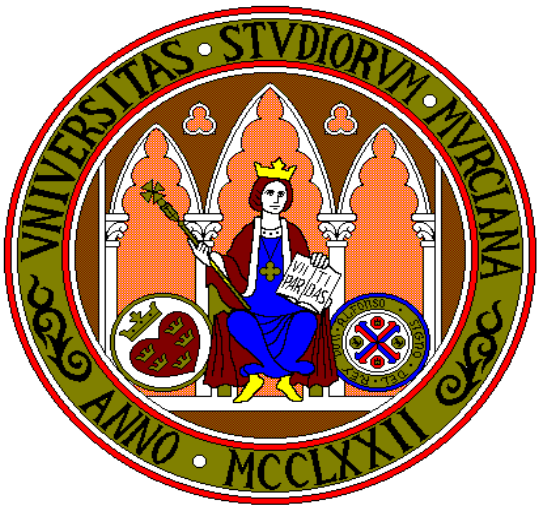


UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

Characterisation and antioxidant and antienzymatic bioactivities of essential oils of lavender, thyme and oregano from Murcia

Caracterización y bioactividad antioxidante y antienzimática de aceites esenciales de lavandas, tomillos y orégenos de Murcia.

D. Alejandro Carrasco Ruiz

2015

Este trabajo es resultado del proyecto de investigación (19545/PI/14, *Enzimas y biomoléculas con aplicaciones biotecnológicas de plantas labiadas de la Región de Murcia*) financiado por la Fundación Séneca – Agencia de Ciencia y Tecnología de la Región de Murcia, en el marco del PCTIRM 2011-2014. Investigador Principal Dr. José Tudela Serrano e Investigadora Colaboradora Dra. Virginia Tomás Martínez, GENZ-Grupo de investigación *Enzimología*, Universidad de Murcia.

El autor de esta Memoria ha disfrutado de las siguientes ayudas:

1. Beca de Colaboración para la Investigación, financiada con cargo a proyectos en la Universidad de Murcia: "*Dermocosméticos naturales basados en extractos de plantas aromáticas*".

2. Formación Práctica en el ámbito de actividades de I+D+I del Plan Propio de la Universidad de Murcia "*Enzimas y biomoléculas nutricéuticas y cosmecéuticas*".

3. Beca ERASMUS Prácticas de 01/06/14 a 30/09/14 para estancia investigadora predoctoral en el Departamento de química y farmacia de la Facultad de ciencias y tecnología de la Universidad del Algarve en Faro, Portugal. Tutorizado por la Prof. Dra. María Graça Miguel.

En Murcia, a 30 de Octubre de 2015

Fdo.: **D. Alejandro Carrasco Ruiz**
Alumno de doctorado
Depto. de Bioquímica y Biología Molecular-A
Facultad de Biología
Universidad de Murcia

Fdo.: **Dr. José Tudela Serrano**
Investigador principal de los proyectos
Catedrático de Bioquímica y Biología Molecular
Facultad de Biología
Universidad de Murcia

Agradecimientos

"El amigo ha de ser como la sangre,
que acude luego a la herida sin esperar que le llamen."
Francisco de Quevedo.

Tras varios años de laborioso trabajo, finalmente, ha llegado el día en el que esta memoria ve la luz. Sin embargo, el fruto de este trabajo no es exclusivamente mío, aunque sea mi mano la que le ha dado su forma final. Haber llegado hasta este punto se lo debo a muchas personas que, con sus distintas aportaciones, han hecho que el rumbo de mi camino me haya traído hasta aquí y haya aguantado los rigores de la tesis.

Por eso quiero agradecer su paciencia, su buen hacer y enorme conocimiento y guía a mis directores de tesis José Tudela, Virginia Tomás y Maria Graça Miguel. También a los catedráticos del grupo de investigación GENZ que me han hecho sentir como en casa: Francisco García y José Neptuno Rodríguez y a todos los profesores con los que he compartido esta segunda vivienda que es el departamento. I also want to thank the group of Faro, Portugal (especially Graça and Custódia) for their kindness and hard work in the University of Algarve. And I'd like to thank Kenneth from Gent, Belgium for his fantastic willing to help and for all the great moments in the old times.

La gran familia que formamos los alumnos del grupo ha sido fuente inagotable de experiencias, alegrías y amigos. Son estos amigos los que hacen que los éxitos compartidos se multipliquen y las pequeñas penas también compartidas se diluyan. Todos juntos hemos descubierto que la ciencia se forja a base de fallar pero que duelen sólo las cien primeras veces y que un día malo lo tiene cualquiera pero un día bueno podemos crearlo entre todos.

Por eso no quiero olvidarme de agradecer el fantástico trabajo y la meticulosidad de Ana que es una persona noble y entregada, que hace la vida más fácil a quienes la rodeamos, la profundidad e información siempre actual de Antonio, su sentido del humor y algún que otro despiste que nos ha alegrado a todos la vida por aquí (thermomix incluida). El aire fresco y la gran generosidad de Mar, la positividad y sinceridad de Rafa, la alegría que siempre nos aporta Mar (la otra Mar), el compromiso y lo muchísimo que aprendí con Xavi, aunque se suponía que yo era el profesor, la paciencia y la gran disposición de Delma y la visión entregada y el humor de Quique. De ninguna manera me voy a olvidar tampoco de los "mayores" que tanto me enseñaron en los primeros años y que se ganaron mi gratitud día a día compartiendo conmigo su extensa experiencia: compañeros de fatigas y algunas buenas juergas también, Magda y Jesús, compañeros del té, Paquito y José Luís, donde de tantas y tantas cosas hemos hablado todo el grupo. También los compis de pasillo y memorables cenas de "fin de trimestre": Maria Piedad, Marisa, Fernanda y Sole.

Y si de todos mis compañeros y compañeras de faenas guardo tan grato recuerdo, ¿qué puedo decir de Vanessa? Lo único que puedo decir, a estas alturas, es que no hubiera llegado hasta aquí sin sus consejos, sin nuestras interminables charlas en el pasillo de casa, sin las risas, sin el apoyo mutuo todos estos años y sin la luz maravillosa que desprende y que disfrutamos quienes la conocemos. Que me ha crecido de repente la familia, porque tengo una hermana más y se lo agradezco en el alma.

También merece especial mención Yolanda, mi Yoli, la Zipi de este Zape desde que nos conocimos en el curso cero antes de empezar siquiera la carrera y mira que hemos vivido cosas juntos… ¡hasta países hemos conquistado! Gracias por todos estos años de energía súper positiva que me das y prepárate para los que vienen porque la cosa sólo puede ir a mejor, acabemos geográficamente donde acabemos,

siempre encontraremos el momento y la excusa perfecta para reunirnos, como hemos hecho hasta ahora. Y no me voy a olvidar de Ana, mi Anica, que es una luchadora y siempre está cuando hay que estar apoyándome, a veces no sé si me lo merezco. Gracias también a Aurora, Samanta y Mª Dolores por los cines, por los libros y por tantas historias vividas, compañeras de tantos momentos maravillosos, gracias por vuestra amistad.

A mi Antonia, sabes que te quiero un montón, gracias por estar siempre a mi lado, que en estos últimos años me ha aguantado todos mis rollos de tesis y que siempre proponía plan para los sábados noche con la genial Esther. A Karima por las alegrías vividas juntos, por las noches de fiesta y los disfraces semi-improvisados. A Sonia, Fran y Blas por todas las juergas, viajes y risas. A Raúl y Agustín por una amistad que sobrevive a tantos y tantos años, aunque yo sea un perro total, por tener la perseverancia de no darme por perdido, porque jamás me hubiera perdonado perderos.

A mi familia, a todos en general, a mis tíos y primos, pero en particular a Luci porque, aunque no me recomendabas hacer esto me has apoyado en mi tesis y en mi vida. Y a Rubén y a Nati porque somos los tres mosqueteros, porque siempre estáis ahí cuando os necesito y porque por mucho que pasen los años, siempre seguiremos ahí. Y a Cloti y Manolo porque sin vuestro apoyo, yo no sé que habría sido de nosotros. Y no puedo seguir porque esto no puede ser eterno, pero a toda mi familia porque sois fieles, tenéis espíritu de ayuda y lo demostráis a cada momento y con cada giro de la vida. Os quiero desde lo más profundo de mi corazón. Y a mi familia de Vera, por vuestro cariño y vuestra perspectiva y por saber estar tan cerca estando lejos.

A mi padre por su ejemplo de trabajo, de honestidad y de entrega y por ser un moderno. A mi madre por su valor, su empuje y su fuerza, por aprender a renacer y conservar la sonrisa y por ser una perfeccionista con el estilo de la que día a día sigo aprendiendo. Y a mi hermano por su alegría, por su ilusión, por las risas, por las locuras y los pianos y porque, aunque todo el mundo opina que somos totalmente opuestos, lo cierto es que somos complementarios. Porque sois fantásticos, porque, sin vosotros, de ninguna manera hubiera conseguido llegar hasta aquí. Gracias por aguantarme, por escucharme, por consolarme y espolearme cuando no sé qué hacer, por alabar mis virtudes y criticar mis defectos, por ser, en definitiva, la mejor familia que jamás hubiera soñado tener. Os adoro y esta tesis es tan vuestra como mía.

Y ya por último quiero recordar y agradecer a quienes ya no están, a mis dos abuelas. Gracias. Gracias por ser modelos de conducta, por tener el gran corazón y la sonrisa enorme que teníais. Gracias por ser nobles, valientes y luchadoras. Gracias por intentar transmitirme tanta sabiduría y tanto amor. Espero que os podáis sentir orgullosas de mí desde allá desde donde ahora estáis y día a día lo seguiré intentando. Os quiero y jamás os olvido.

Publicaciones

El contenido de esta Tesis Doctoral ha originado la publicación de los siguientes artículos de investigación y comunicaciones en congresos científicos.

Artículos de investigación:

1. Carrasco, A.; Ortiz-Ruiz, V.; Martinez-Gutierrez, R.; Tomas, V.; Tudela, J. (2015) *Lavandula stoechas* essential oil from Spain: Aromatic profile determined by gas chromatography–mass spectrometry, antioxidant and lipoxygenase inhibitory bioactivities. *Industrial Crops and Products*. dx.doi.org/10.1016/j.indcrop.2015.03.088, Factor de impacto (JCR-2014): 2.837, Categoria: Agronomy, posición: 9 de 81, cuartil: Q1.

2. Carrasco, A.; Martinez-Gutierrez, R.; Tomas, V.; Tudela, J. (2015) Lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur) essential oil from Spain: determination of aromatic profile by gas chromatography–mass spectrometry, antioxidant and lipoxygenase inhibitory bioactivities. *Natural Product Research*. dx.doi.org/10.1080/14786419.2015.1043632, Factor de impacto (JCR-2014): 0.919, Categoria: Applied chemistry, posición: 46 de 70, cuartil: Q3.

3. Carrasco, A.; Martinez-Gutierrez, R.; Tomas, V.; Tudela, J. (2015) *Lavandula angustifolia* and *Lavandula latifolia* essential oils from Spain: aromatic profile and bioactivities. *Planta Medica*. dx.doi.org/10.1055/s-0035-1558095, Factor de impacto (JCR-2014): 2.152, Categoría: Plant Sciences, posición: 60 de 200, cuartil: Q2.

4. Carrasco, A.; Tomas, V.; Tudela, J.; Miguel, M.G. (2015) Comparative study of GC-MS characterization, antioxidant activity and hyaluronidase inhibition of different species of *Lavandula* and *Thymus* essential oils. *Flavour and Fragrance Journal*. dx.doi.org/10.1002/ffj.3283, Factor de impacto (JCR-2014): 1.970, Categoría: Applied Chemistry, posición: 22 de 70, cuartil: Q2.

5. Carrasco, A.; Perez, E.; Cutillas, A.B.; Martinez-Gutierrez, R.; Tomas, V.; Tudela, J. (2015) *Origanum vulgare* and *Thymbra capitata* essential oils from Spain: determination of aromatic profile and bioactivities. *Natural Product Communications*. dx.doi.org//, Factor de impacto (JCR-2014): 0.906, Categoría: Food Science & Technology, posición: 76 de 123, cuartil: Q3. Aceptado.

Comunicaciones en congresos internacionales:

1. Carrasco, A.; Ortiz, V.; Martinez-Ruiz, J.; Parra, M.; Martinez, J.A.; Sanchez, M.; Tudela, J. (2010) Spike lavender extracts: aromatic profile and antioxidant capacity. En New Biotrends in Green Chemistry. Exploring the potential of synthetic biology for natural product discovery and production. Harenberg, Dortmund (Alemania). TU Dortmund. Laboratory of Technical Biochemistry.

2. Carrasco, A.; Medina, X.; Tomas, V.; Martinez-Gutierrez, R.; Martinez, FJ.; Sanchez, M.;Tudela, J. (2012) *Lavandin Grosso* from Spain: aromatic profile by enantioselective gas chromatography-mass spectrometry. ANQUE's International Congress of Chemical Engineering. Sevilla (Spain). ANQUE.

3. Carrasco, A.; Medina, X.; Martinez-Gutierrez, R.; Martinez, JA.; Sanchez, M.; Tomas, V.; Tudela, J. (2012) *Lavandula angustifolia* Mill. from Spain: aromatic profile by enantioselective gas chromatography-mass spectrometry. Congress 22nd IUBMB & 37th FEBS "From single molecules to systems biology". Sevilla (Spain). FEBS, IUBMB, SEBBM.

4. Medina, X.; Carrasco, A.; Tomas, V.; Martinez-Gutierrez, R.; Martinez, JA.; Sanchez, M.; Tudela, J. (2012) *Lavandin Super* From Spain: Aromatic Profile By Enantioselective Gas Chromatography-Mass Spectrometry. 15th European Congress on Biotechnology, "Bio-Crossroads". Istambul (Turkey). European Federation of Biotechnology and Biotechnology association of Turkey.

5. Espinoza, D.; Carrasco, A.; Tomas, V.; Martinez-Gutierrez, R.; Martinez, J.A.; Sánchez, M.; Tudela, J. (2013) *Lavandula stoechas* from Spain: aromatic profile by enantioselective gas chromatography-mass spectrometry. 13th International Conference on Antioxidants. Marrakech (Morocco). International Society of Antioxidant in Nutrition and Health (ISANH).

Abreviaturas

AA – Ácido ascórbico

AAPH – 2,2'-azobis(2-metilpropionamidina) dihidrocloruro

ABTS – ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico)

AOH – Antioxidante

$AO^{\bullet}$ – Antioxidante oxidado

AUC – Área bajo la curva

BHA – Butilhidroxianisol

BHT – Butilhidroxitolueno

ChP – Poder quelatante

DOI – Identificador digital de objeto (código único para cada artículo publicado)

DPPH – 2,2-difenil-1-picrilhidracilo

ECM – Matriz extracelular

EDTA – Ácido etilendiaminotetraacético

EI – Impacto electrónico

EIC – Cromatograma de ión extraído

EsGC – Cromatografía de gases enantioselectiva

FerroZine – Hidrato de la sal monosódica del ácido 3-(2-piridyl)-5,6-difenil-1,2,4-triazina-p,p′-disulfónico

FGC – Cromatografía de gases rápida

FID – Detección por ionización en llama

GC – Cromatografía de gases

GPX – Enzima glutatión peroxidasa

GSH – Glutatión reducido

GSSG – Glutatión oxidado

HETP – Altura equivalente de placa teórica

ISO – Organización internacional de normalización

λ_{ex} – Longitud de onda de excitación

λ_{em} – Longitud de onda de emisión

LOD – Límite de detección

LOQ – Límite de cuantificación

LRI – Índice de retención lineal

MDA – Malondialdehído

MS/MSD– Detección por espectrometría de masas

NADPH – Nicotinamida adenina dinucleótido fosfato reducida

ORAC – Capacidad de absorción de radicales de oxígeno

PBS – Tampón fosfato salino

pDMAB – p-Dimetilaminobenzaldehído

PTFE – Politetrafluoroetileno (Teflon ®)

RdP – Poder reductor

RNS – Especies reactivas de nitrógeno (radicales libres nitrogenados)

ROS – Especies reactivas de oxígeno (radicales libres oxigenados)

RSD – Desviación estándar relativa

SDS – Dodecilsulfato sódico

SIM – Monitorización selectiva de iones

SOD – Enzima superóxido dismutasa

STD – Estándar interno

TBA – Ácido tiobarbitúrico

TBARS – Sustancias reactivas con el ácido tiobarbitúrico

TEAC – Capacidad antioxidante equivalente a Trolox

TIC – Cromatograma de iones totales

TID – Detección de iones traza

TOF – Espectrometría de masas por tiempo de vuelo

Trolox – ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxilico

Summary index

This work has been partially conducted in English in order to qualify for the International Doctor certificate. The following index is made to help find the English content of this work, preceded by an English summary of the developed study.

Índice

Introducción

“Tenga cuidado. A la gente le gusta que les digan lo que ya saben. Recuerde eso. Se ponen incómodos cuando uno les cuenta cosas nuevas. Las cosas nuevas... bueno, las cosas nuevas no son lo que se esperan.”

La Verdad. Terry Pratchett.

1. INTRODUCCIÓN

Desde los albores de la humanidad, se ha hecho uso de plantas aromáticas y brebajes hechos con éstas para aliviar dolores y enfermedades así como para condimentar y conservar comidas. Con el paso del tiempo y el avance de la técnica, además de las plantas en bruto, se comenzaron a utilizar los aceites esenciales extraídos de éstas.

Aún hoy en día, estas plantas y sobre todo sus aceites esenciales, son ampliamente utilizados en diversas industrias como la alimentaria, cosmética y farmacéutica. Sin embargo, la composición y calidad exacta de estos aceites esenciales no están absolutamente determinadas; asimismo, las propiedades que pueden presentar tampoco están todas comprobadas y verificadas.

1.1 Plantas aromáticas

La productividad total de un bosque es la suma de una serie de contribuciones parciales, relacionadas con la formación de distintos productos vegetales que se originan en árboles, arbustos, plantas herbáceas, etc. En todos ellos, suele aparecer una flora aromática que ha sido objeto, y lo sigue siendo en la actualidad, de un aprovechamiento industrial que viene practicándose desde tiempo inmemorial en muchos países del mundo (Garcia-Estringana et al., 2010; Hendrawati et al., 2010; Herrera y Bazaga, 2008; Herrera y Jovani, 2010; Singh, 2011; Vinogradova y Azimova, 2011). Refiriéndonos a Europa, destacan los países de la Cuenca Mediterránea. Entre ellos, desde el punto de vista aromático, la península Ibérica ocupa, indiscutiblemente, un puesto prominente. La diversidad, riqueza y finura de los aceites esenciales contenidos en nuestra flora espontánea son notorias, convirtiendo a España, en ciertos casos, en el principal o único proveedor mundial de algunos de ellos (Gaviña Mújica y Torner Ochoa, 1966).

Así, las plantas aromáticas vienen siendo usadas por el aroma que desprenden en campos tan dispares y necesarios como la cocina, siendo parte imprescindible de gastronomías típicas, la perfumería, habitualmente en la forma de sus aceites esenciales, o la medicina por las propiedades curativas que tradicionalmente se les atribuyen.

Pese a los múltiples usos que se dan a estas plantas, el conocimiento de los factores que influyen en la calidad y cantidad de aceites esenciales que se extraen de las mismas son poco concretos. Es evidente que las características climáticas y

edáficas de las distintas regiones españolas varían mucho, pudiendo cambiar con ellas los rendimientos y calidades de los aceites conseguidos en las mismas (Gaviña Mújica y Torner Ochoa, 1966). Modelos válidos para el estudio de variaciones sobre la composición final del aceite esencial, así como para comprobar las posibles propiedades medicinales o aromáticas que puedan mostrar, son las plantas aromáticas espliego, lavanda, lavandín, cantueso, tomillo rojo, tomillo de invierno, orégano español y orégano italiano. El género *Lavandula*, de la familia de las *Lamiaceae*, cuenta con más de 35 especies y más de 100 variedades de lavandas, que muestran diferencias en hábitos de crecimiento, caracteres morfológicos, incluyendo forma de la hoja, distribución de las flores en los verticilos y composición química (Lis-Balchin, 2004). El género *Thymus*, también de la familia de las *Lamiaceae*, es uno de los ocho géneros más importantes habida cuenta del número de especies que incluye, más de trescientas incluyendo híbridos, variedades y ecotipos (Figueiredo et al., 2008). Finalmente, los géneros *Thymbra* y *Origanum*, también de la familia de las *Lamiaceae* están representados en este estudio por las especies *Thymbra capitata* y *Origanum vulgare*.

1.1.1 Espliego

El espliego (*Lavandula latifolia* Medikus) es una especie mediterránea, que crece silvestre en los montes españoles (Figura 1.1), aunque también es frecuente en el sur de Francia e Inglaterra. Es un arbusto con tallos florales ramificados y más largos que los de la lavanda fina o verdadera (*Lavandula angustifolia*); así mismo, las flores son más pálidas y las hojas más largas y anchas que las de la lavanda verdadera. Su cultivo es óptimo entre los 700 y los 1000 metros de altura, bajo un clima mediterráneo semiárido de inviernos fríos y veranos secos. El espliego vive espontáneamente sobre suelos pardo-calizos, pobres en materia orgánica, pedregosos, secos y que drenen bien. Esta especie se puede multiplicar por esqueje y por semilla, siendo su recolección recomendada a mediados de agosto, cuando se encuentra en plena floración (Luna Lorente, 1980).

España es el mayor productor de aceite esencial de espliego con 150-200 toneladas por año (Lesage-Meessen et al., 2015). El aceite esencial obtenido de las flores es un aroma de nota media o cuerpo, fuertemente alcanforado, de base herbácea y ligeramente floral. El aceite está considerado como antibacteriano, antiséptico, cicatrizante, regulador digestivo, antiespasmódico, antihipertensivo y relajante, en las bajas dosis recomendables para evitar efectos perjudiciales. Recientes estudios parecen apoyar bioactividades adicionales como ansiolítico, antidepresivo, antinociceptivo, antiinflamatorio, antitumoral y regulador inmunitario

(Azimova et al., 2011; Cavanagh y Wilkinson, 2002; Herraiz-Peñalver et al., 2013; Roller et al., 2009; Woronuk et al., 2011).

Existen variedades de espliego adaptadas al cultivo en distintos continentes, con sus respectivos bioquimiotipos (Alatrache et al., 2007; Barazandeh, 2002; Eikani et al., 2008; Seino et al., 2008; Tschiggerl y Bucar, 2010). En España, se han realizado estudios sobre la composición molecular de los aceites esenciales de espliego cultivado, en un estudio promedio por algunas partes del territorio nacional y en las regiones de Vitoria, Jaén y Valencia (Muñoz-Bertomeu et al., 2007; Parejo et al., 2002; Salido et al., 2004). Sin embargo, no existen publicaciones sobre los bioquimiotipos de los aceites esenciales de espliego cultivado en la Región de Murcia.

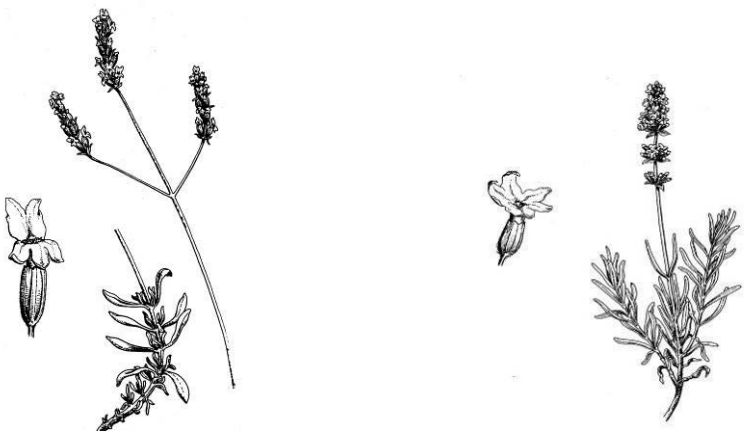


Figura 1.1
Dibujos clásicos de espliego (*Lavandula latifolia*, izquierda) y lavanda (*Lavandula angustifolia*, derecha)
Imágenes libres de copyright: commons.wikimedia.org

1.1.2 Lavanda

La lavanda (*Lavandula angustifolia* Miller), anteriormente conocida como *L. officinalis* Chaix ex. Vill., *L. spica* L., *L. vera* D.C., lavanda verdadera, fina o lavanda inglesa, es un arbusto perenne nativo del sur de Europa y la zona mediterránea, donde se cultiva ampliamente (Da Porto y Decorti, 2008). Su período de floración es de junio a julio y nace espontáneamente en altitudes de 800 a 1500 m. El arbusto tiene hojas lineales, estrechas de verde pálido o ligeramente argénteas con llamativas flores (Figura 1.2) de color azul-violeta (Lis-Balchin, 2004).

Francia, España, Portugal, Hungría, Reino Unido, Bulgaria, Australia, China y EEUU cultivan esta planta, su uso se ha incrementado en los últimos años debido a su agradable aroma y a las presuntas propiedades de su aceite esencial (Verma et al., 2010). El aroma del aceite esencial obtenido de las flores se enmarca como nota alta-media o cabeza-cuerpo, dulcemente floral y con un frescor relajante. El aceite esencial

de lavanda es uno de los ingredientes más preciados en la producción de aromas para cosmética, siendo también usado en la industria farmacéutica por sus propiedades

Figura 1.2
Fotografías de arbusto y detalle de flor de espliego (*Lavandula latifolia*, arriba) y lavanda (*Lavandula angustifolia*, abajo).
Imágenes del Real Jardín Botánico www.anthos.es

funcionales (Lubbe y Verpoorte, 2011). La producción de aceite de lavanda es de 200 toneladas por año, siendo los países que dominan el mercado Bulgaria, Reino Unido, Francia, China, Ucrania, España y Marruecos. Bulgaria es el mayor productor de aceite de lavanda con 3700 ha de cultivos de lavanda y hasta 100 toneladas por año, seguida de las 40 toneladas por año y 3500 ha de Francia, el aceite de lavanda tuvo un precio de 85-150€/kg en 2013 (Lesage-Meessen et al., 2015).

En varios países como China (Cong et al., 2008), Australia (Danh et al., 2013), India (Verma et al., 2010) o España (Castilla La Mancha) (Santana et al., 2012) se han realizado estudios sobre la composición de los aceites esenciales de *Lavandula angustifolia*. Sin embargo, no existen publicaciones sobre los aceites esenciales de lavanda cultivada en la Región de Murcia.

1.1.3 Lavandín

El híbrido natural y estéril de la lavanda y el espliego es conocido como lavandín o lavanda holandesa (*Lavandula* x *intermedia* Emeric ex Loisel, también *L. hybrida* L.). El lavandín (Figura 1.3) es un arbusto aromático, más vigoroso y productivo que sus progenitores, la forma de la planta es más desarrollada y en forma

de bola. Los tallos florales tienen una longitud de 60 a 80 cm y la espiga es grande, puntiaguda y de color violeta, caracterizándose por la existencia de dos espiguillas laterales situadas en la base (Moré, 2008).

Figura 1.3
Fotografías de lavandín arbusto y detalle de flor (*Lavandula* x *intermedia*).
Imágenes de Royal Horticultural Society www.rhs.org.uk

El lavandín tiene un alto rendimiento de aceite esencial, al cual se le atribuyen propiedades como antibacteriano, antifúngico, antiparasitario e insecticida (Jianu et al., 2013; Moon y Shibamoto, 2009; Papachristos et al., 2004; Torras-Claveria et al., 2007). Actualmente, el aceite esencial, encuentra aplicación en la producción de comida, bebida y jabones, y es especialmente importante en la manufactura de ciertos "eau de cologne" y perfumes (Torras-Claveria et al., 2007).

Se han estudiado las composiciones de los aceites esenciales de lavandín de distintas localizaciones como Grecia (Papachristos et al., 2004), Francia (Bombarda et al., 2008), Japón (Seino et al., 2008), Turquía (Baydar y Kineci, 2009) e Italia (Cosimi et al., 2009). Ninguno de estos informes tiene en cuenta el caso del lavandín cultivado en la costa mediterránea española, específicamente la Región de Murcia. Tampoco hay muchos estudios de distribución quiral del aceite esencial de esta planta aromática, y el existente (Flores et al., 2005) sólo tiene en cuenta algunas biomoléculas específicas.

1.1.4 Cantueso

El cantueso (*Lavandula stoechas* L.), también conocido como lavanda francesa o lavanda coronada es un arbusto perenne, aromático que generalmente crece hasta un metro de altura con flores violetas en espiga (Figura 1.4). Crece en el medio natural en los países mediterráneos y ha sido usado como especia de cocina y componente en las industrias de la perfumería, alimentaria, de bebidas, jabones, cosmética y farmacéutica, en muchos casos en forma de aceite esencial (Benabdelkader et al.,

2011; Goren et al., 2002; Kaya et al., 2012; Msaada et al., 2012; Zuzarte et al., 2013). Estas aplicaciones tienen relación con las bioactividades como antibacteriano natural, antifúngico, insecticida y posible antioxidante y antiinflamatorio con baja toxicidad para las células epiteliales humanas, que presenta el aceite esencial de cantueso (Kirmizibekmez et al., 2009; Matos et al., 2009).

Figura 1.4
Fotografías de cantueso, arbusto y flor (*Lavandula stoechas*).
Imágenes del Real Jardín Botánico www.anthos.es

La composición del cantueso se ha estudiado en muestras de distintos países como: Argelia (Benabdelkader et al., 2011; Dob et al., 2006), Turquía (Giray et al., 2008; Goren et al., 2002; Inan et al., 2013; Kaya et al., 2012; Kirmizibekmez et al., 2009; Sertkaya et al., 2010; Topal et al., 2008), Italia (Angioni et al., 2006; Marongiu et al., 2010; Zuzarte et al., 2013), Francia (Ristorcelli et al., 1998), Grecia (Hassiotis, 2010; Tzakou et al., 2009), Portugal (Matos et al., 2009), Túnez (Msaada et al., 2012) e Irán (Ebadollahi et al., 2010). No existen datos en la bibliografía de estudios de la composición de cantueso cultivado en la Región de Murcia.

1.1.5 Tomillo rojo

El género *Thymus* se encuentra predominantemente en la región Mediterránea, Asia, el sur de Europa y el norte de África, el género comprende más de trescientas especies, incluyendo híbridos, variedades y ecotipos. El tomillo rojo (*Thymus zygis* Loefl. ex L.) es una planta endémica de la Península Ibérica (Figura 1.5), un arbusto perenne que puede llegar hasta los 0.2 m de altura, aromático, abundante en suelos con buen drenaje. Se han encontrado, al menos, ocho bioquimiotipos en la región sureste de España, donde las variaciones estacionales y estados fenológicos también son factores que contribuyen a la variación de los aceites esenciales de estas plantas (Moldao-Martins et al., 1999; Saez, 1995b). La creciente demanda de aceite esencial de tomillo para perfumería, cosmética y farmacología (Hazzit et al., 2009), se suma al

uso consolidado del mismo como conservante en la industria alimentaria, encontrándose entre los 10 aceites esenciales más utilizados a nivel mundial (Ehivet et al., 2011).

Figura 1.5
Fotografías de tomillo rojo, arbusto y flor (*Thymus zygis*).
Imágenes del Real Jardín Botánico www.anthos.es

Se han informado ciertas bioactividades en los aceites esenciales de tomillo rojo: antimicrobiana, antigiardiásica, antiviral, inhibidora de enzimas y antioxidante (Ballester-Costa et al., 2013; Blanco Salas et al., 2012; Dandlen et al., 2010; Dandlen et al., 2011a; Dandlen et al., 2011b; Gonçalves et al., 2010; Machado et al., 2010; Perez-Sanchez et al., 2012; Pina-Vaz et al., 2004; Youdim et al., 2002).

La composición del aceite esencial de tomillo rojo peninsular se ha determinado en ciertas localizaciones, como es el caso de los estudios de Portugal (Dandlen et al., 2010; Gonçalves et al., 2010; Moldão-Martins et al., 2002; Moldao-Martins et al., 1999; Pina-Vaz et al., 2004), los de Almería (Saez, 1995b), Jaén (Ballester-Costa et al., 2013), Córdoba (Penalver et al., 2005) y estudios sobre la quiralidad de alguna molécula relevante a nivel global (Stahl-Biskup y Sáez, 2002) y en Israel (Ravid et al., 1996). Sin embargo, el caso de Murcia no se ha estudiado todavía.

1.1.6 Tomillo de invierno

El tomillo de invierno (*Thymus hyemalis* Lange) se encuentra principalmente en la zona sureste de España: Alicante, Murcia y Almería (Figura 1.6), es un arbusto perenne, aromático, con floración en los meses de invierno, puede llegar a una altura de 0.5 m y se suele encontrar en lugares con alta insolación. La variabilidad química de esta especie ha sido estudiada por medio de su aceite esencial, detectando la presencia de al menos 4 bioquimiotipos, estables frente a cambios estacionales y condiciones edáficas y climáticas (Jordan et al., 2006; Saez, 1995a).

Figura 1.6
Fotografías de tomillo de invierno, arbusto y flor (*Thymus hyemalis*).
Imágenes del Real Jardín Botánico www.anthos.es

En 2008 se demostró la capacidad antimicrobiana del aceite esencial de *T. hyemalis*, estando relacionada con la cantidad de timol presente en el aceite y mostrándose el bioquimiotipo timol más activo que el bioquimiotipo carvacrol, también mostró cierta capacidad antioxidante (Rota et al., 2008; Tepe et al., 2011).

La composición del aceite esencial de tomillo de invierno ha sido estudiada en algunas localizaciones, como es el caso de Turquía (Tepe et al., 2011), Almería (Saez, 1995a) y campos de cultivo experimentales del sureste (Jordan et al., 2006; Rota et al., 2008).

1.1.7 Orégano español

El orégano español (*Thymbra capitata* [L.] Cav. syn. Coridothymus capitatus (L.) Rchb.f. y Thymus capitatus [L.] Hoffmanns. & Link) es un arbusto de hasta 0.4 m (Figura 1.7), perenne y herbáceo, comúnmente utilizado como especia culinaria en planta seca y como aditivo en forma de diversos extractos, entre ellos es especialmente relevante el aceite esencial por su facilidad de uso, su intenso aroma y sus diversas propiedades que se aplican en industrias como las de alimentación y cosmética, entre otras (Castroviejo, 1986-2012).

Adicionalmente se han descubierto otras posibles aplicaciones del aceite esencial de orégano español como antibacteriano (Nabavi et al., 2015), antifúngico (Tabti et al., 2014), citotóxico e inhibidor de la adhesión de patógenos al colon (Džamić et al., 2015), fitotóxico (Saoud et al., 2013) y antioxidante en sistemas biológicos (Hortigón-Vinagre et al., 2014).

Figura 1.7
Fotografías de orégano español, arbusto y flor (*Thymbra capitata*).
Imágenes del Real Jardín Botánico www.anthos.es

El aceite esencial de orégano español se ha estudiado en localizaciones como Italia (Napoli et al., 2010; Russo et al., 2013), Grecia (Economou et al., 2011; Skoula y Grayer, 2005), Portugal (Palmeira-de-Oliveira et al., 2012), Marruecos (Bakhy et al., 2013), Túnez (Ali et al., 2013; Amri et al., 2014; Hosni et al., 2013), Argelia (Tabti et al., 2014) y España (Ballester-Costa et al., 2013).

1.1.8 Orégano italiano

El orégano italiano (*Origanum vulgare* L.) es un pequeño arbusto achaparradeo de hasta 1.3 m de altura (Figura 1.8). Se puede encontrar en toda la península Ibérica y países mediterráneos, mostrando preferencia por los substratos básicos (Castroviejo, 1986-2012). La planta se usa principalmente seca para condimentar comidas y sus extractos, como en el caso del orégano español, también encuentran aplicación en diversas industrias. Entre todos los extractos, el aceite esencial es especialmente útil como aromatizante en las industrias alimentarias.

Figura 1.8
Fotografías de orégano italiano, arbusto y flor (*Origanum vulgare*).
Imágenes del Real Jardín Botánico www.anthos.es

La investigación de la aplicación de este aceite esencial también lo ha propuesto como potencial nematicida (Oka et al., 2000), antibacteriano y antioxidante aplicado sobre carne cruda (Krishnan et al., 2015).

La composición del aceite esencial de orégano italiano se ha estudiado en países como: Turquía (Koldas et al., 2015), Hungría (Veres et al., 2007), Bosnia (Stoilova et al., 2008), Italia (Bonfanti et al., 2012; de Falco et al., 2013; Lukas et al., 2008), Austria (Lukas et al., 2013), India (Bisht et al., 2009), Argentina (Farias et al., 2010), Méjico (Hernandez-Hernandez et al., 2014), Brasil (Mallet et al., 2014), Túnez (Mechergui et al., 2010) y Serbia (Bozin et al., 2006).

1.2 Aceites esenciales

El aprovechamiento de las plantas aromáticas se viene realizando desde la antigüedad por medio de extractos de las mismas. Las extracciones líquido-sólido más comunes, que aún hoy se realizan a diario en millones de hogares, son las infusiones acuosas de estas plantas. Sin embargo, industrialmente, los procesos extractivos sólido-líquido más comunes son los que involucran disolventes orgánicos, seguidos de su posterior destilación y reciclado, como es el caso de los concretos (extractos de planta en n-hexano u otros hidrocarburos), absolutos (extractos de concretos en etanol puro) y las tinturas (extractos de planta en etanol). También en los últimos años se han empezado a utilizar las extracciones con fluidos supercríticos de CO_2 líquido a altas presiones, que muestran una alta apolaridad similar a la del hexano. No obstante, si lo que deseamos es extraer únicamente la parte volátil, tendremos que recurrir a métodos de destilación, como es el caso de los aceites esenciales (Azimova et al., 2011; Rubiolo et al., 2010).

Los aceites esenciales son líquidos fuertemente olorosos, volátiles, oleosos y por tanto menos densos que el agua. El aceite esencial se puede producir en todos los órganos aéreos de la planta (flores, brotes, semillas, hojas, ramas, corteza y frutos), excepcionalmente en raíz como en el caso del gengibre, y se almacena en células secretoras, cavidades, canales, células epidérmicas o tricomas glandulares (Schwab et al., 2008) (Figura 1.9). Se extrae de plantas aromáticas como las anteriormente descritas, utilizando distintos métodos como destilación por arrastre en corriente de vapor (Figura 1.10), hidrodestilación, o destilación seca, de toda la planta o partes de la misma (hojas, tallos, frutos, etc.). También se puede obtener por prensado en frío, para el caso de las cortezas de los frutos cítricos (Biswas et al., 2009; Gounaris, 2010; Rubiolo et al., 2010).

La destilación por arrastre con vapor (extracción sólido-gas) se realiza en una sola etapa y es ampliamente utilizada en entornos industriales, se beneficia de la

capacidad extractora del vapor de agua sobrecalentado, pero sufre los problemas de realizarse en una única etapa, lo cual no es óptimo para aprovechar el máximo de los componentes a extraer, y puede provocar la degradación del producto obtenido por las altas temperaturas y presiones utilizadas en el proceso. De forma análoga actúa la extracción con fluido supercrítico, donde al variar la proporción de CO_2 y etanol en el fluido, se pueden conseguir extractos de menor o mayor polaridad en los componentes mayoritarios, aunque siempre con altas presiones y temperaturas crecientes al aumentar la polaridad con etanol (Azimova et al., 2011).

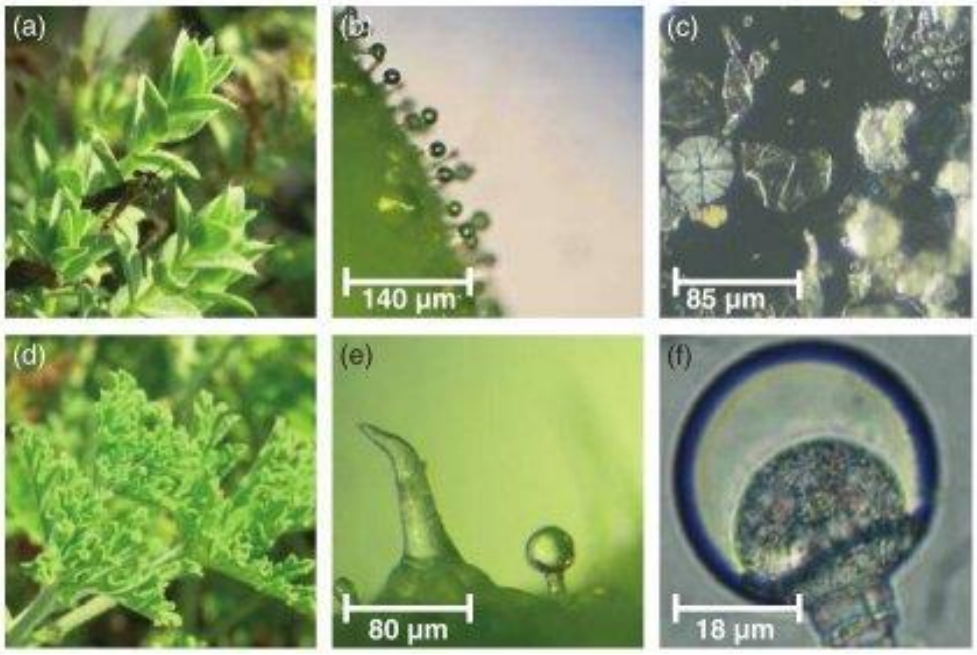


Figura 1.9
Tricomas glandulares con aceites esenciales en *Origanum dayi* (a-c) y *Pelargonium graveolens* (d-f) (Schwab et al., 2008)

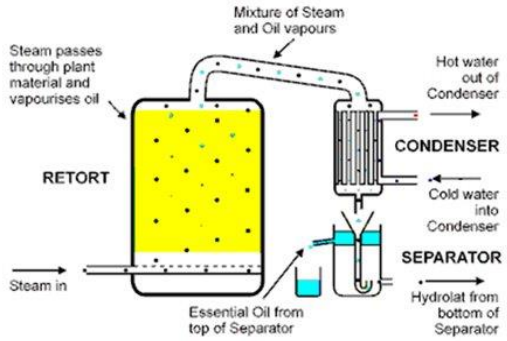


Figura 1.10
Esquema de un equipo industrial para destilación de aceites esenciales por arrastre de vapor de agua

Por otro lado, la hidrodestilación más usual en laboratorio con dispositivos tipo Clevenger, es una destilación en continuo en la que el material vegetal se encuentra en contacto directo con el agua, es decir, sumergido en ésta. Gracias a ser un proceso en continuo, permite aprovechar mejor la totalidad de aceite esencial contenido en el material vegetal. En este tipo de procesos continuos, el disolvente, es decir vapor de agua condensado, se recircula para realizar el proceso varias veces. La hidrodestilación es una extracción mixta sólido-líquido, sólido-gas y líquido-gas. En ella las burbujas de vapor de agua durante la ebullición extraen y/o arrastran los volátiles y los transportan hasta el condensador. La presencia de agua líquida sobre la masa vegetal provoca un choque osmótico, que actúa a favor de la extracción debilitando las resistencias que oponen las paredes celulares y la fibra vegetal en general (Baser and Buchbauer, 2010). Mejoras a este proceso como la hidrodestilación a vacío, para conseguir el mismo efecto con menores temperaturas, se encuentran aún a día de hoy en investigación, para optimizar su viabilidad económica a escala industrial.

En la naturaleza, los aceites esenciales juegan un papel importante en la atracción de los insectos para promover la dispersión del polen o las semillas. Además, debido a ciertas propiedades antibacterianas, antivirales, antifúngicas, insecticidas, herbicidas o disuasorias frente a herbívoros o insectos dañinos, se consideran también agentes defensivos de la planta (Azimova et al., 2011).

En general, los aceites esenciales son útiles en aromaterapia, además de mostrar propiedades bioactivas como potenciales agentes analgésicos, bacteriostáticos, fungicidas, diuréticos o expectorantes. Por ello se utilizan en las industrias de producto químico, perfumes, cosméticos, alimentos y fármacos (Adorjan y Buchbauer, 2010; Baser y Buchbauer, 2010; Burdock, 2009; Reineccius, 2005; Romeo et al., 2008).

Los aceites esenciales tienen una composición muy compleja que puede variar desde una docena hasta cientos de ingredientes por aceite. Están constituidos principalmente por fenilpropanoides y terpenoides volátiles, formados éstos últimos por unidades de isopreno enlazadas en estructuras con 10 (monoterpenoides) ó 15 (sesquiterpenoides) carbonos, que pueden contener una gran variedad de grupos funcionales como alcoholes, éteres, aldehídos, cetonas, ésteres, fenoles, óxidos, etc. (Figura 1.11) Las proporciones de las biomoléculas presentes en los aceites esenciales son altamente variables. Los componentes mayoritarios pueden constituir hasta un 85% del aceite esencial, mientras que el resto de ingredientes pueden alcanzar niveles de trazas (Baser y Buchbauer, 2010).

El aroma de cada aceite resulta de la combinación de los aromas de sus constituyentes mayoritarios y minoritarios, puesto que todos ellos pueden tener

propiedades organolépticas de gran importancia (Miguel, 2010a, b). La evaluación de la autenticidad de los aceites esenciales es clave para garantizar la seguridad y trazabilidad de los productos industriales que los contienen como ingredientes (Bauermann et al., 2008).

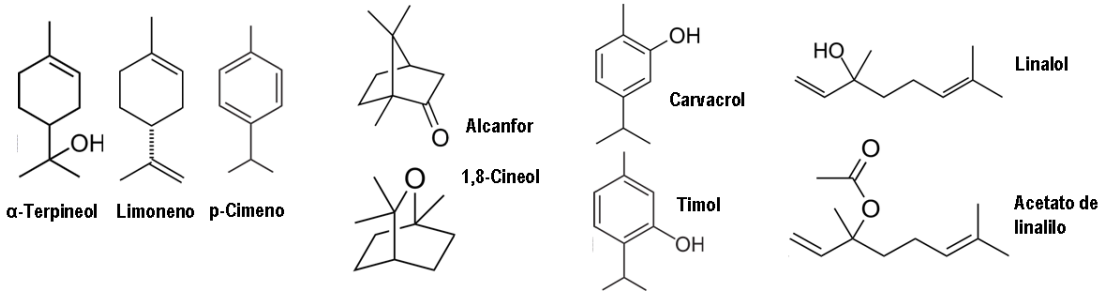


Figura 1.11
Estructuras de algunas biomoléculas presentes en aceites esenciales de plantas aromáticas

1.2.2 Cromatografía de gases

La técnica instrumental más apropiada para la identificación y determinación cuantitativa de los aceites esenciales es la cromatografía de gases (GC) con detección mediante espectrometría de masas (MS), puesto que es una técnica de alta resolución para moléculas volátiles. No obstante, esta técnica es lenta con instrumentos clásicos realizando cromatogramas de una o dos horas de duración para separar los numerosos ingredientes de los aceites esenciales. Además, predomina la cuantificación relativa de las biomoléculas mediante la simple integración de las áreas de pico de los cromatogramas, sin determinar la concentración absoluta de los principales ingredientes (Adams, 2007; AENOR, 2006; Berger, 2007; ISO, 2002, 2012).

Aunque la detección por ionización en llama (FID) se ha usado tradicionalmente por su amplio rango lineal de respuesta instrumental, actualmente, la detección por espectrometría de masas es más popular gracias a su adecuado rango lineal de respuesta instrumental y al gran valor de los espectros de masas obtenidos que podemos comparar con las bases de datos espectrales (Rubiolo et al., 2010; Smelcerovic et al., 2013).

Dependiendo de los compuestos a separar en la cromatografía se elige una u otra fase estacionaria para la columna. Para el caso común de compuestos con diferentes polaridades es útil recurrir a columnas polares o apolares.

En el caso de los volátiles orgánicos mayormente liposolubles que están contenidos en los aceites esenciales, la fase estacionaria más apropiada es la apolar

de silfenileno polimerizado altamente entrecruzada, tanto por su elevada resolución como por su bajo sangrado y prolongada vida útil.

La elección del diámetro interno y la longitud de la columna condicionan la rapidez de la cromatografía y su resolución. Las columnas de gran diámetro interno y elevada longitud proporcionan tradicionalmente muy buenas resoluciones cromatográficas y tienen capacidad para aceptar grandes volúmenes de muestra, pero consumen mucho tiempo para desarrollar un cromatograma (valor típico alrededor de 1 hora). Sin embargo, las columnas cortas de pequeño diámetro interno requieren menos tiempo (alrededor de 10 minutos), tienen capacidad para aceptar menor volumen de muestra (por lo que se pueden usar en detección de trazas) y peores resoluciones. Pero si se trata de columnas cortas capilares de fase estacionaria altamente entrecruzada (para evitar el sangrado en lo posible), y con poco espesor de película estacionaria (para mejorar la resolución), se pueden lograr resultados de alta resolución en bajos tiempos de cromatografía, en cromatógrafos cuya microfluídica admita elevada contrapresión (SUPELCO, 2010).

La demanda de cromatografías de gases rápidas se incrementa continuamente. Aunque ha sido investigada teóricamente desde el principio de los años 60, la cromatografía de gases rápida (Cramers et al., 1999) ha comenzado a usarse en la práctica para el análisis cromatográfico de aceites esenciales en esta última década.

La forma más efectiva de acelerar una separación por cromatografía de gases, es acortar la longitud de la columna manteniendo un poder de resolución suficiente. Para ello se han utilizado columnas cortas cada vez con menor diámetro interno e inferior espesor de fase estacionaria.

En la actualidad está aceptado que una cromatografía es “rápida” (Fast Gas Chromatography, FGC) cuando se resuelve en 15 minutos o menos (Figura 1.12), con columnas de diámetro interno entre 0.25 y 0.1 mm, espesor de fase estacionaria entre 0.25 y 0.1 µm, longitud entre 5 y 15 m, programas de temperatura con velocidades de entre 20 y 60 ºC/min y anchuras de pico en el rango de 0.5-5.0 s.

El uso práctico de la FGC se ha hecho posible gracias a la introducción gradual, en los últimos 10 años, de crecientes avances en la microfluídica de los cromatógrafos, el control electrónico de presión de la fase móvil, los detectores tipo FID, detectores por espectrometría de masas de alta velocidad con cuadrupolo o TOF, así como el desarrollo de equipos informáticos capaces de controlar estos dispositivos (Hübschmann, 2008; Pasikanti et al., 2008; Rubiolo et al., 2010).

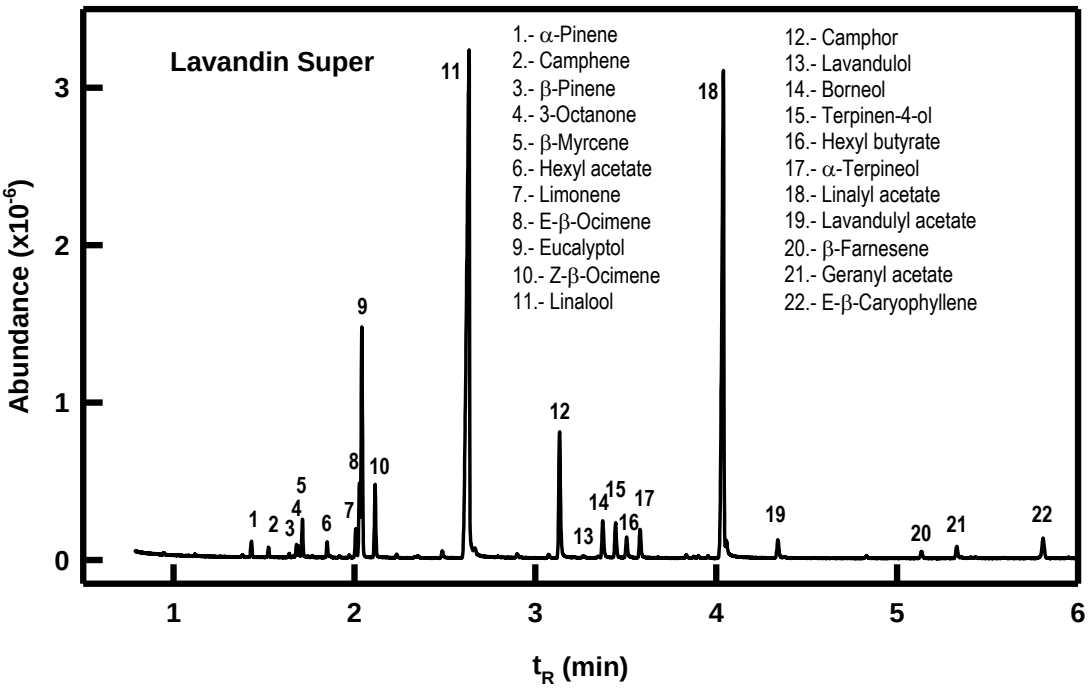


Figura 1.12
Comatograma FGC típico de lavandín var. Super con 22 compuestos mayoritarios identificados

Las fases móviles habituales son nitrógeno (N_2) y helio (He), situándose el hidrógeno (H_2) como fase móvil emergente, con la peligrosidad consabida de su reactividad y la capacidad explosiva que conlleva su acumulación en espacios cerrados (≥4% v/v).

De entre estas tres fases móviles de elección, sólo el helio y el hidrógeno se recomiendan para las cromatografías rápidas en columnas capilares, siempre secos y con la mayor pureza posible, debido a su mayor facilidad para ocupar el espacio de la columna capilar de forma homogénea y creando un frente de fase móvil lo más recto posible. El nitrógeno presenta perfiles parabólicos de avance en el frente de fase móvil cuando se utiliza en columnas capilares de bajo diámetro interno.

