithiolane	26.158	1171	1157	138.275	C ₃ H ₆ S ₃
3-Vinyl-1,2-dithiacyclohex-4-ene	27.394	1203	1191	144.258	C ₆ H ₈ S ₂
4H-1,2,3-Trithiine	27.922	1218	1202	136.259	C ₃ H ₄ S ₃
3-Vinyl-1,2-dithiacyclohex-5-ene	28.340	1229	1224	144.258	C ₆ H ₈ S ₂
Diallyl trisulfide	31.505	1318	1313	178.339	C ₆ H ₁₀ S ₃
Allyl propyl trisulfide	31.856	1328	1321	180.354	C ₆ H ₁₂ S ₃
Allyl-1-propenyl trisulfide	32.305	1341	1333	178.339	C ₆ H ₁₀ S ₃
5-Methyl-1,2,3,4-tetrathiane	34.094	1394	1370	170.340	C ₃ H ₆ S ₄
Diallyl tetrasulfide	39.504	1567	1560	210.404	C ₆ H ₁₀ S ₄
Alcohols					
2-Propen-1-ol	3.148	566	568	58.0791	C ₃ H ₆ O
1-Propanol	3.319	572	568	60.0950	C ₃ H ₈ O
2,3-Butanediol	9.830	776	782	90.1210	C ₄ H ₁₀ O ₂
1-Hexanol	13.589	867	867	102.1748	C ₆ H ₁₄ O
Phenylethyl alcohol	24.115	1118	1118	122.1644	C ₈ H ₁₀ O
Esters					
Methyl acetate	2.930	558	559	74.0785	C ₃ H ₆ O ₂
Ethyl hexanoate	19.192	997	996	144.211	C ₈ H ₁₆ O ₂

Table S1. Cont.

Aldehydes and ketones					
2-Propenal	2.440	<500	480	56.063	C ₃ H ₄ O
Propanal	2.516	543	506	58.079	C ₃ H ₆ O
Butanal	3.967	595	595	72.106	C ₄ H ₈ O
2-Butenal	5.529	651	648	70.089	C ₄ H ₆ O
(<i>E</i>)-3-Penten-2-one	8.321	737	735	84.116	C ₅ H ₈ O
2-Methyl-2-butenal	8.52	742	748	84.116	C ₅ H ₈ O
2-Methylene-4-pentenal	8.811	750	763	96.127	C ₆ H ₈ O
5-Hexen-2-one	9.750	774	747	98.143	C ₆ H ₁₀ O
Hexanal	10.731	800	800	100.159	C ₆ H ₁₂ O
2-Methyl-2-pentenal	12.125	832	831	98.143	C ₆ H ₁₀ O
2-Hexenal	13.001	853	850	98.143	C ₆ H ₁₀ O
Benzaldehyde	17.800	965	965	106.122	C ₇ H ₆ O
Benzeneacetaldehyde	21.339	1049	1049	120.149	C ₈ H ₈ O
Furran derivatives and pyrazines					
2,5-Dihydrofuran	3.645	584	571	70.089	C ₄ H ₆ O
2-Methyl-pyrazine	11.700	823	824	94.115	C ₅ H ₆ N ₂
Furfural	12.140	833	833	96.084	C ₅ H ₄ O ₂
2-Furanmethanol	13.004	854	850	98.099	C ₅ H ₆ O ₂
5-Methyl-2(3H)-furanone	13.700	869	869	98.099	C ₅ H ₆ O ₂
2,5-Dimethyl-pyrazine	15.565	913	913	108.141	C ₆ H ₈ N ₂
Trimethyl-pyrazine	19.464	1003	1005	122.168	C ₇ H ₁₀ N ₂
1-(2-Pyridinyl)-ethanone	20.814	1037	1030	121.137	C ₇ H ₇ NO
2,3,5-Trimethyl-6-ethylpyrazine	25.880	1163	1163	150.221	C ₉ H ₁₄ N ₂

^a Compounds identified by comparing experimental and literature LRI values and experimental MS data with those of mass spectral libraries (Wiley, NIST).

^{b-d} LRI values on non-polar phases reported in the NIST database; and by Yang *et al.* (2019) and Setiyoningrum *et al.* (2021), respectively.

Table S2. Volatile concentrations ($\mu\text{g g}^{-1}$ dry sample) determined by SPME-GC-MS in fresh garlic (FG), aged garlic samples processed for different times (14, 21, 28 and 40 days) from bulbs (AGCB) and crushed bulbs (AGC) and commercial black garlic samples (CBG).

LRI ¹	Compound ²	FG	14 days		21 days		28 days		40 days		CBG1	CBG2
			AGB	AGCB	AGB	AGCB	AGB	AGCB	AGB	AGCB		
Thiols												
598	Allyl mercaptan	0.87 (0.04)*	0.88 (0.05)	0.86 (0.03)	0.54 (0.03)	0.37 (0.03)	0.50 (0.02)	0.33 (0.05)	0.23 (0.02)	0.22 (0.03)	-	0.08 (0.04)
726	Thiazole	0.5 (0.04)	0.19 (0)	0.08 (0.02)	0.16 (0.01)	0.06 (0.01)	0.13 (0.03)	0.05 (0.01)	0.07 (0.01)	0.028 (0.006)	0.002 (0)	0.002 (0)
964	3H-1,2-Dithiole	3.3 (1.1)	4.6 (1.5)	4 (1)	2.9 (0.6)	1.5 (0.2)	2.6 (0.4)	1.1 (0.2)	1.0 (0.1)	0.5 (0.1)	0.006 (0.003)	0.38 (0.04)
Monosulfides												
698	Allyl methyl sulfide	1.2 (0.6)	0.28 (0.06)	0.2 (0.1)	0.24 (0.06)	0.17 (0.06)	0.2 (0.1)	0.16 (0.04)	0.10 (0.04)	0.3 (0.1)	0.09 (0.03)	0.142 (0.001)
862	Diallyl sulfide	5.4 (0.4)	3.5 (0.3)	3.7 (0.9)	2.5 (0.8)	2 (1)	2.0 (0.8)	1.3 (0.5)	1.0 (0.4)	1.0 (0.3)	0.02 (0.02)	0.24 (0.07)
Disulfides												
746	Dimethyl disulfide	0.47 (0.01)	0.06 (0.01)	0.04 (0.01)	0.054 (0.004)	0.03 (0.01)	0.06 (0.02)	0.03 (0.01)	0.03 (0.01)	0.08 (0.02)	0.4 (0.1)	0.22 (0.02)
922	Allyl methyl disulfide	15.1 (0.5)	3.1 (0.1)	2 (0.2)	2.7 (0.1)	1.4 (0.3)	2.5 (0.5)	1.1 (0.1)	1.3 (0.1)	1.8 (0.2)	0.5 (0.2)	1.4 (0.5)
1092	Diallyl disulfide	38 (7)	24 (5)	23 (3)	19 (3)	14 (1)	18 (1)	11 (1)	10 (1)	7 (1)	0.2 (0.1)	3 (2)
1103	(Z)-1-Propenyl propyl disulfide	3 (1)	0.4 (0.1)	0.24 (0.04)	0.12 (0.02)	0.03 (0.03)	0.08 (0.01)	0.03 (0.01)	0.02 (0.01)	0.016 (0.003)	0.001 (0.001)	0.02 (0.01)
1109	(E)-1-Propenyl propyl disulfide	7 (2)	1.0 (0.2)	0.4 (0.1)	0.14 (0.03)	0.07 (0.01)	0.08 (0.01)	0.04 (0.01)	0.02 (0.01)	0.02 (0.01)	-	0.01 (0.01)

Table S2. Cont.