Descartado el nitrógeno, la fase móvil más habitual es el helio por su seguridad y buenas prestaciones pero existen dos factores que desaconsejan su uso. Por un lado está la necesidad de extracción de este helio, que se encuentra en los yacimientos de gas natural en una proporción que ronda el 1%. Si bien aun existen reservas mundiales de este gas, el agotamiento progresivo ya está dejando notar una escalada en los precios del mismo, que irá aumentando gradualmente hasta su agotamiento (Manning, 2008). Por otro lado, tal como muestran los estudios representados con curvas de Van Deemter (Figura 1.13), el comportamiento del helio a velocidades altas de fase móvil repercute en un menor rendimiento cromatográfico

que el del hidrógeno. Así podemos observar una menor altura equivalente de placas teóricas (HETP, mejor separación) a una velocidad lineal mayor con el gas portador hidrógeno.

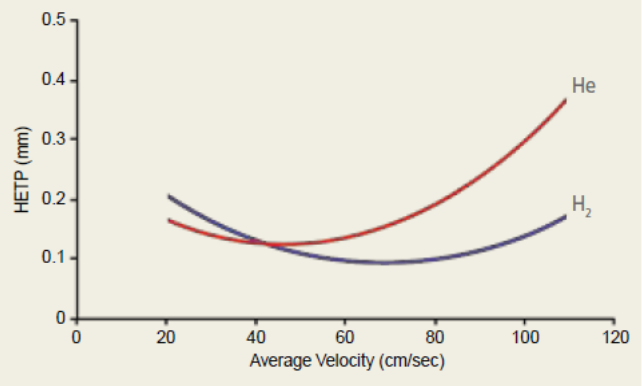


Figura 1.13
Curvas de Van Deemter de helio e hidrógeno
Columna de diámetro interno 0.1 mm (SUPELCO Analytical, 2010)

Las curvas de Van Deemter plasman empíricamente los resultados teóricos de la teoría de Golay de las columnas tubulares abiertas por la que la velocidad óptima del gas es proporcional a su difusión, el hidrógeno tiene mayor difusión que el helio por lo que su velocidad lineal óptima es mayor y puede ser utilizado con un caudal más alto sin afectar negativamente a la eficacia. Para que la cromatografía de gases sea efectivamente rápida es necesario que, dentro de la medida de lo posible, la fase móvil se desplace con la mayor rapidez por dentro de la columna, sin perder resolución. Esto lleva a que, a igualdad de resolución, se prefiera una velocidad lineal de gas portador lo más elevada posible para acortar el tiempo total de la cromatografía. En este caso, el hidrógeno permite trabajar con mejor resolución (menos de 0.1 mm de altura equivalente de placa teórica) que el helio, a velocidades de alrededor de 80 cm/s.

Una vez descartado el helio se presentan dos opciones con respecto al hidrógeno, éste puede ser comprado en balas o generado *in situ* por medio de hidrólisis electrolítica de agua ultra-pura. Los problemas principales de la compra de balas y su almacenamiento son:

- La peligrosidad de tener almacenadas grandes cantidades de un gas altamente explosivo.
- El riesgo de grandes pérdidas de gas explosivo en caso de fugas en el circuito de conducciones.
- El inconveniente de tener que cambiar las balas de gas cuando se agotan, pudiendo suceder esto durante una serie de experimentos.

Por otro lado, en el caso de generar hidrógeno, sólo se genera el necesario para el experimento en cuestión en cantidades capilares, reduciendo el riesgo de acumulación y explosión, puesto que no se puede disponer de altas presiones de este gas. Por otro lado, la pureza del mismo es muy alta y garantizada en todo momento. Además, el propio conjunto de generador y cromatógrafo ya dispone de sistemas de control electrónico de presión y seguridad que interrumpe la generación de hidrógeno en el caso de fugas, evitando acumulaciones imprevistas de hidrógeno. Otro elemento de seguridad es el uso de detectores automáticos de hidrógeno en el laboratorio, así como el continuo funcionamiento de un extractor de aire hacia el exterior del edificio.

Split es la voz inglesa con la que se denomina la relación de división que se da en el inyector. Cuando se introduce una muestra en el inyector, se puede elegir si se desea que esa muestra se diluya con el propio gas portador en una relación dada, o si se prefiere introducir la muestra completa en la columna. De esta manera existen los distintos tipos de inyección según el *split*, a saber: Inyección *split*, inyección *splitless* e inyección *on-column*. Estos tipos de inyección son susceptibles de variación para dar lugar a tipos de inyecciones derivadas como es el caso del *pulsed-split* y *pulsed-splitless*.

Fundamentalmente se utilizan las inyecciones *split* y *splitless*, la inyección *split* funciona de la siguiente manera: la muestra es inyectada y el flujo de gas portador es calculado, de manera que el volumen de gas portador que entra en relación con el volumen de muestra, produzca la dilución deseada. Hay que entender que lo que se produce no es una dilución al uso en ningún caso, sino más bien una distribución. El gas portador arrastra la muestra y la deposita en la cabeza de columna, desde donde la va arrastrando cuando la temperatura de la columna es la adecuada, así que no se trata de un gas que actúe como disolvente. Pero en el caso del *split*, el gas portador se mezcla con la muestra y se desecha parte de esa mezcla, de manera que se introduce en la columna sólo parte de la muestra inyectada. Esta disminución del volumen de muestra inyectada es la que se compara con una dilución de la misma, evitando incorporar un frente de disolvente que pueda enmascarar sustancias altamente volátiles.

Así, si queremos diluir la muestra 100 veces especificaremos un *split* de 100:1 lo que quiere decir que entrarán 104 ml/min de gas portador, de los cuales 3 ml/min se desecharán por la purga del *septum* (tiene la finalidad de evitar sobrepresiones), 1 ml/min entrará a la columna y 100 ml/min se eliminarán por la purga del *septum*. Habitualmente interesa trabajar de este modo ya que elimina la necesidad de hacer diluciones manuales, y permite inyectar muestra más concentrada sin saturar la columna cromatográfica. Por otro lado, está la inyección *splitless* que funciona

cerrando la purga de *split*. De esta manera, toda la muestra inyectada pasa a la columna. Este método se emplea en la detección de trazas, donde la concentración de la muestra es beneficiosa, no para determinar sus componentes mayoritarios, sino aquellos que podrían salir del rango de detección del aparato, ante una dilución de la muestra. En el caso de usar *splitless*, el inyector lo que hace es llenarse de gas portador y mezclarlo con la muestra vaporizada, dejando un tiempo largo y modificable para que toda la muestra pase a la columna, según su diámetro interno. Pasado este tiempo, se abre la válvula de *split* para limpiar el inyector (aquí cabe la posibilidad de una ligera pérdida de muestra). Este comportamiento es el que diferencia el *splitless* de la inyección *on-column*, en la que igualmente se inyecta toda la muestra pero sin acción del gas portador, es directamente la aguja la que deposita la muestra en la cabeza de la columna, evitando sobrecalentamientos que puedan dañar los compuestos termolábiles.

Las inyecciones derivadas "*pulsed*" incluyen la modificación de generar un pulso de sobrepresión controlada de gas portador, para acelerar y garantizar la entrada de la muestra deseada en la cabeza de columna, pero conllevan la desventaja de estropear la columna cromatográfica, aumentando significativamente el sangrado y reduciendo la vida útil de la cabeza de la columna. En el caso de trabajar con una columna EsGC las inyecciones *pulsed* están absolutamente contraindicadas por la fragilidad de la columna y el uso de la inyección *splitless* está desaconsejada por la ligera sobrepresión que se genera al principio del ensayo.

Tanto las temperaturas del inyector y las máxima y mínima del horno en todas las programaciones de temperatura, tienen que ser determinadas basándose en distintas variables. La limitación de temperatura máxima tanto del horno como del inyector está determinada, en un primer momento, por la máxima temperatura útil que soporta columna. Si se prevé que las sustancias a analizar pueden descomponerse o deteriorarse con la temperatura se pueden reducir estas temperaturas, principalmente la del inyector.

El flujo de gas portador óptimo se determina empíricamente basándose en el diámetro interno de la columna capilar, la máxima presión que soporta en cabeza de columna y la velocidad lineal que desarrolla dentro de la columna. Como se comentó anteriormente, es muy importante trabajar a un flujo que permita obtener una velocidad lineal que minimice la altura de placa teórica.

Un paso clave para poder realizar una cromatografía rápida de alta resolución es optimizar la programación de temperaturas. Una vez tenemos la muestra volatilizada por el inyector, ésta se carga en la columna que está "fría" y condensa parcialmente en la cabeza de la columna, asegurando un frente de avance de la

muestra homogéneo. La clave para elegir una temperatura mínima a la que empezar la cromatografía, está en prever el ingrediente más volátil y menos retenido por la columna, comenzando la cromatografía al menos 20ºC antes de alcanzar su punto de ebullición. En general ese ingrediente será el disolvente en el que esté preparada la muestra con el cual será necesario no sobrecargar la capacidad del detector, alargando su vida útil.

Para hacer la optimización completa del programa de temperaturas hay que basarse en resultados empíricos, repitiendo la cromatografía del tipo de muestra a analizar hasta ajustar un tiempo corto de cromatograma con una buena resolución, que sea capaz de separar todos los picos del cromatograma lo mejor posible, bajo las condiciones que permita el cromatógrafo. Otro parámetro importante para alagar la vida útil del detector, protegiendo del desgaste los filamentos y del envejecimiento al electromultiplicador es el retardo del disolvente, que consiste en no encender el detector hasta que haya pasado el pico correspondiente al disolvente, que suele ser especialmente grande y contribuye en gran medida a acortar la vida del detector, si es analizado rutinariamente. Este tiempo depende del tipo de disolvente y de la programación de temperatura de la cromatografía, así que debe determinarse nuevamente cada vez que cambien estas variables.

Los iones generados por impacto electrónico se seleccionan, enfocan y dirigen al electromultiplicador del detector, gracias al cuadrupolo de cuarzo monolítico hiperbólico recubierto de oro y trabajado a alta temperatura. El factor de ganancia constante garantiza que la respuesta del electromultiplicador será procesada por el software, de manera que el envejecimiento del detector no afecte a la señal obtenida por el usuario. Funciona aumentando la tensión a la que está sometido el detector cuando, en el sintonizado del equipo, se observan signos de pérdida de sensibilidad. De la misma manera, utilizar este factor permite ajustar la sensibilidad del equipo a voluntad, teniendo en cuenta que mayores sensibilidades provocan un rápido desgaste del mismo, aunque pueden ser necesarias en determinados casos.

Es de especial importancia determinar la velocidad de barrido y el intervalo de masas sobre el cual se realiza el barrido. El rango de masas puede variar desde la masa de 1 uma hasta la masa 1050 uma, teniendo en cuenta que cuanto más rango se quiera barrer, menor será la velocidad de barrido. A este respecto las claves están en el uso que se quiera dar a la información obtenida. A saber, para cuantificar un compuesto se requieren un mínimo de 15 barridos por pico (aquí habrá de tenerse en cuenta la anchura del pico más estrecho del cromatograma), mientras que si la intención es únicamente de cualificación se puede trabajar con 10 barridos por pico. La

velocidad de barrido se puede modificar, ajustando el rango de masas que queremos analizar, a las masas reales que pueden surgir.

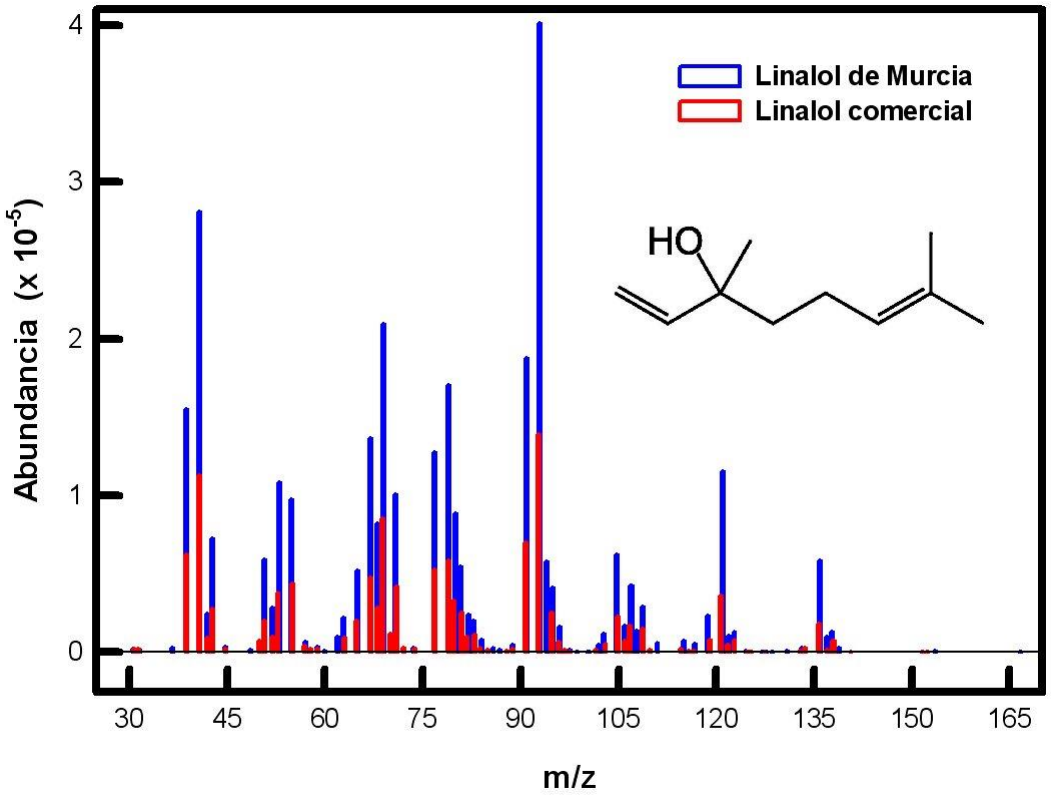


Figura 1.14
Espectro de masas de una biomolécula característica, linalol.

Hay dos parámetros que se usan como indicadores de la identidad de las moléculas presentes en los cromatogramas, el tiempo obtenido por la inyección individual de los patrones comerciales de los ingredientes de los aceites esenciales, lo cual es imprescindible para su cuantificación, y el contraste de los espectros de masas de todos los picos resueltos con los disponibles en bases de datos espectrales (NIST 08 y WILEY 7), y con los obtenidos en el laboratorio mediante una biblioteca de datos tomados en nuestro propio dispositivo de medida instrumental. La comparación con el programa NIST MS Search 2.0 (NIST, Gaithersburg, MD), confirma la identidad de la biomolécula correspondiente a cada pico del cromatograma. En la Figura 1.14 se ilustra un ejemplo de la satisfactoria identificación de un constituyente característico de los aceites esenciales de las plantas estudiadas, en concreto, linalol.

La identificación por coincidencia del tiempo de retención o coelución con patrones, se trata usando los índices de retención (LRI, linear retention indices) (van Den Dool y Dec. Kratz, 1963), este tratamiento facilita y universaliza los datos de retención de los compuestos, permitiendo su comparación relativa con otras columnas

de fases estacionarias similares. Estos datos también figuran en algunas bases de datos espectrales como la NIST 08 y Wiley 7. En el caso de aquellas sustancias de las que se dispone de patrón comercial (Figura 1.15), los LRI confirman la identificación tentativa junto con el espectro de masas. En este caso los LRI se calculan usando un patrón comercial consistente en una dilución de una serie de n-alcanos homólogos, siguiendo las recomendaciones de la IUPAC (IUPAC, 1997). El índice *I* correspondiente a cada compuesto se calcula usando la ecuación (1.1) para cromatografías con programa de temperatura, recomendada por la IUPAC:

$$I = 100\left[\frac{t_{Ri} - t_{Rz}}{t_{R(z+1)} - t_{Rz}} + z\right] \qquad (1.1)$$

Donde t_{Ri} es el tiempo de retención del compuesto problema, t_{Rz} es el tiempo de retención del alcano anterior en elución a *i* y $t_{R(z+1)}$ es el tiempo de retención del alcano siguiente en elución a *i*, *z* es el número de carbonos del alcano anterior en elución a *i*. Sin embargo, el uso de tiempos de retención o índices de los mismos (LRI) no es un factor determinante para la identificación inequívoca, puesto que distintos compuestos pueden llegar a colegir, y también porque algunos compuestos pueden variar su tiempo de retención ligeramente, por causa de la matriz de compuestos de la mezcla.

Gracias a la información obtenida se pueden realizar las comparativas de las proporciones de los compuestos principales con las normas internacionales para distintos aceites esenciales, que ha publicado la Organización Internacional de Normalización (ISO). El conjunto de biomoléculas presentado en estas normas es apropiado para identificar diferentes plantas a través de sus aceites esenciales, para distinguir variedades dentro de cada especie, y para detectar adulteraciones de aceites esenciales naturales con componentes sintéticos. Debe resaltarse que las normas ISO aplican únicamente el criterio de cuantificación relativa, utilizando porcentajes relativos de cada biomolécula. Por ello, es posible que dos aceites esenciales que cumplan la norma, contengan concentraciones absolutas distintas de sus ingredientes. Sin embargo, tendrá mayor valor añadido el aceite esencial cuyas biomoléculas se encuentren en mayor concentración absoluta, razón por la cual es recomendable realizar su laboriosa pero útil determinación.

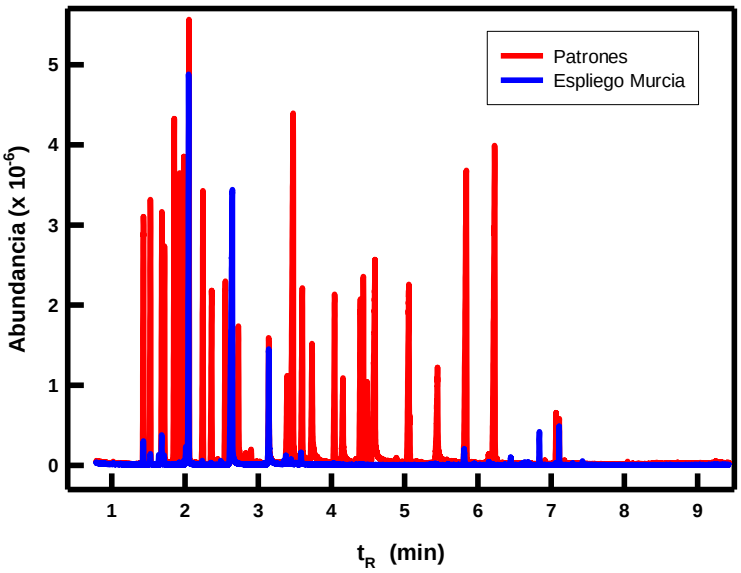


Figura 1.15
Cromatograma para identificación de los compuestos volátiles presentes en el aceite esencial de espliego de Murcia por coelución con patrones comerciales (GC/MS)

Para el caso de la cuantificación absoluta de los ingredientes, es imprescindible tener rectas de calibrado de los patrones con el consiguiente estudio que conlleva, puesto que en un detector por espectrometría de masas se debe asegurar la linealidad en el rango de concentraciones analizadas. Las rectas de calibrado se preparan a partir de los patrones puros de la siguiente manera: primero se selecciona el grupo de sustancias de interés no mutuamente interferentes, después se mezclan con la precaución de que, al final del método, todas las sustancias estén dentro del rango de linealidad del aparato. Esta disolución “madre” se diluye para abarcar todo el rango de concentraciones que interese dentro de la linealidad. La recta de calibrado es útil sólo durante el tiempo que el equipo funciona sin requerir una resintonización. La resintonización es un calibrado interno del espectrómetro de masas que se realiza cada semana o dos semanas, dependiendo del trabajo al cual el equipo ha estado sometido, y que modifica los parámetros electrónicos del equipo para dar siempre los resultados más precisos y veraces posibles, así aseguramos que las masas detectadas son siempre correctas y adecuadas. De esta manera la recta de calibrado requiere que se vuelva a realizar cada vez que se resintoniza el equipo para seguir trabajando con estas muestras. Además este tipo de rectas de calibrado de componentes volátiles no se deben mantener, ni siquiera en viales sellados guardados en frío puesto que la concentración de los componentes en las mismas puede variar, generando errores importantes en las determinaciones, así la recta de calibrado debe hacerse desde cero con cada resintonía del equipo.

Las mezclas y el manejo de estas sustancias se deben realizan con equipo de vidrio, ya que los aceites esenciales pueden corroer el plástico, y con micro-jeringas con émbolo de politetrafluoroetileno (PTFE, teflón) estancas a gas (Hamilton), para asegurar la precisión y la ausencia de posibles fugas. Para mejorar la reproducibilidad y la precisión de los cromatogramas obtenidos siempre se debe trabajar con las técnicas y materiales más adecuados a cada caso, como por ejemplo, en el inyector se debe usar un septum de bajo sangrado certificado para espectrometría de masas, *focus-liner* con lana de vidrio y *O-Ring* de alta temperatura de Supelco. Frascos de vidrio ámbar de 1.5 mL con tapones con septum de silicona/PTFE para los de rosca y PTFE/silicona/PTFE para los de sellado para muestras de larga duración, insertos de vidrio con interior mandrilado y pie polimérico para muestras de menos de 300 µL de Agilent. Todas las muestras deben ser filtradas con microfiltros de jeringa de 0.2 µm de PTFE de Agilent.

Para aumentar la reproducibilidad del método, en todos los cromatogramas se debe utilizar el método del estándar interno, que consiste en añadir una o más sustancias conocidas en cantidad determinada y siempre fija a todas las muestras a inyectar, bien sean aceites o patrones. Así se refieren todas las áreas que se obtengan de los picos del cromatograma a este área fija del estándar interno. De esta manera se evitan todas las posibles fluctuaciones debidas a la inyección de la muestra.

El programa AMDIS permite hacer la deconvolución espectral de los picos calibrados de un cromatograma, lo cual es necesario en el caso de que tengamos sustancias que casi coeluyan o picos que se solapen por cercanía. Este programa es capaz de separar esos picos basándose en unos potentes algoritmos de software, según los espectros de masas que definen ambos picos.

El programa de control instrumental ChemStation ayuda en la tarea de calibración, teniendo en cuenta la adición del estándar interno. Además, mejora el método de medida de áreas, enfocándose a la determinación de áreas de los iones representativos de cada sustancia (EIC – Extracted Ion Chromatogram), en lugar de trabajar sobre el cromatograma de iones totales (TIC – Total Ion Chromatogram). Del mismo modo, se le puede indicar que guarde las rectas de calibración, para que calcule automáticamente la concentración del analito tras integrar el pico. Al realizar la integración manual de cada pico en cada cromatograma, trabajando sobre el ión cuantificador del mismo, aseguramos la precisión del método.

El programa ya tiene en cuenta los factores de corrección de la calibración debida al área del estándar interno (STD), ya que las curvas de calibración las

representa como relación de respuestas $\left(\frac{\text{respuesta analito}}{\text{respuesta STD}}\right)$ frente a relación de concentraciones $\left(\frac{\text{concentración analito}}{\text{concentración STD}}\right)$.

Para asegurar la fiabilidad de los experimentos se calculan los parámetros de desviación estándar relativa (RSD), límite de detección (LOD) y límite de cuantificación (LOQ) que evalúan la repetibilidad y adecuación del ensayo sobre todos los patrones. Siguiendo las recomendaciones de la farmacopea europea (European-Pharmacopoeia, 2011), el RSD se calcula como el porcentaje de desviación de cinco medidas de muestras de patrones 3 mM. El LOD se calcula como la concentración de cada compuesto que da una relación señal/ruido de 3 y el LOQ se determina como la concentración de cada patrón que da una relación señal/ruido de 10.

1.2.3 Cromatografía de gases enantioselectiva.

La distribución quiral de los componentes de los aceites esenciales también es un aspecto muy importante de la composición real de los mismos, ya que permite identificar los aceites esenciales originales de aquellos que hayan sido adulterados por adición de mezclas racémicas sintéticas de los componentes naturalmente presentes, lo que aumenta algunas propiedades que mejoran la venta del producto a bajo precio. La distribución quiral de los componentes puede incluso ayudar a identificar cuándo los aceites son de diferentes puntos del planeta ya que cada situación de cultivo de las

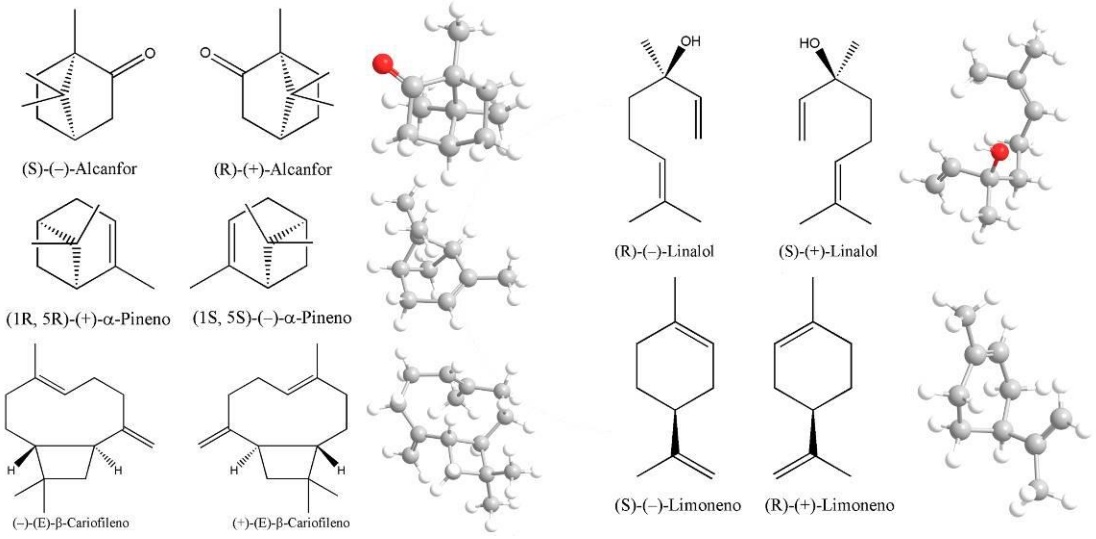


Figura 1.16
Algunos compuestos enantioméricos abundantes, presentes en aceites esenciales

plantas puede tener sus propias distribuciones quirales peculiares (Bicchi et al., 2010; Bicchi et al., 2008; del Castillo et al., 2004; Smelcerovic et al., 2013). Además, la

quiralidad de los terpenos (Figura 1.16) tiene gran importancia, ya que da lugar a diferentes bioactividades y propiedades organolépticas con cada enantiómero, esto encuentra su aplicación en fármacos, fragancias y aromas, entre otros (Baser y Buchbauer, 2010).

Las columnas enantioselectivas permiten obtener cromatogramas quirales de los componentes de los aceites esenciales. Las ciclodextrinas (Figura 1.17) son azúcares cíclicos hidrofílicos con forma troncocónica en el espacio que dejan una cavidad central hidrofóbica en su estructura, así, dependiendo del número de azúcares que conformen el ciclo tendremos mayores o menores huecos en el centro de la estructura. Partiendo de tres tipos de ciclodextrinas naturales, la α de 6 glucosas, la β de 7 glucosas y la γ de 8 glucosas, se han sintetizado numerosos derivados con distintas propiedades, como los selectores quirales introducidos en las columnas cromatográficas enantioselectivas. Las ciclodextrinas, como selectores quirales de la columna, se asocian con una cierta energía diferente a cada uno de los enantiómeros, formando complejos de inclusión, esta pequeña diferencia de energía es la que se traduce en distintos tiempos de retención y permite determinar la distribución enantiomérica. No obstante, para conseguir ver esta diferencia de tiempos de retención es necesaria una eficiencia cromatográfica muy alta y un preciso control de la temperatura, ya que el proceso, gobernado por la termodinámica, está basado en cinéticas muy rápidas (Bicchi et al., 2008).

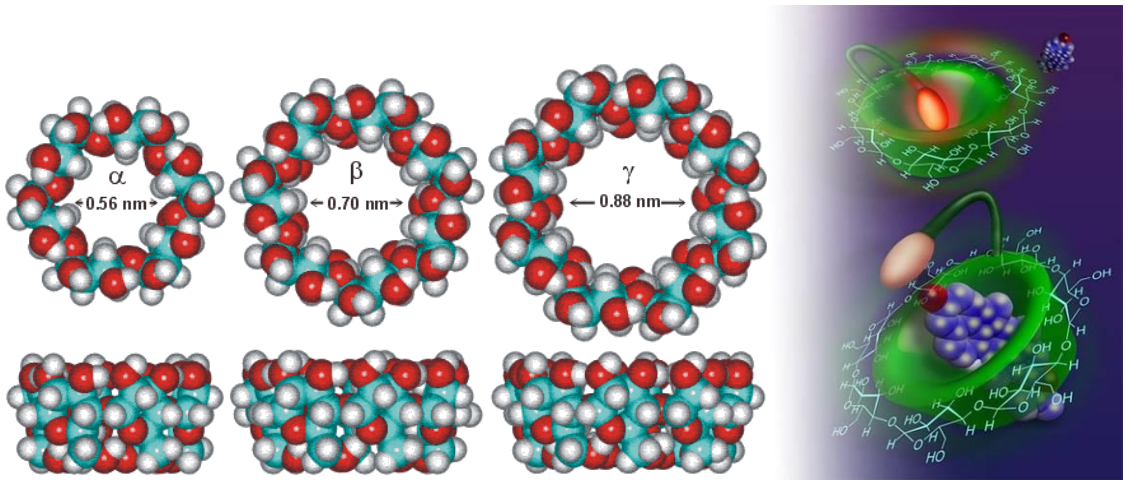


Figura 1.17
Ciclodextrinas α, β y γ según su tamaño. Inclusión temporal de una molécula en la ciclodextrina.
Imágenes de www.cyclolab.hu y www.isbu.ac.uk

Los compuestos quirales se determinan por comparación del tiempo de retención de los patrones enantiómeros puros comerciales y se comprueban por su espectro de masas (Figura 1.18).

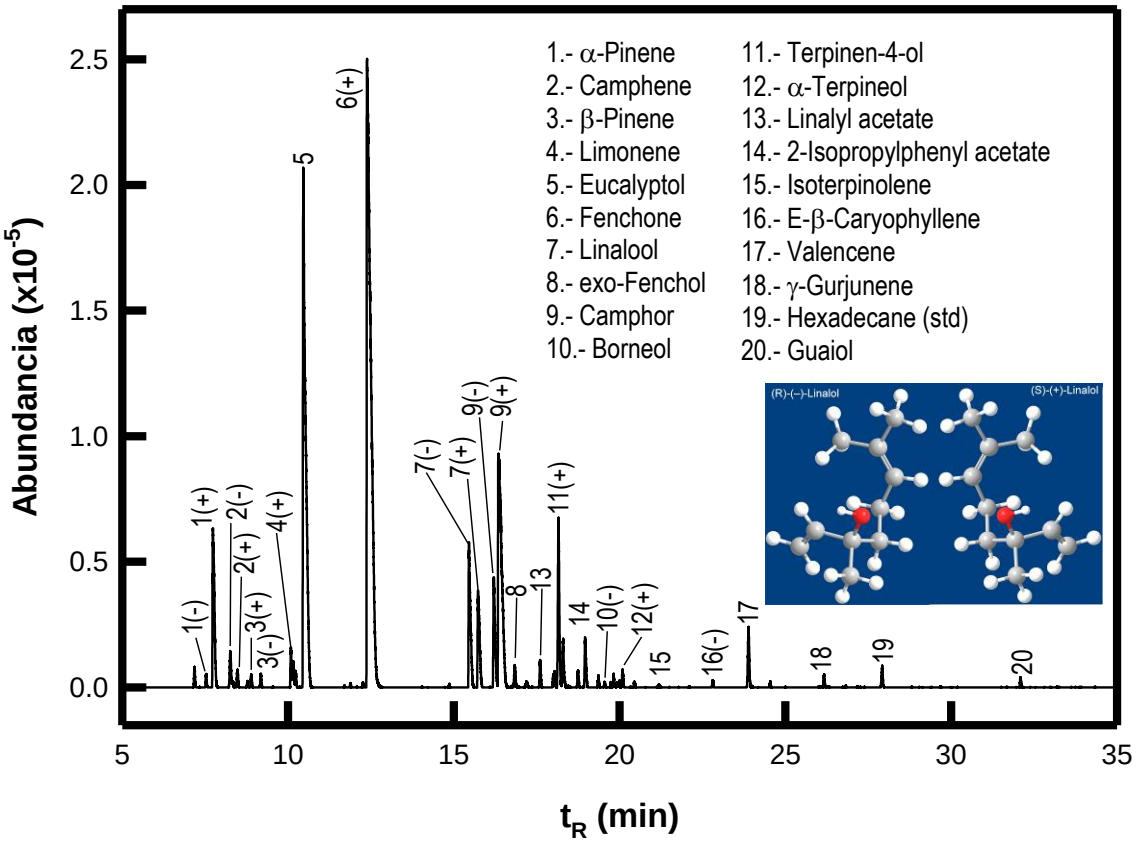


Figura 1.18
Cromatograma de gases enantioselectivo típico de cantueso. Enantiómeros de linalol en 3D

1.3 Capacidad antioxidante

En los últimos años, se han desarrollado muchos estudios sobre las propiedades biológicas de los aceites esenciales. Gracias a ellos se ha comprobado que los aceites esenciales pueden ser útiles como antinociceptivos, anticancerígenos, antiinflamatorios, mejoradores de la penetración epidérmica, repelentes de insectos, antivirales y antioxidantes. Sin embargo, cada aceite esencial tiene una composición particular, lo que le otorga unas propiedades determinadas cuyos mecanismos de acción habrán de ser estudiados (Adorjan y Buchbauer, 2010; Augustyniak et al., 2010; Krishnaiah et al., 2011; Niwano et al., 2011; Obrenovich et al., 2011; Wojcik et al., 2010).

La evaluación de las bioactividades de los aceites esenciales, comúnmente usados en medicina tradicional y aromaterapia, ha tomado relevancia por sus presuntos efectos terapéuticos. Los mecanismos por los cuales los aceites esenciales demuestran sus actividades en sistemas biológicos son muy complejos (Chao et al., 2005). El uso de ensayos *in vitro*, como los usados para medir la eliminación de radicales libres o la reducción de iones metálicos, sirve como modelo para la

evaluación preliminar de la bioactividad farmacológica (Huang et al., 2005). Los resultados relevantes obtenidos en esos ensayos se pueden utilizar para apoyar los presuntos efectos medicinales de los aceites esenciales.

De entre todas estas propiedades son especialmente reseñables las que se refieren a la capacidad antioxidante, fuertemente relacionadas con campos tan útiles y diferentes como pueden ser la salud humana, la cosmética y la alimentación.

La potencial actividad o capacidad antioxidante de los aceites esenciales es una propiedad biológica de gran interés, porque permite preservar los alimentos de los efectos tóxicos de los agentes oxidantes. Estos agentes oxidantes son habitualmente radicales libres (Figura 1.19) u otras especies reactivas. Cuando los aceites esenciales son capaces de neutralizar estas especies, pueden jugar un papel importante en alimentación pero también en sanidad ayudando a la prevención de diversos procesos y enfermedades relacionados con el estrés oxidativo, como: envejecimiento, arteriosclerosis, cáncer, enfermedades cardiovasculares, Alzheimer, disfunción cerebral, enfermedades neurológicas y depresiones del sistema inmunitario (Lu et al., 2010; Masaki, 2010; Mates et al., 2011).

Puesto que algunos aceites esenciales son capaces de actuar como antioxidantes, se piensa que también podrán ser agentes antiinflamatorios, ya que una de las respuestas inflamatorias está relacionada con la alteración oxidativa que ocurre en diversas células. En la fagocitosis de las bacterias, que ocurre durante la inflamación (Figura 1.19), se produce un uso intenso de oxígeno que resulta en la formación del anión radical superóxido ($O_2^{\bullet -}$), el cual es rápidamente transformado en peróxido de hidrógeno (H_2O_2) por la enzima superóxido dismutasa (SOD). El peróxido de hidrógeno entonces puede ser reducido por iones de metales de transición al radical hidroxilo ($HO^{\bullet}$), a través de reacciones de tipo Fenton. Radicales oxigenados como $O_2^{\bullet -}$ e $HO^{\bullet}$ pueden reaccionar rápidamente con ácidos grasos poliinsaturados (lípidos), resultando en la producción de radicales peroxilo ($ROO^{\bullet}$). Estos radicales se conocen comúnmente como ROS (especies reactivas de oxígeno). Además, existen otras especies reactivas que se producen durante los procesos inflamatorios, las llamadas RNS (especies reactivas de nitrógeno).

Tanto las ROS como las RNS se generan en los fagocitos para neutralizar a los organismos invasores, lo cual demuestra la gran importancia biológica que tienen estas especies. Sin embargo, una sobreproducción de estas especies es la responsable del daño sobre los tejidos en los lugares inflamados (Leopoldini et al., 2011; Miguel, 2010b; Perron y Brumaghim, 2009).

Los antioxidantes actúan como:

- Barreras físicas para prevenir la generación de especies radicales o su acceso a lugares biológicamente importantes.
- Sumideros de electrones aceptando los electrones desapareados de las especies radicales.
- Sistemas enzimáticos que neutralizan o desvían las especies radicales.
- Complejantes que se enlazan a iones metálicos para impedir la generación de especies radicales.
- Terminadores de las reacciones en cadena neutralizando y destruyendo las especies radicales.

Los antioxidantes primarios donan un electrón o un átomo de hidrógeno a un radical lipídico eliminándolo, y convirtiéndose a su vez en otro radical más estable y menos perjudicial, el cual puede ser neutralizado posteriormente a través de reacciones de desproporción, dimerización, adición, etc. Un importante ejemplo fisiológico es el tocoferol o vitamina E (Figura 1.19), cuya liposolubilidad favorece su actuación para la reducción de peróxidos lipídicos en las membranas celulares. A su vez, la vitamina E oxidada es reducida por el ácido ascórbico o vitamina C, hidrosoluble, en la interfase de las membranas celulares con el citosol.

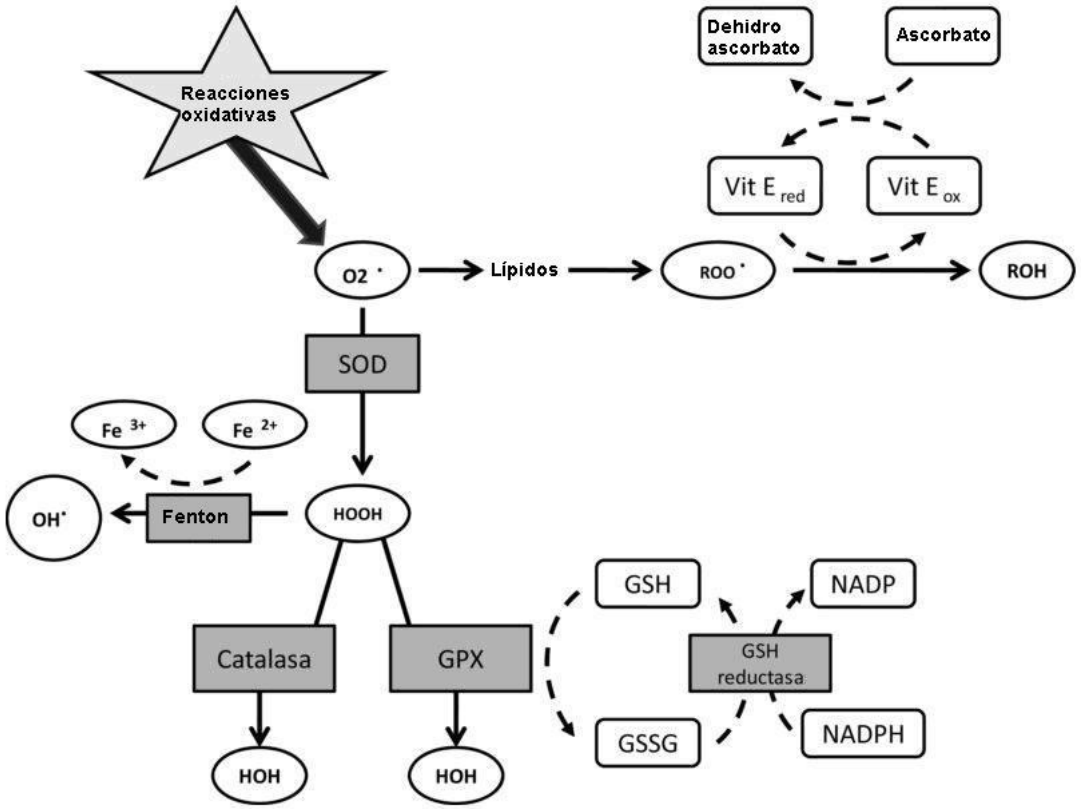


Figura 1.19
Metabolismo de especies de oxígeno reactivo (ROS) y antioxidantes fisiológicos

Por otra parte, los antioxidantes secundarios inhiben a las enzimas iniciadoras, reaccionan con radicales iniciadores o reducen el nivel de oxígeno en el medio. Así, los antioxidantes secundarios (o co-antioxidantes) retrasan el comienzo de la reacción de oxidación, por eliminación de los iniciadores. Ejemplos fisiológicos de estos antioxidantes son enzimas que reducen H_2O_2 a agua (Figura 1.19), como catalasa y glutatión peroxidasa (GPX). Ésta última consume glutatión reducido (GSH), cuya forma oxidada (GSSG) es reducida a su vez por glutatión reductasa, enzima que utiliza NADPH producido por la ruta de las pentosas fosfato. Tanto catalasa como GPX consumen el H_2O_2 presente en el medio impidiendo que se forme el radical $HO^{\bullet}$ por reacciones de tipo Fenton.

La capacidad antioxidante se atribuye a biomoléculas capaces de donar electrones o átomos de hidrógeno a radicales libres, las cuales se convierten transitoriamente en otro radical libre, menos reactivo y susceptible de rápida eliminación. Este proceso consiste en su conversión en especies no radicales, más estables y menos perjudiciales para el organismo, a través de reacciones de desproporción, dimerización, adición, etc. (Krishnaiah et al., 2011; Leopoldini et al., 2011; Lu et al., 2010) Esta actividad antioxidante se considera más intensa, en general, cuando la biomolécula contiene ciertos grupos reactivos electrodonadores, o deslocalizadores de electrones desapareados (Figura 1.20). También se ha de tener en cuenta que cada método antioxidante mide un aspecto distinto y será más sensible con distintos tipos de moléculas antioxidantes.

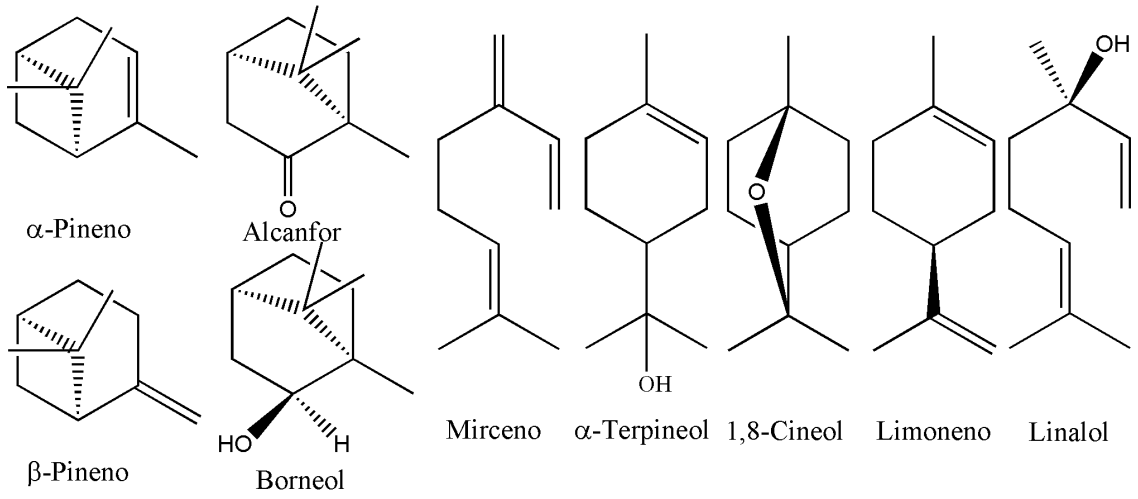


Figura 1.20
Estructura de biomoléculas características de los aceites esenciales

Estas propiedades se pueden usar para medir la actividad de estos antioxidantes, a través de diversos métodos (Armstrong, 2010; Celik et al., 2010; Huang et al., 2005; Karadag et al., 2009; Laguerre et al., 2010; Moon y Shibamoto, 2009; Niki, 2010a, b; Wang et al., 2011). Entre ellos se encuentran los métodos de medida indirectos, que informan acerca de la capacidad de ciertos antioxidantes, para neutralizar determinadas especies reactivas, como ABTS y DPPH. Por otra parte, existen métodos de medida directos que observan el descenso de oxidación del sistema al añadir antioxidantes.

Destacan los métodos directos basados en la autooxidación espontánea de la muestra, seguidos de la determinación de dienos conjugados, o bien de la reacción con ácido tiobarbitúrico (TBARS). Otros métodos de medir el poder antioxidante de una sustancia es comprobar la capacidad de la misma para reducir o quelatar iones metálicos como el hierro. Estos iones metálicos presentan actividad prooxidante, al reducir los iones de hierro o retirarlos del medio gracias a reacciones de quelatación, se observa una disminución de la oxidación del mismo. Los métodos más utilizados para medir estos parámetros son los que determinan la “capacidad quelatante” (ChP) y la “capacidad reductora” (RdP). El método “Oxygen Radical Absorbance Capacity” (ORAC) es directo y utiliza azo-compuestos termolábiles para iniciar, modificar y controlar las condiciones del ensayo. El método ORAC (Ou et al., 2001; Ou et al., 2002; Prior et al., 2003) es aceptado internacionalmente para la elaboración de tablas y bases de datos sobre la capacidad antioxidante de alimentos (Stockham et al., 2011; USDA, 2010; Wu et al., 2004).

Los tejidos tanto animales como vegetales contienen ácidos grasos insaturados, principalmente en la fracción de los fosfolípidos de membrana celular. Estos lípidos son especialmente susceptibles a la oxidación, puesto que tienen dobles enlaces deficientes en electrones. Los productos de ruptura oxidativa de estos ácidos grasos producen nuevos sabores y olores, a menudo desagradables, así como pérdida de nutrientes y deterioro del color. Así, es imprescindible el uso de antioxidantes capaces de interceptar radicales libres. En las industrias alimentaria y cosmética (Andre et al., 2010; Brewer, 2011; Gasperlin y Gosenca, 2011) es frecuente el uso de antioxidantes sintéticos como sulfitos, BHA, BHT o ácidos p-hidroxibenzoicos, con crecientes restricciones internacionales.

La actividad antioxidante de los aceites esenciales está en investigación debido a su potencial como conservantes, cosmecéuticos o nutracéuticos en las industrias alimentaria, cosmética y farmacéutica. Se especula que pueden actuar previniendo la peroxidación de lípidos, neutralizando radicales o, en algunos casos, quelando o

reduciendo iones metálicos. Los estudios sobre sinergias y antagonismos no se han desarrollado aún, debido a la complejidad resultante de las múltiples reacciones posibles (Miguel, 2010a; Wang et al., 2011). Entre ellos se encuentran biomoléculas con uno o más anillos aromáticos y uno o más grupos –OH, capaces de donar hidrógenos, así como biomoléculas alifáticas oxigenadas e hidrocarbonadas. Estos compuestos están presentes en los aceites esenciales que tienen, además, la ventaja de ser moléculas naturales, lo que les proporciona un valor añadido frente a los antioxidantes sintéticos.

1.3.1 Método ORAC

En este ensayo se mide la capacidad antioxidante de una sustancia frente las especies reactivas de oxígeno (ROS), de ahí proviene su nombre: Oxygen Radical Absorbance Capacity (ORAC). En este caso, partimos de un ensayo inicial en el que la intensidad de la fluorescencia de la fluoresceína decrece con el tiempo, oxidada por un radical peroxilo ($ROO^{\bullet}$) formado por la descomposición térmica de AAPH en disolución acuosa tamponada (Betigeri et al., 2005; Werber et al., 2011). En presencia de una muestra que contenga antioxidantes, capaces de romper la cadena oxidativa o capaces de neutralizar el radical peroxilo, el descenso de la intensidad de fluorescencia se verá retardado (Figura 1.21). A partir de las curvas de descenso de fluorescencia se determina la capacidad antioxidante, a mayor área bajo la curva (AUC), mayor capacidad antioxidante. Todas las medidas de muestras son comparadas con una muestra de Trolox, análogo estructural de la vitamina E con mayor solubilidad en agua, en las mismas condiciones para poder expresar la capacidad antioxidante en unidades TEAC (Trolox Equivalent Antioxidant Capacity) (Huang et al., 2005; Miguel, 2010a; Roginsky y Lissi, 2005). Los valores de AUC se calcularon usando la ecuación (1.2).

$$\mathrm{AUC} = 1 + \sum_{i=1}^{i=n} \frac{f_i}{f_0} \qquad (1.2)$$

Donde f_0 es la fluorescencia inicial medida a 0 min y f_i es la fluorescencia medida a cada instante *i*, hasta la medida *n* (normalmente *n* = 50–200).