Trisulfides													
978	Dimethyl trisulfide	3.31 (0.03)	0.35 (0.03)	0.17 (0.01)	0.25 (0.01)	0.09 (0.02)	0.22 (0.01)	0.09 (0.01)	0.1 (0.01)	0.20 (0.05)	0.2 (0.1)	0.4 (0.1)	
1153	Allyl methyl trisulfide	18 (4)	9 (2)	6 (1)	6 (2)	1.7 (0.2)	4.7 (0.4)	1.5 (0.3)	1.7 (0.2)	1.4 (0.3)	0.12 (0.08)	2.4 (0.02)	
1318	Diallyl trisulfide	22 (3)	30 (2)	26 (2)	21 (1)	11 (1)	18 (0.5)	9 (1)	7.2 (0.3)	3.1 (0.2)	-	2 (0.5)	
1341	Allyl-1-propenyl trisulfide	5 (1)	0.24 (0.02)	0.16 (0.02)	0.08 (0.01)	0.04 (0.002)	0.06 (0.002)	0.03 (0.002)	0.02 (0.001)	0.012 (0.001)	-	0.014 (0.001)	
Cyclic sulfides													
1171	4-Methyl-1,2,3-trithiolane	2.4 (0.4)	6 (1)	4.6 (0.4)	3.6 (0.3)	1.8 (0.2)	3.3 (0.2)	1.6 (0.2)	1.4 (0.1)	0.7 (0.1)	0.01 (0.008)	0.98 (0.01)	
1229	3-Vinyl-1,2-dithiacyclohex-4-ene	3.7 (0.5)	1.6 (0.6)	0.9 (0.3)	1 (0.3)	0.4 (0.1)	0.8 (0.2)	0.3 (0.1)	0.3 (0.1)	0.1 (0.03)	-	0.07 (0.04)	
1218	4H-1,2,3-Trithiine	2 (0.3)	2.6 (0.2)	1.9 (0.1)	1.7 (0.1)	0.7 (0.2)	1.3 (0.3)	0.6 (0.2)	0.5 (0.1)	0.3 (0.1)	-	0.2 (0.1)	
1229	3-Vinyl-1,2-dithiacyclohex-5-ene	9 (1)	5 (3)	1.5 (0.1)	3 (1)	1.0 (0.2)	2.3 (0.4)	0.8 (0.2)	0.8 (0.2)	0.4 (0.1)	-	0.2 (0.1)	
Alcohols													
566	2-Propen-1-ol	0.78 (0.01)	2.9 (0.1)	2.4 (0.2)	7.3 (0.2)	4.6 (1.0)	9.5 (0.7)	4 (0.5)	8 (1)	2.1 (0.4)	0.16 (0.01)	1.2 (0.03)	
572	1-Propanol	0.12 (0.03)	0.6 (0.02)	0.9 (0.2)	1.0 (0.2)	0.7 (0.2)	1.0 (0.2)	0.6 (0.1)	0.9 (0.2)	0.16 (0.04)	-	0.3 (0.1)	
776	2,3-Butanediol	-	0.09 (0.05)	0.13 (0.06)	0.1 (0.01)	0.3 (0.2)	0.12 (0.05)	0.18 (0.05)	0.15 (0.03)	0.4 (0.1)	0.09 (0.03)	0.01 (0.001)	
1118	Phenylethyl alcohol	-	0.02 (0.01)	0.06 (0.01)	0.05 (0.01)	0.16 (0.02)	0.07 (0.01)	0.20 (0.06)	0.06 (0.002)	0.13 (0.03)	-	-	
Acids and esters													
518	Acetic acid	4.9 (0.3)	3.8 (6)	4.4 (0.5)	4.1 (0.5)	4.8 (0.2)	5.3 (0.2)	4.2 (0.3)	4.1 (0.3)	4.4 (0.3)	5.5 (0.9)	5.1 (0.6)	

Table S2. Cont.

997	Ethyl hexanoate	0.02 (0)	0.26 (0.03)	0.37 (0.05)	0.7 (0.1)	0.5 (0.1)	0.8 (0.2)	0.3 (0.05)	1.1 (0.1)	0.07 (0.01)	-	-
Aldehydes/ketones												
541	2-Propenal	0.4 (0.1)	0.13 (0.02)	0.15 (0.01)	0.2 (0.01)	0.09 (0.01)	0.18 (0.01)	0.08 (0.01)	0.12 (0.01)	0.04 (0.008)	0.01 (0.001)	0.02 (0.001)
543	Propanal	0.39 (0.03)	0.10 (0.02)	0.09 (0.01)	0.34 (0.02)	0.13 (0.02)	0.20 (0.06)	0.1 (0.01)	0.16 (0.04)	0.07 (0.004)	0.04 (0.02)	0.03 (0.02)
651	2-Butenal	13 (1)	03 (0.1)	1.7 (0.3)	1.6 (0.2)	0.05 (0.002)	0.21 (0.01)	0.034 (0.007)	0.081 (0.002)	0.024 (0.007)	-	-
750	2-Methylene 4-pentenal	0.46 (0.07)	0.20 (0.07)	0.25 (0.04)	0.22 (0.04)	0.069 (0.002)	0.18 (0.004)	0.03 (0.001)	0.09 (0.001)	0.025 (0.002)	-	0.005 (0.001)
965	Benzaldehyde	0.39 (0.04)	0.5 (0.2)	1.2 (0.3)	1.2 (0.3)	1.2 (0.1)	1.4 (0.2)	0.8 (0.1)	0.6 (0.08)	0.18 (0.04)	0.05 (0.02)	0.08 (0.03)
Pyrazines and furan derivatives												
823	Methyl-pyrazine	-	0.015 (0.003)	0.012 (0.001)	0.06 (0.01)	0.12 (0.01)	0.10 (0.01)	0.16 (0.01)	0.12 (0.003)	0.1 (0.002)	0.007 (0.001)	0.27 (0.05)
833	Furfural	0.005 (0.001)	0.01 (0.003)	0.10 (0.01)	0.07 (0.01)	0.19 (0.01)	0.15 (0.01)	0.22 (0.03)	0.14 (0.01)	0.21 (0.02)	1.5 (0.3)	2.2 (0.6)
854	2-Furanmethanol	-	-	0.01 (0.002)	0.004 (0.001)	0.15 (0.004)	0.02 (0.0003)	0.26 (0.04)	0.03 (0.003)	0.32 (0.05)	0.015 (0.002)	0.015 (0.005)
1163	2,5-Dimethyl-pyrazine	-	0.04 (0.01)	0.16 (0.02)	0.041 (0.008)	2.1 (0.1)	0.09 (0.002)	1.04 (0.09)	0.13 (0.008)	0.38 (0.04)	0.01 (0.003)	0.29 (0.08)

¹ Experimental linear retention index (LRI) on DB-5 column, calculated from retention data of *n*-alkanes (C₆-C₃₀) analysed under identical experimental conditions.

² Identifications as provided in Table S1.

* Average ($n = 3$) and standard deviation in brackets.

-: Non detected.

Anexo 6: Correspondiente a la sección 4.3.

Simultaneous microwave-assisted extraction of bioactive compounds from aged garlic

Supplementary material

Table S1. Retention times (t_R) and MS and MS/MS data of organosulfur compounds detected by HPLC-QToF MS in aged garlic AG1.

Compounds	t_R (min)	MS [M+H] ⁺	MS/MS
Cycloalliin	3.97	178.0526	118.0875; 72.0836
Alliin	4.32	178.0532	88.0400; 137.0171; 120.0135
S-allyl-L-cysteine	5.46	162.0583	145.0293; 120.0076
S-1-propenyl-L-cysteine	6.46	162.0583	145.0321; 116.0524
γ -Glutamyl-S-methylcysteine	6.91	265.0853	130.0495; 121.0509
S-allylmercaptocysteine	9.59	194.0304	84.0437; 166.0861; 120.0801
γ -Glutamyl-S-allyl-L-cysteine	15.95	291.1009	145.0290; 162.0571; 274.0705
γ -Glutamyl-S-1-propenyl-cysteine	18.75	291.1009	145.0295; 162.0563; 274.0728
γ -Glutamyl-S-allylmercaptocysteine	21.53	323.0720	121.0501; 149.0224; 97.9686

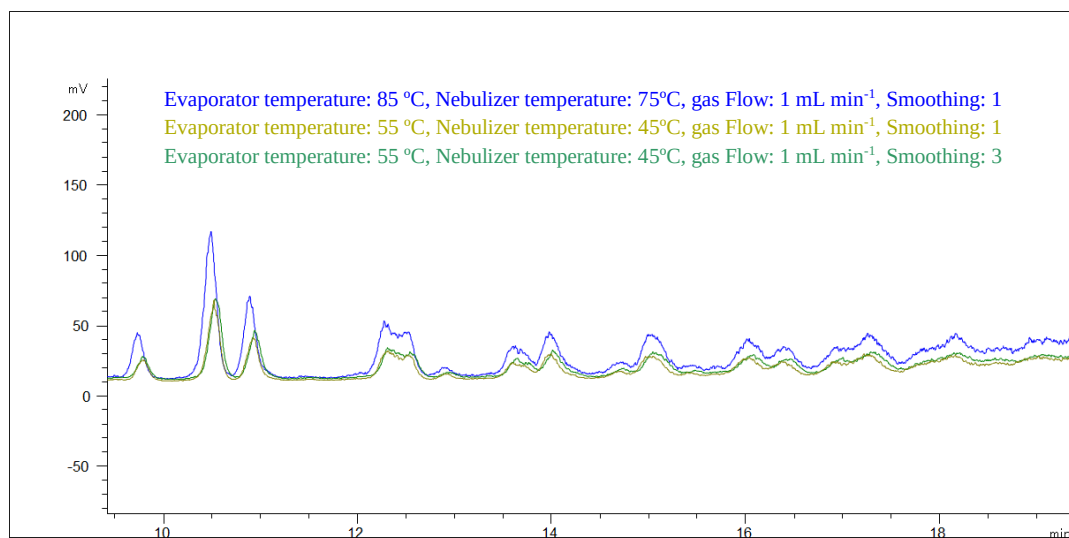
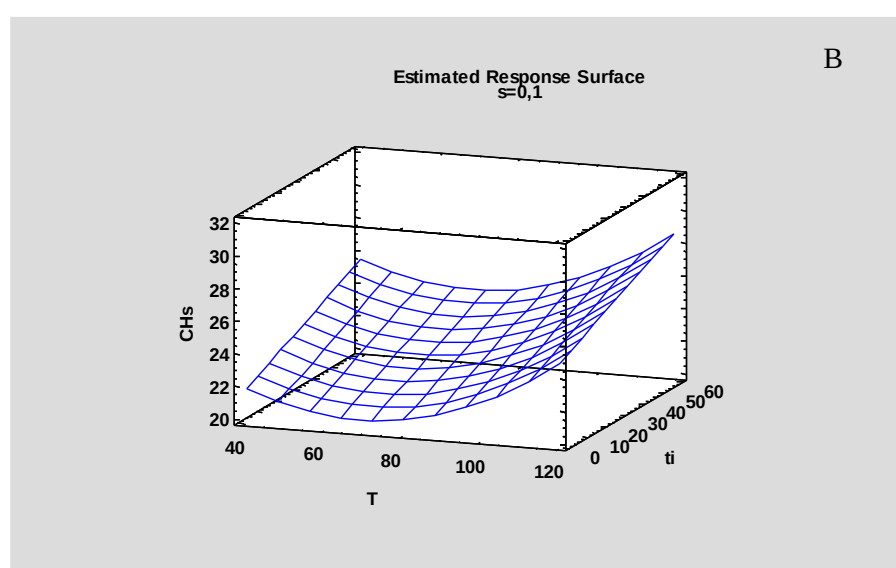
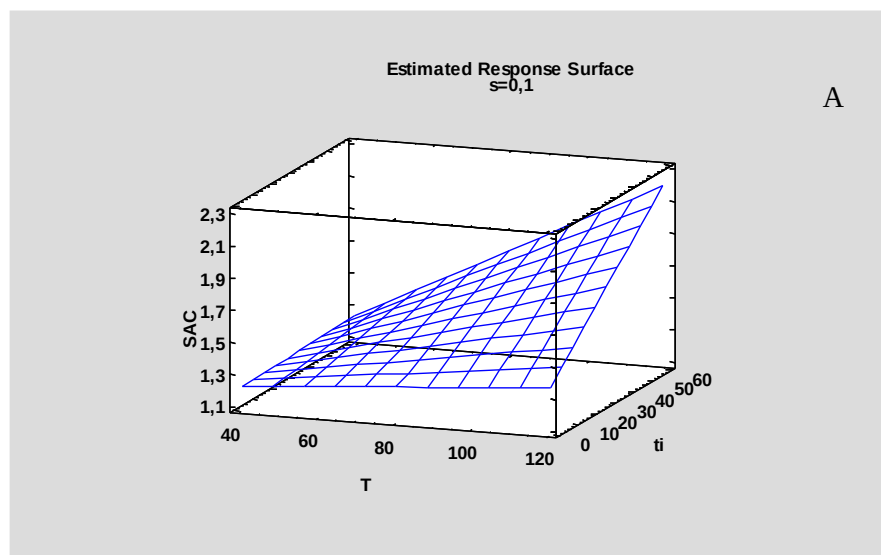
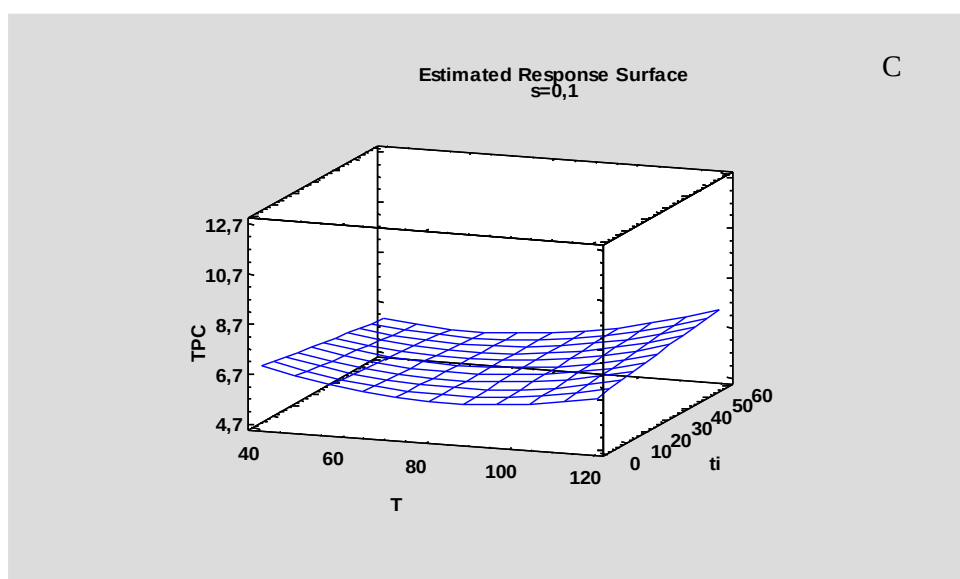
Figure S1. HPLC-ELSD profile of AG1 using different detector conditions.