Este método proporciona una curva de descenso de la fluorescencia, en ensayos sin (blanco) y con distintas concentraciones de antioxidante (muestra). A la correspondiente área bajo la curva se le resta la del blanco para cada concentración de muestra. Así se pueden representar los datos de área bajo la curva frente a la concentración de aceite esencial, y ajustarlos mediante regresión lineal. Trabajando de igual forma sobre el Trolox obtendremos su pendiente (Figura 1.22). Las unidades

TEAC que quedan para este método se expresan como “cantidad de Trolox equivalente por volumen de aceite esencial”(Ou et al., 2001; Ou et al., 2002; Prior et al., 2003). Así el valor ORAC relativo a Trolox se calcula con la siguiente ecuación (1.3):

$$\mathrm{ORAC} = \frac{\mathrm{AUC_{Muestra}} - \mathrm{AUC_{Blanco}}}{\mathrm{AUC_{Trolox}} - \mathrm{AUC_{Blanco}}} \times \frac{[\mathrm{Trolox}]}{[\mathrm{Muestra}]} \qquad (1.3)$$

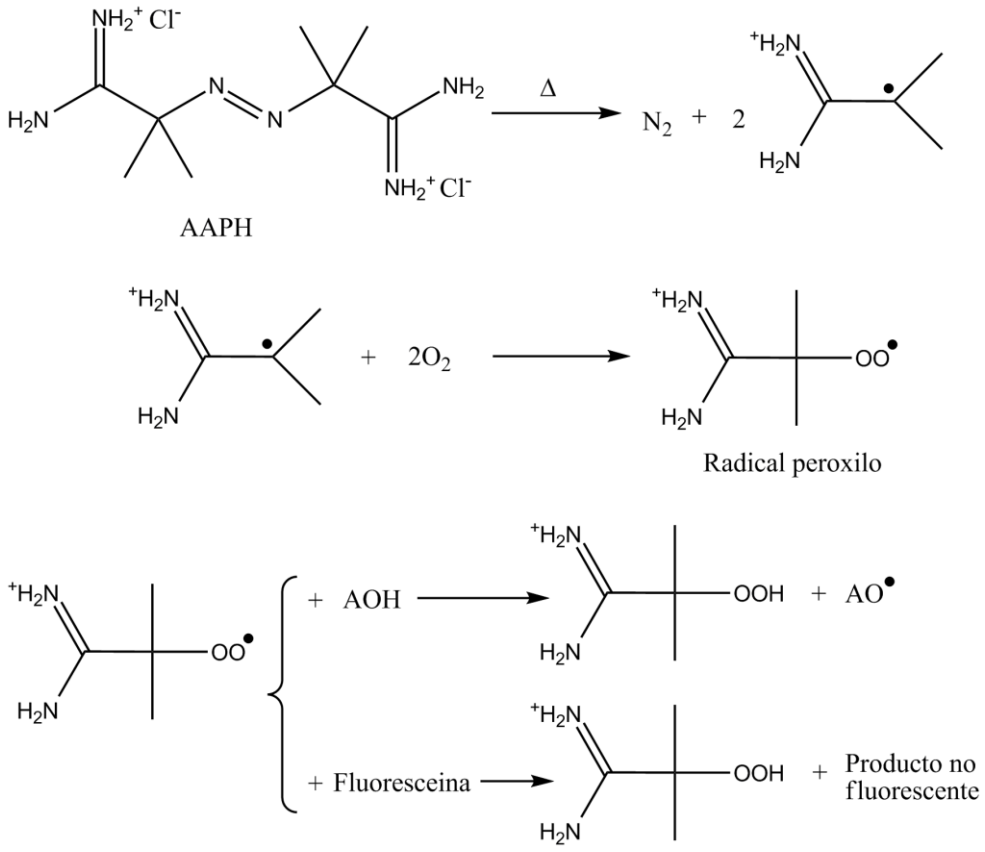


Figura 1.21
Descomposición térmica del AAPH, reacción con el oxígeno disuelto para formar el radical peroxilo y ataque del radical a fluoresceína o neutralización por un antioxidante (AOH)

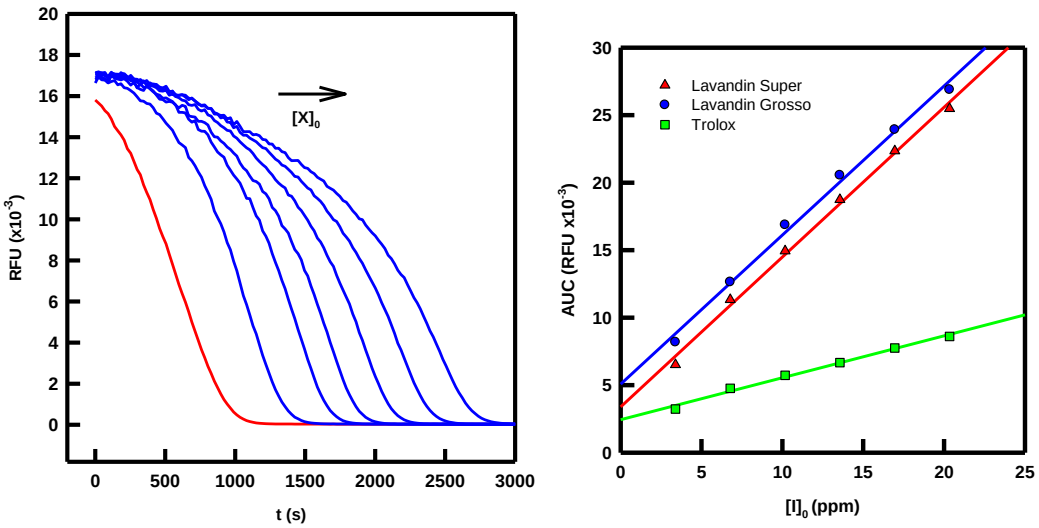


Figura 1.22
Registro de descenso de fluorescencia con el tiempo (izquierda) y pendientes obtenidas de datos de concentración frente a AUC de aceites esenciales comparados con Trolox (derecha)

1.3.2 Método ABTS

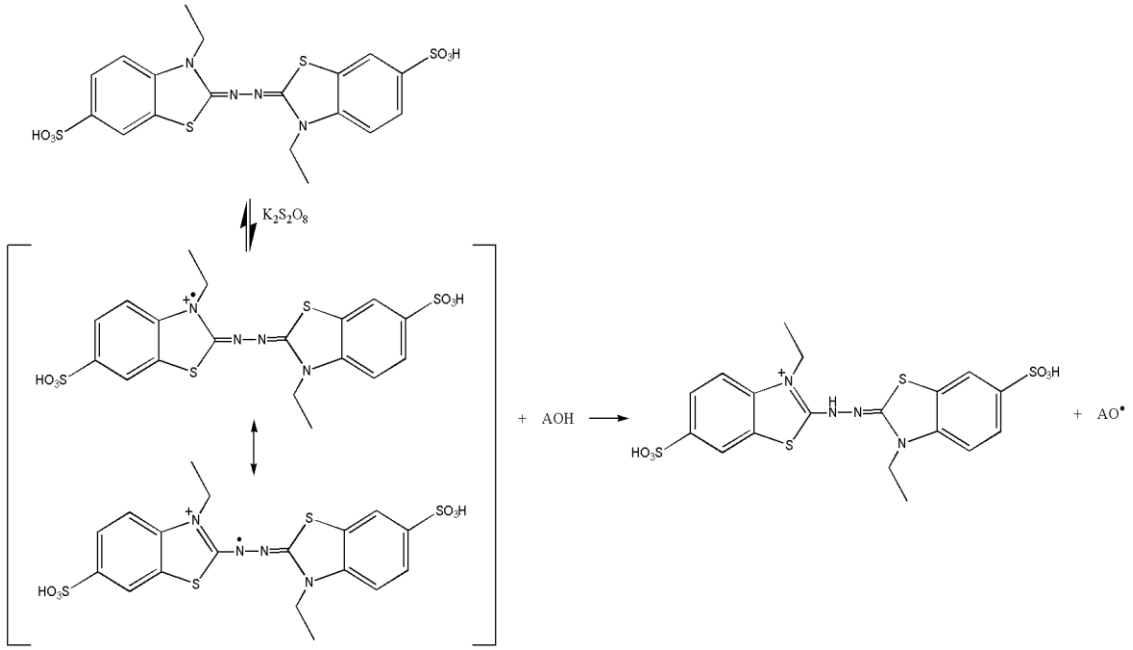


Figura 1.23
Oxidación de ABTS con persulfato potásico para la obtención del catión radical ABTS$^{\bullet+}$ y su reacción con un antioxidante (AOH)

La determinación de la capacidad neutralizadora de catión radical de ABTS se mide por decoloración del ABTS$^{\bullet+}$ (Re et al., 1999) (Figura 1.23) que es de color verde oscuro. El radical ABTS$^{\bullet+}$ se genera por oxidación con persulfato del compuesto ABTS y tiene dos máximos de absorbancia, uno mayor a 420 y otro menor 734 nm. Se usa

420 nm en métodos de oxidación enzimática ya que permite seguir la aparición de pequeñas cantidades del catión radical. Usamos 734 nm pues esta reacción es masiva, pasando la disolución de un color verde casi negro hasta un color verde muy claro o casi incoloro, así un máximo de menores absorbancias nos permite seguir la reacción sin saturar el detector.

1.3.3 Método DPPH

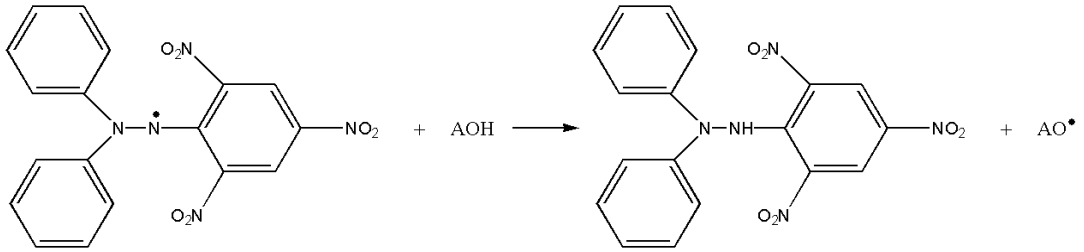
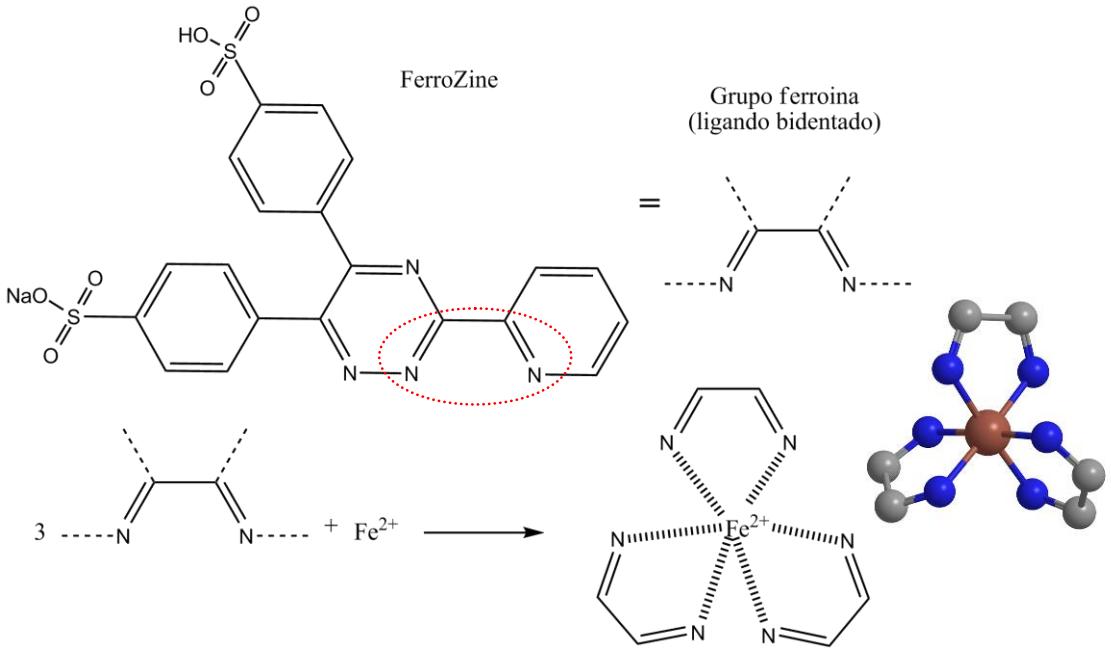


Figura 1.24
Reacción del radical DPPH• con un antioxidante (AOH)

La determinación de la actividad neutralizadora de radicales libres de DPPH• se mide con la decoloración de una disolución de este radical sólido de color violeta (Brandwilliams et al., 1995), que tras decolorarse da un ligero color amarillo (Figura 1.24).

1.3.4 Evaluación de la capacidad quelatante



Figuras 1.25
Reacción de FerroZine con hierro (II) (izquierda) y FerroZine 3D quelatando hierro (II) (derecha)

El grado de complejación de iones ferrosos obtenido por los aceites esenciales se valoró con el método usado por la doctora Miguel *et* al. (Miguel et al., 2010). Este método se fundamenta en la reacción de quelatación que da FerroZine con los iones de hierro (II). La reacción se debe al grupo ferroina constituido por los átomos –N=C–C=N– que constituye un ligando bidentado. Cuando tres unidades de este grupo se coordinan con átomos de hierro (II), aparece un color rosa intenso (Thompsen y Mottola, 1984). La coordinación es tridimensional como se ve en la Figura 1.25.

1.3.5 Evaluación de la capacidad reductora

El método se basa en la capacidad de la muestra de reducir el hierro (III) del ferricianuro potásico $K_3[Fe(CN)_6]$ en medio ligeramente ácido y en caliente, posteriormente, se acidifica fuertemente el medio y se separan partículas que puedan haber y se añade hierro (III) ión (en forma de cloruro) para que el ferrocianuro formado pueda reaccionar con el hierro (III) libre y formar el complejo “azul de Prusia” (Figura 1.26) (Oyaizu, 1986). La cantidad formada de azul es proporcional a la capacidad que tiene la muestra de reducir el ferricianuro y, por ende, el hierro (III). La medición espectrofotométrica a 700 nm debe hacerse con rapidez, ya que el azul de Prusia precipita pasado el tiempo.

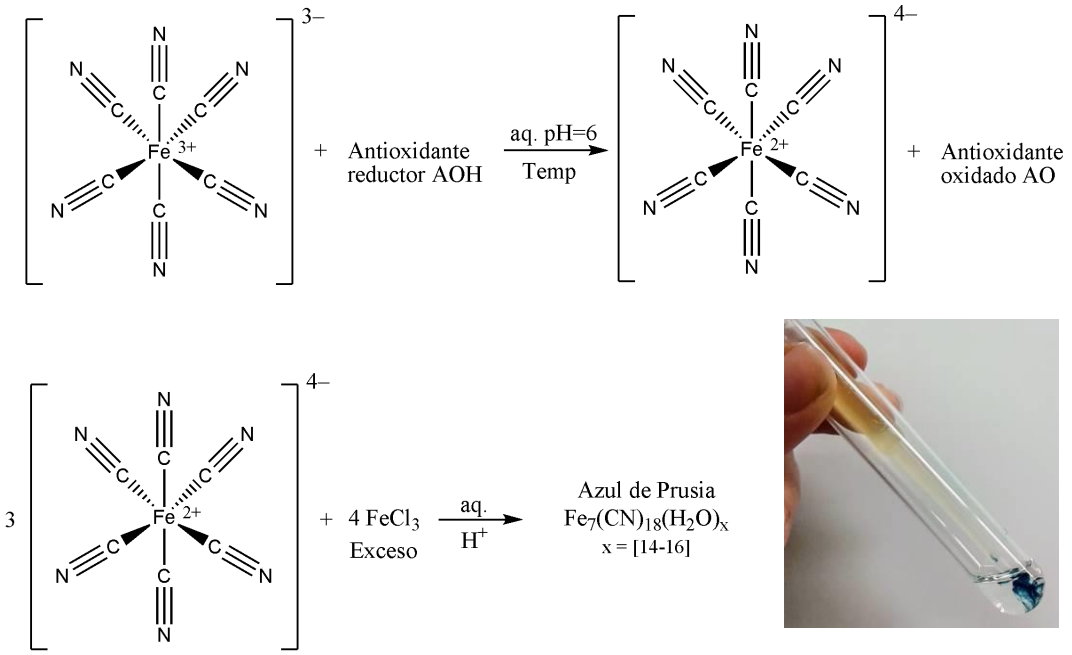


Figura 1.26
Reacción del ferricianuro con un antioxidante reductor (AOH) y generación del azul de Prusia. Foto de azul de Prusia mientras se forma en un tubo de ensayo.

1.3.6 Método TBARS

El método que evalúa la cantidad de sustancias reactivas con el ácido tiobarbitúrico (TBARS) se realizó con pequeños cambios con respecto a la bibliografía (Dorman et al., 1995). Las grasas, sometidas a estrés térmico y lumínico en presencia de oxígeno, pueden degradarse por peroxidación. En este proceso, los ácidos grasos poliinsaturados tienden a degradarse formando malondialdehído (MDA). El MDA se presenta como una tautomería ceto-enol, donde la forma enol es predominante, este producto del estrés oxidativo en organismos es capaz de causar daños reaccionando con el ADN y las proteínas. El MDA es reactivo con TBA en medio ácido, dando lugar a un aducto MDA-TBA_2 (Figura 1.27) de color rojo con λ_{abs} = 532 nm y que exhibe fluorescencia con λ_{ex} = 530 nm y λ_{em} = 550 nm. Así, cuando la adición de un antioxidante hace disminuir la cantidad de aducto rojo formado, es porque ha conseguido disminuir las consecuencias del estrés térmico sobre la matriz lipídica y se ha formado menos MDA (Janero, 1990).

1.3.7 Método de captura de radicales hidroxilo

El método que mide la capacidad de los antioxidantes de neutralizar los radicales hidroxilo ($OH^{\bullet}$) se realizó con pequeñas modificaciones al método original (Chung et al., 1997). El método se basa en producir radicales hidroxilo mediante la reacción de Fenton, para ello quelatamos el Fe^{2+} del sulfato de hierro (II) con EDTA para conseguir el catalizador y añadimos H_2O_2 para comenzar la reacción de generación de radicales hidroxilo, aunque esta reacción debería hacerse en medio ácido con Fe^{2+}, se puede llevar a cabo en medio neutro gracias a que el hierro quelatado es mejor catalizador. La 2-desoxirribosa es un modelo de la base de ADN que podría ser atacada por los radicales en un sistema biológico. Al degradarse esta base da lugar a MDA, el cual podemos ponerlo de manifiesto con una reacción del tipo TBARS para el cual necesitamos TBA, 95 ºC y medio ácido (Figura 1.27).

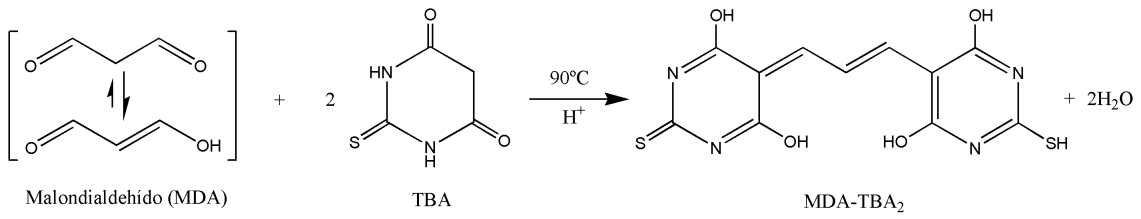


Figura 1.27
Reacción de dos moléculas de TBA con MDA para dar lugar al aducto coloreado.

1.3.8 Método de captura de óxido nítrico

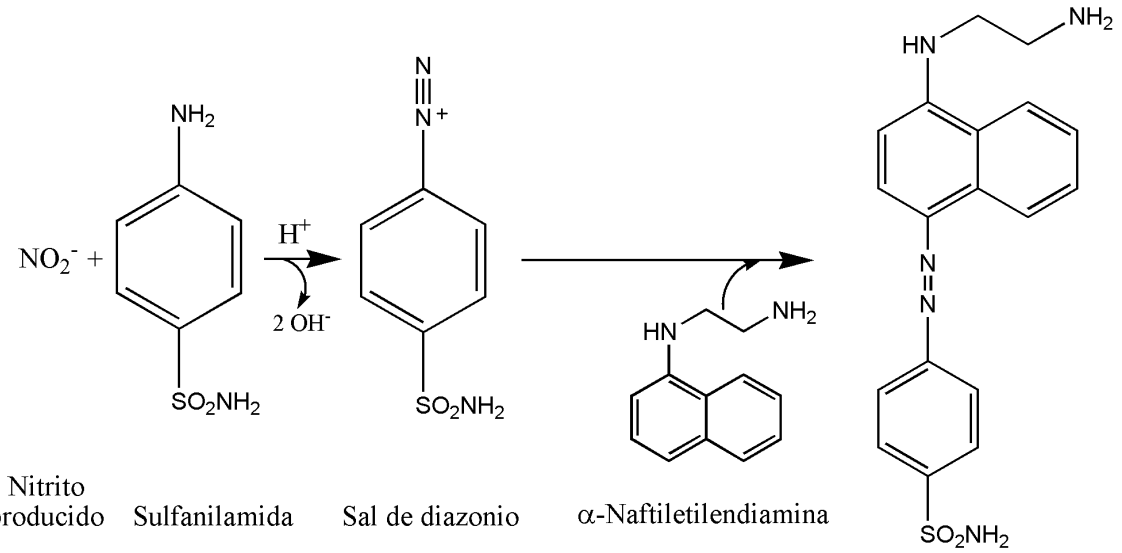


Figura 1.28
Reacción de Griess para la generación del producto azo coloreado.

La capacidad de capturar el radical óxido nítrico ($NO^{\bullet}$) que poseen las muestras se evaluó de acuerdo con el método establecido en la bibliografía (Ho et al., 2010). Este método se basa en que el nitroprusiato, al ser expuesto a luz y/o temperatura libera el ligando $NO^{\bullet}$, que es inestable en disolución acuosa y en presencia de oxígeno y evoluciona hacia la formación de ácido nitroso y por tanto a nitritos estables. Si la muestra es capaz de impedir que esta especie evolucione a nitrito, se considera que es una especie antioxidante, capaz de capturar y estabilizar el óxido nítrico (Friederich y Butterworth, 1995). El resultado final de este ensayo se revela al medir la cantidad de nitritos producidos gracias a la reacción de diazotación de Griess: con la sulfanilamida en medio fosfórico, los nitritos forman una sal de diazonio que da una coloración rosada al añadir la α-naftiletilendiamina (Figura 1.28).

1.4 Capacidad inhibidora de enzimas

Son muchos los factores que deben concurrir para que una molécula sea capaz de inhibir una enzima determinada. Tenemos el factor de solubilidad en un medio común adecuado para la actividad enzimática y a una concentración suficiente para competir con el sustrato, el factor estérico para que la molécula sea capaz, por tamaño y disposición de cargas, de entrar en el sitio activo de la enzima o en algún centro capaz de desactivarla, así como el factor de reactividad para que la molécula sea capaz de interactuar mediante enlaces con la enzima y el efecto de inhibición se mantenga en el tiempo. Dependiendo de la enzima encontraremos diversas estructuras que puedan interactuar con la misma, pero investigar que esas moléculas

inhibidoras sean de origen natural es un trabajo laborioso de ensayo y error, que se está desarrollando hoy en día, para conseguir que distintos productos naturales puedan ser utilizados con fundamento científico, en el tratamiento contra diversas dianas terapéuticas enzimáticas.

1.4.1 Lipoxigenasa

La lipoxigenasa (LOX) (linoleato oxidoreductasa, EC 1.13.11.12) es una enzima que contiene hierro no heme, y cataliza la dioxigenación, con oxígeno molecular o peróxido de hidrógeno como oxidantes, de ácidos grasos poliinsaturados a lípidos con una estructura *cis*,*cis*-1,4-pentadieno. Las lipoxigenasas se encuentran en plantas, hongos y animales y están implicadas en varias funciones celulares (Anwar et al., 2014; Fang et al., 2014; Lin et al., 2014). En plantas, diferentes isoenzimas de LOX están implicadas en diversos aspectos fisiológicos como el crecimiento y desarrollo, resistencia a plagas, senescencia y respuesta a heridas, en el caso de la isoenzima 1-LOX de soja, tiene como sustrato específico el ácido linoleico (Figura 1.29).

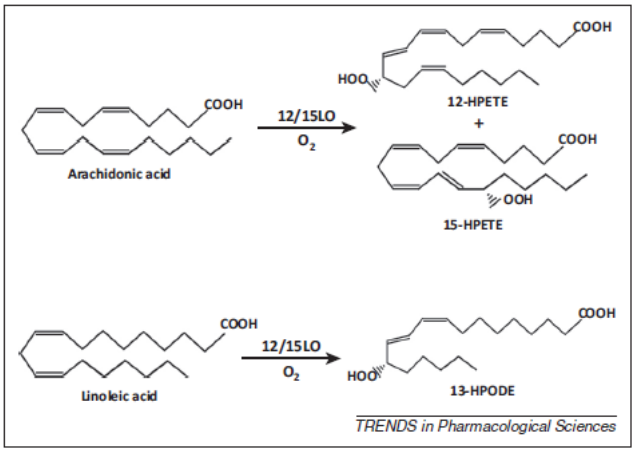


Figura 1.29
Transformación de los sustratos linoleico y araquidónico por acción de LOX.
(Joshi et al., 2015)

En mamíferos, las diferentes isoenzimas 5-LOX, 12-LOX (eritrocito y leucocito) y 15-LOX (leucocito y epidérmica) tienen como sustrato específico el ácido araquidónico (20:4 ω-6) y también otros eicosanoides precursores de leucotrienos (Figura 1.30). Al estar involucrada en la biosíntesis de leucotrienos, la 5-LOX (codificada en el gen ALOX5) transforma ácido araquidónico en ácido 5-hidroperoxieicosatetraenoico (5-HPETE), que espontáneamente se reduce a ácido 5-hidroxieicosatetraenoico (5-HETE). Pero antes de reducirse el 5-HPETE puede ser transformado, a su vez, por la enzima leucotrieno A sintasa (LTA) en leucotrieno A_4 (LTA_4) que puede ser transformado en LTB_4 o conjugado con glutatión para transformarse en LTC_4, que será transportado fuera de la célula y transformado en

LTD_4 y LTE_4. Todos estos leucotrienos forman parte de la cascada de señalización de diversos procesos inflamatorios, principalmente LTB_4 es un quimiotáctico importante en la adhesión de leucocitos y en la generación de superóxido en neutrófilos, mediando para conseguir vasoconstricción e hinchazón por acumulación de plasma en los lugares inflamados. Así la inhibición de lipoxigenasa, existente en muchos tejidos en mamíferos, puede ser útil contra procesos inflamatorios en enfermedades como asma, psoriasis, arteriosclerosis, diabetes, enfermedades renales, obesidad y malignizaciones tumorales (Kuhn et al., 2015).

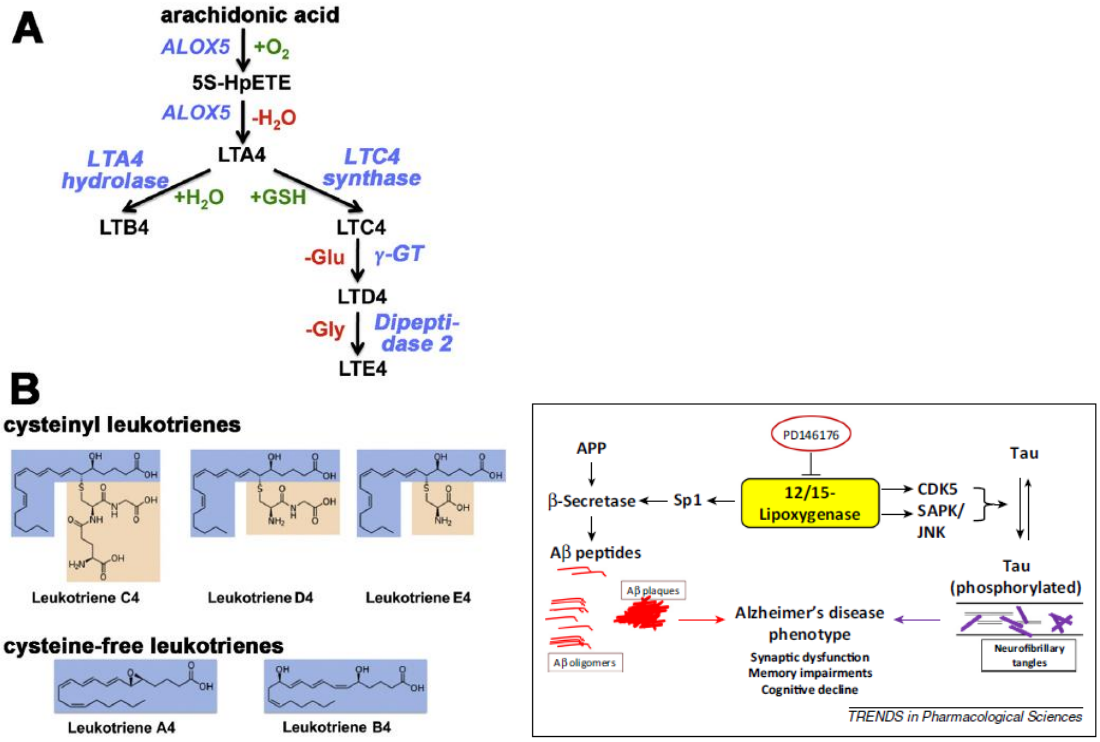


Figura 1.30
Izquierda: Implicación de 5-LOX en los procesos de creación leucotrienos. (Kuhn et al., 2015)
Derecha: 12/15-LOX en la creación de marañas fibrilares y placas β-amiloides. (Joshi et al., 2015)

Recientemente se ha comenzado a relacionar la actividad de 12/15-LOX, que juega un papel importante en el sistema nervioso central en varias enfermedades neurodegenerativas, incluyendo la enfermedad de Alzheimer (Figura 1.30). En el cerebro, las reacciones de estrés oxidativo se han mostrado como eventos tempranos de dicha enfermedad. Así, se ha descubierto que 12/15-LOX modula la producción de placas β-amiloides a través de la β-secretasa, actuando sobre el factor de transcripción Sp1. Además, LOX actúa sobre quinasas que fosforilan la proteína tau, favoreciendo su incorporación a las marañas fibrilares intracelulares, en los axones neuronales. Así, tanto la sobreexpresión de 12/15-LOX mostrada en ratones, frente al grupo mutante con el gen silenciado para expresar 12/15-LOX, como la acción del

inhibidor PD146176, demuestran que se puede mitigar la enfermedad de Alzheimer actuando directamente sobre LOX (Joshi et al., 2015).

De la misma forma, se han encontrado evidencias de que las diferentes isoenzimas de LOX están relacionadas con varios tipos de enfermedades, entre ellas varios tipos de cáncer (colo-rectal, mama, próstata, esofágico, epitelial). Su sobreexpresión condiciona una mayor generación de tumores, y una mayor facilidad para la dispersión de los mismos en el organismo (Mashima y Okuyama, 2015).

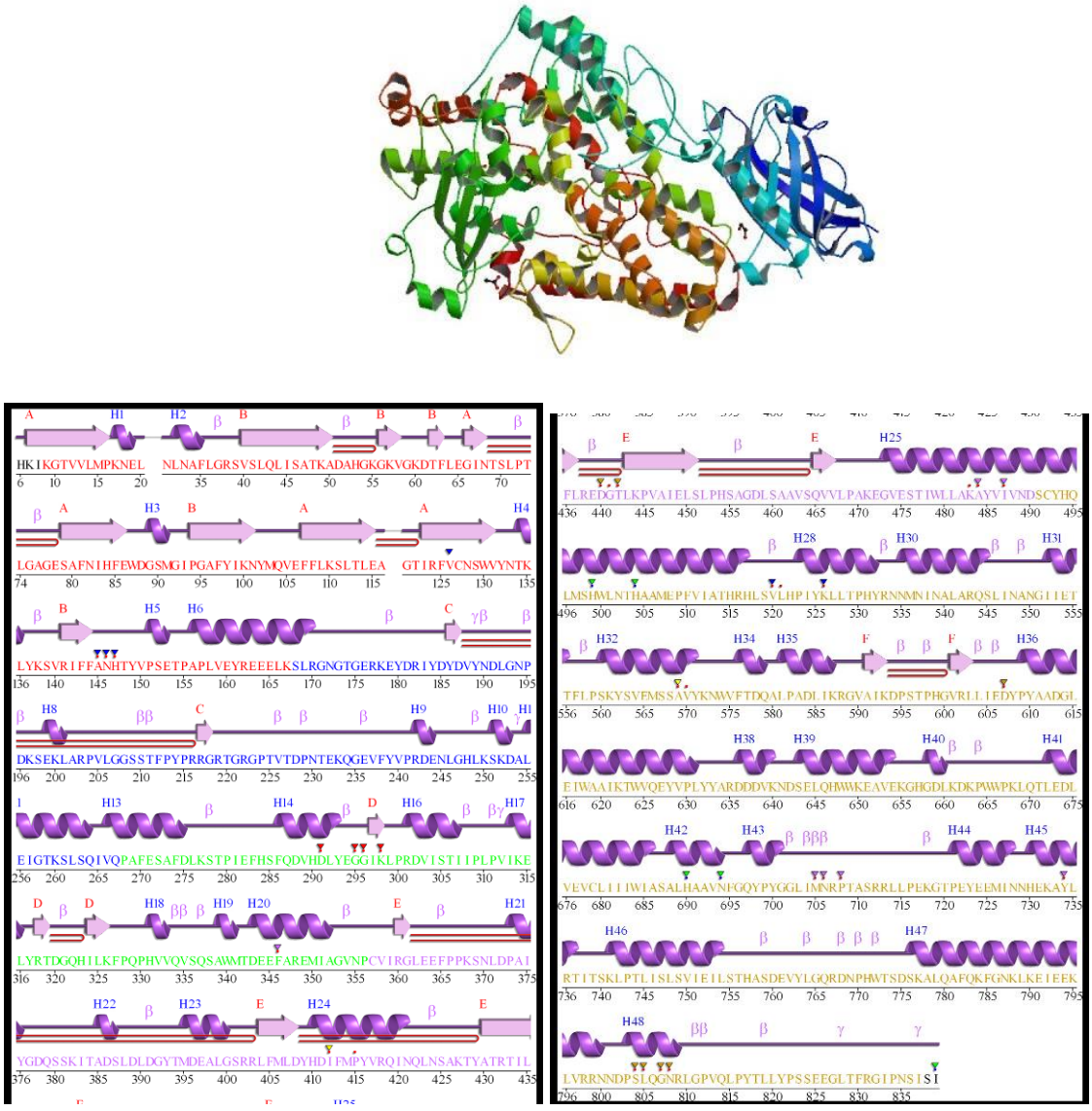


Figura 1.31
Arriba: 1-lipoxigenasa de soja. Abajo: Estructura secundaria de 1-lipoxigenasa de soja.
Imágenes dePDB y PDBsum ID: 3PZW

La proteína (Figura 1.31) está formada por una cadena con un pequeño dominio PLAT N-terminal y un dominio mayor catalítico C-terminal, con 18-22 hélices alfa y 1-2 láminas beta antiparalelas, que contiene el sitio activo (Figura 1.31). El sitio

activo contiene un átomo de hierro hexacoordinado que está unido al N^t de tres histidinas, al O del carbonilo C-terminal, al O de la cadena lateral de Asn y a un grupo hidroxilo o molécula de agua. Las dos hélices largas centrales se cruzan en el sitio activo y dos cavidades en el dominio mayor se extienden desde el sitio activo hasta la superficie en el caso de la LOX de soja, mientras que la de mamíferos sólo tiene una cavidad (Mashima y Okuyama, 2015).

La actividad enzimática de lipoxigenasa se puede determinar con un método espectrofotométrico (Christop.J et al., 1970), basado en la absorción a 234 nm de los hidroperóxidos con dienos conjugados (ε = 25000 $M^{-1}cm^{-1}$), que se forman cuando el ácido linoleico (18:2, usado como sustrato) se oxida en presencia de oxígeno y LOX.

Dado el sustrato específico, ácido linoleico, altamente hidrofóbico de esta enzima, es lógico suponer que los componentes de los aceites esenciales, en gran medida terpenoides, puedan entrar en la cavidad de la enzima y no sean repelidos por interacciones polares. Así, ensayar la actividad inhibidora de los aceites esenciales en LOX parece una opción razonable, respaldada por algunos estudios previos de bioactividad antiinflamatoria presente en algunos aceites esenciales (Gautam et al., 2014; Lesgards et al., 2014; Rubio et al., 2013). Así, la inhibición de lipoxigenasa de soja es utilizada como modelo de la actividad antiinflamatoria de un aceite esencial (Kim et al., 2014; Mandal et al., 2014; Mohamed et al., 2014).

1.4.2 Hialuronidasa

El ácido hialurónico o hialuronano es un polisacárido, compuesto por ácido D-glucurónico y D-N-acetilglucosamina, unido por enlaces glicosídicos alternantes β-1,4 y β-1,3, con un tamaño molecular de 5 a 20 MDa *in vivo*. El ácido hialurónico embebe agua, proporciona resiliencia a los cartílagos, reemplaza las fibras de colágeno degradado y mejora la regeneración de colágeno en la piel y la matriz extracelular (ECM).

La hialuronidasa (Hialuronoglucosaminidasa EC 3.2.1.35) (Figura 1.33) degrada aleatoriamente el ácido hialurónico por el enlace β-1,4 glicosídico entre N-acetil-D-glucosamina y D-glucuronato, dando lugar a cadenas de polímero, principalmente tetrasacáridos (hyal-1 humana) o fragmentos de 20 kDa (hyal-2 humana), con glucosamina terminal. La enzima actúa a pH ácido y tiene un tamaño de alrededor de los 40-80 kDa. Aproximadamente un tercio del ácido hialurónico presente en el cuerpo de una persona adulta y sana, se degrada y vuelve a sintetizar diariamente, en un ciclo homeostático con hialuronidasa y hialuronato sintetasa (Stern, 2004). Las hialuronidasas se encuentran ampliamente distribuidas en la naturaleza, estando presentes en mamíferos, insectos, invertebrados como sanguijuelas, hongos y

bacterias. En mamíferos han sido identificadas en diversos órganos como testículos, hígado, riñones, sistema linfático y piel. En otros animales han sido detectadas en los venenos de serpientes y abejas (El-Safory et al., 2010).

Al catalizar la hidrólisis de hialuronato, un constituyente de la ECM, la hialuronidasa reduce la viscosidad del hialuronato, incrementándose así la permeabilidad del tejido. De esta manera, este hecho se usa en medicina para aumentar la velocidad de dispersión de fármacos. Sin embargo, la ruptura de la ECM está relacionada con el estrés oxidativo, la degradación de proteínas como el colágeno y la elastina, y la hidrólisis catalizada por hialuronidasa de ácido hialurónico y otros glicosaminoglicanos.

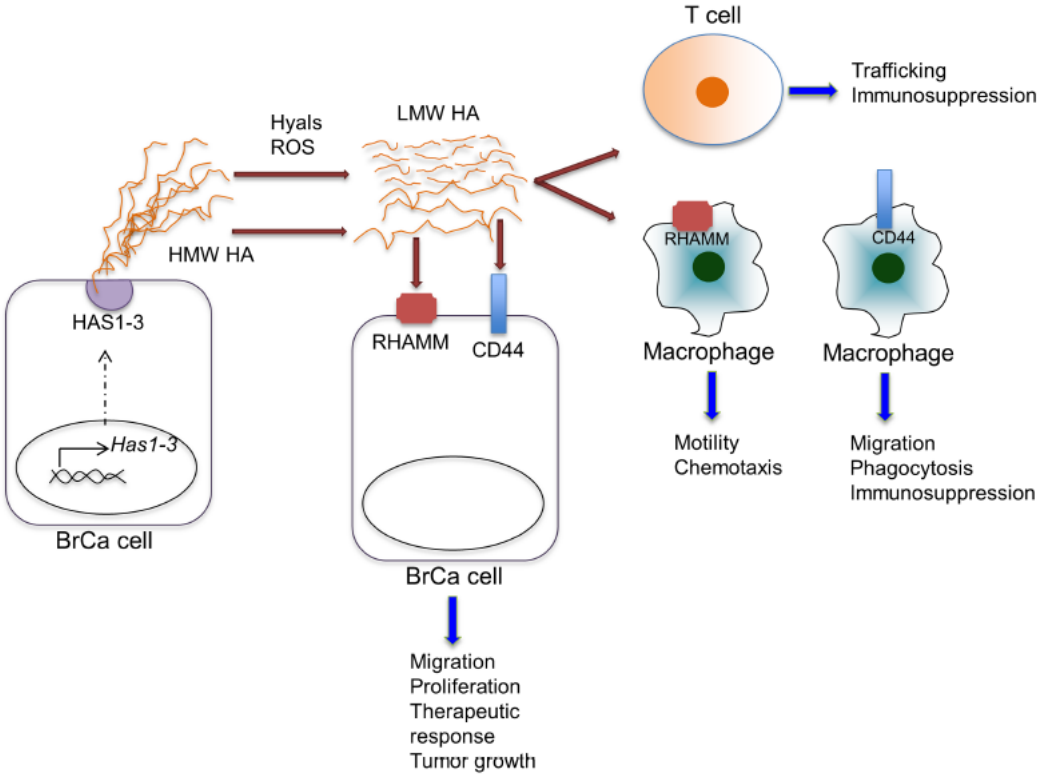


Figura 1.32
Hialuronidasa humana implicada en la progresión del cáncer de mama.
(Schwertfeger et al., 2015)

El sistema ácido hialuronano/hialuronidasa participa también en muchas condiciones fisiopatológicas, como la acción de venenos de serpiente, la reacción acrosómica (la fertilización del óvulo), la patogénesis microbiana y la progresión del cáncer. Se piensa que la hialuronidasa degrada la ECM alrededor de los tumores y ayuda a las células cancerígenas a escapar de la masa del tumor primario. Así, se ha demostrado que el polisacárido hialuronano es un ejemplo de componente de la ECM en el microentorno cancerígeno que promueve la progresión del cáncer de mama de dos formas, primero por su sobreproducción en células tumorales que se sirven del

mismo para protegerse frente a los ataques de los agentes quimioterápicos y los ROS/RNS del sistema inmune, y segundo por la función de los fragmentos del mismo una vez degradado por los radicales libres o la hialuronidasa (Figura 1.32).

Los fragmentos de la degradación de hialuronano, liberados al medio por la acción de los ROS/RNS y hialuronidasa, son pro-inflamatorios y activan señalizaciones celulares que inducen la supervivencia, migración, vascularización e invasión de las células tumorales. De esta manera, agentes que limiten la producción aberrante de hialuronano, su fragmentación o que bloqueen las interacciones con sus receptores, se perfilan como muy prometedores para el avance en el desarrollo de nuevas terapias para pacientes oncológicos (Schwertfeger et al., 2015).

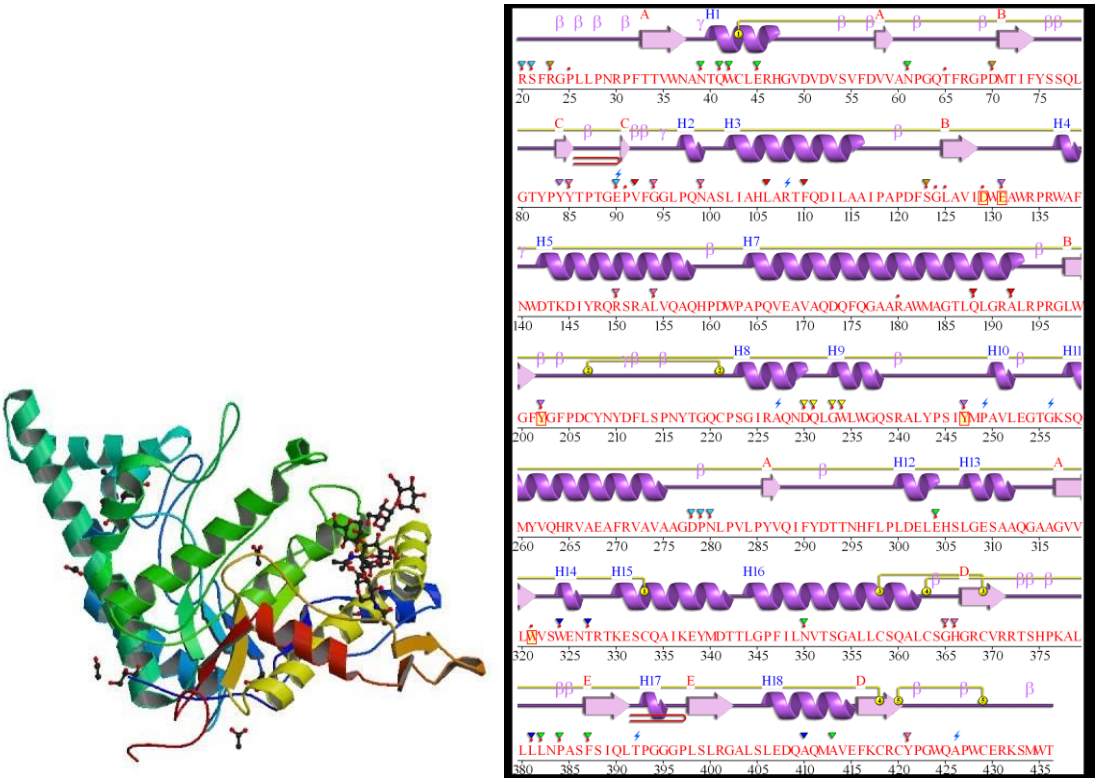


Figura 1.33
Izquierda: Hialuronidasa humana. Derecha: Estructura secundaria de hialuronidasa humana.
Imágenes de PDB y PDBsum ID: 2PE4

La enzima está conformada por una sola cadena, con un dominio catalítico principal en el que destacan grandes hélices-α y 5 puentes disulfuro (Figura 1.33). La hialuronidasa bovina tiene un 61% de similitud con la humana, de forma que se configura como un buen modelo para investigar las interacciones con distintos sustratos en inhibidores (El-Safory et al., 2010).

Los inhibidores de hialuronidasa pueden servir como agentes anticonceptivos al interferir con la degradación de la ECM presente en las capas externas del ovocito, contravenenos/toxinas al retardar el efecto dispersivo de las toxinas de éstos,

antimicrobianos al impedir la movilidad y alimentación de las bacterias que degradan hialuronano y que lo usan como fuente de energía, anti-envejecimiento al impedir, localmente, la degradación del hialuronano con efecto estructural de la dermis, antiinflamatorios al impedir la generación de fragmentos de la degradación del hialuronano que son pro-inflamatorios y anticancerígenos al inhibir la progresión y señalización necesaria para la supervivencia de las células tumorales (McAtee et al., 2014).

Teniendo en cuenta que el sustrato específico es un polímero polar, tan sólo los terpenos más polares de los aceites esenciales tendrán una ventaja para poder aproximarse al sitio activo de la enzima. Sin embargo, dado que el hialuronano es mucho más voluminoso que los terpenos, éstos pueden aprovechar la situación y el volumen interno del sitio activo para introducirse e interactuar con el mismo.

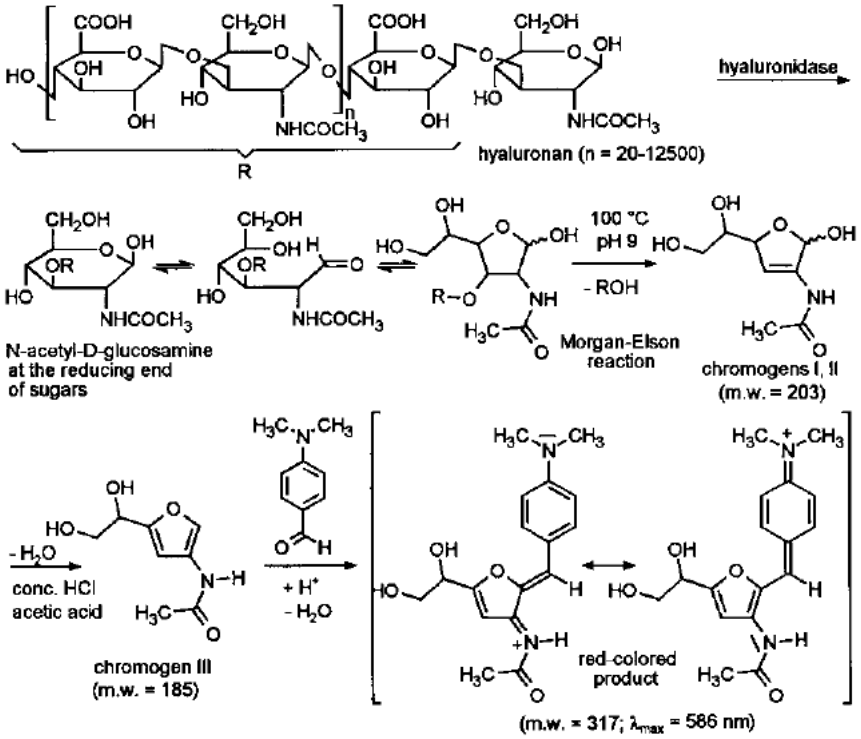
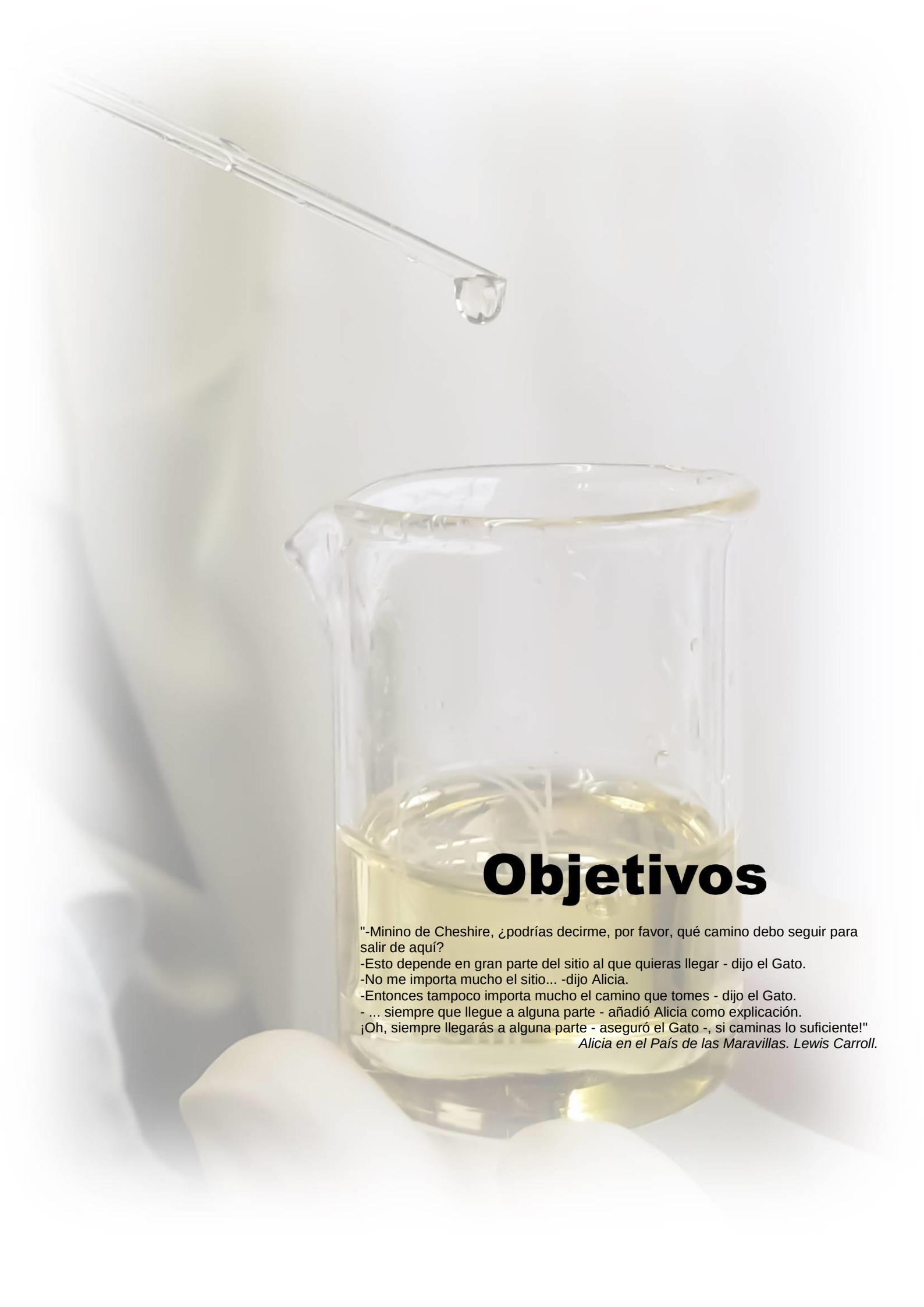


Figura 1.34
Método de detección de acetilaminoazúcares con pDMAB
(Muckenschnabel et al., 1998)

La inhibición de hialuronidasa se determina midiendo la cantidad de N-acetilglucosamina, formada por hidrólisis enzimática del hialuronato sódico en presencia y en ausencia de inhibidores (Sahasrabudhe y Deodhar, 2010). Tras ese paso, se realiza la medida de los fragmentos con acetilamino azúcares hidrolizados terminales. Esta medida se basa en la reacción de Morgan-Elson, que utiliza el

pDMAB (p-dimetil amino benzaldehído) para generar el compuesto cromofórico final (Figura 1.34) (Reissig et al., 1955).



Objetivos

"-Minino de Cheshire, ¿podrías decirme, por favor, qué camino debo seguir para salir de aquí?
-Esto depende en gran parte del sitio al que quieras llegar - dijo el Gato.
-No me importa mucho el sitio... -dijo Alicia.
-Entonces tampoco importa mucho el camino que tomes - dijo el Gato.
- ... siempre que llegue a alguna parte - añadió Alicia como explicación.
¡Oh, siempre llegarás a alguna parte - aseguró el Gato -, si caminas lo suficiente!"

Alicia en el País de las Maravillas. Lewis Carroll.

2. OBJETIVOS

2.0 Objectives

The present work aims to develop and use fast gas chromatography (FGC) and enantioselective gas chromatography (EsGC), coupled to mass spectrometry detection (MSD) methods, to determine qualitatively and quantitatively the composition, as well as the enantiomeric distribution, of the main components of the essential oils extracted from aromatic plants cultivated in Murcia. The developed method will be tested in essential oils, obtained from plants belonging to the *genus Lavandula*, *Thymus*, *Thymbra* and *Origanum*, all of them members of the *Lamiaceae* family.

Thus, the natural character and the absence of adulterations, as well as the commercial applicability of these essential oils, will be checked with the use of FGC-MSD and EsGC-MSD methods here developed.

Furthermore, this work aims to evaluate the possible antioxidant bioactivity of these essential oils, which will be tested by several different methods, in order to check their efficiency against several oxidant agents and reactive oxygen and nitrogen species.

Finally, inhibitory activities on hyaluronidase and lipoxygenase enzymes will also be studied, due to their relation with the degradation of hyaluronic acid, inflammatory processes, as well as several diseases such as the progression of cancer.

2.1 Objetivo general

El presente trabajo pretende utilizar métodos de cromatografía de gases rápida (FGC) y cromatografía de gases enantioselectiva (EsGC), para el análisis de aceites esenciales extraídos de plantas aromáticas, e ilustrar su aplicabilidad caracterizando aceites esenciales de muestras de plantas de los géneros *Lavandula*, *Thymus*, *Thymbra* y *Origanum*, todos ellos de la familia *Lamiaceae*, cultivados en Murcia, así como evaluar sus posibles bioactividades antioxidante e inhibidora de enzimas con aplicaciones biotecnológicas.

2.2 Objetivos específicos

El desarrollo del objetivo general sobre los aceites esenciales de plantas cultivadas en Murcia, abarca como objetivos específicos, la realización de los siguientes estudios experimentales:

- Establecimiento de métodos de cromatografía de gases rápida (FGC) y enantioselectiva (EsGC), con detección por espectrometría de masas (MSD), para el análisis de aceites esenciales de plantas aromáticas.
- Identificación cualitativa y determinación cuantitativa de sus biomoléculas constituyentes, mediante FGC-MSD, caracterización imprescindible para evaluar su potencial aplicabilidad comercial.
- Determinación cuantitativa relativa de la distribución enantiomérica de sus principales componentes, aplicando EsGC-MSD, útil para garantizar su carácter natural, la ausencia de adulteraciones y potenciar su comercialización.
- Evaluación de su posible bioactividad antioxidante global utilizando varios métodos, ilustrativos de su eficacia frente a diversos agentes oxidantes y especies reactivas de oxígeno y nitrógeno.
- Evaluación de su posible capacidad inhibidora de hialuronidasa y lipoxigenasa, enzimas relacionadas con la degradación de ácido hialurónico, procesos inflamatorios y otras enfermedades como la progresión del cáncer.

Materiales y Métodos

"Prácticamente todas las cosas dan problemas, es su naturaleza. Lo único que podemos hacer es cantar por el camino."

La Verdad. Terry Pratchett.

3. MATERIALES Y MÉTODOS

3.1 Material vegetal

Las muestras de plantas (300 g cada una), cultivadas en Murcia en campos de cultivo ecológicos dedicados para la extracción de aceite esencial a nivel industrial, se cultivaron en las zonas bioclimáticas: Bajo Meso-Mediterránea, Alto Meso-Mediterránea y Supra-Mediterránea. Las características de las zonas bioclimáticas (Rivas-Martínez, 1987) se presentan en la Tabla 3.1.

Tabla 3.1

Descripción de los rasgos ecológicos de las zonas bioclimáticas a estudio.