Figure S2. Response surface plot to maximize the extraction of SAC (A), bioactive carbohydrates (B) and TPC (C) from aged garlic.





Analysis of Variance of Experimental design

- Analysis of Variance for SAC (R_I)

Source	Sum of Squares	Df	Mean	Square F-ratio	P-value
T	0.678613	1	0.678613	15.73	0.0027
t	0.32	1	0.32	7.42	0.0214
$T.t$	0.140625	1	0.140625	3.26	0.0997
$t.s$	0.172225	1	0.172225	3.99	0.0736
Total error	0.431298	10	0.0431298		
Total (corr.)	1.74276	14			

R-squared = 75.252 percent

PRESS = 1.29838

R-squared (predicted) = 25.4986 percent

Standard Error of Est. = 0.207677

Mean absolute error = 0.141367

Durbin-Watson statistic = 1.69569 (P=0.2024)

Lag 1 residual autocorrelation = 0.0585166

- Analysis of Variance for bioactive carbohydrates (R_2)

Source	Sum of Squares	Df	Mean	Square F-ratio	P-value
T	18.4528	1	18.4528	7.81	0.0175
t	25.1695	1	25.1695	10.65	0.0076
T^2	26.4049	1	26.4049	11.17	0.0066
Total error	26.0006	11	2.36369		
Total (corr.)	96.0279	14			

R-squared = 72.9239 percent

PRESS = 48.4632

R-squared (predicted) = 49.5322 percent

Standard Error of Est. = 1.53743

Mean absolute error = 1.03729

Durbin-Watson statistic = 2.27838 (P=0.6870)

Lag 1 residual autocorrelation = -0.238135

- Analysis of Variance for TPC (R_3)

Source	Sum of Squares	Df	Mean	Square F-ratio	P-value
T	0.775013	1	0.775013	7.32	0.0241
s	47.1906	1	47.1906	445.99	0.0000
T^2	2.18792	1	2.18792	20.68	0.0014
$T.t$	0.585225	1	0.585225	5.53	0.0432
s^2	4.93352	1	4.93352	46.63	0.0001
Total error	0.9523	9	0.105811		
Total (corr.)	56.1894	14			

R-squared = 98.3052 percent

PRESS = 4.03912

R-squared (predicted) = 92.8116 percent

Standard Error of Est. = 0.325286

Mean absolute error = 0.206167

Durbin-Watson statistic = 1.72248 (P=0.3299)

Lag 1 residual autocorrelation = 0.0629709

Anexo 7: Correspondiente a la sección 4.4.

Evaluation of advanced extraction techniques to obtain enriched extracts from garlic (*Allium sativum* L.) by-products

Supplementary material

Table S1. Information of the different subproducts, including skins (Sk), roots (R), stem (S) and leaves (L), from different garlic plants (G) used in this study.

Sample	Geographical origin	Garlic ecotype	Developed bulb	Subproducts
G1	Talavera de la Reina (Castilla La-Mancha, Spain)	white	No	S1
G2	'Las Pedroñeras' (Castilla La-Mancha, Spain)	purple	No	S2
G3	Colmenar de Oreja (Madrid, Spain)	purple	No	S3
G4	Colmenar de Oreja (Madrid, Spain)	white	No	S4
G5	'Las Pedroñeras' (Castilla La-Mancha, Spain)	purple	Yes	Sk5, R5, S5 and L5
G6	Talavera de la Reina (Castilla La-Mancha, Spain)	white	Yes	S6
G7	'Las Pedroñeras' (Castilla La-Mancha, Spain)	purple	Yes	S7
G8	Colmenar de Oreja (Madrid, Spain)	white	Yes	S8

Figure S1. Estimated response surfaces of UAE (A) and PLE (B).

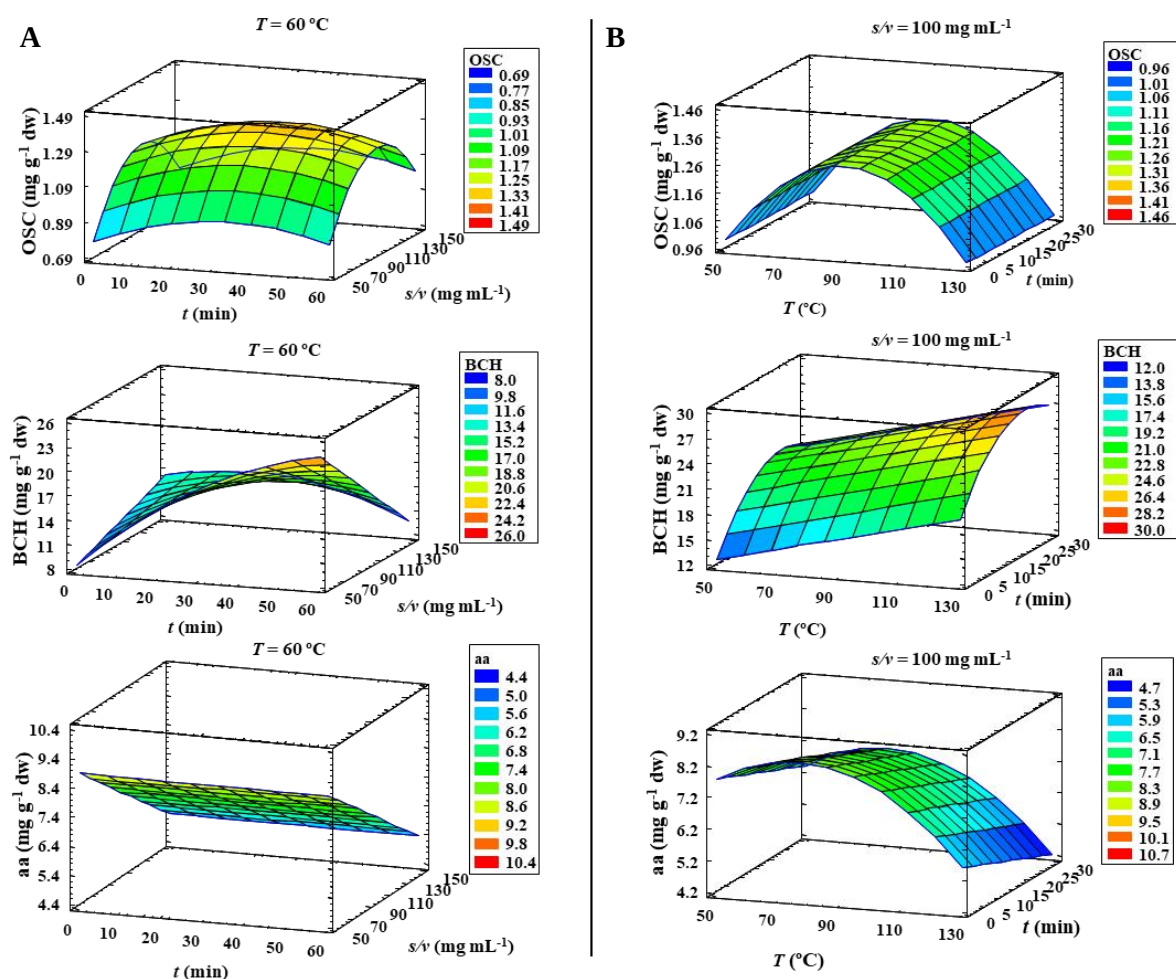
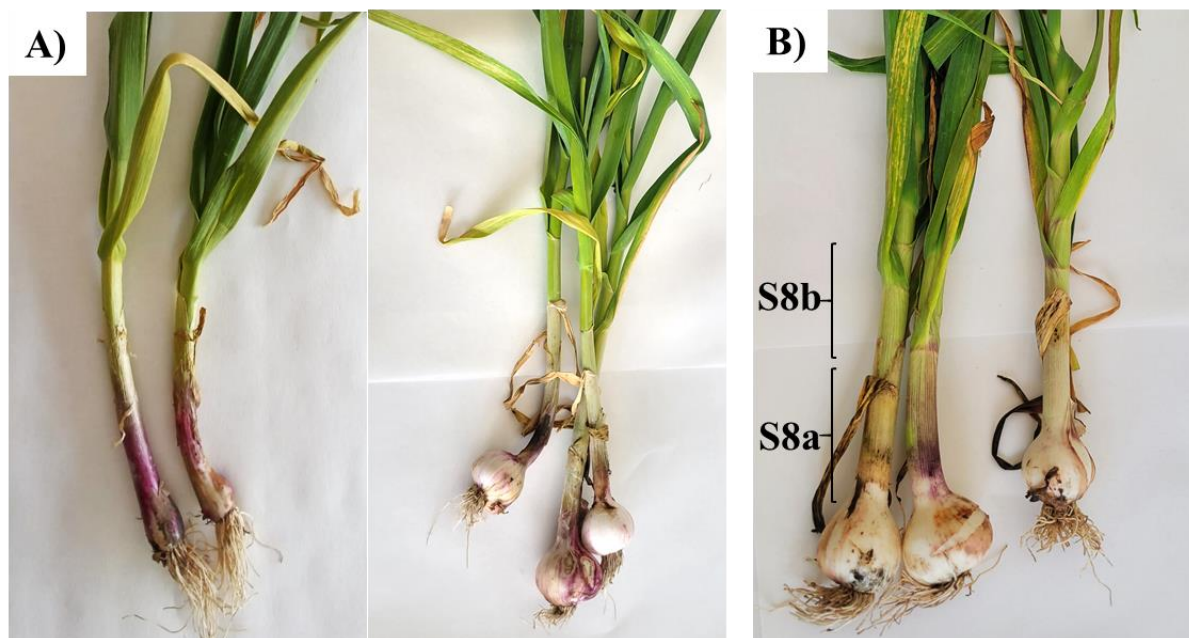