Zona bioclimática	Índice de termicidad (ºC)	Pluviosidad (mm/año)
Bajo Meso-Mediterránea	301 – 350	350 – 600
Alto Meso-Mediterránea	211 – 300	>600
Supra-Mediterránea	61 – 210	>600

Índice termicidad = 10 * (T + M + m), (T, temperatura anual media; M, media de las temperaturas más altas del mes más frío; m, media de las temperaturas más bajas en el mes más frío)

Las muestras secas se sometieron a hidrodestilación en un dispositivo Clevenger. Los aceites esenciales obtenidos se secaron con sulfato sódico anhidro obteniendo aceites olorosos de color amarillo pálido. Las muestras se conservaron a – 20 ºC hasta su uso.

3.2 Reactivos

Los siguientes compuestos fueron adquiridos a Sigma-Aldrich: patrones puros (>95%) para identificación por GC, fluoresceína, AAPH [2,2'-azobis(2-metilpropionamidina) dihidrocloruro], Trolox [ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxilico] (pureza 99.3% por titración con NaOH), butilhidroxitolueno (BHT, pureza 99.9% por cromatografía de gases), DPPH [2,2-difenil-1-picrilhidracilo], ABTS [ácido 2,2'-azino-bis(3-etilbenzotiazolina-6-sulfónico)], manitol [(2R, 3R, 4R, 5R)-Hexano-1,2,3,4,5,6-hexol] (pureza 98.4% por GC), rutina [quercetin-3-rutinósido] (pureza 95.9% por HPLC), tampón fosfato salino (PBS), 2-desoxirribosa, hidróxido sódico, borato sódico, cloruro cálcico, hialuronidasa bovina, p-dimetilaminobenzaldehído

(pDMAB), cloruro potásico, dodecilsulfato sódico (SDS), n-butanol, persulfato potásico, ferricianuro potásico, ácido tricloroacético, cloruro de hierro (III), FerroZine [hidrato de la sal monosódica del ácido 3-(2-piridyl)-5,6-difenil-1,2,4-triazina-p,p′-disulfónico] y cloruro de hierro (II) (Figura 3.1).

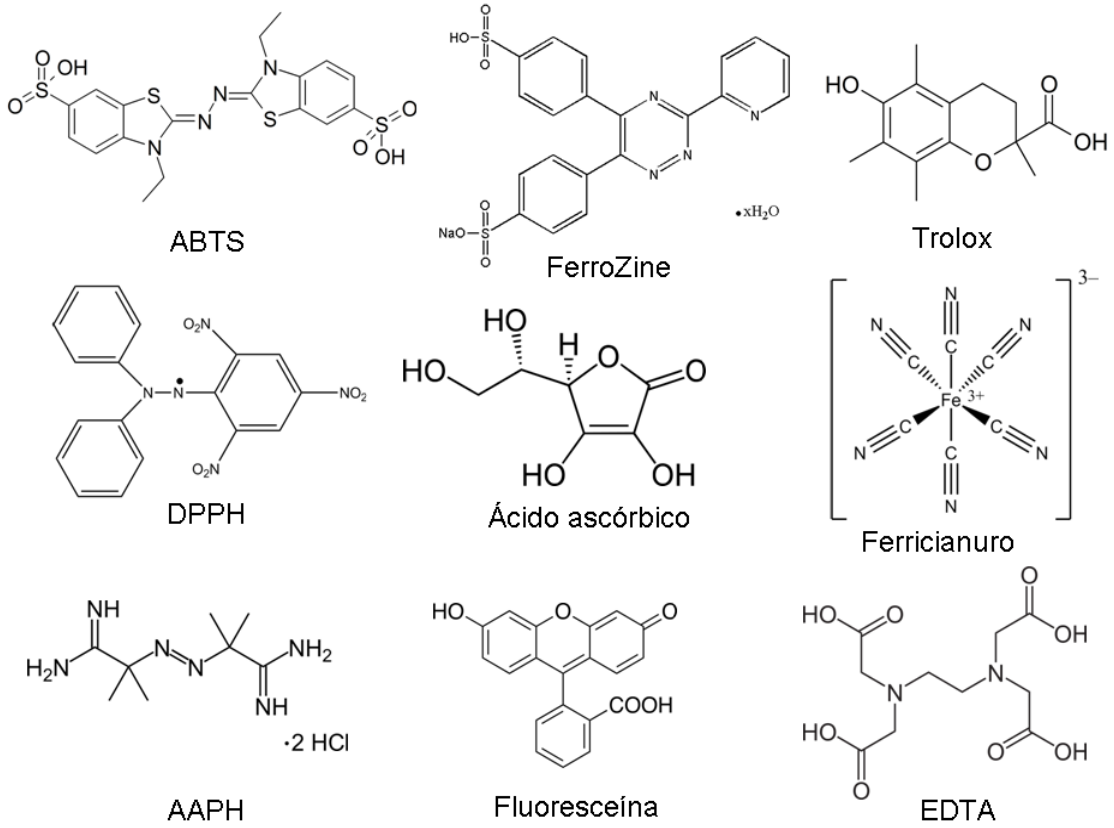


Figura 3.1
Algunos reactivos de estructura compleja utilizados en este trabajo

El ácido ascórbico (AA, pureza 99.7% por ensayo acidimétrico) fue adquirido a Scharlau y el ácido etilendiaminotetraacetico (EDTA, pureza 99% por titulación complejométrica) fue adquirida a Carl Roth. Los disolventes calidad cromatografía de gases, el peróxido de hidrógeno, el ácido tiobarbitúrico (TBA) tampones y otros reactivos de grado analítico fueron adquiridos a Merck. El hialuronato de sodio se compró a Acros Organics, el nitroprusiato sódico se compró a Riedel-de-Haen y el kit del reactivo de Griess se compró a Promega. El ácido tricloroacético se compró a VWR. El sulfato de hierro (II) se adquirió en Panreac. El agua de laboratorio tipo I (18 MΩ cm) fue desionizada con un equipo MilliQ-Reference de Millipore (Figura 3.2).

Figura 3.2
MilliQ-Reference

Figura 3.3
Generador de hidrógeno electrolítico Parker-Domnick-Hunter

3.3 Cromatografía de gases rápida-Espectrometría de masas

Figura 3.4
Cromatógrafo Agilent GC7890, espectrómetro de masas MS5975 y automuestreador Gerstel MPS-2XT

Los análisis mediante FGC-MSD se efectuaron en un cromatógrafo Agilent GC7890 con un detector MS5975, un automuestreador Gerstel MPS-2XT (Figura 3.4) y una columna cromatográfica capilar de sílice fundida SLB-5ms de Sigma-Aldrich-Supelco, utilizando gas impulsor hidrógeno producido por un generador electrolítico Parker-Domnick-Hunter (Figura 3.3). Este equipo modular fue controlado con los programas ChemStation, MS-Search y AMDIS, además de las bases de datos con espectros de masas NIST 08 y Wiley 7.

La presión en cabeza de columna también está limitada por las características del cromatógrafo, de la columna y de la capacidad de generación de hidrógeno (máximo 85 psi). Conjugando estas ventajas y limitaciones, la columna elegida fue la SLB5-ms de Sigma-Aldrich SUPELCO de dimensiones 15m x 100µm x 0.1µm

(longitud x diámetro interno x espesor fase estacionaria), de fase estacionaria silfenilen polímero, virtualmente equivalente en polaridad al poli (5% difenil / 95% metil siloxano) de bajo sangrado (disminuye el ruido de fondo), con salida a vacío dentro del espectrómetro de masas. La pequeña longitud proporciona tiempos cortos de cromatograma, mientras que los bajos valores de diámetro interno y espesor de película aumentan la resolución.

En nuestro caso concreto contamos con una cromatografía de gases rápida cuyo gas impulsor es el hidrógeno (fase móvil), generado por electrolisis de agua de calidad MilliQ-Reference (Figura 3.2). El generador de hidrógeno (Figura 3.3) suministra un caudal máximo que supera los 80 ml/min a una presión de 6 bar.

El automuestreador Gerstel MPS-2XT cuenta con una jeringa GERSTEL de 10 µL y ejecuta una inyección tipo sándwich compuesta de 0.2 µL de aire, 0.2 µL de isooctano, 0.2 µL de aire, 0.3 µL de muestra y 0.2 µL de aire por este orden desde el émbolo hasta la punta de la aguja. Tras cada toma de muestra, la jeringa se limpia con varios lavados de dos disolventes distintos.

La jeringa utilizada fue la que mejor resultado arrojaba en un estudio de reproducibilidad entre inyecciones. Al contrario de lo que se pueda pensar, jeringas de volúmenes menores no conseguían mejores resultados que la de 10 µL. Esto es achacable a las distintas geometrías y materiales usados por las distintas empresas suministradoras de este material.

Debido a la alta concentración de compuestos esperados en los aceites esenciales, el tipo de inyección más apropiado es la inyección con *split*. El *split* tiene limitaciones por el caudal de hidrógeno máximo que puede proporcionar el generador, siendo habitual trabajar con *split* máximo de 300:1. Desde el *split* 1:1 hasta el 300:1, se hicieron pruebas para obtener la mejor relación de *split*, que nos permitiese tener los picos de los compuestos mayoritarios dentro del rango de respuesta del detector, perdiendo la menor información posible de los compuestos minoritarios. Esto se consiguió utilizando un *split* 100:1. En la Figura 3.5 se observa la incidencia del *split* sobre el cromatograma.

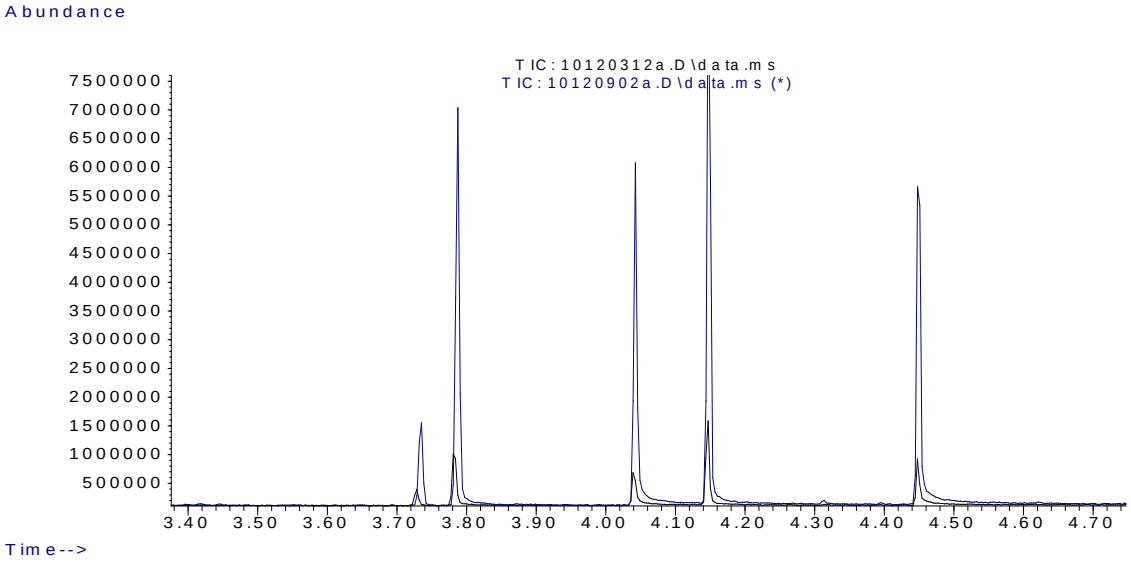


Figura 3.5
Detalle de los cromatogramas FGC-MSD para patrones.
Cromatogramas de las mismas sustancias a distintas concentraciones, en este caso la línea negra (10120312a) es un split de 150:1 mientras que la línea azul (10120902a) es un split de 30:1.

La temperatura de volatilización del inyector fue de 300 ºC, con una presión inicial de 46.345 psi, donde se efectúa una purga de *septum* de 3 mL/min, encargada de limpiar la parte baja del *septum*, ayudar a controlar las sobrepresiones, evitar la retromezcla y prevenir una posible inundación por exceso de muestra vaporizada. Las cromatografías se realizan con una relación de división o *split* 100:1, sacando por la purga de *split* 80 mL/min. Pasados dos minutos del comienzo de la cromatografía, se activa el sistema de ahorro de gas portador, pasando por la purga de *split* sólo 20 mL/min. Estas cromatografías se realizan a flujo constante de 0.8 mL/min, obteniendo así un flujo total de 83.8 mL/min al inicio de la cromatografía y 23.8 mL/min pasados dos minutos.

En el caso de la columna SLB5-ms: 350 ºC. Esta temperatura no debe ser alcanzada para no dañar la fase estacionaria de la columna, de forma que por seguridad se ha fijado la temperatura del inyector a 300 ºC, así como la máxima temperatura del horno. En el caso que nos ocupa no se han determinado temperaturas de descomposición a 300 ºC, de los compuestos potencialmente presentes en los aceites esenciales de espliego y lavanda.

Esta velocidad para este tipo de columnas y el gas portador hidrógeno es de alrededor de 80 cm/s. Esto se consigue con una presión en cabeza de columna de unos 46 psi al comienzo de la programación de temperatura y un flujo de 0.8 mL/min. De esta manera, trabajar a flujo constante de 0.8 mL/min optimiza el rendimiento de la separación.

Cada muestra debe ser evaluada de forma particular pero, en rasgos generales, los aceites esenciales se diluyeron 10 ó 1000 veces antes de proceder a la inyección de la muestra. En el caso concreto de las cromatografías mostradas en este trabajo la temperatura de inicio fue de 60 ºC, porque a esa temperatura aún no salía el frente de disolvente, en nuestro caso isooctano (99 ºC), pero estaba suficientemente cercana como para que comenzase la cromatografía.

En nuestro caso, el horno es capaz de subir la temperatura de forma controlada, hasta una velocidad de calentamiento máxima de 60 ºC/min, y es capaz de enfriar la columna a una velocidad no controlada mucho mayor. Utilizando isooctano como disolvente, el retardo de disolvente es de 0.7 min.

La programación de temperatura a la que se somete la cromatografía, se obtuvo tras un proceso de exploración de todas las muestras involucradas y un esfuerzo de síntesis de un proceso único que tuviese en cuenta las peculiaridades de todos los compuestos presentes en las muestras para poder separar todos los componentes con una sola programación de temperaturas. Es la que se observa en la Tabla 3.2.

Tabla 3.2

Programación de temperatura en cromatografía FGC-MSD

Velocidad de calentamiento (ºC/min)	Temperatura (ºC)	Tiempo de espera (min)	Tiempo (min)
	60.0		0.0
20	92.0		1.6
2	94.0		2.6
20	121.0		4.0
2	123.0		5.0
20	133.0		5.5
2	135.0		6.5
20	136.0		6.5
30	141.0		6.7
20	141.2		6.7
5	142.0		6.8
40	300.0	0.5	11.3

La línea de transferencia del cromatógrafo al detector está permanentemente termostatizada a 280 ºC para homogeneizar las condiciones de entrada de los compuestos al detector.

El detector por espectrometría de masas tiene un vacío interno de $\approx 3 \cdot 10^{-5}$ torr, conseguido por las bombas de vacío y turbomolecular de 25000 rps. Se trata de un espectrómetro de masas de ionización por impacto electrónico (EI), operado a 70 eV y

con detección de simple cuadrupolo, estas condiciones son imprescindibles para tener una buena compatibilidad con las bases de datos espectrales NIST 08 y Wiley 7.

El cuadrupolo se termostata a 150 ºC y la fuente de iones a 230 ºC, utilizando 1129 V en el electromultiplicador. Para aprovechar al máximo la vida útil del equipo, al mismo tiempo que se garantiza la reproducibilidad de los cromatogramas en el tiempo, se ha activado el factor de ganancia y se ha optimizado al valor 5.

En este caso, las masas seleccionadas son las del intervalo entre 30 y 300 uma, porque los terpenos de los aceites esenciales de más de 300 uma no son volátiles a 300 ºC, y los fragmentos de menos de 30 uma, contribuyen más al ruido de fondo que a la determinación exacta de la sustancia.

Así, se ha trabajado en modo barrido (SCAN) a velocidad de 10000 uma/s, en el rango de 30 a 300 uma, garantizando los 21.035 barrido/s. De esta manera, los picos más pequeños de hasta 0.72 s, se definen por al menos 15 puntos. Esto permite cuantificar en base al área obtenida de cada pico en el cromatograma, con la certeza de que el pico se ha obtenido de forma ajustada a la realidad. Por otro lado, estos análisis se han realizado con la función TID (Trace Ion Detection) activada. Este filtro digital original del software permite bajar el límite de detección, reducir el nivel de ruido, mejorar las formas de los picos y los coeficientes de correlación con las bases de datos espectrales. Ocasionalmente, se ha utilizado el modo de monitorización selectiva de iones (SIM), unas diez veces más sensible para el análisis de algunas biomoléculas minoritarias, consideradas como características de un aceite esencial por su respectiva norma ISO (AENOR, 2006).

Trabajando en estas condiciones se obtienen los cromatogramas procesados mediante el programa MSD ChemStation Data Analysis (Agilent Technologies, Santa Clara, CA). Una vez llegados a este punto se han utilizado dos métodos de identificación. El contraste de espectros de masas y de LRIs calculados con una serie de alcanos (C7 – C30) homólogos de Supelco.

En este caso se hicieron diez diluciones o niveles de concentración, que se representan como diez puntos en cada curva de calibración. Cada uno de esos niveles de concentración fue preparado, inyectado y analizado por triplicado. Cada muestra se evaluó en dos diluciones diferentes 1:10 y 1:1000 en isooctano y por triplicado para poder determinar las concentraciones exactas de los diferentes compuestos presentes en los aceites esenciales.

Por otro lado, todos los cromatogramas se hacen e integran por triplicado, pudiendo tomar valores medios de las medidas y controlando un coeficiente de

variación de Pearson, definido como desviación estándar dividido entre la media por cien, siempre menor del 15%. Se escogieron 3 alcanos como estándar interno, uno para las moléculas de alta volatilidad, nonano (MS **43**, 57, 71, 85), otro para las de media volatilidad, tetradecano (MS 43, **57**, 71, 85) y otro para las de baja volatilidad, hexadecano (MS 41, 43, **57**, 71). Cada patrón comercial se asignó a uno de esos tres grupos y quedó referenciado al estándar interno más cercano.

En cada caso se escogen 4 iones para cada molécula, un ión para cuantificar (en negrita) y otros 3 cualificadores para verificar que estén en las proporciones adecuadas, lo cual asegura la identidad de la molécula.

3.4 Cromatografía de gases enantioselectiva-Espectrometría de masas

La cromatografía enantioselectiva (EsGC) nos proporciona la distribución enantiomérica de las principales moléculas de los aceites esenciales. El mismo dispositivo analítico se utilizó para la EsGC, esta vez con una columna Astec Chiraldex B-DM (30 m longitud x 0.25 mm diametro interno x 0.12 µm de espesor de película estacionaria) de Supelco. Esta columna capilar está hecha de silicio fundido con 2,3-di-O-metil-6-t-butilsilil β-ciclodextrina como fase estacionaria especial, no unida covalentemente con el cuerpo de la columna. Por esta imposibilidad de unir el derivado de ciclodextrina al cuerpo de la columna hay que operar con ella en condiciones menos exigentes que en el caso de la columna de FGC. Esta columna no permite ni rápidas subidas de temperatura (más de 15 ºC/min) ni temperaturas muy altas (más de 200 ºC). Asimismo es especialmente sensible a las sobrepresiones por lo que debe limitarse la presión de cabeza y no es recomendable usar inyecciones splitless o pulsed split. Sin embargo el flujo de gas portador de trabajo ha de ser mayor que en el caso del FGC puesto que el diámetro interno es mayor. También es mucho más sensible al contenido en agua de la muestra, de forma que requiere unas condiciones de operación, mantenimiento y almacenamiento en sequedad.

Las áreas de pico de los triplicados fueron integradas para determinar las distribuciones enantioméricas de los compuestos levorotatorios (–) y dextrorotatorios (+).

Las condiciones de operación se detallan a continuación: caudal de hidrógeno 2.5 mL/min, presión en cabeza de columna inicial 8 psi, temperatura del inyector 200 ºC, temperatura de la línea de transferencia 200 ºC, relación de split 100:1, volúmenes de sándwich: 0.2 µL de aire, 0.2 µL de acetona, 0.2 µL de aire, 0.5 µL de muestra, 0.2

µL de aire. La programación de temperatura fue: temperatura inicial 35 ºC, subida a 170 ºC a 4 ºC/min y descenso de temperatura controlada a 35 ºC otra vez a 15 ºC/min.

3.5 Capacidad antioxidante

Los espectros de fluorescencia, espectros visibles-ultravioleta y diversos ensayos cinéticos en cubeta se registraron en un espectrómetro de luminiscencia Perkin Elmer LS55 (Figura 3.6), controlado por el programa FL-WinLab y un espectrofotómetro de doble haz Perkin Elmer Lambda 35, controlado con el software UV-WinLab. Otros ensayos cinéticos de absorbancia en espectro visible (Sindhu, 2009) o fluorescencia (Sauer et al., 2011) se efectuaron en lectores de placas de 96 pocillos Molecular Devices, SpectraMax 340PC o Gemini XPS, respectivamente, controlados mediante el software SoftMaxPro. Las placas o microtubos se incubaron en un agitador termostatizado Thermomixer comfort de Eppendorf y las centrifugaciones se llevaron a cabo en una centrífuga Centrifuge 5415D de Eppendorf.

Figura 3.6
Fluorímetro Perkin Elmer LS55 (izda.) y fluoresceína disolviéndose en agua bajo luz ultravioleta (dcha.) (Briksnite/CC-BY-3.0)

3.5.1 Método ORAC

Los ensayos por triplicado se realizaron en el lector de fluorescencia sobre placas opacas de 96 pocillos a 37 ºC y durante 60 min, con λ_{ex}=485 nm y λ_{em}=530 nm (Ou et al., 2001; Ou et al., 2002; Prior et al., 2003). Se utilizó fluoresceína 1 µM, tampón fosfato 10 mM, pH 7.5, AAPH 200mM y concentraciones variables de los aceites esenciales ensayados.

Al tratarse de un medio hidrofílico, los aceites esenciales son insolubles, de manera que es necesario el uso de un codisolvente, en este caso etanol, para conseguir una solubilidad sin turbidez que permitiese la medida de la capacidad antioxidante. Todos los aceites se ensayaron a dilución 1:10000 con un 1% de codisolvente en pocillo. Se usó Trolox como antioxidante de referencia, refiriendo los resultados en unidades TEAC.

3.5.2 Método ABTS

El $ABTS^{\bullet+}$ se produce haciendo reaccionar (Re et al., 1999) volúmenes iguales de ABTS 7 mM (verde claro) con persulfato potásico 2.5 mM y dejando que la reacción se desarrolle en oscuridad a temperatura ambiente por 12–16 horas antes de su uso. La disolución se diluye (1:100) con etanol hasta 0.035 mM para obtener una absorbancia de alrededor de 0.7 a 734 nm.

Se probaron distintas disoluciones en etanol de los aceites esenciales a varias concentraciones (0–0.5 mL/L), los ensayos constaban de dos volúmenes de $ABTS^{\bullet+}$ y uno de muestra. Tras dejar reaccionar a temperatura ambiente durante 6 min en oscuridad, se midió la absorbancia a 734 nm con un espectrofotómetro de placas de 96 pocillos. Absorbancias menores de la mezcla de reacción indican mayor actividad neutralizadora de radicales $ABTS^{\bullet+}$.

La capacidad para reducir el $ABTS^{\bullet+}$ se calculó usando la ecuación (3.1):

$$\text{Capacidad antioxidante (\%)} = \frac{A_{734}^{0} - A_{734}^{1}}{A_{734}^{0}} \times 100 \qquad (3.1)$$

Donde A^0 es la absorbancia del blanco (sin antioxidante) y A^1 es la absorbancia en presencia de la muestra (antioxidante). Análogamente al método ORAC, el antioxidante de referencia fue Trolox y los resultados se expresan en unidades TEAC.

3.5.3 Método DPPH

El ensayo consiste en una reacción (Brandwilliams et al., 1995) durante 60 min, a temperatura ambiente y en oscuridad de dos volúmenes de una disolución metanólica de $DPPH^{\bullet}$ 100 µM violeta oscuro y un volumen de disolución metanólica de la muestra a distintas concentraciones. Después de los seis minutos, se mide la absorbancia a 517 nm y se procede a determinar la capacidad antioxidante según la ecuación (3.2):

$$\text{Capacidad antioxidante (\%)} = \frac{A_{517}^{0} - A_{517}^{1}}{A_{517}^{0}} \times 100 \qquad (3.2)$$

Donde A^0 es la absorbancia del blanco (sin antioxidante) y A^1 es la absorbancia en presencia de la muestra (antioxidante). Análogamente al método ORAC, el antioxidante de referencia fue Trolox y los resultados se expresan en unidades TEAC.

3.5.4 Evaluación de la capacidad quelatante

En este método (Miguel et al., 2010), un volumen de muestra se incuba con un volumen de $FeCl_2$ 2 mM, dejando así un par de minutos con agitación para favorecer la

quelatación de los iones hierro (II) por parte de la muestra. Después se añade el mismo volumen de FerroZine 6 mM iniciando la reacción con los iones no complejados por la muesta, tras 10 minutos en oscuridad se mide la absorbancia a 562 nm (Stookey, 1970). EDTA fue escogido como quelatante de referencia. Los datos se han tratado usando la ecuación (3.3) y la unidad en la que se expresan los resultados hace referencia al complejante de referencia escogido (cantidad de EDTA equivalente / volumen de aceite esencial).

$$\text{Capacidad antioxidante (\%)} = \frac{A_{562}^{0} - A_{562}^{1}}{A_{562}^{0}} \times 100 \qquad (3.3)$$

Donde A^0 es la absorbancia del blanco (sin complejante) y A^1 es la absorbancia en presencia de la muestra (complejante).

3.5.5 Evaluación de la capacidad reductora

Cada muestra de aceite esencial (300 µL) se mezcló con tampón fosfato sódico 0.2 M, pH 6.6, 2.5 mL y ferricianuro potásico 1% (w/v), 2.5 mL (Oyaizu, 1986). La mezcla se incubó a 50 ºC durante 20 min. Después se añadió ácido tricloroacético 10% (w/v), 2.5 mL a la mezcla y se centrifugó a 3000 rpm durante 10 min. La fase superior (2.5 mL) se diluyó con agua (2.5 mL) y se añadió $FeCl_3$ 0.1% (w/v), 0.5 mL y se midió la absorbancia a 700 nm del compuesto azul de Prusia generado. En este caso se miden y representan directamente los valores de absorbancia a 700 nm frente a la concentración de antioxidante, obteniendo las pendientes de las regresiones lineales. Se usó ácido ascórbico (AA) como reductor de referencia. Las unidades se expresan como (cantidad de AA equivalente / volumen de aceite esencial).

3.5.6 Método TBARS

Se utilizó un homogenado de yema de huevo como medio rico en lípidos (Dorman et al., 1995). El homogenado se obtuvo llevando una alícuota de yema de huevo a una concentración de 10% (m/v) en una disolución de KCl (1.15%, m/v), la yema se homogeneizó durante 30 s a mano y se ultrasonicó durante 5 min. En un tubo de ensayo se pusieron 500 µL del homogenato y 100 µL de la muestra disuelta en metanol, completando con agua destilada hasta alcanzar 1 mL, a esto le siguió la adición de 1.5 mL de ácido acético 20% (v/v) consiguiendo pH 3.5 y 1.5 mL de TBA 0.8% (m/v) disuelto en SDS 1.1% (m/v) para lo cual hay que llevarlo a ebullición. Esta mezcla se mantuvo en agitación a 95 ºC durante 60 min. Tras enfriar hasta temperatura ambiente, se adicionaron 5 mL de n-butanol a cada tubo para extraer el aducto rojo, se agitó y centrifugó a 3000 rpm durante 10 min para volver a separar las

fases. La absorbancia del n-butanol con aducto rojo sobrenadante se midió a 532 nm. Obtenemos la capacidad antioxidante utilizando la ecuación (3.4):

$$\text{Capacidad antioxidante}\ (\%) = \frac{A^0_{532} - A^1_{532}}{A^0_{532}} \times 100 \qquad (3.4)$$

Donde A^0 es la absorbancia del blanco (sin antioxidante) y A^1 es la absorbancia en presencia de la muestra (antioxidante). El antioxidante de referencia usado para este medio eminentemente lipofílico es el BHT, así los resultados se expresan con la unidad como (cantidad de BHT equivalente / volumen de aceite esencial).

3.5.7 Método de captura de radicales hidroxilo

La mezcla de reacción (Chung et al., 1997) se preparó con $FeSO_4 \cdot 7H_2O$ 10mM, EDTA 10 mM, 2-desoxi-D-ribosa 10 mM, tampón fosfato 0.1M pH = 7.4 y distintas concentraciones de las muestras en un tubo de ensayo para alcanzar un volumen de 1.8 mL. Finalmente, se añadieron 200 µL de H_2O_2 al 0.1% (v/v) a la mezcla y se incubó a 37 ºC durante 4 horas.

Después, se añadieron al tubo de ensayo 1 mL de ácido tricloroacético 2.8% (m/v) y 1 mL de ácido tiobarbitúrico 1% (m/v). Se llevó la mezcla a ebullición durante 10 min para desarrollar el aducto rosa formado por malondialdehído y ácido tiobarbitúrico (MDA-TBA_2) (Figura 1.19). Después de enfriar, se midió la absorbancia a 520 nm. Se usó manitol como control positivo. Como control negativo se probó el método sin EDTA para comprobar si la capacidad quelatante de las muestras interfería aumentando o disminuyendo significativamente el numero de radicales producidos y se probó también un ensayo sin 2-desoxirribosa para comprobar si las muestras podían degradarse hacia MDA o un compuesto de similares características que diese lugar a un aducto coloreado. En ningún caso se encontraron actividades significativas que pudieran interferir en el buen uso del método. En este caso se expresaron los resultados como IC_{50}, la concentración que reduce a la mitad la capacidad antioxidante máxima (control negativo), expresando en estas mismas unidades el antioxidante de referencia para poder comparar fácilmente.

3.5.8 Método de captura de óxido nítrico

En este método (Ho et al., 2010) usamos 50 µL de diluciones seriadas de la muestra en etanol añadidos a 50 µL de nitroprusiato de sodio 10 mM en tampón fosfato salino. La reacción se llevó a cabo en una placa de 96 pocillos, incubando a temperatura ambiente con luz intensa durante 90 minutos. Finalmente, se añade un volumen igual de reactivo de Griess (Sulfanilamida 1% y α-naftiletilendiamina 0.1% en HPO_3 2.5%) a cada pocillo y se mide la absorbancia a 546 nm. Se usó rutina como

control positivo. En este caso se expresaron los resultados como IC_{50}, la concentración que reduce a la mitad la capacidad antioxidante máxima (control negativo, agua), expresando en estas mismas unidades el antioxidante de referencia para poder comparar fácilmente.

3.6 Inhibición enzimática

3.6.1 Lipoxigenasa

La enzima se compró en Sigma como una preparación de lipoxidasa de *Glycine max* (soja) y estaba certificado como un homodímero de 108 kDa con un pI de 5.65. La enzima se caracterizó en términos de especificidad de sustrato, siendo el sustrato un sistema *cis, cis*-1,4-pentadieno.

El ensayo (Christop.J et al., 1970) se llevó a cabo en un espectrofotómetro a 25ºC. El espectrofotómetro estaba equipado con un par de cubetas de cuarzo de calidad QS, certificadas como transparentes para longitudes de onda desde 195 nm, un camino óptico de 10 mm y volumen de 1 mL, una se usó como referencia para la substracción automática y la otra para la muestra. La referencia contenía todos los componentes excepto el sustrato y los inhibidores. Se llevaron a cabo ensayos no enzimáticos y sus velocidades se restaron de las velocidades de estado estacionario de las respectivas reacciones enzimáticas. Todas las muestras se evaluaron por triplicado. La actividad de la enzima se midió basándose en la pendiente de la porción inicial lineal (velocidad de estado estacionario) de las curvas de progreso de la reacción enzimática.

El ácido linoleico se disolvió usando acetonitrilo de gradiente cromatográfico -210 como codisolvente. Este acetonitrilo se pudo usar como codisolvente gracias a su nula absorbancia a 234 nm, además se comprobó que no interfería en la reacción enzimática significativamente en las bajas proporciones utilizadas. Ambas disoluciones de enzima y sustrato se mantuvieron en hielo durante los ensayos para ralentizar su degradación (Whent et al., 2010).

La mezcla de reacción contenía tampón borato 40 mM a pH 9.0, NaOH 4 mM, concentración variable de muestra (inhibidor) en acetonitrilo 10% (v/v) y ácido linoleico 20 µM. Así se obtiene una disolución en cubeta final con un 6% de acetonitrilo. La reacción comienza con la adición de LOX. LOX se preparó para obtener un disolución de 6.24 mU/mL. Consideramos como una unidad (U) de actividad enzimática, la cantidad de enzima que cataliza la conversión de 1 µmol de ácido linoleico como sustrato por minuto. La K_M de la enzima se determinó usando las condiciones de ensayo obteniendo $K_M = (40 \pm 6)$ µM.

Una vez obtenidos los distintos valores de actividad a las diferentes concentraciones de inhibidor, se calculó el grado de inhibición (ID) usando la ecuación (3.5).

$$\mathrm{ID}(\%) = \frac{v_0 - v_i}{v_0} \times 100 \qquad (3.5)$$

Donde v_0 y v_i son las velocidades de estado estacionario en ausencia (control negativo, agua) y presencia de inhibidor, respectivamente.

Los componentes principales de los aceites esenciales se estudiaron para determinar sus valores de IC_{50}. Se representaron los datos de actividad de 8 concentraciones diferentes de inhibidor y se ajustaron a un modelo de regresión no lineal de dos parámetros de hipérbola rectangular expresada según la ecuación (3.6).

$$y = \frac{ax}{b + x} \qquad (3.6)$$

El parámetro *b* representa el IC_{50}, usando un algoritmo de Gauss-Newton, implementado en el software de Sigma Plot.

En este caso el NDGA se ensayó con este método como control positivo.

3.6.2 Hialuronidasa

Se disolvieron 50 µL de hialuronidasa bovina (7900 unidades/mL) en tampón acetato 0.1 M a pH 3.6 y se mezclaron con 50 µL de muestra a distintas concentraciones, esto se incubó a 37ºC durante 20 min. Después, se añadieron 50 µL de cloruro cálcico 12.5 mM y prosiguió la incubación 20 min más a 37ºC. Esta hialuronidasa activada por Ca^{2+} se trató con 250 µL de hialuronato sódico 1.2 mg/mL y se incubó 40 min a 37ºC. Obteniendo el acetilamino azúcar como producto de la actividad enzimática (Sahasrabudhe and Deodhar, 2010).

Después de la incubación, se añadieron 50 µL de hidróxido sódico 0.4 M y 100 µL de borato sódico 0.2 M buscando un pH ≈ 8.9 y se incubó en agua hirviendo durante 3 min, procurando esta reacción enérgica en medio básico, se forma el intermedio necesario para el siguiente paso (probablemente una glucoxazolina (1,3,5)) y también se desnaturaliza la enzima (Reissig et al., 1955). Tras enfriar a temperatura ambiente, se añadieron 1.5 mL de disolución de pDMAB (4 g de pDMAB disuelto en 50 mL de HCl 10 N y 350 mL de ácido acético glacial), y se leyó la absorbancia a 585 nm.

Una vez obtenidos estos datos se trataron para obtener el grado de inhibición a punto final con la ecuación (3.7):

$$\mathrm{ID}(\%) = \frac{A^0_{585} - A^1_{585}}{A^0_{585}} \times 100 \qquad (3.7)$$

Donde A^0 y A^1 son las absorbancias a 585 nm de los ensayos en blanco (control negativo, agua) y en presencia de muestra, respectivamente.

3.7 Análisis de datos

Todos los datos de este trabajo se obtuvieron de ensayos en triplicado como mínimo y se presentan como media ± desviación estándar. Cada valor de error ha sido magnificado usando las reglas de propagación de errores correspondientes para operaciones aritméticas.

Los ajustes de regresión lineal y no lineal, de las tendencias mostradas por los datos obtenidos, se realizaron con el programa SigmaPlot que cuenta con el algoritmo de optimización de mínimos locales de Levenberg-Marquardt. Este algoritmo, ampliamente utilizado para ajustes de regresión no lineal, interpola entre el algoritmo de Gauss-Newton y el método de la máxima pendiente, resultando un método más robusto que los dos anteriores.

La calidad de los datos fue analizada por ANOVA (Sokal and Rohlf, 2012) y las medias fueron comparadas usando el test de Tukey HSD (Honestly-Significant-Difference), considerando significativas las diferencias para valores $p < 0.05$.

La agrupación aglomerativa jerárquica basada en distancias euclidianas mediante enlace simple por mínimas distancias a las áreas relativas de los componentes, se llevó a cabo usando cada aceite esencial como grupo inicial para decir cuáles eran más parecidos. Los análisis estadísticos se llevaron a cabo usando el programa SPSS (Chicago, SPSS Inc.).

Resultados y Discusión

"El mundo exige resultados, no le cuentes a otros tus dolores de parto, muéstrales al niño."

Indira Gandhi.

4. CARACTERIZACIÓN DE ACEITE ESENCIAL DE *LAVANDULA STOECHAS*

4.1 Resumen

Los aceites esenciales de *Lavandula stoechas*, obtenidos de plantas cultivadas en el sureste de España, han sido analizados por cromatografía de gases con detección por espectrometría de masas para determinar su composición, en ambas concentraciones relativa (por área de pico) y absoluta (utilizando rectas de calibrado). Las moléculas más abundantes halladas entre los componentes principales fueron: fenchona (33-37%), alcanfor (16-24%) y eucaliptol (17-18%). Esta caracterización se completó con el uso de cromatografía de gases enantioselectiva, detectando los componentes principales dextrorotatorios (limoneno, fenchona y alcanfor) y levorotatorios (canfeno, linalol o (*E*)-β-cariofileno). Estos enantiomeros atestiguan el origen natural de los aceites esenciales. La capacidad antioxidante se evaluó con resultados positivos por varios métodos: actividad frente a radicales libres (ORAC, ABTS, DPPH), capacidades quelatante y reductora, principalmente debido a los componentes linalol y timol. Se observó una moderada actividad inhibidora de lipoxigenasa lo cual es indicador de una posible actividad antiinflamatoria, principalmente debida al timol, la fenchona y el alcanfor. Estas propiedades apoyan el uso potencial del aceite esencial de *Lavandula stoechas* como cosmético natural e ingrediente farmacéutico natural para tratar diversos desórdenes cutáneos.

4.2 Summary

Lavandula stoechas essential oils (EOs), obtained from plants grown in the South East of Spain, were analyzed by gas chromatography coupled with mass spectrometry (GC/MS) detection to determine their composition, in both relative (peak area) and absolute (using standard curves) concentrations. Fenchone (33-37%), camphor (16-24%) and eucalyptol (17-18%) were determined as the most abundant molecules among the principal compounds. This characterization was completed with the enantioselective gas chromatography, depicting limonene, fenchone or camphor as the main dextrorotatory components and camphene, linalool or (*E*)-β-caryophyllene as the main levorotatory components. These enantiomers provide proof of the natural origin of the EOs. Antioxidant activity was evaluated positively by several methods: activity against free radicals (ORAC, DPPH, ABTS), chelating and reducing power, probably due to linalool and thymol. Mild inhibitory activity on lipoxygenase (LOX) was observed indicating a possible anti-inflammatory activity, mainly due to thymol, fenchone and camphor. These properties support the potential use of *L. stoechas* EOs as natural cosmetic and natural pharmaceutical ingredients for several skin diseases.

4.3 Introduction

Lavandula stoechas also known as 'cantueso', French lavender, Spanish lavender or topped lavender, is a species of aromatic flowering plant of the Lamiaceae family. The genus *Lavandula*, of the Lamiaceae family, consists of approximately 20 species with more than 100 varieties of lavender (Da Porto and Decorti, 2008).

Lavandula stoechas is an evergreen shrub, it usually grows up to one meter high with spike violet flowers. It occurs naturally in Mediterranean countries. It has been used as cooking spices and fragrance, and its essential oil (EO) is one of the aromatic ingredients in the production of food, drinks, soaps, perfumes, cosmetics and pharmaceuticals (Benabdelkader et al., 2011; Goren et al., 2002; Kaya et al., 2012; Msaada et al., 2012; Zuzarte et al., 2013). These applications have been related with their bioactivities as natural antibacterial, antifungal, insecticide, antioxidant and anti-inflammatory agents, with low toxicity for human skin cells (Benabdelkader et al., 2011; Goren et al., 2002; Kaya et al., 2012; Kirmizibekmez et al., 2009; Matos et al., 2009; Msaada et al., 2012; Zuzarte et al., 2013). To our knowledge the EOs of *Lavandula stoechas* grown in Murcia (Spain) have not been studied yet in the literature.

Generally, EOs are lipophilic extracts composed of many monoterpenoids and sesquiterpenoids, mainly obtained by steam distillation from flowers, herbs and spices, although each species has different percentages of each of them (Baser and Buchbauer, 2010). They have been widely used throughout history in aromatherapy, flavoring and perfumes, but also in traditional treatments as analgesic, anti-inflammatory, bacteriostatic, diuretic, expectorant and fungicide agents. Thus, EOs are nowadays being studied and used in the chemical, cosmetic, food, fragrance and pharmaceutical industries (Baser and Buchbauer, 2010; Bilia et al., 2014; Gautam et al., 2014; Lesgards et al., 2014; Raut and Karuppayil, 2014; Seow et al., 2014; Tongnuanchan and Benjakul, 2014).

The composition of EOs is complex and it can vary from just a dozen to hundreds of compounds per EO (Baser and Buchbauer, 2010). The main molecules of these mixtures are some phenylpropanoids, and many volatile monoterpenoids and sesquiterpenoids (10 or 15 carbon skeleton, respectively) containing a wide range of organic functions like alcohols, ethers, aldehydes, ketones or esters. The evaluation of the authenticity of EOs is the key to guarantee the safety and traceability of the industrial products made up of them (Baser and Buchbauer, 2010; Bauermann et al., 2008). Essential Oils can be adulterated by replacing natural enantiomers with inexpensive synthetic racemates which have different bioactivities and organoleptic properties from those of true EOs, leading to man-made EOs that are not suitable to be certified as natural products.

Gas chromatography (GC), with flame ionization or mass spectrometry (MS) detection, is a powerful technique used for the analysis of volatile components, since it provides qualitative and quantitative data for complex mixtures such as those usually present in EOs. Although flame ionization detection was traditionally used due to its wider linear range of instrumental response, nowadays MS detection has reached high popularity thanks to both its suitable range of instrumental response and the valuable mass spectra provided, comparable with those of standards and spectral libraries (O'Shea et al., 2012; Rubiolo et al., 2010; Smelcerovic et al., 2013; Turek and Stintzing, 2013). Some relative quantitations of the *Lavandula stoechas* composition

have been reported (Benabdelkader et al., 2011; Hassiotis, 2010). None of them has studied the specific conditions of the Spanish Mediterranean coast, specifically the region of Murcia where the biggest aromatic plant diversity is found among all regions of the country.

Furthermore, there are no chiral complete studies on EOs of *Lavandula stoechas*. Chiral distribution is an important aspect of the EO composition, it allows to identify natural EOs from those adulterated with racemic mixtures of synthetic compounds (Bicchi et al., 2010; Konig et al., 1997; Smelcerovic et al., 2013), even when the samples come from different places of the Earth (del Castillo et al., 2004; Tranchida et al., 2012). In addition, the chirality in terpenes is highly important due to the different bioactivities and organoleptic properties of each of the enantiomers (Baser and Buchbauer, 2010; Marchelli et al., 1996; Rubiolo et al., 2010), that is applied to drugs, fragrances and flavors among others.

The healthy properties of EOs have been related with their antioxidant activities (Baser and Buchbauer, 2010; Gupta et al., 2014; Lee and Shibamoto, 2002; Peng et al., 2014; Rajendran et al., 2014; Rubio et al., 2013). The antioxidant potential of EOs can be determined using a representative selection of different antioxidant methods, *i.e.* ORAC method for evaluation of the scavenging capacity of free oxygen (peroxyl) radicals; ABTS or DPPH methods able to measure ability to reduce nitrogen radical cations; as well as the Fe^{2+} chelating power (ChP) and Fe^{3+} reducing power (RdP) methods, accounting for the ability to neutralize oxidant metal ions (Bentayeb et al., 2014; Dawidowicz and Olszowy, 2014; Huang et al., 2005; Rubio et al., 2013).

In addition, the bioactivities of EOs have also been related with their anti-inflammatory properties (Gautam et al., 2014; Lesgards et al., 2014; Rubio et al., 2013). Many inflammatory processes are usually associated with leukotriene production catalized by lipoxygenase (LOX), which can use molecular oxygen or hydrogen peroxide as oxidants (Anwar et al., 2014; Fang et al., 2014; Lin et al., 2014). Thus, the inhibition of soybean lipoxygenase as LOX model, is a hint of anti-inflammatory activity of the EO (Kim et al., 2014; Mandal et al., 2014; Mohamed et al., 2014).

The aim of the present study is to determine, for the first time in literature, the relative, absolute and chiral distribution of each of the EOs main constituents, in three samples of EOs from *L. stoechas* grown in Murcia (Spain). Then, the above mentioned methods (ORAC, ABTS, DPPH, ChP, RdP) will be applied to evaluate the antioxidant capacities of the *Lavandula stoechas* EOs. Furthermore, the degree of inhibition of LOX, at a fixed concentration of the EOs, will serve to characterize the inhibitory activity of LOX by these EOs. The experimental results will be compared with those reported for *L. stoechas* EOs from other countries, and their potential biotechnological applications will be discussed.

4.4 Results and Discussion

4.4.1 FGC-EI/MS study

Sample-1 was grown in Supra Mediterranean bioclimatic zone and Samples-2 and -3 were grown in Upper Meso-Mediterranean bioclimatic zones. The essential oils were obtained by hydrodistillation in yields ranging from 0.3 to 1% (w/w). The components of the studied EOs were determined by FGC-EI/MS using calibration

curves (Table 4.1). Each EO is described with two columns. The first column determines the exact concentration of the terpenes expressed in mM (commercially available standards only, represent >90% of the total area). In the second column, the total area of each compound present in the chromatogram was integrated (>99%). Two EOs with similar component proportion but different solvent concentrations, not detectable by either being too volatile (eluting in the solvent delay time or simply unretained), too heavy to be volatile, or using the same solvent for dilution and for sample preparation, can be determined thanks to the first and second columns because of the higher absolute concentration of the EOs terpenes.

Table 4.1.
Parameters of FGC-EI/MS calibration.

Analyte	Calibration curve*	R^2	Calibration range (mM)	RSD (%)	LOD (mM)	LOQ (mM)	Standard source	Product Reference
Nonane	*Internal standard*						SAFC	442694
(-)-α-Pinene	y =-0.018 + 0.471x	0.997	0.25 - 10.06	4.5	0.08	0.25	Fluka	80599
(+)-Camphene	y =-0.008 + 0.272x	0.995	0.66 - 10.51	0.4	0.14	0.44	SAFC	w222909
Sabinene	y =-0.026 + 0.460x	0.998	0.24 - 9.78	0.9	0.07	0.21	Extrasynthese	5062 S
(-)-β-Pinene	y =-0.016 + 0.400x	0.995	0.26 - 10.29	3.7	0.09	0.26	Fluka	80609
Myrcene	y =-0.024 + 0.188x	0.993	2.24 - 8.97	3.5	0.56	1.70	Fluka	64643
(+)-3-Carene	y =-0.012 + 0.355x	0.999	0.25 - 10.05	1.4	0.08	0.25	Aldrich	441619
p-Cymene	y =-0.020 + 0.830x	0.995	0.25 - 9.95	4.9	0.02	0.05	Aldrich	c121452
(+)-Limonene	y =-0.008 + 0.246x	0.995	0.60 - 9.55	0.9	0.12	0.36	Fluka	62118
Eucalyptol	y =-0.008 + 0.233x	0.995	2.39 - 9.55	8.7	0.60	1.82	SAFC	w246506
γ-Terpinene	y =-0.017 + 0.366x	0.995	0.62 - 9.87	2.8	0.12	0.37	Aldrich	223190
(+)-Fenchone	y =-0.069 + 0.613x	0.997	0.62 - 9.92	0.7	0.11	0.33	Fluka	46208
(-)-Linalool	y =-0.008 + 0.214x	0.994	0.88 - 8.80	4.9	0.22	0.67	Fluka	74856
(+)-Camphor	y =-0.024 + 0.181x	0.994	0.99 - 9.85	1.5	0.25	0.76	Alfa Aesar	A10708
(-)-Borneol	y =-0.020 + 0.266x	0.998	0.57 - 9.06	4.4	0.19	0.57	Alfa Aesar	A12684
(-)-Terpinen-4-ol	y =-0.003 + 0.241x	0.997	0.60 - 9.57	4.3	0.20	0.60	Aldrich	11584
(+)-α-Terpineol	y =-0.003 + 0.175x	0.998	1.02 - 10.23	4.5	0.26	0.79	Fluka	83073
Verbenone	y =-0.013 + 0.093x	0.993	0.99 - 9.88	4.4	0.25	0.76	Aldrich	218251
Tetradecane	*Internal standard*						SAFC	442708
(-)-Linalyl acetate	y =-0.006 + 0.217x	0.998	0.72 - 7.18	0.2	0.18	0.55	SAFC	w263605
(-)-Bornyl acetate	y =-0.003 + 0.268x	0.997	0.82 - 8.16	1.4	0.21	0.65	Fluka	45855
Thymol	y =-0.029 + 0.313x	0.998	0.25 - 10.03	4.8	0.08	0.25	Sigma	T0501
Neryl acetate	y =-0.007 + 0.242x	0.999	0.73 - 7.31	3.8	0.18	0.55	SAFC	w277304
Geranyl acetate	y =-0.009 + 0.249x	0.999	0.46 - 7.42	2.9	0.15	0.46	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(-)-(*E*)-β-Caryophyllene	y =-0.002 + 0.079x	0.998	4.41 - 7.05	6.6	0.71	2.16	Sigma	22075
(+)-Ledene	y =-0.002 + 0.058x	0.999	0.70 - 6.92	5.1	0.17	0.52	Aldrich	61770
Bisabolene	y =-0.043 + 0.111x	0.999	1.74 - 6.97	3.4	0.31	0.94	Alfa Aesar	A18724
(-)-Caryophyllene oxide	y =-0.004 + 0.105x	0.999	2.54 - 10.17	2.7	0.46	1.40	SAFC	w509647

*Response ratio vs. Concentration ratio, internal standard correction applied.
Each internal standard is reference compound for the analytes that follow.

The results depict three different *Lavandula stoechas* samples (Table 4.2) having the same six principal compounds, i.e. α-Pinene, camphene, limonene,

eucalyptol, fenchone and camphor. However, Sample-1 has three other abundant molecules, namely linalool, lavandulyl acetate and valencene and Sample-2 along with Sample-3 show three other common abundant molecules, namely bornyl acetate, thymol and myrtenyl acetate. The 9 principal components in Sample-1 represent 90.5% of the total area; the 9 principal components of Sample-2 represent 88.2% and 89.1% in the case of Sample-3.

69 compounds were studied from these three *Lavandula stoechas* samples (Table 4.2). Sample-1 presented 50 compounds, Sample-2 had 54 and Sample-3 had 58 different molecules. Despite the number of compounds the biggest difference is found between Sample-1 and the group formed by Samples-2 and -3, the principal compounds are not the same and the concentrations of the common compounds differ. This conclusion is supported by a dendrogram representation (Figure 4.1) of agglomerative hierarchical clustering based on Euclidean distance applied to the relative area of components, where Sample-2 and -3 are part of the same group, showing high similarity (90.55%), while Sample-1 remains separate, similarity level of 0.57%.

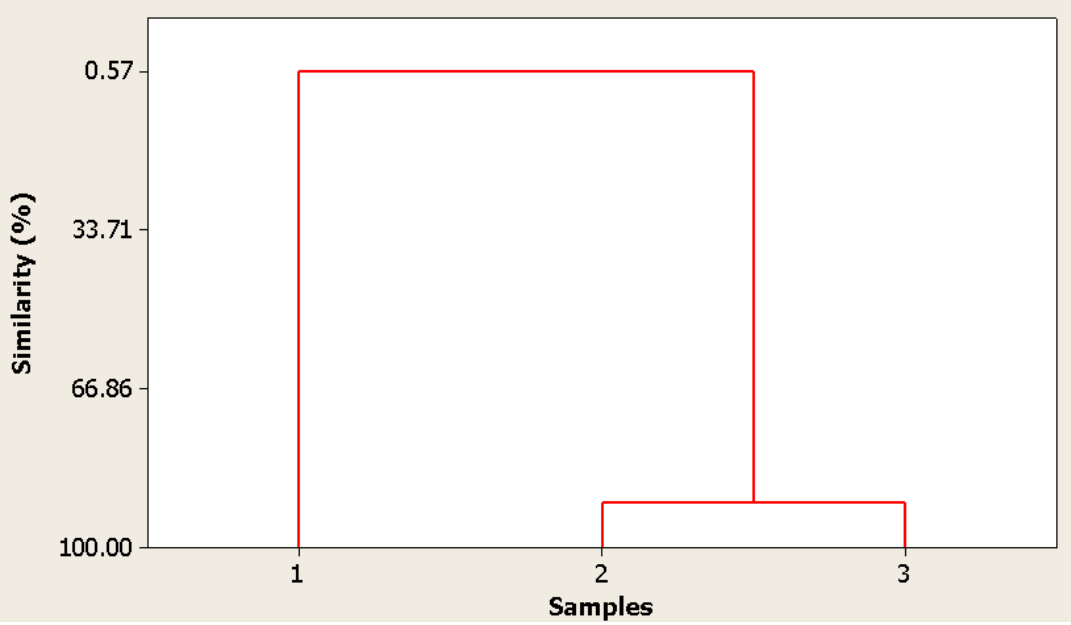


Figure 4.1
Dendrogram of *L. stoechas* samples

Oxygenated monoterpenes are highly predominant in the three samples (Table 4.2), accounting for more than 80% of the total compounds. Ketone is the most abundant organic functional group, exceeding 50% of total molecules, the second organic functional group in abundance is ether almost reaching 20%. The hydrocarbon monoterpenes represent up to 11%, being the second group in abundance. Total terpene hydrocarbons were calculated as the sum of the monoterpene and sesquiterpene hydrocarbons. Total oxygenated terpenes were calculated as the sum of the oxygenated monoterpenes and sesquiterpenes.

Table 4.2. FGC-EI/MS determination of EOs components.

t_r (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Sample-1		Sample-2		Sample-3		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
1.27	890	Bicyclo[4.2.0]oct-7-ene	39, 41, 67, 79				0.1^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS
1.47	929	Tricyclene	79, 93, 121, 136		0.1^{d} ± 0.0		0.2^{e} ± 0.0		0.2^{e} ± 0.0	LRI, MS
1.52	938	α-Pinene	39, 91, 93*, 77	243.3^{a} ± 2.7	4.5^{d} ± 0.0	109.5^{b} ± 2.8	2.3^{e} ± 0.1	109.6^{b} ± 5.5	2.3^{e} ± 0.0	LRI, MS, std
1.62	955	Camphene	67, 79, 93*, 121	66.2^{a} ± 4.2	1.0^{d} ± 0.0	126.8^{b} ± 3.4	2.0^{e} ± 0.1	98.1^{c} ± 8.3	2.0^{e} ± 0.1	LRI, MS, std
1.64	959	Dehydrosabinene	65, 91, 119, 134		0.2^{d} ± 0.0		0.2^{d} ± 0.0		0.2^{d} ± 0.1	LRI, MS
1.75	978	Sabinene	41, 77, 91, 93*	8.4 ± 0.2	0.1^{d} ± 0.0		0.1^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS, std
1.79	985	β-Pinene	41, 69, 79, 93*	32.7^{a} ± 0.9	0.7^{d} ± 0.0	14.6^{b} ± 0.2	0.3^{e} ± 0.0	11.8^{c} ± 0.4	0.4^{e} ± 0.1	LRI, MS, std
1.82	990	Myrcene	39, 41*, 69, 93	24.7^{a} ± 0.1	0.2^{d} ± 0.0	12.5^{b} ± 0.9	0.1^{e} ± 0.0	12.0^{b} ± 0.9	$0.1^{d,e}$ ± 0.1	LRI, MS, std
1.86	997	Isomyrcenol	79, 91, 119, 134						0.1 ± 0.0	LRI, MS
1.98	1012	3-Carene	77, 79, 91, 93*		0.1^{d} ± 0.0	34.3^{a} ± 0.5	0.5^{e} ± 0.0	34.0^{a} ± 1.3	0.5^{e} ± 0.1	LRI, MS, std
2.04	1019	2-Carene	77, 93, 121, 136						0.0 ± 0.0	LRI, MS
2.07	1022	m-Cymene	77, 91, 119, 134						0.1 ± 0.0	LRI, MS
2.11	1026	p-Cymene	77, 91, 119*, 134	22.1^{a} ± 0.3	0.5^{d} ± 0.0	32.2^{b} ± 1.0	0.8^{e} ± 0.0	28.6^{c} ± 1.4	0.7^{e} ± 0.0	LRI, MS, std
2.14	1030	Limonene	67, 68*, 79, 93	128.4^{a} ± 2.0	2.2^{d} ± 0.0	84.1^{b} ± 1.9	1.4^{e} ± 0.1	71.7^{c} ± 4.5	1.5^{e} ± 0.2	LRI, MS, std
2.18	1034	Eucalyptol	43*, 67, 81, 93	835.3^{a} ± 11.8	17.8^{d} ± 0.2	793.2^{a} ± 34.2	17.2^{d} ± 0.2	1500.9^{b} ± 79.8	18.0^{d} ± 0.5	LRI, MS, std
2.25	1043	Ocimene	41, 79, 91, 93*		0.1 ± 0.0					LRI, MS, std
2.39	1059	γ-Terpinene	77, 91, 93*, 136	6.9 ± 0.1	0.1 ± 0.0					LRI, MS, std
2.66	1089	Terpinolene	93, 105, 121, 136		0.2 ± 0.0					LRI, MS
2.71	1095	Fenchone	41, 69, 81*, 152	1472.4^{a} ± 48.7	37.0^{d} ± 0.3	1581.0^{a} ± 59.2	33.6^{e} ± 0.5	2595.6^{b} ± 132.9	33.8^{e} ± 0.9	LRI, MS, std
2.81	1106	Linalool	41, 55, 69, 93*	191.6^{a} ± 6.6	7.5^{d} ± 0.0	38.4^{b} ± 1.7	0.8^{e} ± 0.1	44.7^{b} ± 2.0	0.7^{e} ± 0.2	LRI, MS, std
3.06	1131	*exo*-Fenchol	69, 81, 93, 111		0.5^{d} ± 0.0		0.3^{e} ± 0.1		0.3^{e} ± 0.1	LRI, MS
3.31	1157	Camphor	41, 81, 95*, 108	1279.0^{a} ± 10.7	15.6^{d} ± 0.4	931.5^{b} ± 54.8	24.0^{e} ± 0.4	2014.7^{c} ± 119.9	24.1^{e} ± 1.0	LRI, MS, std
3.46	1172	Lavandulol	41, 69, 111, 123		0.2 ± 0.0					LRI, MS
3.54	1180	1-Butenylidenecyclohexane	79, 93, 107, 136		0.3^{d} ± 0.1		0.2^{d} ± 0.1		0.2^{d} ± 0.1	LRI, MS
3.57	1183	Borneol	41, 93, 95*, 121	22.7^{a} ± 0.6	0.5^{d} ± 0.1	21.1^{a} ± 1.1	0.3^{e} ± 0.0	22.4^{a} ± 0.1	0.3^{e} ± 0.1	LRI, MS, std
3.63	1189	Terpinen-4-ol	71, 77, 91, 93*	16.3^{a} ± 1.6	0.4^{d} ± 0.0	18.4^{a} ± 1.3	0.3^{d} ± 0.0	32.0^{b} ± 0.0	0.4^{d} ± 0.1	LRI, MS, std

Table 4.2. continued

t_r (min)	LRI	Analyte	Qualifying and quantitation ions* (m/z)	Sample-1		Sample-2		Sample-3		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
3.66	1192	Eucarvone	91, 107, 135, 150		$0.2^{d} \pm 0.0$		$0.2^{d,e} \pm 0.0$		$0.1^{e} \pm 0.0$	LRI, MS
3.71	1198	p-Cymenene	65, 91, 117, 132		$0.3^{d} \pm 0.0$		$0.4^{e} \pm 0.0$		$0.4^{e} \pm 0.0$	LRI, MS
3.77	1204	α-Terpineol	59, 68, 79, 93*	$32.9^{a} \pm 1.0$	$0.6^{d} \pm 0.0$	$25.5^{b} \pm 0.7$	$0.5^{d,e} \pm 0.0$	$20.0^{c} \pm 1.2$	$0.5^{e} \pm 0.1$	LRI, MS,std
3.80	1207	Myrtenol	79, 91, 119, 134				$0.6^{d} \pm 0.0$		$0.7^{d} \pm 0.2$	LRI, MS
3.89	1216	Verbenone	41, 79, 91, 107*	$19.2^{a} \pm 0.4$	$0.1^{d} \pm 0.0$	$16.2^{b} \pm 0.1$	$0.1^{d} \pm 0.1$	$23.4^{c} \pm 0.5$	$0.1^{d} \pm 0.0$	LRI, MS,std
3.93	1220	Isopulegone	67, 81, 109, 137		$0.1^{d} \pm 0.0$		$0.2^{e} \pm 0.0$		$0.2^{d,e} \pm 0.0$	LRI, MS
3.99	1227	Carveol	91, 105, 119, 134		$0.1^{d} \pm 0.0$		$0.1^{d} \pm 0.0$		$0.1^{d} \pm 0.0$	LRI, MS
4.20	1248	Linalyl acetate	41, 69, 93*, 121	$19.2^{a} \pm 0.4$	$0.4^{d} \pm 0.0$	$6.4^{b} \pm 0.3$	$0.1^{e} \pm 0.0$	$5.1^{c} \pm 0.0$	$0.1^{e} \pm 0.1$	LRI, MS, std
4.27	1256	Phenethyl acetate	65, 77, 91, 104				0.1 ± 0.0			LRI, MS
4.42	1271	Dihydrocarveol acetate	43, 93, 107, 121		$0.3^{d} \pm 0.0$				$0.1^{e} \pm 0.0$	LRI, MS
4.54	1284	Lavandulyl acetate	43, 69, 93, 121		3.5 ± 0.0					LRI, MS
4.57	1287	Bornyl acetate	79, 93*, 95, 121	$16.2^{a} \pm 0.3$	$0.4^{d} \pm 0.0$	$87.1^{b} \pm 4.0$	$1.8^{e} \pm 0.0$	$72.5^{c} \pm 3.4$	$1.9^{e} \pm 0.1$	LRI, MS, std
4.61	1291	2,6-Dimethyl-1,3,5,7-octatetraene	91, 105, 119, 134		0.3 ± 0.0					LRI, MS
4.71	1301	Isoterpinolene	79, 93, 121, 136		0.3 ± 0.0					LRI, MS
4.82	1311	Thymol	91, 115, 135*, 150	$4.7^{a} \pm 0.2$	$0.1^{d} \pm 0.1$	$195.7^{b} \pm 0.8$	$2.0^{e} \pm 0.1$	$127.0^{c} \pm 2.2$	$1.7^{e} \pm 0.4$	LRI, MS, std
4.90	1318	Cuminol	105, 119, 135, 150				$0.1^{d} \pm 0.0$		$0.1^{d} \pm 0.1$	LRI, MS
5.01	1329	Myrtenyl acetate	91, 119, 134, 152				$3.8^{d} \pm 0.1$		$3.8^{d} \pm 0.0$	LRI, MS
5.24	1350	α-Cubebene	105, 119, 161, 204				$0.1^{d} \pm 0.0$		$0.1^{d} \pm 0.0$	LRI, MS
5.33	1359	Neryl acetate	41, 69, 79, 93*	4.6 ± 0.1	0.1 ± 0.0					LRI, MS, std
5.48	1373	Cycloisosativene	119, 133, 161, 204				$0.2^{d} \pm 0.0$		$0.2^{d} \pm 0.0$	LRI, MS
5.54	1379	Geranyl acetate	41, 69*, 79, 93	$3.4^{a} \pm 0.1$	$0.1^{d} \pm 0.0$	$9.7^{b} \pm 0.2$	$0.3^{e} \pm 0.0$	$14.0^{c} \pm 0.2$	$0.3^{e} \pm 0.0$	LRI, MS, std
6.05	1428	(*E*)-β-Caryophyllene	41, 69, 79, 93*	$20.1^{a} \pm 0.9$	$0.2^{d} \pm 0.0$	$18.1^{a} \pm 1.0$	$0.2^{d} \pm 0.0$	$24.3^{b} \pm 0.4$	$0.2^{d} \pm 0.0$	LRI, MS, std
6.42	1466	Humulene	41, 67, 80, 93		$0.1^{d} \pm 0.0$				$0.1^{d} \pm 0.0$	LRI, MS
6.47	1471	Aromadendrene	119, 133, 161, 204				$0.1^{d} \pm 0.0$		$0.1^{d} \pm 0.0$	LRI, MS
6.71	1495	Valencene	147, 161, 189, 204		$1.4^{d} \pm 0.0$		$0.2^{e} \pm 0.0$		$0.1^{e} \pm 0.0$	LRI, MS
6.75	1499	Ledene	41, 91, 105, 107*	$13.5^{a} \pm 0.3$	$0.1^{d} \pm 0.0$	$10.8^{b} \pm 0.7$	$0.1^{d} \pm 0.0$	$17.2^{c} \pm 0.8$	$0.1^{d} \pm 0.0$	LRI, MS, std

Table 4.2. continued

t_r (min)	LRI	Analyte	Qualifying and quantitation ions* (m/z)	Sample-1		Sample-2		Sample-3		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
6.77	1502	Seychellene	161, 175, 189, 204				0.1^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS
6.78	1504	α-Selinene	161, 175, 189, 204				0.2^{d} ± 0.0		0.2^{d} ± 0.0	LRI, MS
6.89	1522	Germacrene D	105, 119, 161, 204		0.1 ± 0.0					LRI, MS
6.92	1527	δ-Cadinene	134, 161, 189, 204		0.1^{d} ± 0.0		0.5^{e} ± 0.1		0.5^{e} ± 0.0	LRI, MS
6.94	1530	Calamenene	105, 129, 159, 202		0.1^{d} ± 0.0		0.2^{e} ± 0.0		0.1^{d} ± 0.0	LRI, MS
7.01	1542	α-Gurjunene	147, 161, 189, 204		0.1 ± 0.0					LRI, MS
7.05	1549	Bisabolene	41, 79, 93*, 119	17.0^{a} ± 0.4	0.2^{d} ± 0.0	12.6^{b} ± 0.3	0.1^{e} ± 0.0	12.3^{b} ± 0.7	0.1^{e} ± 0.0	LRI, MS, std
7.08	1554	γ-Gurjunene	161, 175, 189, 204		0.3^{d} ± 0.0		0.2^{e} ± 0.0		0.1^{e} ± 0.0	LRI, MS
7.13	1562	Sesquisabinenhydrate	69, 119, 161, 207				0.2^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS
7.22	1577	Spathulenol	91, 105, 119, 159				0.2^{d} ± 0.0		0.2^{d} ± 0.0	LRI, MS
7.32	1594	Caryophyllene oxide	41, 79, 91*, 105	7.2^{a} ± 0.7	0.1^{d} ± 0.0	11.3^{b} ± 0.4	0.1^{e} ± 0.0	11.7^{b} ± 0.6	$0.1^{d,e}$ ± 0.0	LRI, MS, std
7.39	1607	Viridiflorol	147, 161, 189, 204		0.1^{d} ± 0.0		0.5^{e} ± 0.0		0.5^{e} ± 0.0	LRI, MS
7.44	1618	Cubenol	119, 161, 189, 204				0.3^{d} ± 0.2		0.1^{d} ± 0.0	LRI, MS
7.47	1625	Mayuron	91, 161, 189, 204				0.2^{d} ± 0.0		0.2^{e} ± 0.0	LRI, MS
7.54	1642	δ-Cadinol	105, 119, 161, 204				0.1^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS
7.67	1671	Guaiol	133, 161, 189, 204		0.3^{d} ± 0.0		0.1^{e} ± 0.0		0.1^{e} ± 0.0	LRI, MS
7.83	1710	Z-Lanceol	105, 119, 159, 202				0.1^{d} ± 0.0		0.1^{d} ± 0.0	LRI, MS

Table 4.2. continued

t_r (min)	LRI	Analyte	Qualifying and quantitation ions* (m/z)	Sample-1		Sample-2		Sample-3		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
		Oxygenated terpenes:								
		Alcohol			10.26		7.26		6.02	
		Ketone			53.09		58.39		58.48	
		Aldehyde			0.00		0.00		0.00	
		Ester			4.69		6.08		6.27	
		Ether			17.86		17.36		18.13	
		Monoterpene hydrocarbons			11.08		8.33		8.56	
		Oxygenated monoterpenes			85.35		86.56		87.51	
		Sesquiterpene hydrocarbons			2.66		2.25		2.09	
		Oxygenated sesquiterpenes			0.54		2.53		1.39	
		Total terpene hydrocarbons			13.74		10.58		10.66	
		Total oxygenated terpenes			85.89		89.09		88.91	
		Non isoprenoid components			0.37		0.33		0.44	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). *Quantitation ion. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations.

[a, b, c]Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$. [d, e]Different letters in the same compound area mean statistically significant differences with $p < 0.05$.

Several compounds show peak area percentages similar to those reported in literature for *L. stoechas subsp stoechas* (Table 4.3). Some main terpenes like fenchone show similar concentrations to samples from Algeria (Benabdelkader et al., 2011; Dob et al., 2006), Turkey (Giray et al., 2008; Gursoy et al., 2009; Kirmizibekmez et al., 2009; Topal et al., 2008) and Italy (Angioni et al., 2006; Marongiu et al., 2010; Zuzarte et al., 2013) while some others like camphor show similarities to Turkey (Giray et al., 2008; Inan et al., 2013; Sertkaya et al., 2010; Topal et al., 2008), France (Ristorcelli et al., 1998) and Greece (Tzakou et al., 2009). Molecules like eucalyptol have been determined in higher concentrations in Spanish samples than in any other location.

A literature search provided 19 articles, with plant material from 8 different countries, accounting for the main constituent components of *Lavandula stoechas* (Table 4.3). The reported and obtained data of compound concentrations showed high variability even in the most abundant molecules, i.e. fenchone (0 - 66%) and camphor (0 - 49%). This fact highlights the importance of studying the biochemotypes existing in the different locations. This information is useful to establish a common pattern of *L. stoechas* volatile composition and also to obtain the higher profit of each specific cultivar.

Generally, the most abundant and present terpenes are α-pinene, camphene, β-pinene, p-cymene, limonene, eucalyptol, fenchone, camphor, borneol, myrtenol, verbenone, α-fenchyl acetate, carvone, bornyl acetate, myrtenyl acetate, δ-cadinene, caryophyllene oxide and viridiflorol, which constitute the common basis to identify the species.

However, the absence of some of the above mentioned compounds makes the difference to distinguish among biochemotypes of *L. stoechas*. In this way, only the EO reported by Topal *et al.* (Topal et al., 2008), among the Turkish ones, shows a lack of the most volatile compounds (α-pinene to limonene), myrtenyl acetate and viridiflorol. In another case, the EO reported by Gursoy *et al.* (Gursoy et al., 2009) is the only one where p-cymene is detected among all the Turkish EOs.

Other components may be indicators of biochemotypes linked to the geographical location. This is the case of β-pinene and viridiflorol, only reported in samples from Turkey, Italy and Algeria, or caryophyllene oxide only reported in samples from Turkey and Algeria.

Some specific molecules present in isolated cases have been reported determining unique biochemotypes i.e. menthone, menthol and pulegone (Goren et al., 2002); high concentration of linalool, β-thujone and linalyl acetate (Msaada et al., 2012); and α-thujone (Sertkaya et al., 2010).

There are also small amounts of special terpenes reported, that may be used as exclusive biomolecular markers due to their presence in few EOs, e.g. tricyclene (Ristorcelli et al., 1998; Zuzarte et al., 2013); or, alternatively, α-thujene, Z-pinane, myrcene, β-phellandrene and ocimene (Angioni et al., 2006).

Table 4.3. GC determination (%) of EOs components, summary of literature review.

Molecule	Turkey								Italy			Algeria		Greece		France	Portugal	Iran	Tunisia
(X)	[X]1	[X]2	[X]3	[X]4	[X]5	[X]6	[X]7	[X]8	[X]9	[X]10	[X]11	[X]12	[X]13	[X]14	[X]15	[X]16	[X]17	[X]18	[X]19
Tricyclene											0.2					0.5			
α-Thujene									0.01										
α-Pinene		2.94		0.67	6.1	0.45	0.56		1.83		0.4	1	0.06			4.13			
Camphene		2.08		1.05		0.78	0.49		2.39		2.8		0.11			3.18			
Z-Pinane									0.01										
β-Pinene		1.64					0.07		0.05				0.03						
Myrcene									0.02										
p-Cymene				0.41					0.4		0.4	6.5	0.21			0.8			
Limonene		2.52		0.19			0.17		1.01		0.2		tr			1.28			
Eucalyptol		7.67	0.65	13.94	9.7	7.76	9.72	19.47	0.13	10.9	6		4.82		9	6.73		7.02	
β-Phellandrene									0.19										
(*Z*)-Ocimene									0.04										
(*E*)-Ocimene									0.01										
D-Fenchone		32.03	20.49	27.66	40.55	0.25	45.81	45.47	66.23	20.7	37	31.6	26.18	21	46.7	43.63	44		
Linalool				0.46					0.36				0.43			1.64			20.25
α-Thujone						65.78			0.03				0.17						
β-Thujone																			8.97
Camphor		14.71	31.4	49.08	9	18.44	34.14	17.23	12.31	31.7	27.3	22.4	11.72	26.3	9.9	23.27	36		
Menthone	12.6																		
Borneol		0.25	0.19						1.14		0.8		0.6			1.1			
Menthol	18.1																		
Myrtenol		1.95	0.29								0.5		0.62			1.48			
Verbenone		0.1	0.25						0.07		0.3		1.55						
α-Fenchyl acetate			0.35			1.15	0.15						0.24			1			
Pulegone	40.4																		
Linalyl acetate							0.09												64.3
Bornyl acetate		1.68	1.85	1.31		2.83	0.02		3.76		6.2		2.19			2.02			
Myrtenyl acetate		11.7		0.95	9.5		3.84		4.36		1.7		1.72			2.88			
δ-Cadinene		0.58	1.72			0.49					0.3		0.07					5.33	
Caryophyllene oxide	0.56	1.83			0.2	0.11						0.74							
Viridiflorol		2.32		1.45	4.1		0.71		0.01		2.6		5.03						

1(Goren et al., 2002); 2(Giray et al., 2008); 3(Topal et al., 2008); 4(Gursoy et al., 2009); 5(Kirmizibekmez et al., 2009); 6(Sertkaya et al., 2010); 7(Kaya et al., 2012); 8(Inan et al., 2013); 9(Angioni et al., 2006); 10(Marongiu et al., 2010); 11(Zuzarte et al., 2013); 12(Dob et al., 2006); 13(Benabdelkader et al., 2011); 14(Tzakou et al., 2009); 15(Hassiotis, 2010); 16(Ristorcelli et al., 1998); 17(Matos et al., 2009); 18(Ebadollahi et al., 2010); 19(Msaada et al., 2012)

Table 4.4
EsGC-EI/MS determination of EOs components. Enantiomeric ratios.

t_R (min)			Sample-1		Sample-2		Sample-3	
(+) – X	(-) – X	Analyte (X)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)
7.79	7.52	α-Pinene	95	5	95	5	95	5
8.47	8.24	Camphene	31	69	31	69	25*	75*
8.89	9.16	β-Pinene	50*	50*	95*	5*	74*	26*
9.46	-	3-Carene	N/D	N/D	95	5	95	5
10.52	10.00	Limonene	95	5	95	5	95	5
12.51	12.39	Fenchone	95	5	95	5	95	5
15.73	15.57	Linalool	5	95	5	95	44*	56*
16.72	16.46	Camphor	80	20	84	16	74*	26*
-	17.25	Linalyl acetate	5	95	5	95	5	95
18.02	18.18	Bornyl acetate	42*	58*	95	5	95	5
18.32	18.51	Terpinen-4-ol	55	45	53	47	77*	23*
20.10	19.76	α-Terpineol	95	5	95	5	92*	8*
20.15	19.58	Borneol	42*	58*	5	95	5	95
23.92	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95
-	28.81	Caryophyllene oxide	5	95	5	95	5	95

*Biomolecular marker of the EO biochemotype. All results show standard deviation lower than ±5%.

4.4.2 EsGC-EI/MS study

High t_R values were obtained in the EsGC-EI/MS chromatograms, due to lack of commercial availability of fast chiral columns with 0.1mm i.d. x 0.1µm film thickness. The determined enantiomeric ratios of components of EOs from *Lavandula stoechas* are shown in Table 4.4. The (+)-enantiomer predominates in the case of: α-pinene, β-pinene, limonene, fenchone, camphor, terpinen-4-ol, α-terpineol, bornyl acetate and 3-carene, while the (-)-enantiomer predominates in: camphene, linalool, borneol, linalyl acetate, (*E*)-β-caryophyllene and caryophyllene oxide. The enantiomeric distribution of β-pinene is markedly different for each sample and could be useful for their characterization. There are several biomolecular markers of the biochemotype origin of Sample-1: β-pinene, bornyl acetate and borneol; Sample-2: β-pinene; Sample-3: camphene, β-pinene, linalool, camphor, terpinen-4-ol and α-terpineol. This data could be useful to assess the origin and the authenticity of the EOs. To our knowledge, this is the first chiral characterization of the EOs from *Lavandula stoechas* grown in Spain.

The high proportion of (+)-camphor in EOs grown in Spain (Table 4.4), is higher than that reported for the EOs, of *L. stoechas* from Corsica (Ristorcelli et al., 1998) and from Greece (Tzakou et al., 2009). A very low proportion of (-)-fenchone has been detected in the *L. stoechas* EOs from Spain whereas it has not been previously reported for the EOs of this plant grown in Corsica (France) (Ristorcelli et al., 1998), neither in Greece (Tzakou et al., 2009).

4.4.3 Antioxidant activity

The ORAC antioxidant activity of the three samples of *Lavandula stoechas* is expressed in TEAC units (µmol TE/µL EO) and resulted (Table 4.5) as follows: Sample-1^{ORAC} > Sample-2^{ORAC} ≈ Sample-3^{ORAC}.

The antioxidant activity of each EO must be related to its composition and the intrinsic antioxidant activity of each of the compounds. Generally, the rise in composition of oxygenated terpenes is correlated with higher ORAC antioxidant activity. Two oxygenated components may be highly relevant to explain the ORAC value of the EO, namely linalool and thymol, for their high ORAC value (Bentayeb et al., 2014) and moderate concentration in the EO.

Abundant molecules, like linalool or thymol, exhibiting high ORAC values contribute significantly to the global EO ORAC value. However, the total ORAC value of the EO may be determined not just by the main components, but by the whole group of compounds with antioxidant activity present in the EO.

The ABTS antioxidant activity of the three samples of *Lavandula stoechas* is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 4.5) as follows: Sample-1^{ABTS} < Sample-3^{ABTS} < Sample-2^{ABTS}. Correlating these results with the concentration of thymol, higher in Samples-2 and -3 than in Sample-1, we might attribute a high response of this method to phenolic compounds (Osman et al., 2006).

The DDPH antioxidant activity of the three samples of *Lavandula stoechas* is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 4.5) as follows: Sample-1^{DPPH} > Sample-2^{DPPH} ≈ and Sample-3^{DPPH}. Different molecules, present in high quantity in Sample-1 may explain the better performance in DPPH antioxidant assay (Dawidowicz and Olszowy, 2014), *i.e.* α-pinene, limonene, linalool, lavandulyl acetate or valencene.

The chelating power activity is expressed in EDTA units (mg EDTA equivalents / mL EO) and resulted (Table 4.5) as follows: Sample-1^{ChP} < Sample-2^{ChP} ≈ Sample-3^{ChP}. This behavior could be explained by the low concentrations of the series of bicyclic compounds: camphene-camphor-bornyl acetate, present in Sample-1.

The reducing power antioxidant activity of the three samples of *Lavandula stoechas* is expressed in ascorbic acid units (mg Ascorbic acid equivalents/mL EO) and resulted (Table 4.5) as follows: Sample-1^{RdP} > Sample-2^{RdP} > Sample-3^{RdP}. Following the same case as in DPPH, some abundant molecules might show a mild reducing power, like the previously reported linalool (Liu et al., 2012).

Table 4.5
Antioxidant evaluation of *Lavandula stoechas* by the five reported methods.

Samples	ORAC (µmol TE/µL EO)	ABTS (µmol TE/mL EO)	DPPH (µmol TE/mL EO)	ChP (mg EDTA eq/mL EO)	RdP (mg Ascorbic acid eq/mL EO)
Sample-1	2.22a ± 0.07	14.8c ± 0.6	1.3a ± 0.1	1.1b ± 0.1	0.45a ± 0.01
Sample-2	1.42b ± 0.04	175.3a ± 3.3	0.61b ± 0.04	2.0a ± 0.2	0.22b ± 0.02
Sample-3	1.55b ± 0.04	160.9b ± 3.6	0.62b ± 0.05	2.3a ± 0.2	0.18c ± 0.01

a, b, cDifferent letters in the same antioxidant method mean statistically significant differences with $p < 0.05$

4.4.4 Inhibitory activity on LOX

The results of the LOX inhibitory activity were obtained as previously described. Inhibition degree (%) at 0.3 µL(EO)/mL was measured for Sample-1^{LOX} $(29.3 \pm 0.6)^a$ < Sample-2^{LOX} $(43.5 \pm 0.7)^b$ ≈ Sample-3^{LOX} $(44.5 \pm 0.9)^b$. Tukey's HSD test revealed significant differences between Sample-1 and the rest (different superscripts).

For a deeper understanding of the LOX inhibitory activity of the EO, the main commercially available compounds had their LOX inhibitory activity tested, obtaining their IC_{50} (µM) value: thymol (150 ± 11), limonene (356 ± 30), bornyl acetate (405 ± 207), p-cymene (486 ± 32), camphor (805 ± 271), linalool (3346 ± 289) and fenchone (4622 ± 616).

According to the results, thymol, limonene, bornyl acetate, p-cymene and camphor showed the highest inhibitory activity. Special interactions are usually present in natural mixtures when measuring enzymatic bioactivity. These phenomena modify the expected properties derived from the single components, making them greater (synergy) or smaller (antagonism) in the mixture. In this case, the inhibitory activity of the *Lavandula stoechas* EO is clearly due to a combination of high inhibitory activity and more abundant compounds, namely thymol, fenchone and camphor. The first one exhibits a great LOX inhibition and low concentration, while the two others show a low and moderate LOX inhibition, respectively, and very high concentration.

The low inhibition of LOX by Sample-1 regarding Samples-2 and -3, is explained by the fact that thymol and fenchone are found in the smallest absolute concentrations.

4.5 Conclusions

The different *Lavandula stoechas* EOs evaluated showed the same common six principal terpenes, i.e. α-Pinene, camphene, limonene, eucalyptol, fenchone and camphor. Oxygenated monoterpenes account for more than 80% of the total compounds, ketone being the most abundant functional group. Two statistically significant groups were found among the samples, Sample-2 and -3 were found highly similar whereas Sample-1 had more differences in composition. Among the samples of different countries, high variability in concentration of molecules was found, even in the main components: fenchone (0 - 66%) and camphor (0 - 49%). This fact highlights the importance of studying the biochemotypes existing in the different locations. Molecules like eucalyptol have been determined in higher concentrations in Spanish samples than in any other location.

The enantiomeric profile shows dextrorotatory predominance in fenchone and camphor, which are highly representative of the typical EOs from these aromatic plants, but also in α-pinene, β-pinene, limonene, terpinen-4-ol, α-terpineol, bornyl acetate and 3-carene. Levorotatory enantiomers predominate in: camphene, linalool, borneol, linalyl acetate, (*E*)-β-caryophyllene and caryophyllene oxide. However, the proportions of several substances and their enantiomers can be used as biomolecular markers for discrimination of EOs from different origins. These enantiomers help to discriminate natural EOs from those adulterated with synthetic racemates of the main components. High proportion of (+)-camphor and low proportion of (-)-fenchone (not reported before) were found in EOs grown in Spain compared to other countries. A general trend is found, showing that Samples-2 and -3 have important similarities.

Lavandula stoechas essential oils showed moderate antioxidant activities compared to the reference antioxidants, due to the whole complex mixture of their compounds. Nevertheless, the main contributions to the global bioactivities have been identified and assigned to some compounds, *i.e.* antioxidant activity due mainly to linalool and thymol. Also, interesting similarities among antioxidant methods were observed for *L. stoechas* EOs. ORAC, DPPH and reducing power showed a higher

value with Sample-1 (around double compared to Samples-2 and -3), whereas ABTS and chelating power methods showed lower values evaluating Sample-1. Despite the variability of antioxidant activities, due to the measurement of different radicals or properties of the antioxidant probes, the similarities of Samples-2 and -3 were clearly evident.

Regarding potential anti-inflammatory properties, LOX inhibitory activities of *L. stoechas* EOs were due to the complex mixture of the LOX inhibitory activities of individual components. In this case, they were due mainly to thymol, fenchone and camphor, higher in Samples-2 and 3. In this case Samples-2 and -3 are better inhibitors and, again, more similar to each other than to Sample-1.

L. stoechas is a unique species within the *Lavandula* genus, due to its EOs with low proportion of linalool and linalyl acetate, its presence of thymol as *Thymus zygis*, high content of camphor and eucalyptol as *Rosmarinus officinalis* and very high concentration of fenchone as *Foeniculum vulgare* (Baser and Buchbauer, 2010; Bilia et al., 2014; Gautam et al., 2014; Lesgards et al., 2014; Raut and Karuppayil, 2014; Seow et al., 2014; Tongnuanchan and Benjakul, 2014). This biochemical composition could be related to the bioactivities of *L. stoechas* EOs such as antibacterial, antifungal, insecticide, antioxidant and anti-inflammatory agents. These properties support the potential use of L. stoechas EOs as natural cosmetics and natural pharmaceutical ingredients for dermatophytosis, such as onychomycosis, ringworm, athlete's foot and jock itch (Benabdelkader et al., 2011; Goren et al., 2002; Kaya et al., 2012; Kirmizibekmez et al., 2009; Matos et al., 2009; Msaada et al., 2012; Zuzarte et al., 2013).

4.6 References

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5. CARACTERIZACIÓN DE ACEITE ESENCIAL DE *LAVANDULA* X *INTERMEDIA*

5.1 Resumen

Los aceites esenciales de lavandín (*Lavandula* x *intermedia*) de las variedades Abrial, Super y Grosso, cultivados y extraidos en el sureste de España, han sido analizados por cromatografía de gases con detección por espectrometría de masas para determinar su composición, en ambas concentraciones relativa (por área de pico) y absoluta (utilizando rectas de calibrado). Las moléculas más abundantes halladas entre los componentes principales fueron: linalol (34-47%), acetato de linalilo (17-34%), alcanfor (4-9%) y eucaliptol (3-7%). Esta caracterización se completó con el uso de cromatografía de gases enantioselectiva, detectando (–)-linalol, (+)-alcanfor y (–)-acetato de linalilo como componentes principales. La capacidad antioxidante se evaluó con resultados positivos por varios métodos: actividad frente a radicales libres, capacidades quelatante y reductora, principalmente debido a los componentes linalol y acetato de linalilo. Se observó una moderada actividad inhibidora de lipoxigenasa lo cual es indicador de una posible actividad antiinflamatoria, principalmente debida al linalool y el alcanfor. Estas propiedades apoyan el uso potencial del aceite esencial de *Lavandula* x *intermedia* como cosmético natural e ingrediente farmacéutico natural para tratar diversos desórdenes cutáneos.

5.2 Summary

Lavandin (Lavandula x intermedia Emeric ex Loiseleur) essential oils (EOs), from Abrial, Super and Grosso cultivars, cultivated and extracted in the South East of Spain, were analysed by GC/MS to determine their composition, in both relative (peak area) and absolute (using standard curves) concentrations. Linalool (34-47%), linalyl acetate (17-34%), camphor (4-9%) and eucalyptol (3-7%) were determined as the main molecules. This characterisation was completed with the enantioselective gas chromatography, where (-)-linalool, (+)-camphor and (-)-linalyl acetate were determined as the main components. Antioxidant activity was evaluated positively by several methods: activity against free radicals, chelating and reducing power, probably due to linalool and linalyl acetate. Mild inhibitory activity on lipoxygenase (LOX) was observed supporting potential anti-inflammatory activity, mainly due to linalool and camphor. These properties support the potential use of L. x intermedia EOs as natural cosmetic and natural pharmaceutical ingredient to fight several skin diseases.

5.3 Introduction

Lavandula x *intermedia* Emeric ex Loiseleur, also known as lavandin, *Lavandula hybrida* or Dutch lavender is a hybrid between English lavender (*Lavandula angustifolia* Miller) and spike lavender *(Lavandula latifolia* Medikus), resulting in an aromatic flowering plant of the *Lamiaceae* family. The genus *Lavandula*, of the *Lamiaceae* family, consists of approximately 20 species with more than 100 varieties of lavender (Da Porto and Decorti, 2008). *L.* x *intermedia* essential oil (EO) is one of the aromatic ingredients in the production of drinks, food, perfumes and soaps, especially important in the manufacture of “eau de cologne” and perfumes (Torras-Claveria et al., 2007).

Some GC relative quantitation of the *L.* x *intermedia* composition have been reported (Baydar and Kineci, 2009; Bombayda et al., 2008; Cosimi et al., 2009; Papachristos et al., 2004; Seino et al., 2008). None of them has studied the specific conditions of the Spanish Mediterranean coast, specifically the region of Murcia where the biggest aromatic plant diversity among all regions of Spain is found. Furthermore, there are few chiral studies on EOs of *L.* x *intermedia* (Flores et al., 2005)and itaccounts for some specific molecules. Chiral distribution is an important aspect of the EO composition, it helps identifying natural EOs from those adulterated (Smelcerovic et al., 2013), even when the samples come from different places of the Earth (Tranchida et al., 2012). In addition, the chirality in biomolecules is highly important due to the different bioactivities and organoleptic properties of each of the enantiomers (Baser and Buchbauer, 2010).

Apart from the aromatic usages of the *L.* x *intermedia* EO, some bioactivities may be relevant according to the popular uses of some plants of the *Lavandula* genus. The antioxidant potential of EOs can be determined using a representative selection of different antioxidant methods (Bentayeb et al., 2014; Dawidowicz and Olszowy, 2014; Huang et al., 2005; Rubio et al., 2013). Many inflammatory processes are associated with leukotriene production catalysed by lipoxygenase (LOX) (Rubio et al., 2013), which can use molecular oxygen or hydrogen peroxide as oxidants (Anwar et al., 2014) Thus, the inhibition of soybean lipoxygenase as LOX model, is a hint of anti-inflammatory activity of the EO.

The aim of the present study is to determine the relative, absolute and chiral distribution of each of the EO main components in 5 samples grown in Murcia (Spain). Then, five antioxidant methods (ORAC, ABTS, DPPH, Chelating Power and Reducing Power) will be applied to evaluate the antioxidant capacities of the *L.* x *intermedia* EOs. Furthermore, the inhibitory activity of these EOs on LOX will be characterised. The experimental results will be compared with those reported for *L. hybrida* EOs from other countries, and their potential biotechnological applications will be discussed.

5.4 Results and Discussion

5.4.1 Fast gas chromatography/mass spectrometry (FGC/MS) study

Abrial1, Super1 and Grosso1 were grown in Supra Mediterranean bioclimatic zone and Super2 and Grosso2 were grown in Upper Meso-Mediterranean bioclimatic zones. The essential oils were obtained by hydrodistillation in yields ranging from 0.2 to 1.3% (w/w). FGC/MS was used, to determine, using triplicate analysis of the samples

(Sokal and Rohlf, 2012), the components of the studied EOs (European-Pharmacopoeia, 2011; IUPAC, 1997; van Den Dool and Dec. Kratz, 1963) (Table 5.1).

Abrial cultivar sample (Abrial1) is shown together with the two Super cultivar samples (Super1-2) because of its similarity in composition (Table 5.2). Nevertheless, some components make the difference between cultivars acting as biomolecular markers. This is the case of the high relative concentration of β-pinene, *cis*- and *trans*-β-ocimene, camphor and (*E*)-β-caryophyllene in the Abrial cultivar. Focusing on the Super cultivar samples, some components perform as biomolecular markers accounting for the different environmental conditions (Rivas-Martínez, 1987), i.e. high concentration of linalyl acetate in Super1 and Z-β-farnesene in Super2; and low concentration of terpinen-4-ol and α-terpineol in Super1. Results obtained about Grosso cultivar (Table 5.3) (Grosso1-2), show some biomolecular markers. Grosso1 has low relative concentration of p-cymene. β-pinene, linalyl acetate, Z-β-farnesene and γ-muurolene are found in low proportion in Grosso2. High concentrations are found in Grosso1 for the constituents (*Z*)-β-ocimene and lavandulyl acetate; and in Grosso2 for hexyl alcohol, linalool and lavandulol.

Global results show five different *L.* x *intermedia* samples having the same 11 principal molecules, i.e. (*Z*)-β-ocimene, eucalyptol, linalool, camphor, borneol, terpinen-4-ol, α-terpineol, linalyl acetate, lavandulyl acetate, (*E*)-β-caryophyllene and Z-β-farnesene. Specific molecules are found in high concentration for each specific cultivar. In Abrial/Super cultivars, β-myrcene, limonene and (*E*)-β-ocimene arise; whereas in Grosso cultivar, lavandulol is found in higher concentration.

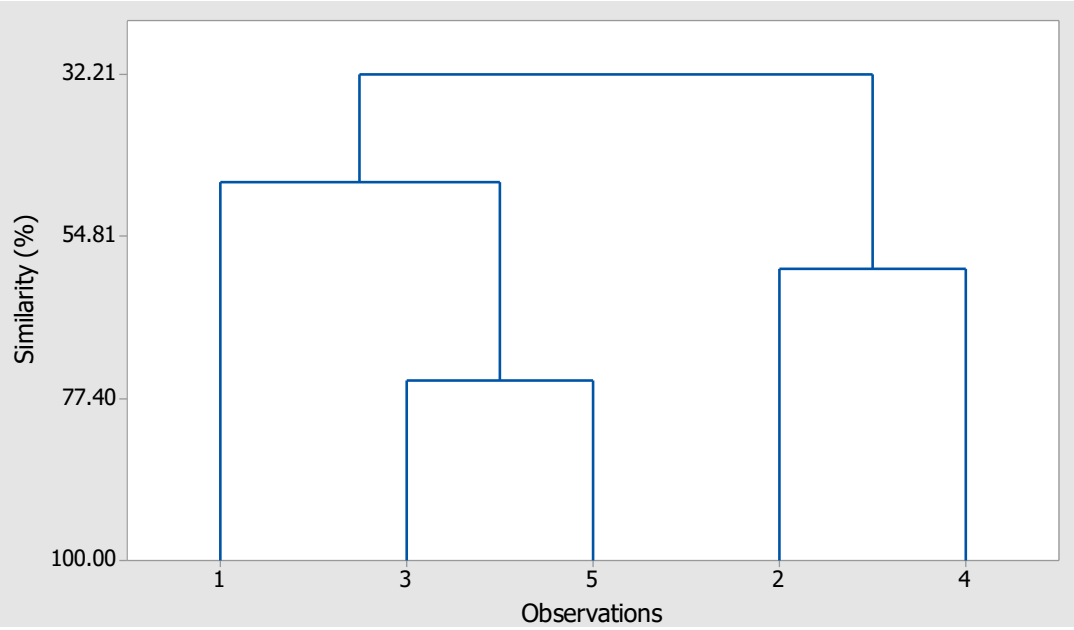

Figure 5.1
Dendrogram for *L.* x *intermedia* samples
The samples in X axis are: 1 = Abrial1, 2 = Super1, 3 = Super2, 4 = Grosso1, 5 = Grosso2

Table 5.1. Parameters of FGC-EI/MS calibration

Analyte	Calibration curve*	R^2	Calibration range (mM)	RSD (%)	LOD (mM)	LOQ (mM)	Standard source	Product Reference
Nonane	*Internal standard*						SAFC	442694
(-)-α-Pinene	y = 0.471x - 0.018	0.997	0.25 - 10.06	4.5	0.08	0.25	Fluka	80599
(+)-Camphene	y = 0.272x - 0.008	0.995	0.66 - 10.51	0.4	0.14	0.44	SAFC	w222909
Sabinene	y = 0.460x - 0.026	0.998	0.24 - 9.78	0.9	0.07	0.21	Extrasynthese	5062 S
(-)-β-Pinene	y = 0.400x - 0.016	0.995	0.26 - 10.29	3.7	0.09	0.26	Fluka	80609
3-Octanone	y = 0.309x - 0.031	0.996	2.56 - 10.24	4.1	0.64	1.95	SAFC	w280305
Myrcene	y = 0.188x - 0.024	0.993	2.24 - 8.97	3.5	0.56	1.70	Fluka	64643
Hexyl acetate	y = 0.452x - 0.057	0.992	2.40 - 9.59	1.7	0.32	0.96	Fluka	25539
(+)-Limonene	y = 0.246x - 0.008	0.995	0.60 - 9.55	0.9	0.12	0.36	Fluka	62118
(*Z*)-β-Ocimene	y = 0.218x - 0.011	0.996	0.70 - 6.97	2.2	0.16	0.48	SAFC	w353901
Eucalyptol	y = 0.233x - 0.008	0.995	2.39 - 9.55	8.7	0.60	1.82	SAFC	w246506
γ-Terpinene	y = 0.366x - 0.017	0.995	0.62 - 9.87	2.8	0.12	0.37	Aldrich	223190
Sabinene hydrate	y = 0.268x - 0.007	0.996	0.63 - 10.00	2.1	0.18	0.56	Fluka	96573
(-)-Linalool	y = 0.214x - 0.008	0.994	0.88 - 8.80	4.9	0.22	0.67	Fluka	74856
(+)-Camphor	y = 0.181x - 0.024	0.994	0.99 - 9.85	1.5	0.25	0.76	Alfa Aesar	A10708
(-)-Borneol	y = 0.266x - 0.020	0.998	0.57 - 9.06	4.4	0.19	0.57	Alfa Aesar	A12684
(-)-Terpinen-4-ol	y = 0.241x - 0.003	0.997	0.60 - 9.57	4.3	0.20	0.60	Aldrich	11584
Hexyl butyrate	y = 0.332x - 0.034	0.991	4.84 - 7.74	0.6	0.77	2.34	SAFC	w256811
(+)-α-Terpineol	y = 0.175x - 0.003	0.998	1.02 - 10.23	4.5	0.26	0.79	Fluka	83073
Tetradecane	*Internal standard*						SAFC	442708
(-)-Linalyl acetate	y = 0.217x - 0.006	0.998	0.72 - 7.18	0.2	0.18	0.55	SAFC	w263605
Neryl acetate	y = 0.242x - 0.007	0.999	0.73 - 7.31	3.8	0.18	0.55	SAFC	w277304
Geranyl acetate	y = 0.249x - 0.009	0.999	0.46 - 7.42	2.9	0.15	0.46	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(-)-(*E*)-β-Caryophyllene	y = 0.079x - 0.002	0.998	4.41 - 7.05	6.6	0.71	2.16	Sigma	22075
α-Humulene	y = 0.541x - 0.019	0.999	0.40 - 6.35	2.2	0.13	0.40	Aldrich	53675
α-Bisabolene	y = 0.111x - 0.043	0.999	1.74 - 6.97	3.4	0.31	0.94	Alfa Aesar	A18724

*Response ratio vs. Concentration ratio, internal standard correction applied.
Each internal standard is reference compound for the analytes that follow.

Table 5.2. FGC/MS determination of components in *L.* x *intermedia* Abrial and Super cultivars.

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Abrial 1		Super 1		Super 2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
1.01	825	Hexyl methyl ether	45, 56, 69, 84		0.1d ± 0.0		0.1e ± 0.0			LRI, MS
1.15	860	4-Hexen-1-ol	31, 41, 55, 67						0.1 ± 0.0	LRI, MS
1.20	873	Hexyl alcohol	43, 56, 69, 82		0.1d ± 0.0		0.1d ± 0.0		0.2e ± 0.0	LRI, MS
1.47	929	Tricyclene	79, 93, 121, 136		0.1d ± 0.0		tr e			LRI, MS
1.53	939	α-Pinene	39, 91, **93**, 77	29.5a ± 0.3	0.5d ± 0.0	16.9b ± 0.5	0.3e ± 0.0	13.4c ± 0.2	0.2f ± 0.0	LRI, MS, std
1.63	957	Camphene	67, 79, **93**, 121	31.3a ± 0.4	0.4d ± 0.0	18.7b ± 0.6	0.3e ± 0.0	14.2c ± 0.3	0.2f ± 0.0	LRI, MS, std
1.75	978	Sabinene	41, 77, 91, **93**	10.2 ± 0.0	0.1d ± 0.0		0.1e ± 0.0		0.1f ± 0.0	LRI, MS, std
1.78	983	β-Pinene	41, 69, 79, **93**	27.9a ± 0.3	**0.6d ± 0.0**	12.8b ± 0.4	0.2e ± 0.0	13.6b ± 0.3	0.3f ± 0.0	LRI, MS, std
1.81	989	3-Octanone	**43**, 57, 71, 99	15.7a ± 0.5	0.1d ± 0.0	35.4b ± 1.7	0.4e ± 0.0	18a ± 0.5	0.1f ± 0.0	LRI, MS, std
1.83	992	β-Myrcene	39, **41**, 69, 93	89.1a ± 2.0	0.8d ± 0.0	62.4b ± 1.4	0.6e ± 0.0	77.1c ± 1.4	0.7f ± 0.0	LRI, MS, std
1.89	1002	3-Octanol	41, 59, 83, 101				0.1 ± 0.0			LRI, MS
1.97	1011	Hexyl acetate	**43**, 56, 84, 61	21.0a ± 0.3	0.2d ± 0.0	37.9b ± 0.6	0.4e ± 0.0	16.6c ± 0.5	0.1f ± 0.0	LRI, MS, std
1.98	1012	3-Carene	77, 79, 91, 93		tr d		0.1e ± 0.0			LRI, MS
2.04	1019	2-Carene	77, 93, 121, 136		0.1 ± 0.0					LRI, MS
2.11	1026	p-Cymene	77, 91, 119, 134		0.1d ± 0.0		0.1d ± 0.0		0.1e ± 0.0	LRI, MS
2.16	1032	Limonene	67, **68**, 79, 93	62.4a ± 2.0	1.0d ± 0.0	44.1b ± 0.9	0.7e ± 0.0	39.8c ± 0.5	0.6f ± 0.0	LRI, MS, std
2.17	1034	(*Z*)-β-Ocimene	41, 79, 91, **93**	218.9a ± 18.9	**2.9d ± 0.3**	144.5b ± 2.3	1.5e ± 0.0	122.7b ± 3.9	1.4e ± 0.1	LRI, MS, std
2.19	1036	Eucalyptol	**43**, 67, 81, 93	536.7a ± 17.9	7.6d ± 0.2	355.3b ± 28.9	3.9e ± 0.0	362.5b ± 18.0	4.8f ± 0.1	LRI, MS, std
2.26	1044	(*E*)-β-Ocimene	41, 79, 93, 121		**6.1d ± 0.0**		2.0e ± 0.0		1.9f ± 0.0	LRI, MS
2.40	1060	γ-Terpinene	77, 91, **93**, 136	10.0a ± 0.2	0.1d ± 0.0			8.6b ± 0.2	0.1e ± 0.0	LRI, MS, std
2.53	1074	Sabinene hydrate	77, 91, **93**, 121	6.5a ± 0.2	0.2d ± 0.0	6.1a ± 0.2	0.2d ± 0.0		0.2d ± 0.0	LRI, MS, std
2.66	1089	Terpinolene	93, 105, 121, 136		0.4d ± 0.0		0.2e ± 0.0		0.3f ± 0.0	LRI, MS
2.83	1108	Linalool	41, 55, 69, **93**	2079.2a ± 61.0	37.0d ± 0.0	2168.7a ± 37.1	39.4e ± 0.1	2439.7b ± 31.4	45.8f ± 0.1	LRI, MS, std
2.97	1122	(*E*)-2-Nonenal	41, 55, 70, 83				0.1 ± 0.0			LRI, MS
3.08	1133	allo-Ocimene	91, 105, 121, 136		0.2d ± 0.0		0.1e ± 0.0		0.1e ± 0.0	LRI, MS
3.25	1151	n-Hexyl isobutyrate	43, 56, 71, 89		0.1d ± 0.0		0.1d ± 0.0		0.1d ± 0.0	LRI, MS
3.33	1159	Camphor	41, 81, **95**, 108	808.4a ± 31.2	**9.0d ± 0.0**	642.0b ± 4.7	4.8e ± 0.0	711.5c ± 3.3	7.6f ± 0.0	LRI, MS, std
3.46	1172	Lavandulol	41, 69, 111, 123		0.7d ± 0.0		0.2e ± 0.0		0.8f ± 0.0	LRI, MS

Table 5.2. Continued

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Abrial 1		Super 1		Super 2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
3.55	1181	1-Butenylidene cyclohexane	79, 93, 107, 136		0.1d ± 0.0				0.1d ± 0.0	LRI, MS
3.60	1186	Borneol	41, 93, **95**, 121	164.0a ± 4.2	1.9d ± 0.0	183.1b ± 4.9	2.3e ± 0.0	390.2c ± 8.7	2.7f ± 0.0	LRI, MS, std
3.63	1189	Terpinen-4-ol	71, 77, 91, **93**	61.9a ± 2.9	1.0d ± 0.0	24.1b ± 1.6	**0.4e ± 0.0**	96.9c ± 1.4	1.5f ± 0.0	LRI, MS, std
3.69	1196	Hexyl butyrate	**43**, 56, 71, 89	25.6a ± 0.6	0.3d ± 0.0	46.1b ± 1.5	0.7e ± 0.0	34.8c ± 1.3	0.5f ± 0.0	LRI, MS, std
3.71	1198	p-Cymenene	65, 91, 117, 132		0.1 ± 0.0					LRI, MS
3.77	1204	α-Terpineol	59, 68, 79, **93**	66.1a ± 0.3	1.0d ± 0.0	45.9b ± 5.0	**0.7e ± 0.0**	104.1c ± 2.7	1.6f ± 0.0	LRI, MS, std
4.03	1231	Perillol	68, 79, 93, 121		0.1d ± 0.0		0.1d ± 0.0		0.2e ± 0.0	LRI, MS
4.05	1232	Isobornyl formate	93, 95, 121, 136		0.1d ± 0.0		0.1e ± 0.0		0.1e ± 0.0	LRI, MS
4.06	1234	Hexyl 2-methylbutyrate	41, 57, 85, 103				0.1d ± 0.0		0.1d ± 0.0	LRI, MS
4.12	1240	Hexyl valerate	43, 56, 85, 103		0.1d ± 0.0		0.1d. e ± 0.0		0.1e ± 0.0	LRI, MS
4.21	1249	Linalyl acetate	41, 69, **93**, 121	1215.6a ± 19.8	19.4d ± 0.1	2107.0b ± 30.7	**34.5e ± 0.1**	1198.9a ± 30.2	19.8f ± 0.1	LRI, MS, std
4.27	1255	Geraniol	41, 69, 93, 121		0.4d ± 0.0		0.5e ± 0.0		0.6f ± 0.0	LRI, MS
4.54	1284	Lavandulyl acetate	43, 69, 93, 121		1.5d ± 0.0		1.2e ± 0.0		1.2e ± 0.0	LRI, MS
5.04	1331	6-Undecanol	41, 55, 83, 101		0.1d ± 0.0		0.1e ± 0.0		0.1f ± 0.0	LRI, MS
5.34	1359	Neryl acetate	41, 69, 79, **93**	12.8a ± 0.4	0.2d ± 0.0	13.3a ± 0.4	0.3d ± 0.0	19.9b ± 0.4	0.4e ± 0.0	LRI, MS, std
5.54	1379	Geranyl acetate	41, **69**, 79, 93	23.2a ± 0.5	0.4d ± 0.0	23.1a ± 1.1	0.4d ± 0.0	35.6b ± 0.8	0.7e ± 0.0	LRI, MS, std
5.57	1381	β-Cubebene	91, 105, 120, 161		0.1d ± 0.0				0.1e ± 0.0	LRI, MS
5.65	1389	α-Bergamotene	69, 93, 105, 119		0.1d ± 0.0				0.1d ± 0.0	LRI, MS
5.82	1405	α-Cedrene	93, 105, 119, 204		0.1d ± 0.0		0.1e ± 0.0		0.1e ± 0.0	LRI, MS
6.03	1426	α-Santalene	41, 94, 161, 189		0.4d ± 0.0				0.2e ± 0.0	LRI, MS
6.06	1429	(*E*)-β-Caryophyllene	41, 69, 79, **93**	169.2a ± 4.4	**2.0d ± 0.1**	101.3b ± 0.9	1.2e ± 0.0	144.1c ± 1.3	1.8f ± 0.0	LRI, MS, std
6.17	1441	E-α-Bergamotene	41, 69, 93, 119		0.2d ± 0.0		0.1e ± 0.0		0.1f ± 0.0	LRI, MS
6.29	1453	β-Farnesene	41, 69, 93, 133		tr d		tr d			LRI, MS
6.37	1461	(*Z*)-β-Farnesene	41, 69, 93, 133		0.7d ± 0.0		0.7d ± 0.0		**1.2e ± 0.0**	LRI, MS
6.43	1467	α-Humulene	41, 67, 80, **93**	5.2a ± 0.3	0.1d ± 0.0	4.0b ± 0.0	0.1e ± 0.0	5.1a ± 0.1	0.1d ± 0.0	LRI, MS, std
6.57	1481	α-Amorphene	105, 119, 161, 204		0.1 ± 0.0					LRI, MS

Table 5.2. Continued

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Abrial 1		Super 1		Super 2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
6.65	1489	γ-Muurolene	105, 119, 161, 204		0.4d ± 0.0		0.3e ± 0.0		0.4f ± 0.0	LRI, MS
6.78	1504	α-Selinene	161, 175, 189, 204		0.1d ± 0.0		0.1e ± 0.0		0.1d ± 0.0	LRI, MS
6.80	1507	α-Farnesene	41, 93, 107, 121		0.1d ± 0.0		0.1e ± 0.0		0.1e ± 0.0	LRI, MS
6.84	1514	Bergamotene	41, 77, 93, 119						0.1 ± 0.0	LRI, MS
6.89	1521	Germacrene D	105, 119, 161, 204		0.1d ± 0.0		tr e		0.1d ± 0.0	LRI, MS
7.05	1549	α-Bisabolene	41, 79, **93**, 119					35.5 ± 0.3	0.3 ± 0.0	LRI, MS, std
7.32	1593	Caryophyllene oxide	41, 79, 91, 105		tr d		0.1d ± 0.0		0.1d ± 0.0	LRI, MS
7.61	1657	Cadinol	105, 119, 161, 204		tr					LRI, MS
7.77	1695	α-Bisabolol	41, 69, 93, 119		0.1d ± 0.0		0.2e ± 0.0		0.1f ± 0.0	LRI, MS
		Oxygenated terpenes:								
		Alcohol			42.0		43.4		53.0	
		Ketone			9.0		4.8		7.6	
		Aldehyde			0.0		0.0		0.0	
		Ester			21.6		36.5		22.2	
		Ether			7.6		3.9		4.9	
		Monoterpene hydrocarbons			14.1		6.6		6.6	
		Oxygenated monoterpenes			80.1		88.4		87.4	
		Sesquiterpene hydrocarbons			4.7		2.7		4.6	
		Oxygenated sesquiterpenes			0.1		0.3		0.1	
		Total terpene hydrocarbons			18.7		9.3		11.2	
		Total oxygenated terpenes			80.2		88.6		87.5	
		Non isoprenoid components			1.0		2.1		1.3	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). *Quantitation ions are shown in bold. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. a, b, c Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$. d, e, f Different letters in the same compound area mean statistically significant differences with $p < 0.05$. Areas in bold mean "biomolecular marker" of the EO biochemotype, tr = traces (<0.1%)

Table 5.3. FGC-MS determination of components in *L.* x *intermedia* Grosso cultivar.