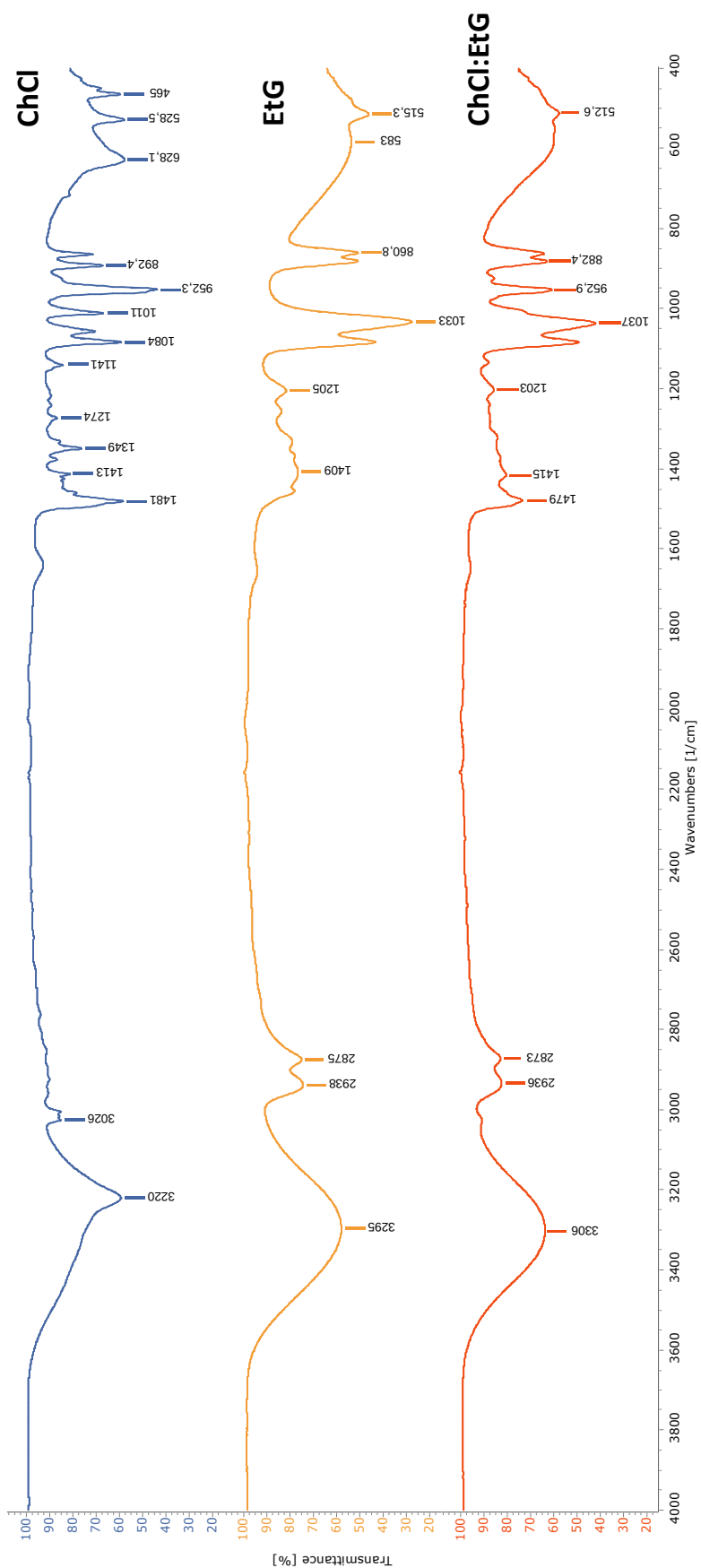
Figure S2. A) Garlic plants at different stage of maturation and B) different parts of the stem considered: i) the closest part (white part) to the bulb (S8a) and ii) the more distal part (green part) to the bulb (S8b).



Anexo 8: Correspondiente a la sección 4.5.

Natural Deep Eutectic Solvents for Solubility and Selective Fractionation of Bioactive Low Molecular Weight Carbohydrates

Supplementary material

Figure S1. FT-IR spectra of chloride choline (ChCl), ethylene glycol (EtG) and NADES1 (ChCl:EtG).

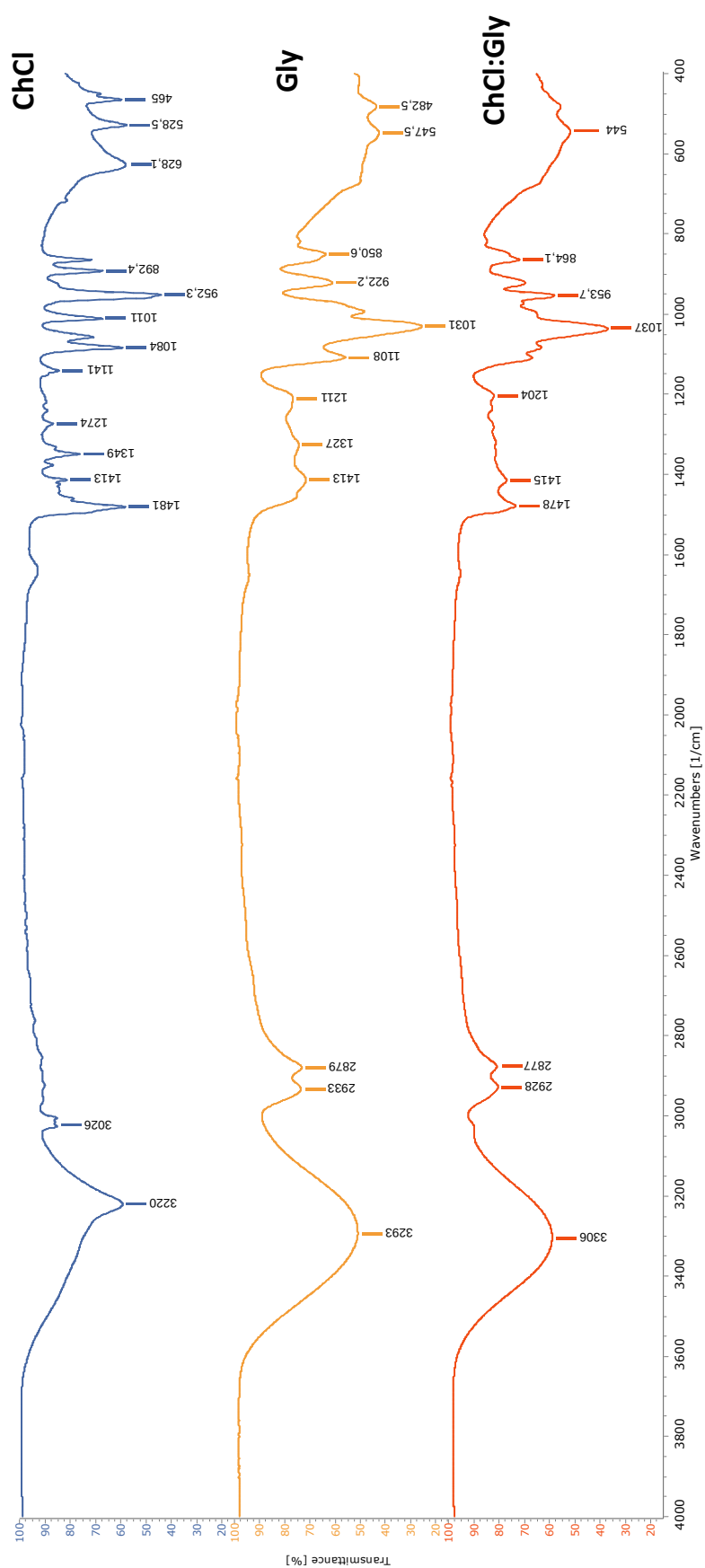
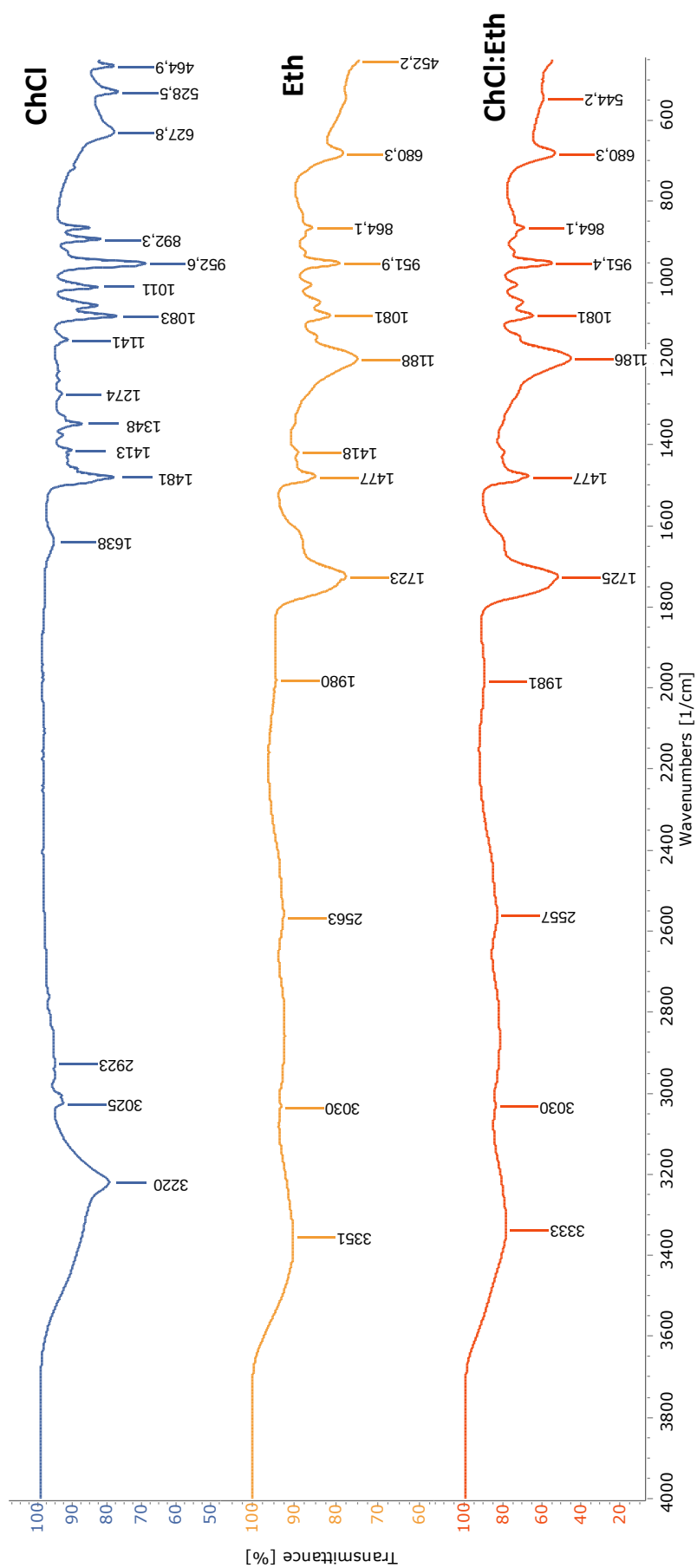