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Grosso 1 Concentration (mM ± SD)	Grosso 1 Area (% ± SD)	Grosso 2 Concentration (mM ± SD)	Grosso 2 Area (% ± SD)	Identification methods
1.15	860	4-Hexen-1-ol	31, 41, 55, 67		tr			LRI, MS
1.20	873	Hexyl alcohol	43, 56, 69, 82		tr c		**0.3d ± 0.0**	LRI, MS
1.47	929	Tricyclene	79, 93, 121, 136		0.1 ± 0.0			LRI, MS
1.53	939	α-Pinene	39, 91, **93**, 77	24.2a ± 1.0	0.4c ± 0.0	19.6b ± 1.5	0.5c ± 0.0	LRI, MS, std
1.63	957	Camphene	67, 79, **93**, 121	20.4a ± 0.6	0.3c ± 0.0	16.1b ± 1.0	0.3d ± 0.0	LRI, MS, std
1.75	978	Sabinene	41, 77, 91, **93**	9.4a ± 0.5	0.1c ± 0.0	51.1b ± 0.5	0.1d ± 0.0	LRI, MS, std
1.78	983	β-Pinene	41, 69, 79, **93**	24.3a ± 1.0	0.6c ± 0.0	14.2b ± 0.2	**0.3d ± 0.0**	LRI, MS, std
1.83	992	β-Myrcene	39, **41**, 69, 93	75.8a ± 5.7	0.7c ± 0.0	49.7b ± 2.1	0.8d ± 0.0	LRI, MS, std
1.97	1011	Hexyl acetate	**43**, 56, 84, 61	15.3a ± 0.8	0.1c ± 0.0	15.9a ± 0.2	0.2d ± 0.0	LRI, MS, std
1.99	1013	3-Carene	77, 79, 91, 93		0.1c ± 0.0		0.1c ± 0.0	LRI, MS
2.04	1019	2-Carene	77, 93, 121, 136		0.1c ± 0.0		0.1d ± 0.0	LRI, MS
2.11	1026	p-Cymene	77, 91, **119**, 134	5.8a ± 0.2	**0.1c ± 0.0**	6.1a ± 0.1	0.1d ± 0.0	LRI, MS, std
2.16	1032	Limonene	67, **68**, 79, 93	49.4a ± 3.0	0.8c ± 0.0	55.1a ± 5.8	0.9d ± 0.0	LRI, MS, std
2.17	1034	(*Z*)-β-Ocimene	41, 79, 91, **93**	79.6a ± 5.3	**1.1c ± 0.1**	52.1b ± 1.6	0.5d ± 0.0	LRI, MS, std
2.19	1036	Eucalyptol	**43**, 67, 81, 93	428.5a ± 33.1	6.1c ± 0.2	214.3b ± 9.5	4.5d ± 0.1	LRI, MS, std
2.26	1044	(*E*)-β-Ocimene	41, 79, 93, 121		0.4c ± 0.0		0.7d ± 0.0	LRI, MS
2.39	1059	γ-Terpinene	77, 91, **93**, 136	10.3a ± 0.2	0.1c ± 0.0	16.9b ± 0.3	0.3d ± 0.0	LRI, MS, std
2.53	1074	Sabinene hydrate	77, 91, **93**, 121	7.6a ± 0.6	0.2c ± 0.0	7.0a ± 0.2	0.2c ± 0.0	LRI, MS, std
2.66	1089	Terpinolene	93, 105, 121, 136		0.3c ± 0.0		0.4d ± 0.0	LRI, MS
2.83	1108	Linalool	41, 55, 69, **93**	1886.1a ± 41.4	34.1c ± 0.3	4283.2b ± 28.5	**47.3d ± 0.0**	LRI, MS, std
3.08	1133	allo-Ocimene	91, 105, 121, 136		0.1c ± 0.0		0.1c ± 0.0	LRI, MS
3.25	1151	n-Hexyl isobutyrate	43, 56, 71, 89		0.1c ± 0.0		0.2d ± 0.0	LRI, MS
3.33	1159	Camphor	41, 81, **95**, 108	718.1a ± 17.8	7.1c ± 0.1	394.8b ± 18.7	7.1c ± 0.0	LRI, MS, std
3.46	1172	Lavandulol	41, 69, 111, 123		0.3c ± 0.0		**1.4d ± 0.0**	LRI, MS
3.55	1181	1-Butenylidene cyclohexane	79, 93, 107, 136		0.1 ± 0.0			LRI, MS
3.60	1186	Borneol	41, 93, **95**, 121	362.3a ± 3.3	2.0c ± 0.0	221.7b ± 0.1	3.9d ± 0.1	LRI, MS, std
3.63	1189	Terpinen-4-ol	71, 77, 91, **93**	217.0a ± 17.4	3.0c ± 0.0	195.3a ± 1.5	4.7d ± 0.2	LRI, MS, std
3.69	1196	Hexyl butyrate	**43**, 56, 71, 89	17.9a ± 1.1	0.2c ± 0.0	24.8b ± 0.7	0.4d ± 0.0	LRI, MS, std

Table 5.3. Continued

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Grosso 1		Grosso 2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
3.77	1204	α-Terpineol	59, 68, 79, **93**	85.8a ± 2.2	1.3c ± 0.0	73.8b ± 1.8	1.4d ± 0.0	LRI, MS, std
4.03	1231	Perillol	68, 79, 93, 121		0.2c ± 0.0		0.2c ± 0.0	LRI, MS
4.12	1240	Hexyl valerate	43, 56, 85, 103		0.1c ± 0.0		0.2d ± 0.0	LRI, MS
4.21	1249	Linalyl acetate	41, 69, **93**, 121	1872.4a ± 34.9	30.7c ± 0.2	1074.6b ± 31.0	**17.1d ± 0.0**	LRI, MS, std
4.27	1255	Geraniol	41, 69, 93, 121		0.6c ± 0.0		0.6c ± 0.0	LRI, MS
4.54	1284	Lavandulyl acetate	43, 69, 93, 121		**2.4c ± 0.1**		1.3d ± 0.0	LRI, MS
5.04	1331	6-Undecanol	41, 55, 83, 101		0.1c ± 0.0		0.1d ± 0.0	LRI, MS
5.34	1359	Neryl acetate	41, 69, 79, **93**	17.0a ± 0.3	0.3c ± 0.0	35.2b ± 0.2	0.3d ± 0.0	LRI, MS, std
5.54	1379	Geranyl acetate	41, **69**, 79, 93	30.8a ± 0.5	0.6c ± 0.0	25.4b ± 0.1	0.5c ± 0.0	LRI, MS, std
5.57	1381	β-Cubebene	91, 105, 120, 161		0.1c ± 0.0		0.1d ± 0.0	LRI, MS
5.65	1389	α-Bergamotene	69, 93, 105, 119		0.1c ± 0.0		0.1c ± 0.0	LRI, MS
5.82	1405	α-Cedrene	93, 105, 119, 204		0.1 ± 0.0			LRI, MS
6.03	1426	α-Santalene	41, 94, 161, 189		0.2 ± 0.0			LRI, MS
6.06	1429	(*E*)-β-Caryophyllene	41, 69, 79, **93**	119.5a ± 3.0	1.4c ± 0.0	57.2b ± 0.2	1.1d ± 0.0	LRI, MS, std
6.17	1441	(*E*)-α-Bergamotene	41, 69, 93, 119		0.1c ± 0.0		0.1d ± 0.0	LRI, MS
6.25	1449	β-Farnesene	41, 69, 93, 133		tr			LRI, MS
6.37	1461	(*Z*)-β-Farnesene	41, 69, 93, 133		0.9c ± 0.0		**0.7d ± 0.0**	LRI, MS
6.43	1467	α-Humulene	41, 67, 80, **93**	5.6 ± 0.2	0.1 ± 0.0			LRI, MS, std
6.57	1481	α-Amorphene	105, 119, 161, 204		0.1 ± 0.0			LRI, MS
6.65	1489	γ-Muurolene	105, 119, 161, 204		0.6c ± 0.0		**0.2d ± 0.0**	LRI, MS
6.78	1504	α-Selinene	161, 175, 189, 204		0.2c ± 0.0		0.1c ± 0.0	LRI, MS
6.80	1507	α-Farnesene	41, 93, 107, 121		0.2c ± 0.0		0.1d ± 0.0	LRI, MS
6.84	1514	Bergamotene	41, 77, 93, 119		0.2c ± 0.0		0.1d ± 0.0	LRI, MS
6.89	1521	Germacrene D	105, 119, 161, 204		0.2 ± 0.0			LRI, MS
6.95	1532	β-Sesquiphellandrene	41, 69, 93, 161		0.1 ± 0.0			LRI, MS
7.32	1593	Caryophyllene oxide	41, 79, 91, 105		tr c		0.1d ± 0.0	LRI, MS

Table 5.3. Continued

t_R (min)	LRI	Analyte (X)	Qualifying and quantitation ions* (m/z)	Grosso 1 Concentration (mM ± SD)	Grosso 1 Area (% ± SD)	Grosso 2 Concentration (mM ± SD)	Grosso 2 Area (% ± SD)	Identification methods
7.61	1658	Cadinol	105, 119, 161, 204		0.1c ± 0.0		0.1d ± 0.0	LRI, MS
7.77	1695	α-Bisabolol	41, 69, 93, 119		0.2 ± 0.0			LRI, MS
		Oxygenated terpenes:						
		Alcohol			41.2		59.1	
		Ketone			7.1		7.1	
		Aldehyde			0.0		0.0	
		Ester			34.0		19.3	
		Ether			6.2		4.5	
		Monoterpene hydrocarbons			5.9		5.8	
		Oxygenated monoterpenes			88.4		90.0	
		Sesquiterpene hydrocarbons			5.0		2.8	
		Oxygenated sesquiterpenes			0.0		0.1	
		Total terpene hydrocarbons			10.9		8.5	
		Total oxygenated terpenes			88.4		90.0	
		Non isoprenoid components			0.7		1.4	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). SD = standard deviation. tr = traces (<0.1%)
MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. *Areas in bold mean "biomolecular marker" of the EO biochemotype. *Quantitation ions are shown in bold.
a, b Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$. c,d Different letters in the same compound area mean statistically significant differences with $p < 0.05$.

Table 5.5. EsGC/MS determination of components in *L.* x *intermedia*.

t_R (min)		Analyte (X)	Abrial 1		Super 1		Super 2		Grosso 1		Grosso 2	
(+) - X	(-) - X		(+) - [X] (%)	(-) - [X] (%)	(+) - [X] (%)	(-) - [X] (%)	(+) - [X] (%)	(-) - [X] (%)	(+) - [X] (%)	(-) - [X] (%)	(+) - [X] (%)	(-) - [X] (%)
7.79	7.52	α-Pinene	95	5	95	5	95	5	95	5	95	5
8.47	8.24	Camphene	35*	65*	30*	70*	14	86	19	81	5	95
8.89	9.16	β-Pinene	66*	34*	89*	11*	73*	27*	80*	20*	95	5
10.52	10.00	Limonene	95	5	95	5	95	5	95	5	80*	20*
15.73	15.57	Linalool	5	95	3	97	5	95	5	95	3	97
16.72	16.46	Camphor	95	5	95	5	95	5	95	5	95	5
18.32	18.51	Terpinen-4-ol	95	5	95	5	95	5	95	5	95	5
20.10	19.76	α-Terpineol	78*	22*	95	5	95	5	95	5	78*	22*
20.15	19.58	Borneol	95	5	95	5	95	5	95	5	95	5
23.92	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	5	95	5	95
-	28.81	Caryophyllene oxide	5	95	5	95	5	95	5	95	5	95

*Biomolecular marker of the EO biochemotype

Despite the important differences found between all the three cultivars, using a dendrogram representation (Figure 5.1) of agglomerative hierarchical clustering based on Euclidean distance applied to the relative area of components, the high similarity between Grosso and Super cultivars was unveiled, whereas both cultivars show a higher distance to the Abrial cultivar. In the dendrogram, samples Super2 and Grosso2 are part of the same group, showing high similarity (74.81%); Super1 and Grosso1 show a similarity of 59.34% and Abrial1 has a similarity of 47.27% with the Super2/Grosso2 group. According to the results, the growing location (bioclimatic zone) becomes more important to composition than the cultivar of the plant material.

Oxygenated monoterpenes are highly predominant in the five samples, accounting for more than 80%, in average, of the total molecules. Alcohol is the most abundant organic functional group, exceeding 40% of total molecules; the second organic functional group in abundance is ester, accounting for more than 20% in average. The hydrocarbon monoterpenes represent a maximum of 14%, in the case of Abrial/Super cultivar.

Total terpene hydrocarbons were calculated as the sum of the monoterpene and sesquiterpene hydrocarbons. Total oxygenated terpenes were calculated as the sum of the oxygenated monoterpenes and sesquiterpenes.

Several ingredients show peak area percentages similar to those reported in literature for plant samples of *L.* x *intermedia* grown in different countries. Some main components like linalool or camphor show a similar concentration to samples from Turkey (Baydar and Kineci, 2009), borneol is similar to the Japanese (Seino et al., 2008) or French reports (Bombayda et al., 2008), linalyl acetate is similar to the one reported from Italy (Cosimi et al., 2009) while some others like α-terpineol show similarities to Greece (Papachristos et al., 2004). Molecules like *cis*- and *trans*-β-ocimene have been determined in higher concentrations in Spanish samples than in any other location.

5.4.2 International Standard comparative

The International Organization for Standardization (ISO) has published International Standards for *L.* x *intermedia* EOs of both Abrial (ISO, 2001) and Grosso (ISO, 2009) cultivars (French type) but not for the Super cultivar. The results showed in (Table 5.4) were obtained taking ISO Standards for comparison and grouping up together Abrial and Super cultivars due to their similarities. Some constituents from the Spanish EOs exceed the maximum relative concentration allowed for the French type. The case of linalool is especially interesting, being found exceeding the limits in all samples. Linalyl acetate is another important molecule due to its pleasant aroma, it has been found in high concentrationin Super1 whereas in Grosso2 has been found beneath limits.

5.4.3 Enantioselective gas chromatography/mass spectrometry (EsGC/MS study)

The enantiomeric determinations of molecules of EOs from *L.* x *intermedia* are shown in (Table 5.5). There are no adulterations with synthetic racemates of the main molecules, such as linalool, linalyl acetate and camphor. The enantiomeric predominance is the same for the three types of cultivars. The (+)-enantiomeric excess is shown in the case of: α-pinene, β-pinene, limonene, camphor, terpinen-4-ol, α-

terpineol and borneol; while the (-)-enantiomeric excess is shown in: camphene, linalool, linalyl acetate, (*E*)-β-caryophyllene and caryophyllene oxide. The enantiomeric distribution of α-terpineol is markedly different for each sample and could be useful for their characterisation. There are several biomolecular markers of the biochemotype origin: camphene for Abrial1 and Super1; β-pinene for Abrial1, Super1, Super2 and Grosso1; α-terpineol for Abrial1 and Grosso2; and limonene for Grosso2. This data could be useful to assess the origin and the authenticity of the EOs. To our knowledge, this is the first chiral wide characterisation of the EOs from *L.* x *intermedia* grown in Spain.

Table 5.4.
GC/MS determination of components in *L.* x *intermedia*, comparative with ISO normative.

	Normative*		Abrial 1	Super 1	Super 2	Normative†		Grosso 1	Grosso 2
Component (X)	Min (%)	Max (%)	Area X (%)	Area X (%)	Area X (%)	Min (%)	Max (%)	Area X (%)	Area X (%)
β-Myrcene						0.30	1.00	0.74	0.80
Limonene	0.50	1.50	1.10	0.72	0.69	0.50	1.50	0.83	0.99
(*Z*)-β-Ocimene	1.50	3.00	3.54	1.75	1.70	0.50	1.50	1.28	0.53
Eucalyptol	6.00	11.00	8.41	4.26	5.41	4.00	8.00	6.64	4.79
(*E*)-β-Ocimene	3.00	7.00	6.97	2.22	2.19	nd	1.00	0.39	0.75
Linalool	26.00	38.00	41.91	43.27	51.98	24.00	37.00	37.74	51.27
Camphor	7.00	11.00	10.29	5.23	8.60	6.00	8.50	7.86	7.68
Lavandulol	0.40	1.20	0.78	0.21	0.87	0.20	1.00	0.38	1.48
Borneol	1.50	3.50	2.17	2.51	3.12	1.50	3.50	2.26	4.33
Terpinen-4-ol	0.30	1.00	1.10	0.41	1.72	1.50	5.00	3.35	5.27
Hexyl butyrate						0.30	0.50	0.31	0.45
α-Terpineol						0.30	1.30	1.45	1.57
Linalyl acetate	20.00	29.00	21.99	38.09	22.39	25.00	38.00	34.15	18.60
Lavandulyl acetate	1.00	2.00	1.73	1.32	1.34	1.50	3.50	2.63	1.47

* ISO normative for lavandin Abrial (ISO, 2001)

† ISO normative for lavandin Grosso (ISO, 2009)

The high proportion of (-)-linalool and (-)-linalyl acetate in EOs grown in Spain (Table 5.5), is similar to those reported for the EOs of *L.* x *intermedia* worldwide. On the contrary, (-)-camphor was reported as the main enantiomeric species obtained from EOs but (+)-camphor was obtained from manufactured products declaring the presence of *Lavandula* EO (Flores et al., 2005). In this study, (+)-camphor was the main enantiomeric species of camphor observed. (1R)-(+)-Camphor has also been reported for some other varieties of lavender, like *L. angustifolia* (Bicchi et al., 2010) or *L. stoechas (Ristorcelli et al., 1998)*, leading to a high probability of being (+)-camphor the predominant enantiomer in all the *Lavandula* genus. The enantiomeric species declared by this study is checked by commercial samples of (+) and (-)-camphor, thus showing a possible mistake in the already mentioned study of Flores *et al*.

5.4.4 Antioxidant activity

The ORAC antioxidant activity (Ou et al., 2001) of the five samples of *L.* x *intermedia* is expressed in TEAC units (µmol TE/µL EO) and resulted (Table 5.6) as follows:

Abrial1^{ORAC} ≈ Super1 ^{ORAC} ≈ Super2 ^{ORAC} ≈ Grosso1 ^{ORAC} ≈ Grosso2 ^{ORAC}.

The antioxidant activity of each EO is related to its composition and the intrinsic antioxidant activity of each of the compounds. Generally, the rise in composition of oxygenated terpenes is correlated to higher ORAC antioxidant activity. That is lowest for Abrial1 and highest for Grosso2 EOs (Tables 5.2, 5.3 and 5.6).Two oxygenated components are highly relevant to explain the ORAC value of the EO, namely linalool and linalyl acetate, the first and the most important molecule because of its high concentration and high ORAC value and the other for its moderate ORAC value and high concentration (Bentayeb et al., 2014).

Linalool is the principal ingredient of *L.* x *intermedia* EO and shows high intrinsic antioxidant activity, thus it holds the main contribution to the antioxidant activity of the EO. Other abundant molecules, like linalyl acetate, exhibiting high ORAC values contribute significantly to the global oil ORAC value. However, the total ORAC value of the EO is determined not just by the main components but by the whole group of ingredients present in the EO.

The ABTS antioxidant activity (Re et al., 1999) of the samples of *L.* x *intermedia* is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 1) as follows:

Abrial1^{ABTS} ≥ Super1^{ABTS} ≈ Grosso2^{ABTS} ≥ Super2^{ABTS} ≥ Grosso1^{ABTS}.

As linalool and (*E*)-β-ocimene show the lowest concentrations in Grosso1 sample and show the highest concentrations in Grosso2 and Abrial1, respectively (Tables 5.2 and 5.3), we can propose a remarkable antioxidant activity of (*E*)-β-ocimene and slightly lower antioxidant activity of linalool against ABTS radical cation.

The DDPH antioxidant activity (Brandwilliams et al., 1995) of the samples of *L.* x *intermedia* is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 5.6) as follows:

Abrial1^{DPPH} > Super2^{DPPH} ≥ Super1^{DPPH} ≥ Grosso1^{DPPH} > Grosso2^{DPPH}.

Different molecules, present in high quantity in Abrial1 and in low quantity in Grosso2 may explain the better performance in DPPH antioxidant assay (Dawidowicz and Olszowy, 2014), *i.e.* ocimene and (*E*)-β-caryophyllene (Tables 5.2 and 5.3).

Table 5.6.
Antioxidant capacity of *L.* x *intermedia* EOs.

Antioxidant method (units)	Abrial1	Super1	Super2	Grosso1	Grosso2
ORAC (µmol TE/µL EO)	1.24a ± 0.08	1.27a ± 0.09	1.28a ± 0.09	1.26a ± 0.07	1.37a ± 0.09
ABTS (µmol TE/mL EO)	1.7a ± 0.1	1.6a,b ± 0.1	1.4b,c ± 0.1	1.3c ± 0.0	1.6a,b ± 0.1
DPPH (µmol TE/mL EO)	0.73a ± 0.05	0.56b,c ± 0.02	0.62b ± 0.04	0.48c ± 0.03	0.31d ± 0.01
ChP (mg EDTA eq/mL EO)	0.9b,c ± 0.0	1.1b ± 0.1	0.8c ± 0.1	1.5a ± 0.1	0.5d ± 0.0
RdP (mg Ascorbic acid eq/mL EO)	0.22c ± 0.01	0.38b ± 0.02	0.41a,b ± 0.02	0.40a,b ± 0.02	0.43a ± 0.02

a, b, c Different letters in the same antioxidant method mean statistically significant differences with $p < 0.05$

The chelating power activity (Miguel et al., 2010) is expressed in EDTA units (mg EDTA equivalents / mL EO) and resulted (Table 5.6) as follows:

Grosso1^{ChP} > Super1^{ChP} ≥ Abrial1^{ChP} ≥ Super2^{ChP} > Grosso2^{ChP}.

The highest concentrations of linalyl acetate and lavandulyl acetate (Tables 5.2 and 5.3) were found in Grosso1 sample. The high electronic densities of the oxygen atoms of the carboxylic ester groups, could be useful for complexation of cations.

The reducing power antioxidant activity (Oyaizu, 1986) of the samples of *L.* x *intermedia* is expressed in ascorbic acid units (mg Ascorbic acid equivalents/mL EO) and resulted (Table 5.6) as follows:

Grosso2^{RdP} ≥ Super2^{RdP} ≈ Grosso1^{RdP} ≥ Super1^{RdP} > Abrial1^{RdP}.

Some abundant alcoholic terpenoids , as the already reported linalool (Liu et al., 2012), might show a mild reducing power.

5.4.5 Inhibitory activity on LOX

The results of the LOX inhibitory activity (Christop.J et al., 1970; Whent et al., 2010) were obtained as described in Experimental section (shown in Supplementary material). Inhibition degree (%) at 0.3 µL(EO)/mL was measured for Grosso2^{LOX}(32.6 ± 0.9)^{a} > Super2^{LOX}(24.0 ± 0.8)^{b} ≥ Grosso1^{LOX}(20.9 ± 1.2)^{b,c} ≥ Super1^{LOX}(18.2 ± 1.1)^{c} ≈ Abrial1^{LOX}(17.5 ± 3.4)^{c}. Tukey's HSD test revealed significant differences between the samples (different superscripts). For a deeper understanding of the LOX inhibitory activity of the EO, the main commercially available compounds, had their LOX inhibitory activity tested, obtaining their IC_{50} (µM) value: limonene (356 ± 30), p-cymene (486 ± 32), camphor (2743 ± 85) and linalool (3346 ± 44).

The inhibitory activity of the *L.* x *intermedia* EO is clearly due to a combination of compounds with high inhibitory activity, and more abundant compounds namely camphor and linalool (Tables 5.2 and 5.3). The high inhibition of LOX by Grosso2 in comparison with the other samples, can be explained by the fact that linalool is found in the biggest absolute concentration.

L. x *intermedia* is an economically key species within the *Lavandula* genus, due to its high yield EO with high proportion of linalool and linalyl acetate, thus it can emulate *L. angustifolia* EO aroma (Tongnuanchan and Benjakul, 2014). Its biochemical composition is the key to understand the bioactivities of *L.* x *intermedia* EOs such as antibacterial, antifungal, antiparasitic, insecticide, antioxidant and anti-inflammatory agents (Jianu et al., 2013; Papachristos et al., 2004; Torras-Claveria et al., 2007). These properties support the potential use of *L.* x *intermedia* EOs as natural cosmetics and natural pharmaceutical ingredients useful for relieving gastrointestinal disorders and for fighting human protozoal pathogens (Baker et al., 2012; Moon et al., 2006).

5.5 Conclusions

The different *L.* x *intermedia* EOs evaluated showed the same common 11 principal constituents, i.e. (*Z*)-β-ocimene, eucalyptol, linalool, camphor, borneol, terpinen-4-ol, α-terpineol, linalyl acetate, lavandulyl acetate, (*E*)-β-caryophyllene and Z-β-farnesene. Some concentrations of important odorant molecules like linalool and linalyl acetate are found exceeding ISO normative limits, highlighting a new cheap raw material for industrial manufacturing of lavender aroma. A new source of pure enantiomers is unveiled showing (-)-linalool, (-)-linalyl acetate and (+)-camphor as highly representative compounds of the typical *L.* x *intermedia* EO. *L.* x *intermedia* EOs showed moderate antioxidant activities, especially due to linalool and linalyl acetate. Regarding potential anti-inflammatory properties, LOX inhibitory activities of *L.* x

intermedia EOs, were especially due to linalool and camphor. *L.* x *intermedia* has a high yield of EO whose properties support the potential use of *L.* x *intermedia* EOs as natural cosmetics and natural pharmaceutical ingredients useful for relieving gastrointestinal disorders and for fighting diseases related to oxidative stress.

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6. CARACTERIZACIÓN DE LOS ACEITES ESENCIALES DE *L. ANGUSTIFOLIA* Y *L. LATIFOLIA*

6.1 Resumen

Los aceites esenciales de lavanda (*Lavandula angustifolia*) y espliego (*Lavandula latifolia*), cultivados y extraidos en la región sureste de España, han sido analizados por cromatografía de gases con detección por espectrometría de masas para determinar su composición, en ambas concentraciones relativa (por área de pico) y absoluta (utilizando rectas de calibrado). Las moléculas más abundantes halladas en *L. angustifolia* fueron: linalol (37-54%), acetato de linalilo (21-36%) y (E)-β-cariofileno (1-3%). Linalol (35-51%), eucaliptol (26-32%), alcanfor (10-18%), α-pineno(1-2%), α-terpineol (1-2%) y α-bisaboleno (1-2%) fueron los componentes más abundantes hallados para *L. latifolia*. Esta caracterización se completó con el uso de cromatografía de gases enantioselectiva, detectando (–)-linalol, (+)-alcanfor y (–)-acetato de linalilo como componentes principales. (S)-(–)-Canfeno, (R)-(+)-limoneno, (1R, 9S)-(–)-(E)-β-cariofileno y (1R, 4R, 6R, 10S)-(–)-óxido de cariofileno se hallaron en este estudio como enantiómeros predominantes en *L. angustifolia* española. La capacidad antioxidante se evaluó con resultados positivos por varios métodos: actividad frente a radicales libres (ABTS, DPPH, ORAC) y capacidades quelatante y reductora. Se observó actividad inhibidora de lipoxigenasa (LOX) lo cual es indicador de una posible actividad antiinflamatoria, principalmente debida a linalool, alcanfor, p-cimeno y limoneno. Estos resultados pueden ser un prometedor comienzo para el estudio futuro de los potenciales usos cosméticos y farmacológicos de los aceites esenciales de *L. angustifolia* y *L. latifolia* como componentes naturales para tratar desórdenes cutáneos.

6.2 Summary

Compositions of lavender (Lavandula angustifolia) and spike lavender (Lavandula latifolia) essential oils (EOs), cultivated and extracted in the South East region of Spain, were determined by gas chromatography coupled with mass spectrometry (GC-MS) detection, obtaining both relative (peak area) and absolute (using standard curves) concentrations. Linalool (37-54%), linalyl acetate (21-36%) and (*E*)-β-caryophyllene (1-3%) were the most abundant components for L. angustifolia. Linalool (35-51%), eucalyptol (26-32%), camphor (10-18%), α-pinene (1-2%), α-terpineol (1-2%) and α-bisabolene (1-2%) were the most abundant components for L. latifolia. This characterization was completed with an enantioselective gas chromatography, the main molecules determined were (-)-linalool, (-)-linalyl acetate and (+)-camphor. (S)-(–)-Camphene, (R)-(+)-limonene, (1R, 9S)-(–)-(*E*)-β-caryophyllene and (1R, 4R, 6R, 10S)-(–)-caryophyllene oxide were found in this study as predominant enantiomers in Spanish Lavandula angustifolia. The characterised EOs were tested for antioxidant activity against free radicals (ABTS, DPPH, ORAC), chelating and reducing power. Inhibitory activity on lipoxygenase (LOX) was observed

indicating possible anti-inflammatory activity, mainly due to linalool, camphor, p-cymene and limonene. These results can be the starting point for a future study of the potential use of L. angustifolia and L. latifolia EOs as natural cosmetic and natural pharmaceutical ingredients for several skin diseases.

6.3 Introduction

Lavandula angustifolia Miller, formerly known as *Lavandula officinalis,* which is also known as lavender, true lavender or English lavender, is native to southern Europe and the Mediterranean area, where it is widely grown (da Porto and Decorti, 2008). France, Spain, Portugal, Hungary, the UK, Bulgaria, Australia, China and the USA cultivate this plant, its use has increased in the last years due to the pleasant aroma and presumed properties of its essential oil (EO) (Verma et al., 2010). Lavender EO is one of the most valuable ingredients in the production of flavourings or cosmetics, it is also used in the pharmaceutical industry due to its functional properties (Lubbe and Verpoorte, 2011).

Lavandula latifolia Medikus, which is also known as spike lavender or Spanish lavender, grows as a domestic plant in the Mediterranean region. It has been traditionally used as a raw material in perfumery and cosmetics due to its fragrance. In addition, a great range of medical uses of this plant have been reported including antispasmodic, sedative, antihypertensive, antiseptic, healing and anti-inflammatory properties, which make it highly appreciated in phytotherapy and aromatherapy (Azimova et al., 2011; Herraiz-Peñalver et al., 2013).

Gas chromatography, coupled to mass spectrometry (GC/MS) is a very powerful technique used for the analysis of volatile components, since it provides qualitative and quantitative data for complex mixtures such as those usually present in natural products (Smelcerovic et al., 2013). Some relative quantitation of *L. angustifolia* and *L. latifolia* compositions have been reported (Cong et al., 2008; Danh et al., 2013; Guillen et al., 1996; Herraiz-Peñalver et al., 2013; Munoz-Bertomeu et al., 2007; Salido et al., 2004; Santana et al., 2012; Seidler-Lozykowska et al., 2014; Smigielski et al., 2013; Verma et al., 2010; Zheljazkov et al., 2013). None of them has studied the specific conditions of the Spanish Mediterranean coast, specifically of the Region of Murcia, where the biggest aromatic plant diversity among all regions of Spain is found.

There are few chiral studies on EOs of *L. angustifolia* and *L. latifolia*, all of them accounting for some specific biomolecules. Nevertheless, chiral distribution is an important aspect of the EO composition, it allows identification of natural EOs from those adulterated with racemic mixtures of synthetic compounds, even when the samples come from different places of the Earth (Bauermann et al., 2008; del Castillo et al., 2004).

The evaluation of bioactivities of plant EOs, commonly used in traditional medicine and aromatherapy, has become relevant due to their presumed safe and therapeutic effects. *In vitro* assays serve as models for preliminary observations in the evaluation of pharmacological bioactivities. The antioxidant potential of EOs can be determined using a representative selection of different antioxidant methods, *i.e.* ORAC method to evaluate the scavenging capacity of free oxygen (peroxyl) radicals, ABTS or DPPH methods to measure the ability of reducing nitrogen radical cations, Fe^{2+} chelating power (ChP) and Fe^{3+} reducing power (RdP) methods, to account for the ability to neutralise oxidant metal ions (Dawidowicz and Olszowy, 2014).

Beneficial applications of EOs have also been related to their anti-inflammatory properties (Rubio et al., 2013). Many inflammatory processes (asthma, allergic rhinitis, psoriasis,…) are associated with leukotriene production catalysed by lipoxygenase (LOX), which can use molecular oxygen or hydrogen peroxide as oxidants (Anwar et al., 2014). Leukotrienes produced by LOX from unsaturated fatty acids, mainly linoleic and arachidonic acids, are a group of highly potent molecules that mediate inflammatory and allergic reactions, their biological effects can be antagonized or prevented by targeting leukotriene production through inhibition of LOX. Thus, the inhibition of soybean lipoxygenase as LOX model is a hint of anti-inflammatory activity of the EO (Kim et al., 2014; Mandal et al., 2014; Mohamed et al., 2014).

The aim of the present study is to determine the relative, absolute and chiral distribution of each of the EO main components in 8 samples grown in Murcia (Spain). Then, the above mentioned methods (ORAC, ABTS, DPPH, ChP, RdP) will be applied to evaluate the antioxidant capacities of *L. angustifolia* and *L. latifolia* EOs. Furthermore, the degree of inhibition of LOX, at a fixed concentration of the EOs, will serve to characterise the inhibitory activity of the EOs, in an attempt to enhance and broaden the potential biotechnological applications of the EOs.

6.4 Results and Discussion

The essential oils were obtained in yields ranging from 0.3 to 1.2% (w/w). Fast Gas Chromatography coupled with Mass Spectrometry detection (FGC/MS) was used to determine the components of the studied EOs (Table 6.1).

Each EO is described with two values: concentration in mM (commercially available standards only, represent >90% of the total area) and peak area (>99% of the total area) of volatile compounds.

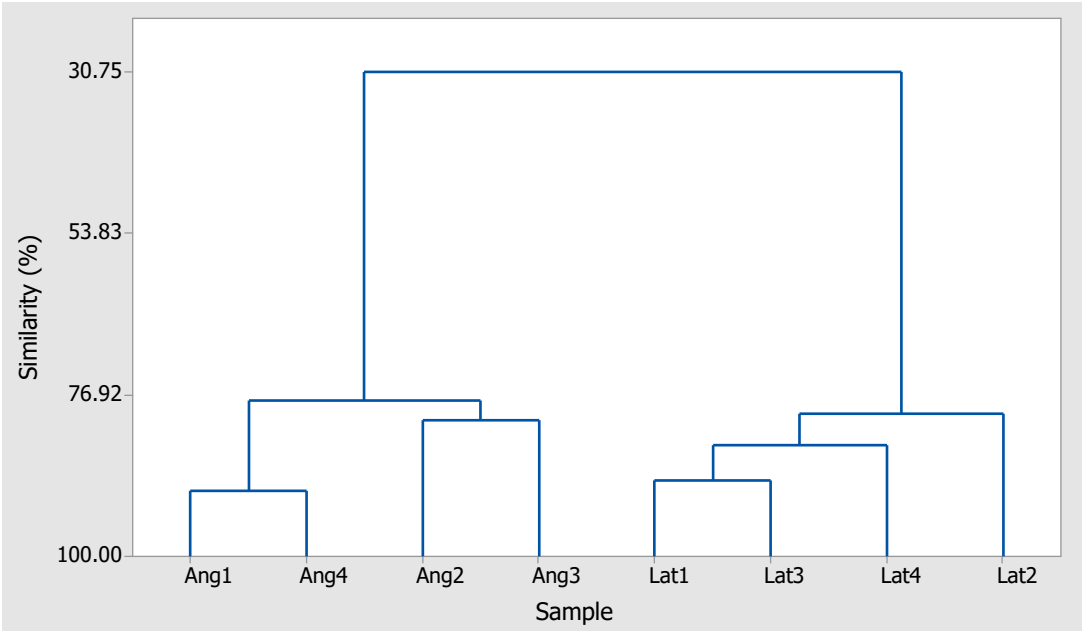

Figure 6.1
Dendrogram showing similarity of *L. angustifolia* and *L. latifolia* samples

Samples Ang1, Ang2, Ang3 and Ang4 are EOs of *L. angustifolia* and samples Lat1, Lat2, Lat3 and Lat4 are EOs of *L. latifolia*. Also, Ang2 and Lat2 were grown in Lower Meso-Mediterranean bioclimatic zone, Ang3 and Lat3 were grown in Upper

Meso-Mediterranean bioclimatic zone and Ang1, Ang4, Lat1 and Lat4 were grown in Supra Mediterranean bioclimatic zone. All the samples were analysed in triplicate.

There are important differences between all the four samples of each species, however, using a dendrogram representation (Figure 6.1) of agglomerative hierarchical clustering based on Euclidean distance applied to the relative area of components, some similarities were unveiled.

Table 6.1
Parameters of FGC/MS calibration

Analyte	Calibration curve[a]	R^2	Calibration range [mM]	RSD [%]	LOD [mM]	LOQ [mM]	Standard source	Product reference
Nonane	*Internal standard*						SAFC	442694
(–)-α-Pinene	y = 0.471x - 0.018	0.997	0.25 - 10.06	4.5	0.08	0.25	Fluka	80599
(+)-Camphene	y = 0.272x - 0.008	0.995	0.66 - 10.51	0.4	0.14	0.44	SAFC	w222909
Sabinene	y = 0.460x - 0.026	0.998	0.24 - 9.78	0.9	0.07	0.21	Extrasynthese	5062 S
(–)-β-Pinene	y = 0.400x - 0.016	0.995	0.26 - 10.29	3.7	0.09	0.26	Fluka	80609
1-Octen-3-ol	y = 0.236x - 0.023	0.994	0.26 - 10.33	0.9	0.09	0.26	Aldrich	o5284
3-Octanone	y = 0.309x - 0.031	0.996	2.56 - 10.24	4.1	0.64	1.95	SAFC	w280305
Myrcene	y = 0.188x - 0.024	0.993	2.24 - 8.97	3.5	0.56	1.70	Fluka	64643
Hexyl acetate	y = 0.452x - 0.057	0.992	2.40 - 9.59	1.7	0.32	0.96	Fluka	25539
(+)-3-Carene	y = 0.355x - 0.012	0.999	0.25 - 10.05	1.4	0.08	0.25	Aldrich	441619
p-Cymene	y = 0.830x - 0.020	0.995	0.25 - 9.95	4.9	0.02	0.05	Aldrich	c121452
(+)-Limonene	y = 0.246x - 0.008	0.995	0.60 - 9.55	0.9	0.12	0.36	Fluka	62118
(*Z*)-β-Ocimene	y = 0.218x - 0.011	0.996	0.70 - 6.97	2.2	0.16	0.48	SAFC	w353901
Eucalyptol	y = 0.233x - 0.008	0.995	2.39 - 9.55	8.7	0.60	1.82	SAFC	w246506
γ-Terpinene	y = 0.366x - 0.017	0.995	0.62 - 9.87	2.8	0.12	0.37	Aldrich	223190
(*trans*)-Sabinene hydrate	y = 0.268x - 0.007	0.996	0.63 - 10.00	2.1	0.18	0.56	Fluka	96573
(–)-Linalool	y = 0.214x - 0.008	0.994	0.88 - 8.80	4.9	0.22	0.67	Fluka	74856
1-Octen-3-yl acetate	y = 0.279x - 0.015	0.995	2.05 - 8.21	1.4	0.38	1.16	SAFC	w358207
(+)-Camphor	y = 0.181x - 0.024	0.994	0.99 - 9.85	1.5	0.25	0.76	Alfa Aesar	A10708
(–)-Borneol	y = 0.266x - 0.020	0.998	0.57 - 9.06	4.4	0.19	0.57	Alfa Aesar	A12684
(–)-Terpinen-4-ol	y = 0.241x - 0.003	0.997	0.60 - 9.57	4.3	0.20	0.60	Aldrich	11584
Hexyl butyrate	y = 0.332x - 0.034	0.991	4.84 - 7.74	0.6	0.77	2.34	SAFC	w256811
(+)-α-Terpineol	y = 0.175x - 0.003	0.998	1.02 - 10.23	4.5	0.26	0.79	Fluka	83073
Tetradecane	*Internal standard*						SAFC	442708
(–)-Linalyl acetate	y = 0.217x - 0.006	0.998	0.72 - 7.18	0.2	0.18	0.55	SAFC	w263605
(–)-Bornyl acetate	y = 0.268x - 0.003	0.997	0.82 - 8.16	1.4	0.21	0.65	Fluka	45855
Thymol	y = 0.313x - 0.029	0.998	0.25 - 10.03	4.8	0.08	0.25	Sigma	T0501
Neryl acetate	y = 0.242x - 0.007	0.999	0.73 - 7.31	3.8	0.18	0.55	SAFC	w277304
Geranyl acetate	y = 0.249x - 0.009	0.999	0.46 - 7.42	2.9	0.15	0.46	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(–)-(*E*)-β-Caryophyllene	y = 0.079x - 0.002	0.998	4.41 - 7.05	6.6	0.71	2.16	Sigma	22075
α-Humulene	y = 0.541x - 0.019	0.999	0.40 - 6.35	2.2	0.13	0.40	Aldrich	53675
(*E*)-α-Bisabolene	y = 0.111x - 0.043	0.999	1.74 - 6.97	3.4	0.31	0.94	Alfa Aesar	A18724
(–)-Caryophyllene oxide	y = 0.105x - 0.004	0.999	2.54 - 10.17	2.7	0.46	1.40	SAFC	w509647

[a]Response ratio vs. Concentration ratio, internal standard correction applied.
Each internal standard is reference compound for the analytes that follow.

Table 6.2. FGC/MS determination of components of *L. angustifolia* EOs.

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Ang1 Concentration [mM ± SD]	Ang1 Area [% ± SD]	Ang2 Concentration [mM ± SD]	Ang2 Area [% ± SD]	Ang3 Concentration [mM ± SD]	Ang3 Area [% ± SD]	Ang4 Concentration [mM ± SD]	Ang4 Area [% ± SD]	Identification methods
1.15	861	4-Hexen-1-ol	31, 41, 55, 67		0.1e±0.0		0.1e±0.0					LRI, MS
1.20	874	n-Hexanol	43, 56, 69, 82				0.2e±0.0		0.1f±0.0			LRI, MS
1.47	929	Tricyclene	79, 93, 121, 136		0.1±0.0							LRI, MS
1.53	939	α-Pinene	39, 91, **93**, 77	8.5a±0.5	0.1e±0.0		0.1g±0.0	7.2b±0.1	0.1f±0.0	0.3c±0.0	0.1g±0.0	LRI, MS, std
1.63	957	Camphene	67, 79, **93**, 121	13.4b±0.8	0.2f±0.0		0.1h±0.0	16.3a±0.1	0.2e±0.0	6.1c±0.2	0.1g±0.0	LRI, MS, std
1.75	978	Sabinene	41, 77, 91, **93**		0.1±0.0							LRI, MS
1.79	985	β-Pinene	41, 69, 79, **93**					6.7a±0.2	0.3e±0.0	2.5b±0.0	0.1f±0.0	LRI, MS, std
1.80	987	1-Octen-3-ol	41, **57**, 67, 81	28.3a±1.1	0.4e±0.0	14.6c±0.8	0.2f±0.0	20.5b±0.4	0.5e±0.0			LRI, MS, std
1.81	989	3-Octanone	**43**, 57, 71, 99			115.3a±1.6	1.3e±0.0	42.5b±1.9	0.1g±0.0	43.7b±1.5	0.2f±0.0	LRI, MS, std
1.83	992	Myrcene	39, **41**, 69, 93	57.2b±2.3	0.5f±0.0	41.9c±3.3	0.4g±0.0	98.6a±0.4	0.9e±0.0	29.9d±0.7	0.3h±0.0	LRI, MS, std
1.89	1002	3-Octanol	41, 59, 83, 101				0.3e±0.0		0.1f±0.0			LRI, MS
1.97	1011	Hexyl acetate	**43**, 56, 84, 61	12.0c±0.1	0.1f±0.0	13.4b±0.3	0.1g±0.0	20.8a±0.5	0.2e±0.0			LRI, MS, std
1.98	1012	β-Phellandrene	77, 93, 121, 136								tr	LRI, MS
2.00	1014	3-Carene	77, 79, 91, **93**	23.1±0.6	0.4e±0.0				0.1f±0.0			LRI, MS
2.06	1021	m-Cymene	77, 91, 119, 134		0.1±0.0							LRI, MS
2.12	1028	p-Cymene	77, 91, **119**, 134	12.9a±0.4	0.3e±0.0	8.1b±0.1	0.2f±0.0	6.5c±0.1	0.1g±0.0	4.2d±0.0	0.1h±0.0	LRI, MS, std
2.16	1032	Limonene	67, **68**, 79, 93	34.5a±0.8	0.5e±0.0	16.6c±0.4	0.2g±0.0	21.6b±0.7	0.3f±0.0	7.4d±1.0	0.2g±0.0	LRI, MS, std
2.17	1034	(*Z*)-β-Ocimene	41, 79, 91, **93**	401.4a±10.4	2.8e±0.0	106.0b±4.1	1.2f±0.0	54.4c±2.0	0.7h±0.0	111.4b±4.6	0.8g±0.0	LRI, MS, std
2.18	1035	Eucalyptol	**43**, 67, 81, 93	101.4b±2.2	2.2f±0.1	134.1a±2.2	2.9e±0.0	10.1d±0.5	0.1h±0.0	78.2c±1.1	1.7g±0.0	LRI, MS, std
2.26	1044	(*E*)-β-Ocimene	41, 79, 93, 121		1.8f±0.0		2.2e±0.0		0.8h±0.0		1.6g±0.1	LRI, MS
2.40	1060	γ-Terpinene	77, 91, **93**, 136	7.2±0.1	0.1e±0.0		0.1f±0.0					LRI, MS, std
2.55	1077	(*trans*)-Sabinene hydrate	77, 91, **93**, 121	6.9c±0.5	0.1g±0.0	9.1b±0.1	0.3f±0.0	7.2c±0.2	0.4e±0.0	15.5a±0.2	0.1g±0.0	LRI, MS, std
2.66	1089	Terpinolene	93, 105, 121, 136		0.1g±0.0		0.1f±0.0		0.2e±0.0			LRI, MS
2.68	1091	(*Z*)-Linalool oxide (furanoid)	43, 59, 68, 111						0.2f±0.0		0.4e±0.0	LRI, MS
2.83	1108	Linalool	41, 55, 69, **93**	2093.8d±68.9	38.0h±0.2	2806.8c±71.0	49.0f±0.1	3201.0b±94.5	54.9e±0.1	5042.2a±185.7	41.0g±0.3	LRI, MS, std
2.86	1111	1-Octen-3-yl acetate	43, **54**, 67, 81	19.1a±2.1	0.1f±0.0	11.5b±0.7	0.1f±0.0	13.1b±1.9	0.6e±0.1			LRI, MS, std
2.98	1123	(*E*)-2-Nonenal	41, 55, 70, 83				0.1±0.0					LRI, MS
3.08	1133	allo-Ocimene	91, 105, 121, 136		0.2e±0.0		0.1f±0.0		0.1g±0.0			LRI, MS

Table 6.2. Continued

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Ang1 Concentration [mM ± SD]	Ang1 Area [% ± SD]	Ang2 Concentration [mM ± SD]	Ang2 Area [% ± SD]	Ang3 Concentration [mM ± SD]	Ang3 Area [% ± SD]	Ang4 Concentration [mM ± SD]	Ang4 Area [% ± SD]	Identification methods
3.25	1150	Hexyl isobutyrate	43, 56, 71, 89				0.1 ± 0.0					LRI, MS
3.33	1159	Camphor	41, 81, **95**, 108	25.1d ± 1.0	0.3g ± 0.0	78.0c ± 2.3	0.9e ± 0.0	68.8b ± 3.4	0.8f ± 0.0	97.6a ± 4.4	0.1h ± 0.0	LRI, MS, std
3.46	1172	Lavandulol	41, 69, 111, 123		0.4f ± 0.0		1.0e ± 0.0		0.2h ± 0.0		0.3g ± 0.0	LRI, MS
3.54	1181	1-Butenylidene cyclohexane	79, 93, 107, 136				0.1f ± 0.0		0.1f ± 0.0		0.4e ± 0.0	LRI, MS
3.60	1186	Borneol	41, 93, **95**, 121	63.5c ± 0.7	0.8g ± 0.0	55.4c ± 0.8	0.8g ± 0.0	130.2b ± 3.4	1.7e ± 0.0	234.1a ± 5.3	1.4f ± 0.0	LRI, MS, std
3.65	1191	Terpinen-4-ol	71, 77, 91, **93**	133.5b ± 4.3	2.1f ± 0.1	173.1a ± 0.8	2.7e ± 0.0	30.5d ± 1.7	0.5h ± 0.0	58.1c ± 0.0	0.9g ± 0.0	LRI, MS, std
3.67	1193	Hexyl butyrate	**43**, 56, 71, 89	11.4c ± 0.6	0.2g ± 0.0	20.5b ± 1.7	0.2f ± 0.0	26.3a ± 1.2	0.4e ± 0.0		0.1g ± 0.0	LRI, MS, std
3.71	1198	p-Cymenene	65, 91, 117, 132						0.1 ± 0.0			LRI, MS
3.79	1206	α-Terpineol	59, 68, 79, **93**	62.0c ± 2.8	1.0g ± 0.0	39.2d ± 0.6	0.6h ± 0.0	108.4b ± 2.8	1.7e ± 0.0	175.5a ± 2.2	1.5f ± 0.0	LRI, MS, std
4.03	1231	Perillol	68, 79, 93, 121		0.1f ± 0.0		0.1f ± 0.0		0.3e ± 0.0		0.1g ± 0.0	LRI, MS
4.21	1249	Linalyl acetate	41, 69, **93**, 121	2242.9b ± 61.9	37.0e ± 0.2	1808.9c ± 51.7	29.1g ± 0.0	1434.9d ± 51.2	23.2h ± 0.0	2489.3a ± 78.1	36.0f ± 0.2	LRI, MS, std
4.27	1255	Geraniol	41, 69, 93, 121		0.4g ± 0.0		0.2h ± 0.0		0.9e ± 0.0		0.6f ± 0.0	LRI, MS
4.54	1284	Lavandulyl acetate	43, 69, 93, 121		1.2f ± 0.0		1.8e ± 0.0		0.4g ± 0.0		1.8e ± 0.1	LRI, MS
4.59	1289	Bornyl acetate	79, **93**, 95, 121	5.2b ± 0.7	0.1f ± 0.0			5.1b ± 0.5	0.1g ± 0.0	79.5a ± 0.3	0.3e ± 0.0	LRI, MS, std
4.80	1309	Thymol	91, 115, **135**, 150					45.1 ± 0.8	0.2 ± 0.0			LRI, MS, std
4.88	1317	Carvacrol	91, 117, 135, 150						0.1 ± 0.0			LRI, MS
5.36	1362	Neryl acetate	41, 69, 79, **93**	13.6c ± 0.1	0.3g ± 0.0	9.8d ± 0.1	0.2h ± 0.0	26.4a ± 0.6	0.5f ± 0.0	18.7b ± 0.7	0.6e ± 0.0	LRI, MS, std
5.56	1380	Geranyl acetate	41, **69**, 79, 93	22.0c ± 0.6	0.4g ± 0.0	15.4d ± 0.3	0.3h ± 0.0	47.1b ± 0.3	1.0f ± 0.0	61.2a ± 0.1	1.1e ± 0.0	LRI, MS, std
5.61	1385	Hexyl caproate	56, 84, 99, 117						0.1f ± 0.0		0.2e ± 0.0	LRI, MS
5.96	1419	Perillyl acetate	91, 119, 134, 152		0.1g ± 0.0		0.1f ± 0.0		0.1g ± 0.0		0.2e ± 0.0	LRI, MS
6.03	1427	α-Santalene	41, 94, 161, 189		0.7f ± 0.0		0.1h ± 0.0		0.5g ± 0.0		0.9e ± 0.1	LRI, MS
6.07	1430	(*E*)-β-Caryophyllene	41, 69, 79, **93**	395.3a ± 27.3	3.5f ± 0.0	292.5b ± 28.1	1.9h ± 0.0	402.9a ± 40.4	2.4g ± 0.0	90.5c ± 6.5	4.0e ± 0.1	LRI, MS, std
6.17	1441	(*E*)-α-Bergamotene	41, 69, 93, 119		0.2f ± 0.0				0.1g ± 0.0		0.4e ± 0.0	LRI, MS
6.29	1453	Coumarin	63, 90, 118, 146						0.1f ± 0.0		0.6e ± 0.0	LRI, MS
6.37	1461	(*Z*)-β-Farnesene	41, 69, 93, 133		1.8e ± 0.0		0.4g ± 0.0		1.2f ± 0.0		0.2h ± 0.0	LRI, MS
6.45	1469	α-Humulene	41, 67, 80, **93**	6.3b ± 0.0	0.1e ± 0.0	4.2d ± 0.1	0.1g ± 0.0	5.5c ± 0.3	0.1e ± 0.0	9.7a ± 0.1	0.1f ± 0.0	LRI, MS, std

Table 6.2. Continued

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Ang1 Concentration [mM ± SD]	Ang1 Area [% ± SD]	Ang2 Concentration [mM ± SD]	Ang2 Area [% ± SD]	Ang3 Concentration [mM ± SD]	Ang3 Area [% ± SD]	Ang4 Concentration [mM ± SD]	Ang4 Area [% ± SD]	Identification methods
6.66	1490	γ-Muurolene	105, 119, 161, 204		0.6e ± 0.0		0.1g ± 0.0		0.1g ± 0.0		0.5f ± 0.0	LRI, MS
6.89	1522	δ-Cadinene	134, 161, 189, 204		0.2f ± 0.0						0.3e ± 0.0	LRI, MS
6.93	1529	β-Sesquiphellandrene	69, 93, 161, 204		0.1f ± 0.0				0.1f ± 0.0		0.3e ± 0.0	LRI, MS
7.34	1597	Caryophyllene oxide	41, 79, **91**, 105	15.5a ± 3.1	0.2e ± 0.0	15.0a ± 1.4	0.2f ± 0.0	17.3a ± 2.4	0.3e ± 0.0	7.5b ± 0.1	0.1g ± 0.0	LRI, MS, std
7.61	1657	δ-Cadinol	105, 119, 161, 204		0.1f ± 0.0						0.1e ± 0.0	LRI, MS
		Oxygenated terpenes:										
		Alcohol			42.9		54.7		63.1		46.7	
		Ketone			0.3		1.0		0.9		0.1	
		Aldehyde			0.0		0.0		0.0		0.0	
		Ester			39.0		31.5		25.2		39.8	
		Ether			2.4		3.1		0.4		1.8	
		Monoterpene hydrocarbons			7.2		4.5		3.8		3.3	
		Oxygenated monoterpenes			84.2		90.0		89.3		88.2	
		Sesquiterpene hydrocarbons			7.2		2.5		4.5		6.7	
		Oxygenated sesquiterpenes			0.3		0.2		0.3		0.2	
		Total terpene hydrocarbons			14.4		7.0		8.3		10.0	
		Total oxygenated terpenes			84.5		90.2		89.5		88.5	
		Non isoprenoid components			1.1		2.7		2.2		1.5	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). [a]Quantitation ions are shown in bold. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations.

tr= traces < 0.1%. a, b, c, d Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$.

e, f, g, h Different letters in the same compound area mean statistically significant differences with $p < 0.05$.

Table 6.3. FGC-MS determination of components of *L. latifolia* EOs.

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Lat1 Concentration [mM ± SD]	Lat1 Area [% ± SD]	Lat2 Concentration [mM ± SD]	Lat2 Area [% ± SD]	Lat3 Concentration [mM ± SD]	Lat3 Area [% ± SD]	Lat4 Concentration [mM ± SD]	Lat4 Area [% ± SD]	Identification methods
1.20	874	n-Hexanol	43, 56, 69, 82				tr		0.1±0.0			LRI, MS
1.47	929	Tricyclene	79, 93, 121, 136		0.1e±0.0		0.1e±0.0		0.1e±0.0			LRI, MS
1.53	939	α-Pinene	39, 91, **93**, 77	114.2a±2.0	2.2e±0.0	103.9b±2.5	2.0f±0.0	56.4c±2.1	1.7g±0.1	121.5a±7.1	1.0h±0.0	LRI, MS, std
1.63	957	Camphene	67, 79, **93**, 121	45.6b±0.9	0.7f±0.0	51.0a±1.1	0.7e±0.0	15.6c±0.7	0.7f±0.0	49.8a,b±2.6	0.3g±0.0	LRI, MS, std
1.64	959	Umbellulol	65, 91, 119, 134								0.1±0.0	LRI, MS
1.75	978	Sabinene	41, 77, 91, **93**	20.4c±0.5	0.4g±0.0	29.5b±0.2	0.5f±0.0	46.9a±0.4	0.6e±0.0	45.9a±3.5	0.3g±0.0	LRI, MS, std
1.79	985	β-Pinene	41, 69, 79, **93**	129.2b±2.0	2.3e±0.0	120.8b±2.4	2.2f±0.0	81.0c±0.6	0.1h±0.0	144.1a±7.6	1.1g±0.0	LRI, MS, std
1.83	992	Myrcene	39, **41**, 69, 93	22.2c±1.3	0.2g±0.0	30.2b±1.7	0.3f±0.0	21.1c±0.3	1.8e±0.0	48.2a±2.1	0.2h±0.0	LRI, MS, std
1.86	997	(*E*)-2-Caren-4-ol	41, 79, 91, 109				tr		0.5e±0.0		0.1f±0.0	LRI, MS
2.04	1019	2-Carene	77, 93, 121, 136				0.1f±0.0		0.2e±0.0		0.1f±0.0	LRI, MS
2.12	1028	p-Cymene	77, 91, **119**, 134	11.5b±0.2	0.2g±0.0	17.7a±0.2	0.4e±0.0	11.1b±0.3	0.2h±0.0	7.4c±0.6	0.3f±0.0	LRI, MS, std
2.16	1032	Limonene	67, **68**, 79, 93	77.3a±2.8	0.8f±0.0	68.3a,b±6.0	0.6g±0.0	32.2c±1.3	1.3e±0.0	62.9b±1.1	0.8f±0.0	LRI, MS, std
2.18	1035	Eucalyptol	**43**, 67, 81, 93	1463.8a±65.9	31.6e±0.0	1547.1a±41.4	31.9e±0.1	906.2c±3.2	27.5f±0.4	1105.4b±47.0	24.5g±0.2	LRI, MS, std
2.26	1044	(*E*)-β-Ocimene	41, 79, 93, 121						0.1±0.0			LRI, MS
2.40	1060	γ-Terpinene	77, 91, **93**, 136			9.1b±0.2	0.1f±0.0	0.6c±0.0	0.3e±0.0	14.7a±1.0	0.1f±0.0	LRI, MS, std
2.55	1077	(*trans*)-Sabinene hydrate	77, 91, **93**, 121	15.5c±0.4	0.3f±0.0	13.8c±0.1	0.3f±0.0	38.6a±0.5	0.2g±0.0	26.8b±2.3	0.4e±0.0	LRI, MS, std
2.66	1089	Terpinolene	93, 105, 121, 136		0.1h±0.0		0.2f±0.0		0.3e±0.0		0.1g±0.0	LRI, MS
2.68	1091	2,6-Nonadien-1-ol	41, 54, 69, 79		0.1e±0.0		0.1f±0.0					LRI, MS
2.83	1108	Linalool	41, 55, 69, **93**	2517.4c±76.2	42.4g±0.0	2128.5d±4.0	35.0h±0.0	6090.8a±8.2	43.7f±0.1	5678.2b±159.6	50.8e±0.1	LRI, MS, std
3.10	1135	6-Camphenol	41, 77, 93, 108				0.1f±0.0		0.1e±0.0			LRI, MS
3.26	1152	Hexyl isobutyrate	43, 56, 71, 89		tr		0.1g±0.0		0.2f±0.0		0.3e±0.0	LRI, MS
3.33	1159	Camphor	41, 81, **95**, 108	874.9d±12.3	10.6g±0.1	1209.2b±15.5	17.8e±0.1	1037.5c±16.2	11.4f±0.3	1311.3a±55.5	10.1g±0.1	LRI, MS, std
3.46	1172	Lavandulol	41, 69, 111, 123				0.2g±0.0		0.4e±0.0		0.3f±0.0	LRI, MS
3.49	1175	Bicyclo[4.1.0]heptane, 7-(1-methylethylidene)-	67, 79, 93, 121		0.1±0.0							LRI, MS
3.54	1180	Myrcenol	59, 68, 79, 93		0.3h±0.0		0.4g±0.0		0.7e±0.0		0.6f±0.0	LRI, MS
3.60	1186	Borneol	41, 93, **95**, 121	93.4d±0.3	1.1f±0.0	105.2c±0.8	1.2e±0.0	124.5b±3.2	0.3g±0.0	141.2a±4.3	0.3g±0.0	LRI, MS, std
3.63	1189	Terpinen-4-ol	71, 77, 91, **93**	17.4b±1.3	0.3f±0.0	29.1a±2.2	0.4e±0.0	27.9a±0.9	0.1g±0.0	28.1a±0.3	0.3f±0.0	LRI, MS, std

Table 6.3. Continued

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Lat1 Concentration [mM ± SD]	Lat1 Area [% ± SD]	Lat2 Concentration [mM ± SD]	Lat2 Area [% ± SD]	Lat3 Concentration [mM ± SD]	Lat3 Area [% ± SD]	Lat4 Concentration [mM ± SD]	Lat4 Area [% ± SD]	Identification methods
3.67	1193	Hexyl butyrate	**43**, 56, 71, 89			11.6 ± 0.2	0.1±0.0					LRI, MS, std
3.71	1198	6-Isopropenyl-5-methylene-3-cyclohexen-1-ol	91, 107, 117, 135		0.1f±0.0		0.1e±0.0					LRI, MS
3.72	1199	Hotrienol	71, 82, 119, 134		0.1f±0.0		0.1e±0.0					LRI, MS
3.79	1206	α-Terpineol	59, 68, 79, **93**	101.1b ± 1.0	1.6e±0.0	82.5c ± 3.1	1.3f±0.0	129.4a ± 0.1	1.0g±0.0	134.2a ± 2.3	1.0g±0.1	LRI, MS, std
3.81	1208	Bicyclo[3.1.0]hexane, 6-isopropylidene-1-methyl-	79, 93, 121, 136		0.1e±0.0		tr		0.1f±0.0			LRI, MS
4.02	1230	Pseudolimonene	69, 79, 93, 136		0.1e±0.0				0.1e±0.0			LRI, MS
4.06	1234	Hexyl 2-methylbutyrate	41, 57, 85, 103		0.1g±0.0		0.1e±0.0		0.1f±0.0			LRI, MS
4.11	1239	Hexyl valerate	43, 56, 85, 103				0.1e±0.0		0.1e±0.0			LRI, MS
4.21	1249	Linalyl acetate	41, 69, **93**, 121	38.1a ± 1.4	0.6e±0.0	4.4d ± 0.1	tr	29.3b ± 0.0	0.2f±0.0	18.4c ± 1.0	0.1f±0.0	LRI, MS, std
4.59	1289	Bornyl acetate	43, **95**, 121, 136	15.0a ± 0.4	0.3f±0.0			5.1c ± 0.1	0.1g±0.0	11.3b ± 0.1	0.4e±0.0	LRI, MS, std
5.57	1381	γ-Elemene	41, 93, 107, 121		0.1f±0.0				0.1e±0.0			LRI, MS
5.63	1387	β-Bourbonene	81, 123, 161, 189		0.1f±0.0		0.1f±0.0		0.2e±0.0		0.2e±0.0	LRI, MS
5.82	1405	α-Farnesene	41, 55, 69, 93		0.1f±0.0		0.1e±0.0		0.1e,f±0.0			LRI, MS
5.91	1414	Cyperene	119, 161, 189, 204		tr		0.1f±0.0		0.1e±0.0			LRI, MS
6.07	1430	(*E*)-β-Caryophyllene	41, 69, 79, **93**	78.4b ± 1.5	0.9h±0.0	86.6a ± 1.3	1.0g±0.0	36.5d ± 0.3	1.7e±0.1	41.2c ± 0.9	1.6f±0.0	LRI, MS, std
6.17	1441	α-Bergamotene	41, 69, 93, 119		0.1f±0.0		0.1f±0.0		0.2e±0.0		0.2e±0.0	LRI, MS
6.37	1461	β-Farnesene	41, 69, 93, 133		0.1g±0.0		0.2f±0.0		0.3e±0.0		0.1h±0.0	LRI, MS
6.45	1469	α-Humulene	41, 67, 80, **93**		0.1g±0.0	4.5b ± 0.2	0.1g±0.0	7.6a ± 0.1	0.6e±0.0	7.6a ± 0.2	0.2f±0.0	LRI, MS, std
6.57	1481	Copaene	105, 119, 161, 189								0.2±0.0	LRI, MS
6.66	1490	Germacrene D	105, 119, 161, 204		0.2f±0.0		0.2g±0.0		0.1h±0.0		0.4e±0.0	LRI, MS
6.78	1504	α-Selinene	161, 175, 189, 204		0.1f±0.0		0.1f±0.0				0.3e±0.0	LRI, MS
6.80	1507	(*Z*)-α-Bisabolene	41, 79, 93, 161		0.1g±0.0		0.1g±0.0		0.1f±0.0		0.3e±0.0	LRI, MS
6.84	1514	β-Bisabolene	41, 69, 93, 204		0.1h±0.0		0.3e±0.0		0.2g±0.0		0.3f±0.0	LRI, MS
6.89	1522	β-Cubebene	105, 120, 161, 204		0.1f±0.0		0.2e±0.0		0.2e±0.0			LRI, MS

Table 6.3. Continued

t_R [min]	LRI	Analyte	Qualifying and quantitation ions[a] [m/z]	Lat1 Concentration [mM ± SD]	Lat1 Area [% ± SD]	Lat2 Concentration [mM ± SD]	Lat2 Area [% ± SD]	Lat3 Concentration [mM ± SD]	Lat3 Area [% ± SD]	Lat4 Concentration [mM ± SD]	Lat4 Area [% ± SD]	Identification methods
6.92	1527	δ-Cadinene	134, 161, 189, 204				0.1 ± 0.0					LRI, MS
7.06	1549	(*E*)-α-Bisabolene	41, 79, **93**, 119	155.6c ± 0.9	1.2g ± 0.0	91.8d ± 1.3	0.7h ± 0.0	383.3a ± 4.7	1.9f ± 0.0	185.8b ± 1.4	2.4e ± 0.0	LRI, MS, std
7.34	1597	Caryophyllene oxide	41, 79, **91**, 105	15.5b ± 0.6	0.2f ± 0.0	16.8b ± 0.8	0.2f ± 0.0	28.4a ± 0.1	0.1g ± 0.0	10.6c ± 0.1	0.3e ± 0.0	LRI, MS, std
7.61	1658	α-Amorphene	94, 105, 161, 204		0.1f ± 0.0		0.1f ± 0.0		0.1f ± 0.0		0.2e ± 0.0	LRI, MS
		Oxygenated terpenes:										
		Alcohol			45.9		39		53.7		46.9	
		Ketone			10.5		17.7		10.3		11.2	
		Aldehyde			0		0		0		0	
		Ester			0.9		0.04		0.45		0.34	
		Ether			31.6		31.9		24.6		28.2	
		Monoterpene hydrocarbons			7.68		7.65		4.4		7.73	
		Oxygenated monoterpenes			88.6		88.4		88.7		86.5	
		Sesquiterpene hydrocarbons			3.46		3.21		6.06		5.64	
		Oxygenated sesquiterpenes			0.2		0.19		0.33		0.13	
		Total terpene hydrocarbons			11.1		10.9		10.5		13.4	
		Total oxygenated terpenes			88.8		88.6		89.1		86.6	
		Non isoprenoid components			0.24		0.54		0.47		0	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). [a]Quantitation ions are shown in bold. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. tr = traces < 0.1%. a, b, c, d Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$.

e, f, g, h Different letters in the same compound area mean statistically significant differences with $p < 0.05$.

The samples coming from a common bioclimatic zone (Ang1 and Ang4) are highly similar and form a cluster because of its similarity (78.88%), whereas Ang2 and Ang3 form a cluster with lower similarity (55.54%) and both clusters have a similarity of 49.49% between them. In the case of *L. latifolia* Lat1 and Lat3 are the most similar samples (70.94%), followed by samples Lat4 (57.63%) and Lat2 (45.95%). Comparing all the species together, they form two differenced clusters, one for *L. angustifolia* and other for *L. latifolia*, having a similarity between clusters of 30.75%.

As the composition results show, both species have high concentration of linalool and moderate concentration of α-terpineol and (*E*)-β-caryophyllene. However, *L. angustifolia* (Table 6.2) has high concentration of linalyl acetate, thus showing a more delicate scent, useful for application in perfumes, whereas *L. latifolia* (Table 6.3) has higher concentrations of eucalyptol and camphor, hence showing an intense aroma, which can be used for masking odours. Furthermore, (*Z*)-β-ocimene, (*E*)-β-ocimene, terpinen-4-ol, lavandulyl acetate and (*Z*)-β-farnesene are found in moderate concentrations in *L. angustifolia* whereas α- and β-pinene, borneol and (*E*)-α-bisabolene are found in moderate concentrations in *L. latifolia*.

Oxygenated monoterpenes are highly predominant in the eight samples accounting for more than 85%, in average, of the total molecules. Alcohol is the most abundant organic functional group, exceeding 40% of total molecules. In the case of *L. angustifolia,* the second organic functional group in abundance is ester, accounting for more than 25% in average. In the case of *L. latifolia,* the second organic functional group in abundance is ether (more than 25%) followed by ketone (more than 10%).

Total terpene hydrocarbons are calculated as the sum of the monoterpene and sesquiterpene hydrocarbons. Total oxygenated terpenes are calculated as the sum of the oxygenated monoterpenes and sesquiterpenes.

Several biomolecules show peak area percentages similar to those reported in literature for plant material of *L. angustifolia* from different countries. Linalool as a main biomolecule shows a similar concentration to samples from China (Cong et al., 2008) and another region of Spain (Santana et al., 2012), most volatile biomolecules i.e. tricyclene, α-pinene, camphene, sabinene or myrcene show similarities to the Australian (Danh et al., 2013) and Indian (Verma et al., 2010) species. Biomolecules like *cis*- and *trans*-β-ocimene or β-caryophyllene are determined in higher concentrations in Spanish samples than in any other location. Linalyl acetate is a biomolecule of relevance, contributing to the pleasant aroma of lavender. This biomolecule is found in high relative concentration in the samples studied in the Indian lavender (Verma et al., 2010) and here. At the same time, the lowest concentrations of camphor and borneol were shown in these lavenders, thus providing a soft floral scent.

The concentration of eucalyptol in *L. latifolia* is lower than the characterizations found from Vitoria (Guillen et al., 1996), Jaén (Salido et al., 2004) and Valencia (Munoz-Bertomeu et al., 2007). The concentrations of camphene, limonene, camphor, borneol and terpinen-4-ol are below the average of Spanish wild species (Herraiz-Peñalver et al., 2013) while linalool and α-terpineol are above it, finding the highest linalool relative concentration in the studied literature.

The International Organization for Standardization (ISO) has published International Standards for *L. angustifolia* (ISO 3515:2002) and *L. latifolia* (ISO 4719:2012) EOs. The results shown in Table 6.4 are obtained taking ISO Standards for comparison. Some constituents from the Spanish EOs exceed the maximum relative concentration allowed.

Table 6.4
GC/MS determination of *L. angustifolia* and *L. latifolia* EOs, ISO normative

Area [%] / Component	Normative*		Ang1	Ang2	Ang3	Ang4	Normative†		Lat1	Lat2	Lat3	Lat4
	Min	Max					Min	Max				
3-Octanone	-	3.0	0.0	1.4	0.1	0.2	-	-				
β-Phellandrene	-	1.0	0.0	0.0	0.0	0.0	-	-				
Limonene	-	1.0	0.6	0.2	0.4	0.3	0.5	3.0	0.9	0.7	1.5	0.9
(*Z*)-β-Ocimene	1.0	10.0	3.3	1.3	0.8	1.0	-	-				
Eucalyptol	-	3.0	2.4	3.1	0.2	2.0	16.0	39.0	35.6	36.5	31.6	27.3
(*E*)-β-Ocimene	0.5	6.0	2.1	2.3	0.9	1.9	-	-				
Linalool	20.0	43.0	43.5	52.7	65.6	47.8	34.0	50.0	47.7	40.1	50.2	56.6
Camphor	-	1.5	0.3	1.0	1.0	0.2	8.0	16.0	11.9	20.4	13.1	11.3
Lavandulol	-	3.0	0.5	1.0	0.2	0.4	-	-				
Terpinen-4-ol	-	8.0	2.4	2.9	0.5	1.0	-	-				
α-Terpineol	-	2.0	1.1	0.6	2.0	1.8	0.2	2.0	1.8	1.5	1.1	1.1
Linalyl acetate	25.0	47.0	42.4	31.3	27.7	41.6	tr	1.6	0.7	tr	0.2	0.1
Lavandulyl acetate	-	8.0	1.4	2.0	0.4	2.0	-	-				
(*E*)-α-Bisabolene	-	-					0.4	2.5	1.4	0.8	2.2	2.7

*Normative for *Lavandula angustifolia* EOs (ISO 3515:2002)
†Normative for *Lavandula latifolia* EOs (ISO 4719:2012)

In the case of Lat2, camphor is found beyond the limits. The case of linalool is especially interesting, being found exceeding the limits in Ang2, Ang3, Ang4 and Lat4, thus the aroma is even more intense than the international regular case. Due to the differences in composition of EOs from the different countries, it is advisable to study the different EO compositions of each location to determine the optimal usages. Also, the impact of seasonal changes is a general limitation of studies with botanicals, which should be taken into account.

Enantioselective Gas Chromatography (EsGC) provided enantiomeric determinations of molecules of EOs from *L. angustifolia* and *L. latifolia* (Table 6.5). There are no adulterations with synthetic racemates of the main molecules, such as linalool, linalyl acetate and camphor as the enantiomeric ratios obtained are far away from the racemate obtained synthetically. The (+)-enantiomer predominates in the case of: α-pinene, β-pinene, limonene, sabinene hydrate, camphor, bornyl acetate, terpinen-4-ol, α-terpineol and borneol; while the (–)-enantiomer predominates in: camphene, linalool, linalyl acetate, (*E*)-β-caryophyllene and caryophyllene oxide.

There are several molecules that could be used as markers of the different origins: α-pinene for Lat3 and Lat4; camphene for Lat1, Lat2 and Lat3; Limonene and bornyl acetate for Ang1; α-terpineol for Ang2 and Ang3; and borneol for Ang2 and Ang4.

The high proportions of (R)-(–)-linalool and (R)-(–)-linalyl acetate in EOs grown in Spain (Table 6.5), are similar to those reported for the EOs, of *Lavandula angustifolia* worldwide (Renaud et al., 2001). (1R, 5R)-(+)-α-Pinene, (R)-(+)-camphor, (R)-(+)-borneol, (S)-(+)-terpinen-4-ol and (R)-(+)-α-terpineol have also been reported for *Lavandula angustifolia* from Italy with the same predominant enantiomer as shown in this study (Bicchi et al., 2010; Bicchi et al., 2008).

Table 6.5. EsGC/MS determination of components of *L. angustifolia* and *L. latifolia* EOs.

t_R [min]		Area [%]	Ang1		Ang2		Ang3		Ang4		Lat1		Lat2		Lat3		Lat4	
(+) - X	(–) - X	Analyte (X)	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]	(+) - [X]	(–) - [X]
7.79	7.52	α-Pinene	95	5	95	5	95	5	95	5	95	5	95	5	66*	34*	67*	33*
8.47	8.24	Camphene	5	95	5	95	5	95	5	95	29*	71*	39*	61*	44*	56*	5	95
8.89	9.16	β-Pinene	N/D	N/D	N/D	N/D	95	5	95	5	34	66	63	37	58	42	57	43
10.52	10.00	Limonene	75*	25*	99	1	95	5	95	5	95	5	95	5	95	5	95	5
14.28	14.51	(*trans*)-Sabinene hydrate	-	-	-	-	-	-	-	-	95	5	95	5	95	5	95	5
15.73	15.57	Linalool	5	95	5	95	5	95	2	98	5	95	5	95	5	95	5	95
16.72	16.46	Camphor	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5
-	17.25	Linalyl acetate	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95
18.02	18.18	Bornyl acetate	55*	45*	N/D	N/D	95	5	95	5	95	5	N/D	N/D	95	5	95	5
18.32	18.51	Terpinen-4-ol	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5
20.10	19.76	α-Terpineol	64	36	94*	6*	95*	5*	72	28	56	44	63	37	62	38	62	38
20.15	19.58	Borneol	53	47	95*	5*	74	26	95*	5*	52	48	77	23	83	17	85	15
23.92	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95
-	28.81	Caryophyllene oxide	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95

*Biomolecular marker of the EO

Furthermore, (S)-(–)-camphene, (R)-(+)-limonene, (1R, 9S)-(–)-(*E*)-β-caryophyllene and (1R, 4R, 6R, 10S)-(–)-caryophyllene oxide were found in this study as predominant enantiomers in Spanish *Lavandula angustifolia*. This data could be useful to assess the origin and the authenticity of the EOs. To our knowledge, this is the first chiral wide characterization of the EOs from *L. angustifolia* and *L. latifolia* grown in Spain.

Table 6.6
Antioxidant capacity of *L. angustifolia* and *L. latifolia* EOs

Sample	ORAC [µmol TE/µL EO]	ABTS [µmol TE/mL EO]	DPPH [µmol TE/mL EO]	ChP [mg EDTAE/mL EO]	RdP [mg AAE/mL EO]
Ang1	1.28b,c ± 0.09	1.3c,d ± 0.1	0.45c ± 0.02	0.1d ± 0.0	0.47d ± 0.03
Ang2	1.39b ± 0.09	0.6e ± 0.1	0.90a ± 0.04	1.7b ± 0.1	0.62c ± 0.04
Ang3	1.65a ± 0.07	1.2d ± 0.1	0.66b ± 0.02	0.4c,d ± 0.0	0.41d ± 0.03
Ang4	1.43b ± 0.07	1.5c ± 0.1	0.32d ± 0.01	2.6a ± 0.2	0.47d ± 0.02
Lat1	1.18c ± 0.07	2.0b ± 0.0	0.35d ± 0.01	2.5a ± 0.2	0.74b ± 0.03
Lat2	1.10c ± 0.05	4.2a ± 0.1	0.22e ± 0.02	1.7b ± 0.2	0.72b,c ± 0.04
Lat3	1.26b,c ± 0.06	0.7e ± 0.1	0.63b ± 0.04	2.3a ± 0.1	1.11a ± 0.05
Lat4	1.25b,c ± 0.07	2.0b ± 0.1	0.61b ± 0.03	0.6c ± 0.0	0.76b ± 0.04

TE = Trolox equivalent, EDTAE = EDTA equivalent, AAE = Ascorbic acid equivalent. a, b, c, d, e Different letters in the same antioxidant method mean statistically significant differences with $p < 0.05$

The ORAC antioxidant activity of the eight samples is expressed in TEAC units (µmol TE/µL EO) and resulted (Table 6.6) as follows:

$Ang3^{ORAC} > Ang4^{ORAC} \approx Ang2^{ORAC} \geq Ang1^{ORAC} \approx Lat3^{ORAC} \approx Lat4^{ORAC} \geq Lat1^{ORAC} \approx Lat2^{ORAC}$

The antioxidant activity of each EO is related to its composition and the intrinsic antioxidant activity of each of the compounds. Generally, the high concentration of oxygenated terpenes is correlated to high ORAC antioxidant activity. A trend is shown, the ORAC value decreases with increasing ether monoterpenoid concentration (Tables 6.2, 6.3 and 6.6). Two oxygenated components may highly relevant to explain the ORAC value of the EO, namely linalool and linalyl acetate, the first and the most important molecule because of its high concentration and high ORAC value and the other for its moderate ORAC value and high concentration, according to the literature (Bentayeb et al., 2014).

The ABTS antioxidant activity is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 6.6) as follows:

$Lat2^{ABTS} > Lat1^{ABTS} \approx Lat4^{ABTS} > Ang4^{ABTS} \geq Ang1^{ABTS} \geq Ang3^{ABTS} > Lat3^{ABTS} \approx Ang2^{ABTS}$

Linalool and limonene are reported to have a moderate impact in ABTS method (Stobiecka et al., 2014; Yang et al., 2010). However the addition of these two compounds is not enough to explain the ABTS results. The whole mixture of the EO should be considered in any case as the cause of the final ABTS value.

The DDPH antioxidant activity is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 6.6) as follows:

$Ang2^{DPPH} > Ang3^{DPPH} \approx Lat3^{DPPH} \approx Lat4^{DPPH} > Ang1^{DPPH} > Lat1^{DPPH} \approx Ang4^{DPPH} > Lat2^{DPPH}$

Very similar values of DPPH were obtained, but different molecules, present in high quantity in Ang2 and in low quantity in Lat2 may explain the better performance in DPPH antioxidant assay (Dawidowicz and Olszowy, 2014). In this case, the molecules that are markedly different between the samples in the extremes of the antioxidant

comparative are: (*E*)-β-ocimene, lavandulol, terpinen-4-ol and lavandulyl acetate (Tables 6.2 and 6.3).

ChP activity is expressed in EDTA units (mg EDTA equivalents / mL EO) and resulted (Table 6.6) as follows:

$Ang4^{ChP} \approx Lat1^{ChP} \approx Lat3^{ChP} > Ang2^{ChP} \approx Lat2^{ChP} > Lat4^{ChP} \geq Ang3^{ChP} \geq Ang1^{ChP}$

The highest concentrations of linalyl acetate and lavandulyl acetate together (Tables 6.2 and 6.3) were found in Ang4, and the lowest concentration of camphor was found in Ang1. The high electronic densities of the oxygen atoms of the carboxylic ester and ketone groups could be useful for complexation of cations.

RdP antioxidant activity is expressed in ascorbic acid units (mg Ascorbic acid equivalents/mL EO) and resulted (Table 6.6) as follows:

$Lat3^{RdP} > Lat4^{RdP} \approx Lat1^{RdP} \geq Lat2^{RdP} \geq Ang2^{RdP} > Ang1^{RdP} \approx Ang4^{RdP} \approx Ang3^{RdP}$

Some abundant terpene alcohol molecules (Tables 6.2 and 6.3) might show a mild reducing power, as the already reported linalool (Liu et al., 2012).

The results of the LOX inhibitory activity are shown below. Inhibition degree (%) at 0.3 µL(EO)/mL was measured for $Lat4^{LOX}(38.0 \pm 0.4)^{a} \geq Lat3^{LOX}(32.9 \pm 0.4)^{a,b} \geq Ang2^{LOX}(32.5 \pm 4.3)^{a,b,c} \geq Lat1^{LOX}(32.1 \pm 0.5)^{b,c} \approx Ang3^{LOX}(31.6 \pm 2.9)^{b,c} \geq Ang1^{LOX}(27.1 \pm 0.3)^{b,c,d} \geq Ang4^{LOX}(26.7 \pm 0.5)^{c,d} \geq Lat2^{LOX}(23.6 \pm 2.3)^{d}$. Tukey's HSD test revealed significant differences between the samples (different superscripts).

For a deeper understanding of the LOX inhibitory activity of the EO, the main commercially available compounds were analysed, obtaining following IC_{50} (µM) value: limonene (356 ± 30), bornyl acetate (380 ± 14), p-cymene (486 ± 32), camphor (2743 ± 85), 1-octen-3-ol (1505 ± 23) and linalool (3346 ± 44). NDGA was used as reference compound showing IC_{50} (339 ± 9) µM, this result agrees with already reported values (Kurihara et al., 2014).

In this case, the inhibitory activities of the *L. angustifolia* and *L. latifolia* EOs are clearly due to a combination of compounds with moderate to high inhibitory activity and high concentration, namely linalool, camphor, p-cymene and limonene (Tables 6.2 and 6.3).

Important bioactivities, apart from the ones stated in this study, have been reported in the literature for *L. angustifolia* and *L. latifolia* EOs. Antimicrobial and cytotoxic properties were demonstrated in 2014 (Nikolic et al., 2014) and cardioprotective effects on myocardial infarction in 2015, the last being related to antioxidant activity (Ziaee et al., 2015).

6.5 Conclusions

Attending to the results of this study, several molecules are acting as biomolecular markers of the growing conditions. In this case linalool, 1,8-cineole and camphor are the main constituents of spike lavender essential oil and linalool and linalyl acetate are the most abundant components in lavender essential oil. Furthermore, the studied samples show high concentrations of linalool (exceeding ISO normative) and linalyl acetate, which is beneficial for the potential uses of these essential oils in flavours, fragrances and antioxidants.

Enantiomers (–)-linalool and (–)-linalyl acetate as well as (+)-camphor have been found abundant and common in lavender and spike lavender essential oils worldwide, whereas the predominance of (+)-limonene, (–)-camphene, (–)-*E*-β-

caryophyllene and (–)-caryophyllene oxide has been found as a typical feature of the Spanish lavender and spike lavender essential oils.

Global antioxidant capacity evaluation shows Lat2 as the sample with the highest antioxidant capacity, also Lat1 and Lat3 have high global values of antioxidant capacity, but below the results of Lat2. Linalool and linalyl acetate have been found specially effective in the different methods. Thus, lavender and spike lavender essential oils show a high potential to be used as antioxidants.

6.6 References

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7. CARACTERIZACIÓN DE LOS ACEITES ESENCIALES DE *THYMUS ZYGIS* Y *THYMUS HYEMALIS*

7.1 Resumen

Se han determinado las composiciones de los aceites esenciales de tomillo rojo (*Thymus zygis* Loefl. ex L.) y tomillo de invierno (*Thymus hyemalis* Lange), cultivados y extraídos en el sureste de España, usando cromatografía de gases acoplado a espectrometría de masas. Se obtuvieron las concentraciones relativas (área de pico) y absolutas (curvas de calibrado) de los componentes, siendo los más abundantes thymol (30-54%), p-cymene (14-27%) y γ-terpinene (8-28%) en *T. zygis* y 1,8-cineole (3-37%), p-cymene (1-29%), linalool (8-13%) y thymol (0-19%) en *T. hyemalis*. Estas caracterizaciones se completaron con una cromatografía enantioselectiva, donde se determinaron como principales enantiómeros (-)-linalool, (-)-borneol and (+)-limonene. Los aceites esenciales caracterizados probaron su capacidad antioxidante frente a radicales libres, capacidad reductora y quelatante principalmente por su contenido en thymol y linalool. La actividad inhibidora de lipoxigenasa observada indica una posible actividad antiinflamatoria por la presencia de thymol, p-cymene y linalool. Estos resultados apoyan el posible uso de los aceites esenciales de las especies del género Thymus como ingredientes de cosméticos y fármacos naturales.

7.2 Summary

Compositions of red thyme (*Thymus zygis* Loefl. ex L.) and winter thyme (*Thymus hyemalis* Lange) essential oils (EOs), cultivated and extracted in the South East region of Spain, were determined by gas chromatography coupled with mass spectrometry (GC-MS) detection, obtaining both relative (peak area) and absolute (using standard curves) concentrations. Thymol (30-54%), p-cymene (14-27%) and γ-terpinene (8-28%) were the most abundant components for *T. zygis*. 1,8-Cineole (3-37%), p-cymene (1-29%), linalool (8-13%) and thymol (0-19%) were the most abundant components for *T. hyemalis*. This characterization was completed with an enantioselective gas chromatography, the main molecules determined were (-)-linalool, (-)-borneol and (+)-limonene. The characterized EOs were successfully tested for antioxidant activity against free radicals, chelating and reducing power, mainly due to thymol and linalool. Inhibitory activity on lipoxygenase (LOX) was observed indicating possible anti-inflammatory activity, mainly due to thymol, p-cymene and linalool. These results support the potential use of *Thymus* sp. EOs as natural cosmetic and natural pharmaceutical ingredients.

7.3 Introduction

The genus *Thymus*, predominantly found in the Mediterranean region, Asia, Southern Europe and North Africa, is one of the eight most important genera regarding the number of species included: more than three hundred, including hybrids, varieties and ecotypes (Figueiredo et al., 2008; Morales, 2002). There are several ecotypes of thyme, which differ in their morphological characteristics and in the composition of their essential oils (EOs), although all are characterized by a moderate odor and some-times a very pronounced balsamic and spicy flavor (Ballester-Costa et al., 2013).

Thymus zygis, also known as red thyme, is a widespread endemic plant in the Iberian Peninsula. At least, eight chemotypes have been found in this region (Saez, 1995b). Seasonal variations and phenological stages are also factors which contribute to the chemical variability of *T. zygis* EOs (Moldao-Martins et al., 1999; Perez-Sanchez et al., 2012).

Thymus hyemalis, winter thyme, can be found mainly in the South East of Spain (Alicante, Murcia and Almeria) (Jordan et al., 2006). Chemical variability has been reported for the essential oils of this species, the presence of at least 4 chemotypes in that region of Spain was stated beyond the seasonal variations and edaphoclimatic conditions (Goodner et al., 2006; Jordan et al., 2006; Martinez et al., 2005; Saez, 1995a; Sáez, 1998).

The demand for EOs from *Thymus* species is increasing for perfumery, cosmetic and medicinal uses (Hazzit et al., 2009). Additionally, thyme EO is among the world's top 10 EOs also used as a preservative for food purposes (Ehivet et al., 2011).

Generally, the composition of EOs is complex and it can vary from just a dozen to hundreds of ingredients per oil, depending on the source plant species. Usually, the main biomolecules of these mixtures are phenylpropanoids, volatile monoterpenoids and sesquiterpenoids (10 or 15 carbon skeleton, respectively), containing a wide range of organic functions like alcohols, ethers, aldehydes, ketones or esters for each species. The evaluation of the authenticity of EOs is the key to guarantee the safety and traceability of the industrial products made up of them (Bauermann et al., 2008). EOs can be adulterated by replacing natural enantiomers with inexpensive synthetic racemates, which have different bioactivities and organoleptic properties from those of true EOs, leading to man-made EOs that are not suitable to be certified as natural products.

Gas chromatography, coupled to mass spectrometry (GC/MS) or flame ionization detection, is a very powerful technique used for the analysis of volatile components, since it provides qualitative and quantitative data for complex mixtures such as those usually present in natural products (Smelcerovic et al., 2013). Although flame ionization detection was traditionally used due to its wider linear range of instrumental response, nowadays MS detection has reached high popularity thanks to both its suitable range of instrumental response and the valuable mass spectra provided, comparable with those of standards and spectral libraries. Some relative quantitations of *T. zygis* and *T. hyemalis* compositions have been reported (Dandlen et al., 2010; Tepe et al., 2011). None of them has studied the specific conditions of the Spanish Mediterranean coast, specifically of the Region of Murcia, where the biggest aromatic plant diversity among all regions of Spain is found.

Furthermore, there are few chiral studies on EOs of *Thymus* sp., all of them accounting for some specific biomolecules. Nevertheless, chiral distribution is an important aspect of the EO composition which allows identification of natural EOs from those adulterated with racemic mixtures of synthetic compounds, even when the samples come from different places of the Earth. Chirality is highly important due to the different bioactivities and organoleptic properties of each enantiomer. Thus, it is usually regarded in the fields of drugs, fragrances and flavors, among others (del Castillo et al., 2004).

Generally, EOs obtained from aromatic plants show potential bioactive properties. The tested properties support its historical use in traditional treatments, aromatherapy, flavoring and perfumes. Thus, EOs are nowadays being studied and used in the chemical, cosmetic, food, fragrance and pharmaceutical industries (Bentayeb et al., 2014; Raut and Karuppayil, 2014).

Antimicrobial, antigiardial, antiviral, anti-enzymatic and antioxidant activities of *T. zygis* EOs have been reported (Albano et al., 2012; Ballester-Costa et al., 2013; Blanco Salas et al., 2012; Dandlen et al., 2010; Dandlen et al., 2011a; Dandlen et al., 2011b; Gonçalves et al., 2010; Machado et al., 2010; Perez-Sanchez et al., 2012; Pina-Vaz et al., 2004; Tepe et al., 2011; Youdim et al., 2002). Also, antimicrobial activity of *T. hyemalis* EO was found in 2008 (Rota et al., 2008), thymol chemotype being more active than carvacrol chemotype. Antimicrobial activity along with antioxidant activity of winter thyme EO were also reported (Tepe et al., 2011).

Antioxidant assays serve as models for preliminary observations in the evaluation of pharmacological bioactivities. The antioxidant potential of EOs can be determined using a representative selection of different antioxidant methods, *i.e.*, Oxygen radical absorbance capacity (ORAC) or thiobarbituric acid reactive substances (TBARS) methods to evaluate the scavenging capacity of free oxygen (peroxyl) radicals, ABTS or DPPH methods to measure the ability of reducing nitrogen radical cations, Fe^{2+} chelating power (ChP) and Fe^{3+} reducing power (RdP) methods, to account for the ability to neutralize oxidant metal ions (Dawidowicz and Olszowy, 2014).

Healthy applications of EOs have also been related to their anti-inflammatory properties (Rubio et al., 2013). Many inflammatory processes are associated with leukotriene production catalysed by lipoxygenase (LOX), which can use molecular oxygen or hydrogen peroxide as oxidants (Anwar et al., 2014). Thus, the inhibition of soybean lipoxygenase as LOX model is a hint of anti-inflammatory activity of the EO.

The aim of the present study is to determine the relative, absolute and chiral distribution of each of the EO main components in 8 samples grown in Murcia (Spain). Then, the above mentioned methods (ORAC, TBARS, ABTS, DPPH, ChP, RdP) will be applied to evaluate the antioxidant capacities of *T. zygis* and *T. hyemalis* EOs. Furthermore, the inhibition of LOX, expressed as IC_{50} values, will serve to characterize the inhibitory activity of the EOs, in an attempt to enhance and broaden the potential biotechnological applications of the EOs.

7.4 Results and Discussion

7.4.1 FGC/MS study

Samples Tzt1, Tzt2, Tzt3 and Tzt4 are EOs of T. zygis with high concentration of thymol and samples Tzl1, Tzl2, Th3 and Th4 are EOs of T. zygis with high

concentration of linalool and T. hyemalis, respectively. Also, Tzt1, Tzt4 and were grown in Lower Meso-Mediterranean bioclimatic zone, Tzt3, Tzl1 and Th2 were grown in Upper Meso-Mediterranean bioclimatic zone and Tzt2, Tzl2 and Th1 were grown in Supra Mediterranean bioclimatic zone. The essential oils were obtained in yields ranging from 0.4 to 0.8% (w/w). FGC/MS was used to determine the components of the studied EOs.

Table 7.1
Parameters of FGC/MS calibration

Analyte	Calibration curve[a]	R^2	Calibration range (mM)	RSD (%)	LOD (mM)	LOQ (mM)	Standard source	Product Reference
Nonane	*Internal standard*						SAFC	442694
(–)-α-Pinene	$y = 0.002 + 0.546x$	0.998	0.35 - 10.06	6.5	0.11	0.34	Fluka	80599
(+)-Camphene	$y = 0.001 + 0.294x$	0.999	0.50 - 11.09	12.7	0.15	0.47	SAFC	w222909
(–)-β-Pinene	$y = –0.007 + 0.630x$	0.999	0.30 - 10.29	2.4	0.10	0.30	Fluka	80609
Myrcene	$y = –0.018 + 0.287x$	0.999	0.55 - 8.97	3.8	0.17	0.51	Fluka	64643
(–)-Phellandrene	$y = –0.012 + 0.430x$	0.998	0.50 - 9.52	0.9	0.16	0.49	Aldrich	77429
α-Terpinene	$y = –0.035 + 0.425x$	0.997	0.45 - 9.45	2.8	0.15	0.44	Aldrich	86473
p-Cymene	$y = –0.008 + 0.986x$	0.999	0.35 - 9.95	3.8	0.11	0.32	Aldrich	c121452
(+)-Limonene	$y = –0.008 + 0.259x$	0.999	0.75 - 9.55	6.6	0.24	0.73	Fluka	62118
Z-β-Ocimene	$y = –0.021 + 0.277x$	0.996	0.75 - 6.97	1.7	0.23	0.7	SAFC	w353901
1,8-Cineole	$y = –0.008 + 0.383x$	0.998	0.30 - 9.55	8.6	0.09	0.28	SAFC	w246506
γ-Terpinene	$y = –0.009 + 0.460x$	0.999	0.45 - 9.87	6.6	0.14	0.42	Aldrich	223190
(+)-E-Sabinene hydrate	$y = –0.016 + 0.295x$	0.999	1.05 - 9.66	0.8	0.33	1.01	Fluka	96573
(–)-Linalool	$y = –0.022 + 0.310x$	0.998	0.55 - 8.80	2.3	0.18	0.55	Fluka	74856
(+)-Camphor	$y = –0.035 + 0.256x$	0.996	1.00 - 9.64	2.2	0.32	0.98	Alfa Aesar	A10708
(–)-Borneol	$y = –0.041 + 0.429x$	0.997	0.70 - 8.99	0.5	0.22	0.67	Alfa Aesar	A12684
(–)-Terpinen-4-ol	$y = –0.024 + 0.656x$	0.999	0.30 - 9.57	1.8	0.09	0.27	Aldrich	11584
(+)-α-Terpineol	$y = –0.009 + 0.211x$	0.999	0.55 - 9.87	7.7	0.17	0.52	Fluka	83073
Verbenone	$y = –0.032 + 0.183x$	0.994	1.30 - 9.88	1.8	0.42	1.28	Aldrich	218251
Tetradecane	*Internal standard*						SAFC	442708
Citronellol	$y = –0.014 + 0.137x$	0.998	0.90 - 8.69	9.0	0.29	0.87	SAFC	S60330
Methyl ether of carvacrol	$y = –0.013 + 0.239x$	0.999	0.75 - 9.06	5.8	0.23	0.7	Fluka	43778
(–)-Linalyl acetate	$y = –0.034 + 0.461x$	0.999	0.60 - 9.03	2.8	0.18	0.55	SAFC	w263605
Geraniol	$y = –0.018 + 0.338x$	0.999	0.70 - 7.18	3.8	0.22	0.66	SAFC	w250716
(–)-Bornyl acetate	$y = –0.029 + 0.317x$	0.997	0.65 - 8.16	5.5	0.20	0.61	Fluka	45855
Thymol	$y = –0.056 + 0.474x$	0.998	1.45 - 10.48	6.3	0.47	1.43	Sigma	T0501
Carvacrol	$y = –0.118 + 1.069x$	0.997	0.65 - 10.35	1.3	0.21	0.65	SAFC	w224502
Geranyl acetate	$y = –0.018 + 0.313x$	0.998	0.60 - 7.42	2.6	0.19	0.58	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(–)-(*E*)-β-Caryophyllene	$y = –0.015 + 0.417x$	0.998	0.35 - 4.35	8.3	0.11	0.33	Sigma	22075
α-Humulene	$y = –0.041 + 0.820x$	0.999	0.50 - 6.35	3.3	0.16	0.49	Aldrich	53675
(–)-Caryophyllene oxide	$y = –0.012 + 0.097x$	0.997	0.80 - 10.10	6.9	0.25	0.76	SAFC	w509647

Each internal standard is reference compound for the analytes that follow.

[a]Response ratio vs. concentration ratio, internal standard correction applied.

Each EO is described with two columns (Tables 7.2, 7.3). The first column determines the exact concentration of the compounds, expressed in mM (commercially available standards only, represent >90% of the total area). In the second column, the peak area of each compound present in the chromatogram was integrated (>99% of the total area). Thanks to the first column, it becomes clear when an EO has been

diluted with any kind of solvent not detectable by GC. The absolute concentration is not based on area percentages alone, but on calibration straights too, with commercial reagents as standards (Table 7.1).

The four samples of *T. zygis* with high concentration of thymol are shown together because of its similarity (Table 7.2). Nevertheless, some components make the difference between samples. That is the case of the high concentration of carvacrol in Tzt1, α-terpinene, p-cymene and (*E*)-β-caryophyllene in Tzt2, β-pinene and γ-terpinene in Tzt3 and thymol in Tzt4. Global results show four different *T. zygis* samples having the same 11 main molecules, *i.e.*, α-thujene, α-pinene, myrcene, α-terpinene, p-cymene, γ-terpinene, linalool, terpinen-4-ol, thymol, carvacrol and (*E*)-β-caryophyllene.

Focusing on *T. zygis* with high linalool and T. hyemalis samples, some components account for the differences among species and locations (Table 7.3). The high concentration of thymol in Tzl1 compared to Tzl2, species being the same, may account for the difference in location. The high concentrations of p-cymene, linalool and thymol in Th1 and 1,8-cineole and camphor in Th2, may account for a difference in location. Global results show two different *T. zygis* with high linalool samples having the same 10 principal molecules, *i.e.*, α-pinene, myrcene, α-terpinene, p-cymene, limonene, γ-terpinene, E-sabinene hydrate, linalool, terpinen-4-ol and thymol. Also the two different samples of *T. hyemalis* have the same 10 principal molecules, *i.e.*, α-pinene, myrcene, α-terpinene, p-cymene, limonene, 1,8-cineole, γ-terpinene, linalool, camphor and terpinen-4-ol.