Figure S2. FT-IR spectra of chloride choline (ChCl), glycerol (Gly) and NADES2 (ChCl:Gly).

Figure S3. FT-IR spectra of chloride choline (ChCl), oxalic acid dihydrate (Eth) and NADES3 (ChCl:Eth).



Anexo 9. Evaluación de un prototipo de UAE para su implementación industrial.

ANEXO 9. EVALUACIÓN DE UN PROTOTIPO DE UAE PARA SU IMPLEMENTACIÓN INDUSTRIAL.

El prototipo de UAE, NextTgen Ultrasonic Platform (Synaptec, Figura 1) ha sido evaluado para explorar su potencial aplicación industrial para la extracción de SAC a partir de ajo envejecido. Se han realizado distintos ensayos con diferentes condiciones de extracción (tiempo y temperatura) y los resultados se han comparado con los obtenidos a escala industrial (Tabla 1). Las condiciones de extracción del segundo experimento (UAE2) reproducían las mismas condiciones que la extracción realizada a escala industrial por la empresa Pharmactive Biotech Products S.L.U (SLE).



Figura 1. Equipo de UAE, NextTgen Ultrasonic Platform, con capacidad de hasta 1 L.

Tabla 1. Contenido de SAC (%) en los extractos obtenidos mediante el prototipo de UAE empleando diferentes condiciones de temperatura y por SLE a escala industrial.

Ensayos	Condiciones	% SAC
UAE1	40°C; 60 min; 150 g ajo en 600 mL	0,34 (0,01) ^{*a}
UAE2	Abg Cool Tech ^{TM**}	0,39 (0,02) ^b
SLE	Abg Cool Tech TM	0,225 (0,003) ^c

* Desviación estándar

** Las condiciones de ABG Cool-TechTM (proceso desarrollado por Pharmactive Biotech Products S.L.U.) no se detallan por confidencialidad

^{a-c} Distintas letras indican diferencias significativas ($P < 0,05$) en el contenido de SAC entre los tratamientos aplicados

Como puede observarse en la Tabla 1, se encontraron diferencias significativas en la concentración de SAC entre los ensayos UAE1 (condiciones óptimas indicadas en la Sección 4.4) y UAE2 (condiciones empleadas a escala industrial), obteniéndose mejores resultados para este último caso. Además, todos los tratamientos de UAE permitieron la obtención de concentraciones de SAC significativamente superiores a las obtenidas mediante SLE a escala industrial (Abg Cool Tech™). En base a los prometedores resultados obtenidos, esta alternativa se evaluará en el futuro para su posible implementación en la empresa Pharmactive Biotech Products S.L.U.

Anexo 10. Currículum vitae

ANEXO 10. CURRÍCULUM VITAE

Listado de publicaciones

Publicaciones incluidas en la tesis doctoral:

- **I. Jiménez-Amezcu**a, S. Rivas Blas, M. Díez-Municio, A.C. Soria, A.I. Ruiz-Matute, M.L. Sanz. Development of a multianalytical strategy for detection of frauds in *Coleus forskohlii* supplements. *Journal of Chromatography A* (2022), 1676, 463198.
- **I. Jiménez-Amezcu**a, M. Díez-Municio, A.I. Ruiz-Matute, A.C. Soria. A comparative study of LC-MS and FIA-(ESI)MS for quantitation of S-allyl-L-cysteine in aged garlic supplements. *Foods* (2024), 13, 2645.
- **I. Jiménez-Amezcu**a, A. González-Prada, M. Díez-Municio, A.C. Soria, A.I. Ruiz-Matute, M.L. Sanz. Simultaneous extraction of bioactive compounds from aged garlic. *Journal of Chromatography A* (2023), 1704, 464128.
- **I. Jiménez-Amezcu**a, M.I. López-Martínez, A.I. Ruiz-Matute, M.L. Sanz. Natural Deep Eutectic Solvents for Solubility and Selective Fractionation of Bioactive Low Molecular Weight Carbohydrates. *Foods* (2023), 12, 4355.
- **I. Jiménez-Amezcu**a, M.I. López Martínez, A.I. Ruiz-Matute, M.L. Sanz. Advances and challenges in the selective extraction of low molecular weight carbohydrates using ionic liquids and deep eutectic solvents. *Trends in Analytical Chemistry* (2024), 171, 117507.
- **I. Jiménez-Amezcu**a, M. Díez-Municio, A.I. Ruiz-Matute, M.L. Sanz. Flow Injection Analysis-Mass Spectrometry for the fast detection of frauds in *Coleus forskohlii* food supplements. *Journal of Chromatography A* (2024) (En revisión).
- **I. Jiménez-Amezcu**a, M.L. Sanz, M. Díez-Municio, J.K. Parker, M.J. Oruna-Concha, A.C. Soria. Changes in the bioactive content and aroma profile of aged garlic with processing conditions. (Enviado a *LWT-Food Science and Technology*)
- **I. Jiménez-Amezcu**a, A.C. Soria, M. Díez-Municio, M.L. Sanz, A.I. Ruiz-Matute. Evaluation of advanced extraction techniques to obtain bioactive enriched extracts from garlic (*Allium sativum* L.) by-products. (Enviado a *Journal of Agriculture and Food Research*)
- **I. Jiménez-Amezcu**a, M.I. López-Martínez, A.I. Ruiz-Matute, M.L. Sanz. Use of choline chloride-based eutectic solvents for the selective separation of bioactive polyalcohols. (Enviado a *Advances in Sample Preparation*)

Otras publicaciones científicas

- F.J. Carmona FJ; **I. Jiménez-Amezcu**a, S. Rojas, C. C. Romão, J.A.R. Navarro, C.R. Maldonado, E. Barea. Aluminum Doped MCM-41 Nanoparticles as Platforms for the Dual

Encapsulation of a CO-Releasing Molecule and Cisplatin. *Inorganic Chemistry* (2017), 56, 17, 10474-10480.

- **I. Jiménez-Amezcu**a, F.J. Carmona, I. Romero-García, M. Quirós, J.L. Cenis, A.A. Lozano-Pérez, C.R. Maldonado, E. Barea. Silk fibroin nanoparticles as biocompatible nanocarriers of a novel light-responsive CO-prodrug. *Dalton Transactions* (2018), 47, 31, 10434-10438.

Comunicaciones a congresos (relacionados con la tesis)

- M.I. López-Martínez, **I. Jiménez-Amezcu**a, S. Rivas-Blas, A.I. Ruiz-Matute, M.L. Sanz. "Evaluation of the use of Deep Eutectic Solvents (DES) for the selective separation of bioactive carbohydrates" (Póster). 23rd International Symposium on Advances in Extraction Technologies (30/06/21-02/07/21).
- **I. Jiménez-Amezcu**a, S. Rivas-Blas, M. Díez-Municio, A.C. Soria, A.I. Ruiz-Matute, M.L. Sanz. "Evaluation of quality of *Plectranthus barbatus* food supplements by chromatographic techniques coupled to mass spectrometry" (Comunicación Oral Web). XX reunión de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2021) (18-19/11/2021).
- **I. Jiménez-Amezcu**a, S. Rivas-Blas, M. Díez-Municio, A.I. Ruiz-Matute, A.C. Soria, M.L. Sanz. "Detección de fraudes en complementos alimenticios de *Coleus forskohlii* mediante espectrometría de masas" (Póster). I Jornadas de Jóvenes Investigadores SEEM (I JJI-SEEM) (24-25/03/22).
- **I. Jiménez-Amezcu**a, M.I. López Martínez, S. Rivas-Blas, A.I. Ruiz-Matute, M.L. Sanz. "Selective fractionation of bioactive carbohydrates using Natural Deep Eutectic Solvents (NADES)" (Póster). 2nd European Sample Preparation e-Conference and 1st Green and Sustainable Analytical Chemistry e-Conference (14-16/03/22).
- L. Ysa, S. Rivas-Blas, **I. Jiménez Amezcu**a, M. Díez-Municio, M. Luz-Sanz, A.I. Ruiz-Matute, A.C. Soria. "Mass fingerprinting by FIA-(ESI)MS for rapid quantitation of S-allyl-L-cysteine in black garlic supplements" (Póster y comunicación Flash). XXI reunión de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2022) (25-27/10/22).
- A. González-Prada, **I. Jiménez-Amezcu**a, M. Díez-Municio, A.I. Ruiz-Matute, A.C. Soria, M.L. Sanz. "Development of a new method for the simultaneous extraction of bioactive compounds from aged black garlic" (Póster). XXI reunión de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2022) (25-27/10/22).
- I. Jiménez-Amezcu, J. Merino, **A. Martín-Ortiz**, M. Díez-Municio, A.C. Soria, M.L. Sanz, A.I. Ruiz-Matute. "Revalorization of garlic (*Allium sativum* L.) by-products by pressurized liquid extraction" (Póster). 25th International Symposium on Advances in Extraction Technologies (18-21/07/23).

- **I. Jiménez Amezcua.** “Metodologías multianalíticas avanzadas para el aseguramiento de la calidad y revalorización de complementos alimenticios de origen vegetal” (Comunicación Oral). VII Simposio Anual en Química Avanzada (SAQA) (18/07/23).
- **I. Jiménez-Amezcua,** D. Herrero, M. Díez-Municio, A.I. Ruiz-Matute, M.L. Sanz, A.C. Soria, J. Parker, M.J. Oruna-Concha. “The effect of aging conditions on the changes of the aroma volatile profile and key odourants in garlic by SPME GC-MS and GC-O” (Comunicación Oral tipo Flash y Póster). XXII reunión de la Sociedad Española de Cromatografía y Técnicas Afines (SECyTA 2023) (16-18/10/23).
- **I. Jiménez-Amezcua,** M.I. López Martínez, A.I. Ruiz-Matute, M.L. Sanz. “Fraccionamiento selectivo de carbohidratos bioactivos mediante disolventes eutécticos profundos naturales” (Comunicación Oral). IV Jornadas Científicas CIAL Fórum (19-20/10/2023).
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Otras comunicaciones a congresos

- I. Jiménez-Amezcua. “Nanopartículas de fibroína de seda como vehículos de un nuevo fármaco fotoactivo de CO” (Comunicación oral). Jornada de Jóvenes Investigadores en Bioinorgánica, 1ª Edición. Asociación Española de Bioinorgánica (AEBIN). Facultad de Ciencias, Universidad de Granada (Granada, España) (11/05/2018).
- I. Jiménez-Amezcua, F.J. Carmona, I. Romero-García, M. Quirós, J.L. Cenis, A.A. Lozano-Pérez, C.R. Maldonado, E. Barea. “Nanopartículas de fibroína de seda como vehículos de un nuevo fármaco fotoactivo de CO” (Póster). QIES Tenerife 2018 (17-20/05/2018). •

Estancias en el extranjero

Estancia predoctoral internacional en el grupo “Food Research” del Departamento “Food and Nutritional Sciences” de la Universidad de Reading (Reading, Inglaterra, Reino Unido), entre el 1 de Mayo de 2023 y el 31 de Julio de 2023, bajo la supervisión de la profesora María José Oruña Concha. En dicha estancia se estudió el perfil aromático y los compuestos activos responsables del olor del ajo envejecido, a través de análisis basados en SPME GC-MS y SPME GC-O.