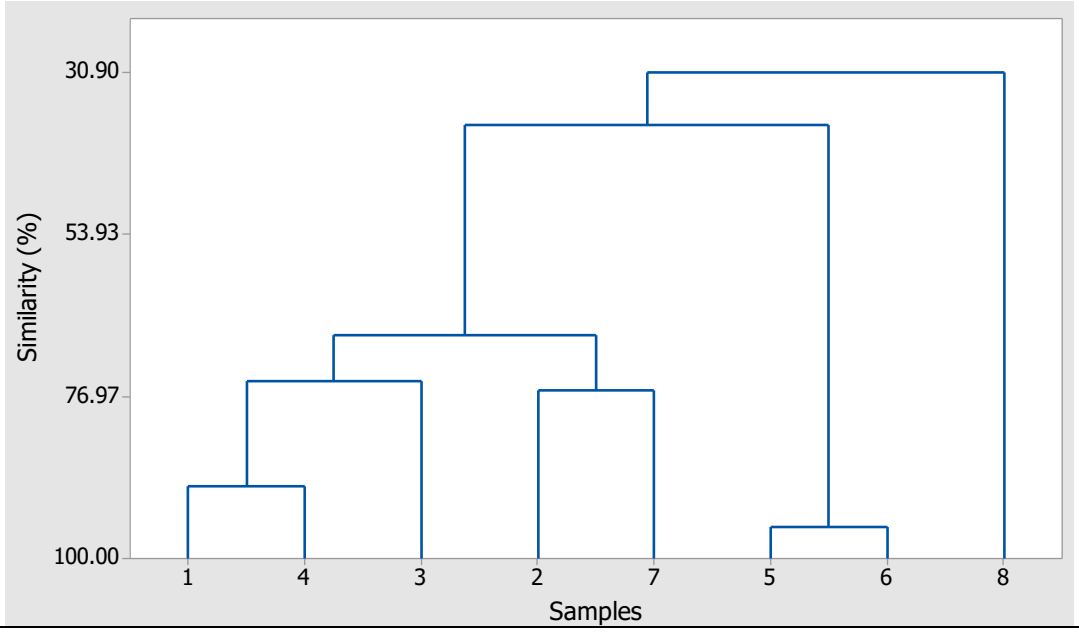

Figure 7.1
Dendrogram showing similarity of *Thymus* sp. samples
(1) Tzt1, (2) Tzt2, (3) Tzt3, (4) Tzt4, (5) Tzl1, (6) Tzl2, (7) Th1, (8) Th2

Using a dendrogram representation (Figure 7.1) of agglomerative hierarchical clustering based on Euclidean distance applied to the relative area of components, some interesting strong relationships are shown. Both *T. zygis* with high linalool samples (Tzl1 and Tzl2) are the most similar samples (95.6% similarity), followed by the pair Tzt1 and Tzt4 (89.7%) and this pair coupled to Tzt3 (74.8%). Tzt2 and Th1

have shown a similarity of 76.1% despite being different species. However, these two samples shared growing location, that fact might have influenced the composition of the final essential oil. Th2 is the most different sample, joining the group formed by the rest of samples at 30.9% of similarity.

Oxygenated monoterpenes are predominant in five of the eight samples (Table 7.2), accounting for more than 60%, in average, of the total molecules. The other three samples, *i.e.*, Tzt2, Tzt3 and Th1, have similar percentage (around 50%) of oxygenated and hydrocarbonated monoterpenes. Alcohol (>40%) is the most abundant organic functional group in all cases except for Th2 where ether is the most abundant functional group (37%) and alcohol is the second most abundant functional group (15.4%). Total terpene hydrocarbons were calculated as the sum of the monoterpene and sesquiterpene hydrocarbons. Total oxygenated terpenes were calculated as the sum of the oxygenated monoterpenes and sesquiterpenes.

T. zygis with high thymol EO is the most studied case in the literature. p-Cymene is found in similar concentration in the studied samples and throughout all the revised literature (Moldão-Martins et al., 2002) except in the case of Almería (Spain) (Saez, 1995b).

Thymol and γ-terpinene were found among the main components in all reported cases except one from Portugal (Dandlen et al., 2010) and another from Almería (Saez, 1995b) respectively, γ-terpinene values of the studied samples are similar to the ones reported in the literature, except in the case of Spanish experimental crops samples (Rota et al., 2008). Portuguese reports (Gonçalves et al., 2010; Pina-Vaz et al., 2004) are the only ones showing similar concentrations to the studied samples regarding myrcene and α-terpinene. The reports from Jaén, Córdoba (Spain) and Portugal have similar concentrations of thymol (Ballester-Costa et al., 2013; Penalver et al., 2005; Pina-Vaz et al., 2004), and all the studies reporting linalool show similar concentrations to the ones obtained in this study (Moldao-Martins et al., 1999). The studied samples show the highest concentrations of p-cymene and γ-terpinene among the revised literature.

In the case of the T. hyemalis EOs, p-cymene, γ-terpinene, thymol and borneol are present in all studied literature, whereas β-pinene, terpinen-4-ol, α-terpineol and geraniol are just present among the main components in the studied samples. This fact may indicate that the last compounds are produced in higher quantities in the studied samples. Just Spanish experimental crops samples (Rota et al., 2008) and the studied sample show linalool as a main component of the EO. The sample from Almería (Saez, 1995a) shows similar concentration of camphene to the studied samples, Spanish experimental crops sample (Jordan et al., 2006; Rota et al., 2008) shows similar concentration of p-cymene and borneol and the Turkish sample (Tepe et al., 2011) shows similar concentration of thymol to Th1. The studied samples show the highest concentrations of camphene, 1,8-cineole and camphor among the revised literature.

Table 7.2. FGC/MS determination of components in *T. zygis* with high thymol EO.

t_R (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Tzt1		Tzt2		Tzt3		Tzt4		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
1.47	929	α-Thujene	39, 77, 93, 136		1.3e ± 0.0		1.3e ± 0.0		0.1g ± 0.0		0.7f ± 0.0	LRI, MS
1.52	938	α-Pinene	77, 93*, 105, 121	76.3b ± 6.4	1.5f ± 0.1	93.9a ± 4.8	1.7e ± 0.1	36.8c ± 1.7	0.7h ± 0.0	38.3c ± 0.5	0.8g ± 0.0	LRI, MS, std
1.62	955	Camphene	79, 93*, 107, 121	51.7b ± 3.7	0.7f ± 0.0	170.3a ± 1.7	2.1e ± 0.1			29.2c ± 0.8	0.4f ± 0.0	LRI, MS, std
1.77	982	β-Pinene	69, 77, 93*, 121	12.1c ± 0.7	0.3f,g ± 0.0	17.3b ± 0.8	0.4f ± 0.1	81.7a ± 3.8	1.7e ± 0.0	7.0d ± 0.1	0.2g ± 0.0	LRI, MS, std
1.79	985	3-Octanone	43, 57, 71, 99				0.3e ± 0.1				0.1f ± 0.0	LRI, MS
1.81	989	Myrcene	41, 69, 79, 93*	145.6a ± 6.4	1.8e ± 0.1	139.9a ± 5.2	1.6f ± 0.1	32.7c ± 0.6	0.4h ± 0.0	90.7b ± 1.3	1.3g ± 0.0	LRI, MS, std
1.89	1002	3-Octanol	41, 59, 83, 101				0.1 ± 0.0				tr	LRI, MS
1.96	1010	α-Phellandrene	77, 93*, 119, 136	17.3a ± 0.9	0.2e ± 0.0	18.0a ± 0.6	0.2e ± 0.0	5.4c ± 0.1	0.1g ± 0.0	12.0b ± 0.1	0.1f ± 0.0	LRI, MS, std
1.98	1012	3-Carene	77, 79, 91, 93		0.1e ± 0.0		0.1e ± 0.0				0.1f ± 0.0	LRI, MS
2.04	1019	α-Terpinene	77, 93*, 121, 136	85.9b ± 4.2	1.7f ± 0.0	102.1a ± 5.5	1.9e ± 0.1	14.6d ± 0.5	0.2h ± 0.0	56.3c ± 1.3	1.2g ± 0.0	LRI, MS, std
2.11	1027	p-Cymene	91, 117, 119*, 134	862.1b ± 29.0	19.4f ± 0.6	1212.8a ± 13.0	27.2e ± 0.7	756.6c ± 12.5	16.1g ± 0.2	705.7c ± 22.9	14.3h ± 0.1	LRI, MS, std
2.13	1029	Limonene	68*, 79, 93, 121	47.1b ± 3.0	0.6f ± 0.0	50.8a,b ± 2.3	0.6f ± 0.0	22.1c ± 0.3	0.3g ± 0.0	53.5a ± 0.8	0.8e ± 0.0	LRI, MS, std
2.15	1031	Z-β-Ocimene	41, 79, 93*, 105	30.3a ± 2.0	0.2e ± 0.0	32.5a ± 1.3	0.2e ± 0.0	23.9b ± 0.6	0.1f ± 0.0	20.0c ± 0.3	0.1f ± 0.0	LRI, MS, std
2.17	1034	1,8-Cineole	43*, 81, 93, 108	26.2b ± 1.5	0.6f ± 0.0	41.0a ± 2.7	0.9e ± 0.0			16.9c ± 0.3	0.4g ± 0.0	LRI, MS, std
2.24	1041	E-β-Ocimene	41, 79, 93, 105		0.1e ± 0.0		0.1e ± 0.0				0.1f ± 0.0	LRI, MS
2.39	1059	γ-Terpinene	77, 93*, 121, 136	448.5c ± 22.4	8.3h ± 0.3	711.1b ± 11.3	13.3f ± 0.4	1462.8a ± 38.2	28.0e ± 0.4	710.8b ± 16.5	12.1g ± 0.1	LRI, MS, std
2.51	1072	E-Sabinene hydrate	77, 93*, 121, 136	36.2a ± 1.0	0.5e ± 0.0	22.2b ± 0.5	0.3f ± 0.0		0.1g ± 0.0	19.6c ± 0.8	0.3f ± 0.0	LRI, MS, std
2.65	1088	Terpinolene	93, 105, 121, 136		0.2e ± 0.0		0.1f ± 0.0		0.2e ± 0.0		0.1g ± 0.0	LRI, MS
2.67	1090	Z-Lilalool oxide	43, 59, 68, 111		0.1f,g ± 0.0		0.1f ± 0.0		0.1e ± 0.0		0.1g ± 0.0	LRI, MS
2.80	1105	Linalool	41, 69, 93*, 121	223.6c ± 2.8	4.1g ± 0.0	361.1b ± 5.6	5.8e ± 0.1		0.1h ± 0.0	386.8a ± 13.6	5.2f ± 0.0	LRI, MS, std
2.85	1110	β-Terpinene	77, 93, 121, 136		0.1e ± 0.0		0.1e,f ± 0.0				0.1f ± 0.0	LRI, MS
3.31	1157	Camphor	81, 95*, 108, 152	13.3c ± 0.5	0.1g ± 0.0	170.4a ± 3.0	2.2e ± 0.1		0.1g ± 0.0	15.7b ± 0.5	0.3f ± 0.0	LRI, MS, std
3.55	1181	Borneol	41, 95*, 110, 121	75.8b ± 0.4	1.0f ± 0.0	440.1a ± 20.2	3.0e ± 0.1			54.9b ± 0.4	0.8g ± 0.0	LRI, MS, std
3.61	1187	Terpinen-4-ol	71, 93*, 121, 136	43.5b ± 0.9	1.5f ± 0.0	35.0c ± 0.5	1.1g ± 0.0	8.9d ± 0.3	0.3h ± 0.0	45.7a ± 0.3	1.8e ± 0.0	LRI, MS, std
3.70	1197	p-Cymenene	65, 91, 117, 132				0.1 ± 0.0					LRI, MS
3.75	1202	α-Terpineol	68, 93*, 121, 136	13.4c ± 0.9	0.2e ± 0.0	21.6b ± 0.2	0.2e ± 0.1		0.1f ± 0.0	33.4a ± 0.3	0.2e ± 0.0	LRI, MS, std
3.77	1204	Citronellene	41, 55, 67, 81		0.2f ± 0.0						0.3e ± 0.0	LRI, MS
4.02	1230	Citronellol	41, 69*, 81, 95			8.5a ± 0.3	0.3e ± 0.0			7.4b ± 0.2	0.1f ± 0.0	LRI, MS, std
4.11	1239	Methyl ether of carvacrol	77, 91*, 117, 134	14.3b ± 0.3	0.2g ± 0.0	30.1a ± 0.5	0.5e ± 0.0			14.7b ± 0.3	0.2f ± 0.0	LRI, MS, std

Table 7.2. (Continued)

t_R (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Tzt1		Tzt2		Tzt3		Tzt4		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
4.27	1256	Geraniol	41, 69, 79, 93*							13.6 ± 0.5	0.2 ± 0.0	LRI, MS, std
4.68	1298	vic-Thymol	91, 115, 135, 150						1.9 ± 0.0			LRI, MS
4.79	1309	Thymol	91, 115, 135*, 150	3147.8b ± 17.8	50.3f ± 0.9	1923.2c ± 27.5	29.9h ± 1.0	3090.8b ± 98.6	48.2g ± 0.5	3636.2a ± 15.2	53.8e ± 0.2	LRI, MS, std
4.86	1315	Carvacrol	77, 91, 135*, 150	112.9a ± 2.5	2.9e ± 0.1	62.3c ± 2.9	1.4g ± 0.1	34.3d ± 1.3	0.4h ± 0.0	80.7b ± 3.6	2.2f ± 0.0	LRI, MS, std
4.98	1327	4-Methyl-2-propylphenol	77, 91, 121, 150						0.1 ± 0.0			LRI, MS
5.00	1328	6-Ethyl-3,4-dimethylphenol	91, 121, 135, 150						0.1 ± 0.0			LRI, MS
6.04	1427	(*E*)-β-Caryophyllene	41, 79, 93*, 133	31.3b ± 0.7	1.4f ± 0.0	50.4a ± 1.0	2.2e ± 0.0		0.7h ± 0.0	24.1c ± 0.3	1.2g ± 0.0	LRI, MS, std
6.23	1447	Aromadendrene	91, 133, 161, 204		0.1e ± 0.0		0.2e ± 0.0		0.2f ± 0.0		0.1f ± 0.0	LRI, MS
6.41	1465	α-Humulene	80, 93*, 121, 147		0.1e ± 0.0		0.1e ± 0.0				0.1e ± 0.0	LRI, MS, std
6.72	1496	Ledene	107, 135, 171, 204		0.1f ± 0.0		0.1f ± 0.0		0.3e ± 0.0		0.1f ± 0.0	LRI, MS
6.92	1527	δ-Cadinene	134, 161, 189, 204		0.1f ± 0.0		0.1e ± 0.0				0.1g ± 0.0	LRI, MS
7.29	1589	β-Vatirenene	131, 145, 159, 202				0.1 ± 0.0				tr	LRI, MS
7.32	1594	Caryophyllene oxide	41, 79, 91*, 105	16.1b ± 0.7	0.1f ± 0.0	24.4a ± 1.5	0.2e ± 0.0			14.3b ± 0.5	0.1f ± 0.0	LRI, MS, std
		Oxygenated terpenes:										
		Alcohol			60.9		42.5		51.6		65.1	
		Ketone			0.1		2.3		0.1		0.2	
		Aldehyde			0.0		0.0		0.0		0.0	
		Ester			0.0		0.0		0.0		0.0	
		Ether			1.0		1.6		0.2		0.8	
		Monoterpene hydrocarbons			36.4		50.4		47.3		32.1	
		Oxygenated monoterpenes			61.8		46.2		51.7		66.0	
		Sesquiterpene hydrocarbons			1.8		2.7		1.0		1.5	
		Oxygenated sesquiterpenes			0.1		0.2		0.0		0.1	
		Total terpene hydrocarbons			38.1		53.1		48.3		33.6	
		Total oxygenated terpenes			61.9		46.3		51.7		66.1	
		Non isoprenoid components			0.0		0.6		0.0		0.3	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). *Ions used for quantitation. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7.
std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. tr = Traces (<0,1%).
a, b, c, d Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$.
e, f, g, h Different letters in the same compound area mean statistically significant differences with $p < 0.05$.

Table 7.3. FGC/MS determination of components in *T. zygis* with high linalool and *T. hyemalis* EOs.

t_R (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Tzl1		Tzl2		Th1		Th2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
1.45	925	4-Methylenecyclohexyl methanol	67, 93, 108, 126		0.2d ± 0.0		0.3c ± 0.0		0.1c ± 0.0		0.1c ± 0.0	LRI, MS
1.47	929	α-Thujene	39, 77, 93, 136		0.3c ± 0.0		0.2d ± 0.0		0.7c ± 0.0		0.5d ± 0.0	LRI, MS
1.52	938	α-Pinene	77, 93*, 105, 121	170.1a ± 11.8	3.0d ± 0.0	185.9a ± 17.8	3.2c ± 0.1	194.8a ± 16.6	3.7d ± 0.1	187.8a ± 11.6	4.0c ± 0.0	LRI, MS, std
1.62	955	Camphene	79, 93*, 107, 121	68.9a ± 4.5	0.8d ± 0.0	75.9a ± 7.5	1.0c ± 0.0	108.0b ± 9.1	1.6d ± 0.1	474.8a ± 7.7	6.5c ± 0.1	LRI, MS, std
1.74	976	Sabinene	41, 77, 91, 93		0.9c ± 0.0		1.0c ± 0.0				1.7 ± 0.0	LRI, MS
1.77	982	β-Pinene	69, 77, 93*, 121	15.6a ± 1.1	0.4d ± 0.0	15.9a ± 0.7	0.4c ± 0.0	18.8b ± 1.3	0.6d ± 0.0	138.7a ± 10.5	2.7c ± 0.0	LRI, MS, std
1.79	985	3-Octanone	43, 57, 71, 99		0.1c ± 0.0		0.1c ± 0.0					LRI, MS
1.81	989	Myrcene	41, 69, 79, 93*	591.8b ± 3.6	7.4c ± 0.1	633.7a ± 18.5	7.5c ± 0.3	316.5b ± 3.9	2.6d ± 0.1	456.3a ± 16.2	4.5c ± 0.0	LRI, MS, std
1.89	1002	3-Octanol	41, 59, 83, 101		0.1c ± 0.0		0.1c ± 0.0					LRI, MS
1.96	1010	α-Phellandrene	77, 93*, 119, 136	33.2a ± 2.1	0.4c ± 0.0	22.4b ± 1.4	0.3d ± 0.0	16.6b ± 1.1	0.2d ± 0.0	25.2a ± 2.8	0.4c ± 0.0	LRI, MS, std
1.98	1012	3-Carene	77, 79, 91, 93						0.1 ± 0.0			LRI, MS
2.04	1019	α-Terpinene	77, 93*, 121, 136	408.2a ± 14.2	4.0c ± 0.0	149.6b ± 0.3	3.1d ± 0.1	107.7a ± 7.6	2.2c ± 0.1	20.4b ± 1.0	0.3d ± 0.0	LRI, MS, std
2.11	1027	p-Cymene	91, 117, 119*, 134	156.7a ± 11.3	3.0c ± 0.0	119.2b ± 7.5	2.3d ± 0.1	1260.7a ± 13.0	29.2c ± 0.9	74.4b ± 4.9	1.3d ± 0.0	LRI, MS, std
2.13	1029	Limonene	68*, 79, 93, 121	230.5a ± 15.0	2.7d ± 0.0	234.9a ± 5.4	3.0c ± 0.1	139.8a ± 12.6	1.9c ± 0.1	67.6b ± 6.9	0.8d ± 0.0	LRI, MS, std
2.15	1031	Z-β-Ocimene	41, 79, 93*, 105	92.2a ± 4.7	0.7c ± 0.0	74.0b ± 5.4	0.5d ± 0.0	55.6b ± 5.0	0.4d ± 0.0	66.6a ± 2.3	0.8c ± 0.1	LRI, MS, std
2.17	1034	1,8-Cineole	43*, 81, 93, 108	12.5a ± 1.1	0.3c ± 0.0	12.8a ± 0.6	0.3c ± 0.0	130.5b ± 11.1	3.1d ± 0.0	1569.7a ± 9.9	36.9c ± 0.3	LRI, MS, std
2.24	1041	E-β-Ocimene	41, 79, 93, 105		tr		tr		0.1d ± 0.0		4.5c ± 0.0	LRI, MS
2.39	1059	γ-Terpinene	77, 93*, 121, 136	413.7a ± 21.4	7.7c ± 0.1	369.5b ± 13.4	6.1d ± 0.2	276.5a ± 13.3	4.8c ± 0.1	117.9b ± 7.7	1.8d ± 0.0	LRI, MS, std
2.51	1072	E-Sabinene hydrate	77, 93*, 121, 136	164.2a ± 10.2	2.4d ± 0.0	186.1a ± 15.0	2.7c ± 0.0		0.1d ± 0.0	31.3 ± 1.8	0.4c ± 0.0	LRI, MS, std
2.65	1088	Terpinolene	93, 105, 121, 136		1.4c ± 0.0		1.3d ± 0.0		0.4c ± 0.0		0.2d ± 0.0	LRI, MS
2.67	1090	Z-Lilalool oxide	43, 59, 68, 111		0.2c ± 0.0		0.2c ± 0.0		0.1 ± 0.0			LRI, MS
2.80	1105	Linalool	41, 69, 93*, 121	1867.8b ± 33.9	41.3d ± 0.2	2064.7a ± 32.6	43.4c ± 0.4	630.7a ± 13.5	12.5c ± 0.1	495.9b ± 14.0	7.8d ± 0.1	LRI, MS, std
2.85	1110	Hotrienol	71, 82, 119, 134		0.7c ± 0.0		0.7c ± 0.0					LRI, MS
2.85	1110	β-Terpinene	77, 93, 121, 136		0.4c ± 0.0		0.5c ± 0.0		0.1d ± 0.0		0.2c ± 0.0	LRI, MS
3.07	1132	6-Camphenol	41, 77, 93, 108		0.5d ± 0.0		0.5c ± 0.0					LRI, MS
3.26	1152	Hexyl isobutyrate	43, 56, 71, 89		0.3c ± 0.0		0.3c ± 0.0		0.1d ± 0.0		0.2c ± 0.0	LRI, MS

Table 7.3. (Continued)

t_R (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Tzl1		Tzl2		Th1		Th2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
3.31	1157	Camphor	81, 95*, 108, 152	18.3b ± 0.4	0.2d ± 0.0	26.5a ± 0.5	0.3c ± 0.0	31.3a ± 0.8	0.4d ± 0.0	803.0a ± 20.1	9.4c ± 0.1	LRI, MS, std
3.53	1179	Lavandulol	41, 69, 111, 123		0.1 ± 0.0				0.1d ± 0.0		0.6c ± 0.0	LRI, MS
3.55	1181	Borneol	41, 95*, 110, 121	115.3b ± 3.9	1.5d ± 0.0	137.6a ± 3.8	1.8c ± 0.1	157.5b ± 4.1	2.1d ± 0.1	419.3a ± 13.3	2.6c ± 0.1	LRI, MS, std
3.61	1187	Terpinen-4-ol	71, 93*, 121, 136	391.4a ± 8.4	13.0c ± 0.1	389.9a ± 15.5	12.9c ± 0.3	155.9a ± 6.2	5.5c ± 0.1	37.0b ± 1.2	1.1d ± 0.0	LRI, MS, std
3.70	1197	p-Cymenene	65, 91, 117, 132		0.1c ± 0.0		0.1c ± 0.0		0.2 ± 0.0			LRI, MS
3.75	1202	α-Terpineol	68, 93*, 121, 136	126.3a ± 6.5	1.6c ± 0.0	137.9a ± 5.5	1.8c ± 0.1	147.8b ± 5.7	2.3c ± 0.0	174.4a ± 8.1	2.4c ± 0.1	LRI, MS, std
3.83	1210	E-Dihydrocarvone	67, 95, 109, 152		0.1d ± 0.0		0.2c ± 0.0					LRI, MS
3.87	1214	Verbenone	91, 107*, 135, 150	12.3 ± 0.1	0.2c ± 0.0		0.1d ± 0.0	69.9 ± 2.1	0.8 ± 0.1			LRI, MS, std
3.97	1224	Carveol	91, 105, 119, 134		0.1c ± 0.0		0.1c ± 0.0					LRI, MS
4.02	1230	Methyl ether of thymol	91, 119, 149, 164		0.1c ± 0.0		0.1c ± 0.0		0.3 ± 0.0			LRI, MS
4.04	1232	Isobornyl formate	93, 95, 121, 136		0.1d ± 0.0		0.2c ± 0.0					LRI, MS
4.11	1239	Methyl ether of carvacrol	77, 91*, 117, 134	8.8a ± 0.3	0.1c ± 0.0	5.4b ± 0.5	0.1d ± 0.0	22.5 ± 0.3	0.3 ± 0.0			LRI, MS, std
4.18	1246	Linalyl acetate	41*, 69, 93, 121	54.0a ± 1.8	0.9c ± 0.0	51.2a ± 1.2	0.9c ± 0.0	42.0a ± 0.7	0.7c ± 0.0	38.7b ± 1.1	0.6d ± 0.0	LRI, MS, std
4.27	1256	Geraniol	41, 69, 79, 93*	8.1a ± 0.7	0.1c ± 0.0	9.3a ± 0.6	0.1c ± 0.0	6.0b ± 0.3	0.1d ± 0.0	15.3a ± 0.4	0.2c ± 0.0	LRI, MS, std
4.53	1283	Lavandulyl acetate	43, 69, 93, 121				0.1 ± 0.0					LRI, MS
4.58	1288	Bornyl acetate	43, 95*, 121, 136		0.1d ± 0.0	8.5 ± 0.2	0.2c ± 0.0	8.8b ± 0.5	0.2d ± 0.0	28.7a ± 0.7	0.5c ± 0.0	LRI, MS, std
4.79	1309	Thymol	91, 115, 135*, 150	101.4a ± 1.5	1.0c ± 0.1	18.1b ± 0.5	0.1d ± 0.0	1210.6 ± 6.6	18.5 ± 0.9			LRI, MS, std
4.86	1315	Carvacrol	77, 91, 135*, 150	9.5 ± 0.0	0.1 ± 0.0			75.1 ± 1.8	1.8 ± 0.1			LRI, MS, std
5.08	1335	Elixene	79, 93, 107, 121				0.1 ± 0.0		0.1d ± 0.0		0.3c ± 0.0	LRI, MS
5.54	1379	Geranyl Acetate	41, 69, 93*, 121		0.1d ± 0.0		0.2c ± 0.0	5.8b ± 0.3	0.1d ± 0.0	23.9a ± 0.6	0.8c ± 0.0	LRI, MS, std
6.00	1423	Zingiberene	41, 69, 93, 119								0.1 ± 0.0	LRI, MS
6.04	1427	(*E*)-β-Caryophyllene	41, 79, 93*, 133	25.5b ± 0.1	1.1c ± 0.0	26.8a ± 0.8	1.2c ± 0.1	25.6b ± 0.4	1.1d ± 0.0	49.4a ± 1.3	2.0c ± 0.1	LRI, MS, std
6.23	1447	Aromadendrene	91, 133, 161, 204						0.1 ± 0.0			LRI, MS
6.41	1465	Conyrine	79, 93, 106, 120								0.1 ± 0.0	LRI, MS
6.43	1467	α-Humulene	80, 93*, 121, 147		tr		0.1 ± 0.0		0.1d ± 0.0	5.8 ± 0.3	0.2c ± 0.0	LRI, MS, std
6.51	1475	α-Amorphene	105, 119, 161, 204								0.3 ± 0.0	LRI, MS
6.64	1488	Germacrene D	91, 105, 119, 161		tr		0.1 ± 0.0				0.2 ± 0.0	LRI, MS

Table 7.3. (Continued)

t_R (min)	LRI	Analyte	Qualifying and quantitation ions (m/z)	Tzl1		Tzl2		Th1		Th2		Identification methods
				Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	Concentration (mM ± SD)	Area (% ± SD)	
6.72	1496	Ledene	107, 135, 171, 204						0.1d ± 0.0		1.2c ± 0.1	LRI, MS
6.76	1500	Seychellene	121, 161, 189, 204		0.1c ± 0.0		0.1c ± 0.0				0.1 ± 0.0	LRI, MS
6.92	1527	δ-Cadinene	134, 161, 189, 204						0.1d ± 0.0		0.6c ± 0.0	LRI, MS
7.13	1562	Sesquisabinenhydrate	69, 119, 161, 207								0.3 ± 0.0	LRI, MS
7.29	1589	β-Vatirenene	131, 145, 159, 202								0.2 ± 0.0	LRI, MS
7.32	1594	Caryophyllene oxide	41, 79, 91*, 105	9.9b ± 0.2	0.1c ± 0.0	11.5a ± 0.8	0.1c ± 0.0	15.9a ± 1.2	0.1c ± 0.0	13.5a ± 4.0	0.1d ± 0.0	LRI, MS, std
7.46	1623	Cedrene	91, 119, 161, 204								0.2 ± 0.0	LRI, MS
7.66	1669	Cycloisosativene	105, 119, 161, 204								0.2 ± 0.0	LRI, MS
7.83	1710	β-Guaiene	105, 119, 161, 204								0.1 ± 0.0	LRI, MS
8.22	1823	E-α-Bergamotene	41, 69, 93, 119						0.1 ± 0.0			LRI, MS
8.44	1895	1,3,3-Trimethyl-2-vinyl-1-cyclohexene	93, 107, 135, 150						0.1 ± 0.0			LRI, MS
8.49	1913	Fusaric acid	39, 65, 92, 135						0.1 ± 0.0			LRI, MS
		Oxygenated terpenes:										
		Alcohol			62.6		64.3		43.1		15.4	
		Ketone			0.5		0.6		1.2		9.4	
		Aldehyde			0.0		0.0		0.0		0.0	
		Ester			1.5		1.9		1.1		2.1	
		Ether			0.8		0.8		3.9		37.0	
		Monoterpene hydrocarbons			33.2		30.5		48.8		30.2	
		Oxygenated monoterpenes			65.1		67.3		49.1		63.5	
		Sesquiterpene hydrocarbons			1.2		1.6		1.7		5.7	
		Oxygenated sesquiterpenes			0.1		0.1		0.1		0.4	
		Total terpene hydrocarbons			34.4		32.1		50.5		35.9	
		Total oxygenated terpenes			65.2		67.4		49.2		63.9	
		Non isoprenoid components			0.4		0.5		0.3		0.2	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). *Quantitation ion. SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7.

std = identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. tr = Traces (<0,1%).

a, b Different letters in the same compound concentration and the same species mean statistically significant differences with $p < 0.05$.

c, d Different letters in the same compound area and the same species mean statistically significant differences with $p < 0.05$.

Table 7.5. EsGC/MS determination of EOs components. Enantiomeric ratios.

t_R		Analyte	Tzt1		Tzt2		Tzt3		Tzt4		Tzl1		Tzl2		Th1		Th2	
(+) – X (min)	(-) – X	(X)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)	(+) – [X] (%)	(-) – [X] (%)
7.79	7.52	α-Pinene	95	5	46[a]	54[a]	95	5	73[a]	27[a]	90	10	85	15	90	10	38[a]	62[a]
8.47	8.24	Camphene	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95
8.89	9.16	β-Pinene	65	35	36[a]	64[a]	95[a]	5[a]	68	32	54	46	53	47	44	56	45	55
10.36	9.39	α-Phellandrene	5	95	5	95	5	95	5	95	5	95	5	95	5	95	5	95
10.52	10.00	Limonene	95	5	95	5	95	5	85	15	95	5	90	10	95	5	88	12
14.28	14.51	E-Sabinene hydrate	95	5	95	5	N/D	N/D	88	12	95	5	95	5	N/D	N/D	95	5
15.73	15.57	Linalool	5	95	5	95	5	95	2	98	2	98	2	98	5	95	5	95
16.72	16.46	Camphor	5	95	5	95	5	95	38[a]	62[a]	5	95	5	95	5	95	5	95
18.02	18.18	Bornyl acetate	–	–	–	–	–	–	–	–	95	5	95	5	95	5	95	5
18.32	18.51	Terpinen-4-ol	63	37	64	36	82[a]	18[a]	61	39	73	27	72	28	71	29	49[a]	51[a]
20.10	19.76	α-Terpineol	95	5	95	5	95	5	40[a]	60[a]	90	10	90	10	39[a]	61[a]	66[a]	34[a]
20.15	19.58	Borneol	5	95	5	95	–	–	7	93	5	95	5	95	5	95	5	95
24.00	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	7	93	5	95	5	95	5	95	5	95
–	28.81	Caryophyllene oxide	5	95	5	95	–	–	5	95	5	95	5	95	5	95	5	95

[a]Biomolecular marker of the EO. N/D = not detectable. All results show standard deviation lower than ± 5%.

Table 7.6. Antioxidant capacity evaluation of *Thymus* sp. EOs by the six reported methods.

Sample	ORAC (µmol TE/µL EO)	ABTS (µmol TE/mL EO)	DPPH (µmol TE/mL EO)	ChP (mg EDTA eq/mL EO)	RdP (mg AA eq/mL EO)	TBARS (mg BHT eq/mL EO)
Tzt1	1.73b ± 0.06	3117.7b ± 179.8	14.5b ± 0.9	-	5.4a ± 0.3	314.4a,b ± 55.0
Tzt2	1.51c ± 0.06	1941.4d ± 91.5	15.4b ± 0.5	-	4.5b ± 0.2	245.0b ± 25.5
Tzt3	1.36c ± 0.05	2560.5c ± 194.6	28.3a ± 2.8	-	2.6c ± 0.1	361.6a ± 35.0
Tzt4	1.98a ± 0.06	3675.6a ± 267.1	14.1b ± 1.2	-	4.7b ± 0.2	324.1a,b ± 32.3
Tzl1	1.34c ± 0.07	60.5f ± 4.5	0.3d ± 0.1	0.73c ± 0.07	0.40e ± 0.04	16.2d ± 1.9
Tzl2	1.38c ± 0.05	14.9f ± 0.2	0.4c,d ± 0.0	1.05b ± 0.07	0.25e ± 0.02	6.1d ± 1.3
Th1	1.44c ± 0.08	1161.9e ± 12.4	3.8c ± 0.3	-	1.6d ± 0.1	142.1c ± 14.8
Th2	0.73d ± 0.04	3.0f ± 0.1	0.4c,d ± 0.0	1.92a ± 0.13	0.65e ± 0.03	3.8d ± 0.4

a, b, c, d, e, f Different letters in the same antioxidant method mean statistically significant differences with $p < 0.05$

In T. zygis with high linalool EO, myrcene, γ-terpinene, linalool and terpinen-4-ol were present in most of the reported samples in the literature. The sample from Jaén (Ballester-Costa et al., 2013) has the most similar values of linalool, p-cymene and myrcene to the studied samples. Jaén sample along with the experimental crops sample (Rota et al., 2008), they have similar values of α-terpinene, limonene and α-terpineol to the studied samples. The samples from Almería (Saez, 1995b) and central Portugal (Gonçalves et al., 2010) are the most different from the studied samples showing higher values of p-cymene, borneol, terpinen-4-ol, thymol and (*E*)-β-caryophyllene and lower values of linalool and myrcene.

Table 7.4.
GC/MS determination of components in *T. zygis* with high thymol (%), comparative with ISO normative

Component	Normative*		Tzt1	Tzt2	Tzt3	Tzt4
	Min	Max				
α-Thujene	0.2	1.5	1.4	1.5	0.1	0.7
α-Pinene	0.5	2.5	1.6	1.9	0.7	0.8
Myrcene	1.0	2.8	1.9	1.8	0.4	1.4
α-Terpinene	0.9	2.6	1.8	2.2	0.2	1.3
p-Cymene	14.0	28.0	20.4	30.8	16.9	15.0
γ-Terpinene	4.0	11.0	8.7	15.1	29.4	12.7
E-Sabinene hydrate	tr	0.5	0.5	0.3	0.1	0.3
Linalool	3.0	6.5	4.3	6.6	0.1	5.5
Terpinen-4-ol	0.1	2.5	1.6	1.2	0.3	1.9
Methyl ether of carvacrol	0.1	1.5	0.2	0.6	0.0	0.2
Thymol	37.0	55.0	53.0	33.9	50.6	56.6
Carvacrol	0.5	5.5	3.1	1.6	0.4	2.3
(*E*)-β-Caryophyllene	0.5	2.0	1.5	2.5	0.7	1.3

*ISO normative for *Thymus zygis* (ISO, 2010)

7.4.2 International Standard comparative

The International Organization for Standardization (ISO) has published an International Standard for *T. zygis* EO (ISO 14715:2010). The results shown in Table 7.4 were obtained taking ISO Standards for comparison. Some constituents from the Spanish EOs exceed the maximum relative concentration allowed. This is the case of γ-terpinene in Tzt2, Tzt3 and Tzt4, p-cymene, linalool and (*E*)-β-caryophyllene in Tzt2 and thymol in Tzt4.

All those differences in concentration of molecules are found frequently when the samples studied come from different countries. That makes research of EO production dependent on the geographical location. Thus, it is advisable to study the different EO compositions of each location to determine the optimal usages.

7.4.3 EsGC/MS study

Enantioselective chromatograms show higher t_R values than FGC (Table 7.5), due to lack of commercial availability of fast chiral columns with 0.1mm i.d. x 0.1µm film thickness.

The enantiomeric determinations of molecules of EOs from *T. zygis* and *T. hyemalis* are shown in Table 7.5. There are no adulterations with synthetic racemates of the main molecules, such as linalool or E-sabinene hydrate. The (+)-enantiomer predominates in the case of: α-pinene, limonene, E-sabinene hydrate, bornyl acetate, terpinen-4-ol and α-terpineol; while the (–)-enantiomer predominates in: camphene, α-phellandrene, linalool, camphor, borneol, (*E*)-β-caryophyllene and caryophyllene oxide.

There are several biomolecular markers of the origin: α-pinene for Tzt2, Tzt4 and Th2; β-pinene for Tzt2 and Tzt3; camphor for Tzt4; terpinen-4-ol for Tzt3 and Th2 and α-terpineol for Tzt4, Th1 and Th2. Thanks to the enantiomeric distribution of the compounds already mentioned for Tzt4, it is possible to differentiate the similar samples Tzt1 from Tzt4.

According to the reported data from Israel (Ravid et al., 1996), (1S,2R)-(–)-borneol has high purity in these *Thymus* sp. Reported variability about α-pinene and limonene enantiomers, present in *Thymus* sp. EOs, were found in some worldwide studies (Stahl-Biskup and Sáez, 2002). In the studied samples, (R)-(+)-α-pinene and (R)-(+)-limonene were found. This data could be useful to assess the origin and the authenticity of the EOs.

To our knowledge, this is the first chiral wide characterization of the EOs from *T. zygis* and *T. hyemalis* grown in Spain.

7.4.4 Antioxidant activity

The ORAC antioxidant activity of the eight samples is expressed in TEAC units (µmol TE/µL EO) and resulted (Table 7.6) as follows:

$Tzt4^{ORAC} > Tzt1^{ORAC} > Tzt2^{ORAC} \approx Th1^{ORAC} \approx Tzl2^{ORAC} \approx Tzt3^{ORAC} \approx Tzl1^{ORAC} > Th2^{ORAC}$

The antioxidant activity of each EO is related to its composition and the intrinsic antioxidant activity of each of the compounds. Generally, the high concentration of oxygenated terpenes is correlated to high ORAC antioxidant activity. Two oxygenated components are highly relevant to explain the ORAC value of the EO, namely thymol and linalool, the first is the most important molecule because of its high concentration and very high ORAC value and the other has a moderate ORAC value and high concentration (Bentayeb et al., 2014). The general trend shows increasing ORAC values with increasing thymol concentrations, however, due to the influence of linalool, this trend is broken in the order of Tzt2 and Tzt3 and in the order of Tzl1 and Tzl2.

The ABTS antioxidant activity is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 7.6) as follows:

$Tzt4^{ABTS} > Tzt1^{ABTS} > Tzt3^{ABTS} > Tzt2^{ABTS} > Th1^{ABTS} > Tzl1^{ABTS} \approx Tzl2^{ABTS} \approx Th2^{ABTS}$

Thymol has a great impact on ABTS method (Osman et al., 2006). The trend followed by this method is exactly the dependence with thymol concentration. The contributions to the ABTS value of the rest of compounds are slightly significant.

The DDPH antioxidant activity is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 7.6) as follows:

$Tzt3^{DPPH} > Tzt2^{DPPH} \approx Tzt1^{DPPH} \approx Tzt4^{DPPH} > Th1^{DPPH} \geq Th2^{DPPH} \approx Tzl2^{DPPH} \geq Tzl1^{DPPH}$

Thymol is not the molecule with the highest response to this method although it contributes significantly to the results. Some other molecules with the highest concentration present in Tzt3 may explain the better performance in DPPH antioxidant assay (Dawidowicz and Olszowy, 2014), *i.e.,* γ-terpinene and p-cymene, mainly.

The chelating power activity is expressed in EDTA units (mg EDTA equivalents / mL EO) and resulted (Table 7.6) as follows:

$Th2^{ChP} > Tzl2^{ChP} > Tzl1^{ChP}$

The highest concentrations of 1,8-cineole and camphor were found in Th2, also, linalool, being higher in Tzl2, makes the main difference between Tzl1 and Tzl2. The high electronic densities of the oxygen atoms of the ether, ketone and alcohol groups could be useful for complexation of cations. Phenolic compounds like thymol, having the electronic density of the oxygen atom delocalized by action of the aromatic ring, may show less chelating power.

The reducing power antioxidant activity is expressed in ascorbic acid units (mg Ascorbic acid equivalents/mL EO) and resulted (Table 7.6) as follows:

$Tzt1^{RdP} > Tzt4^{RdP} \approx Tzt2^{RdP} > Tzt3^{RdP} > Th1^{RdP} > Th2^{RdP} \approx Tzl1^{RdP} \approx Tzl2^{RdP}$

Thymol has a great influence in this assay although the order is not its decreasing trend, some other molecules like the aromatic p-cymene or the already reported alcohol linalool (Liu et al., 2012) might show a mild reducing power.

The thiobarbituric acid reactive substances (TBARS) generated by oxidation were measured and expressed in butylhydroxytoluene units (mg BHT equivalents/mL EO) and resulted (Table 7.6) as follows:

$Tzt3^{TBARS} \geq Tzt4^{TBARS} \approx Tzt1^{TBARS} \geq Tzt2^{TBARS} > Th1^{TBARS} > Tzl1^{TBARS} \approx Tzl2^{TBARS} \approx Th2^{TBARS}$

Thymol has a strong influence in this assay as the order of the TBARS values is the decreasing concentration of thymol except for the Tzt3 case. γ-Terpinene is shown in the highest concentration in Tzt3 and it has been reported to have an important TBARS antioxidant activity (Ruberto and Baratta, 2000), thus, the mixture of thymol and γ-terpinene is responsible for the high response of the TBARS method in Tzt3.

7.4.5 Inhibitory activity on LOX

LOX inhibitory activity resulted as follows: IC_{50} (µL(EO)/L) was measured for $Tzt4^{LOX}(54 \pm 10)^{c} \approx Tzt3^{LOX}(66 \pm 3)^{c} \approx Tzt1^{LOX}(67 \pm 4)^{c} \approx Tzt2^{LOX}(73 \pm 1)^{c} \approx Th1^{LOX}(100 \pm 5)^{c} < Th2^{LOX}(240 \pm 35)^{b} \approx Tzl1^{LOX}(299 \pm 25)^{b} < Tzl2^{LOX}(402 \pm 52)^{a}$. Tukey's HSD test revealed significant differences between the samples (different superscripts).

For a deeper understanding of the LOX inhibitory activity of the EO, the main commercially available compounds had their LOX inhibitory activity tested, obtaining their IC_{50} (µM) value: thymol (150 ± 11), limonene (356 ± 30), bornyl acetate (380 ± 14), p-cymene (486 ± 32), camphor (2743 ± 85) and linalool (3346 ± 44). NDGA was used as reference compound showing IC_{50} (339 ± 9) µM, this result agrees with already reported values (Kurihara et al., 2014).

Special interactions are usually present in natural mixtures when measuring enzymatic bioactivity. These phenomena modify the expected properties derived from the single components, making them greater (synergy) or smaller (antagonism) in the

mixture. In this case, the inhibitory activities of the *T. zygis* and *T. hyemalis* EOs are clearly due to a combination of compounds with high inhibitory activity and high to moderate concentration, namely thymol, p-cymene, limonene and linalool.

Important bioactivities, apart from the ones stated in this study, like antimicrobial, antifungal, cytotoxic, antiprotozoal and inhibition of replication of Epstein–Barr virus (Ballester-Costa et al., 2013; Gonçalves et al., 2010; Raut and Karuppayil, 2014; Rota et al., 2008) have been reported in the literature for *T. zygis* and *T. hyemalis* EOs.

7.5 Conclusions

The different *T. zygis* and *T. hyemalis* EOs showed the same common 7 principal biomolecules, *i.e.*, α-pinene, myrcene, α-terpinene, p-cymene, γ-terpinene, linalool, terpinen-4-ol. *T. zygis* has also thymol as a main biomolecule and *T. hyemalis* has 1,8-cineole. Oxygenated monoterpenes account for more than 46% of the total compounds, alcohol being the most abundant functional group, except for Th2 where ether is the most abundant functional group. Important variability between samples has been found even in the case of the same species, mainly due to the different growing bioclimatic zones. The whole group of Tzt samples is linked by 68.2% similarity and the couple of samples of Th show a 30.9% similarity.

Some concentrations of important bioactive molecules, like γ-terpinene, linalool and thymol are found exceeding ISO normative limits in some samples, highlighting the EOs as good sources of those molecules.

The enantiomeric profile shows predominance of (R)-(–)-linalool, (R)-(+)-limonene and (1S, 2R)-(–)-borneol being highly representative of the typical EOs from the studied aromatic plants. (R)-(+)-α-pinene, (R)-(+)-limonene, (1R, 9S)-(–)-(*E*)-β-caryophyllene and (1R, 4R, 6R, 10S)-(–)-caryophyllene oxide were found in this study as predominant enantiomers in Spanish *Thymus* sp. This data could be useful to assess the origin and the authenticity of the EOs.

T. zygis and *T. hyemalis* EOs showed good antioxidant activities compared to the reference antioxidants, due to the whole complex mixture of their compounds. Nevertheless, the main contributions to the global bioactivities have been identified and assigned to some compounds, *i.e.*, antioxidant activity due mainly to thymol and linalool.

Regarding potential anti-inflammatory properties, LOX inhibitory activities of *T. zygis* and *T. hyemalis* EOs, were due to the complex mixture of the abundance and of the LOX inhibitory activities of individual components. In this study, they were due mainly to thymol, p-cymene, linalool and limonene.

The biochemical compositions and bioactivities of *T. zygis* and *T. hyemalis* EOs, shown in this study, are crucial to understand the several pharmacological (anti-inflammatory, antiprotozoal, cytotoxic, antiviral…) and industrial (antioxidant, antimicrobial, antifungal…) potential uses and applications of the studied EOs.

7.6 References

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8. CARACTERIZACIÓN DE LOS ACEITES ESENCIALES DE *ORIGANUM VULGARE* Y *THYMBRA CAPITATA*

8.1 Resumen

Los aceites esenciales de orégano (*Thymbra capitata* y *Origanum vulgare*) cultivados y extraídos en el sureste de España, se han analizado por GC/MS para determinar su composición. La composición muestra una concentración de los componentes principales de (*E*)-β-caryophyllene (0.5-4.9%), thymol (0.2-5.8%), p-cymene (3.8-8.2%), γ-terpinene (2.1-10.7%) y carvacrol (58.7-77.4%). Esta caracterización se completó con una cromatografía de gases enantioselectiva donde se determinaron los enantiómeros principales como (–)-(*E*)-β-caryophyllene, (+)-α-pinene y (+)-β-pinene. Se evaluó positivamente la capacidad antioxidante por varios métodos, teniendo en cuenta actividad frente a radicales libres y capacidad reductora. Se observó una importante actividad inhibidora de lipoxigenasa (LOX) apoyando la posible actividad antiinflamatoria, principalmente debida a carvacrol. Estas propiedades respaldan el uso potencial de los aceites esenciales de orégano como ingredientes cosméticos y farmacéuticos naturales.

8.2 Summary

Oregano (*Thymbra capitata* and *Origanum vulgare*) essential oils (EOs), cultivated and extracted in the South East of Spain, were analysed by GC/MS to determine their composition. (*E*)-β-Caryophyllene (0.5-4.9%), thymol (0.2-5.8%), p-cymene (3.8-8.2%), γ-terpinene (2.1-10.7%) and carvacrol (58.7-77.4%) were determined as the main molecules. This characterisation was completed with the enantioselective gas chromatography, where (–)-(*E*)-β-caryophyllene, (+)-α-pinene and (+)-β-pinene were determined as the main enantiomers. Antioxidant activity was evaluated positively by several methods, accounting for activity against free radicals and reducing power. Important inhibitory activity on lipoxygenase (LOX) was observed supporting potential anti-inflammatory activity, mainly due to carvacrol. These properties support the potential use of oregano EOs as natural cosmetic and natural pharmaceutical ingredients.

8.3 Introduction

The worldwide used term “oregano” refers to a large number of species that belong to different botanical families and genera. Two main species are commonly used for culinary purposes: Greek/Italian oregano (*Origanum vulgare*) and Spanish oregano (*Thymbra capitata*) (Economou et al., 2011). These aromatic plants have two main commercial uses, *i.e.*, as a fresh or a dehydrated herb added to foods and beverages, and as plant material to obtain different extracts, mainly its essential oil (EO), which is employed in food, cosmetic, aromatherapy and pharmaceutical industries (Farias et al., 2010). *Origanum vulgare* L. and *Thymbra capitata* [L.] Cav. (syn. *Coridothymus capitatus* (L.) Rchb.f. and *Thymus capitatus* [L.] Hoffmanns. & Link) are perennial and herbaceous shrubs, commonly used as spicy herbs that belong to the *Lamiaceae* family.

Some GC relative quantitations of *O. vulgare* and *T. capitata* EOs have been reported, showing high concentrations of thymol and carvacrol (Economou et al., 2011; Farias et al., 2010). None of them has studied the specific conditions of the Spanish Mediterranean coast, specifically the region of Murcia where the biggest aromatic plant diversity among all regions of Spain is found. Furthermore, there are few chiral studies on EOs of *O. vulgare* and *T. capitata* (Bisht et al., 2009; Tateo et al., 1998). Chiral distribution is an important aspect of the EO composition, it helps identifying natural EOs from those adulterated, even when the samples come from different places of the Earth. In addition, the chirality in biomolecules is highly important due to the different bioactivities and organoleptic properties of each enantiomer (Smelcerovic et al., 2013).

Apart from the aromatic usages of *O. vulgare* and *T. capitata* EOs, some bioactivities may be relevant. The antioxidant potential of EOs can be determined using a representative selection of different antioxidant methods (Dawidowicz and Olszowy, 2014). Many inflammatory processes are associated with leukotriene production catalysed by lipoxygenase (LOX), which can use molecular oxygen or hydrogen peroxide as oxidants (Anwar et al., 2014). Thus, the inhibition of soybean lipoxygenase as LOX model is a hint of possible anti-inflammatory activity of the EO. Also, the ability to inhibit acetylcholinesterase (AChE) is interesting, as it can be used as a hint of both, potential insecticidal use (Pundir and Chauhan, 2012) and possible treatment of some nervous illnesses such as Alzheimer’s disease (Murray et al., 2013).

The aim of the present study is to determine the relative and chiral distribution of the main components of each EO in 6 samples grown in Murcia (Spain). Then, five antioxidant methods *i.e.*, Oxygen radical antioxidant capacity (ORAC), thiobarbituric acid reactive substances (TBARS), ABTS, DPPH and Reducing Power (RdP) will be applied to evaluate the antioxidant capacities of *O. vulgare* and *T. capitata* EOs. Furthermore, the inhibitory activity of these EOs on LOX and AChE will be characterised. The experimental results will be compared with those reported for *O. vulgare* and T. *capitata* EOs from other countries, and their potential biotechnological applications will be discussed.

8.4 Results and Discussion

8.4.1 Fast gas chromatography/mass spectrometry (FGC/MS) study

The essential oils were obtained by hydrodistillation in yields ranging from 0.8 to 2.0% (w/w) for *O. vulgare* and 2.8 to 3.5% (w/w) for *T. capitata*. FGC/MS was used,

Table 8.1. FGC/MS determination of components in *T. capitata* and *O. vulgare* EOs.

			Tc1	Tc2	Tc3	Ov1	Ov2	Ov3	
LRI (Lit)	LRI (Exp)	Analyte	Area (% ± SD)	Area (% ± SD)	Area (% ± SD)	Area (% ± SD)	Area (% ± SD)	Area (% ± SD)	Identification methods
928	926	α-Thujene	1.1a±0.0	tr	1.0b±0.0	1.7d±0.0		1.4e±0.0	LRI, MS, std
930	934	α-Pinene	0.8a±0.0	0.7a±0.0	0.8a±0.0	1.1d±0.0	0.5f±0.0	0.9e±0.0	LRI, MS, std
943	951	Camphene	0.1b±0.0	0.3a±0.0	0.1b±0.0	0.3d±0.0	0.2f±0.0	0.2e±0.0	LRI, MS, std
963	980	3-Octanone				0.2d±0.0		0.2e±0.0	LRI, MS, std
972	987	β-Pinene	0.4a±0.0	0.3a±0.0	0.3a±0.0	0.7d±0.0	0.1f±0.0	0.6e±0.0	LRI, MS, std
981	989	β-Myrcene	1.8a±0.0	1.2c±0.0	1.7b±0.0	2.4d±0.0	0.4f±0.0	2.0e±0.0	LRI, MS, std
985	998	3-Octanol		tr	tr	0.1d±0.0		0.1e±0.0	LRI, MS, std
997	1008	α-Phellandrene	0.3a±0.0	0.2c±0.0	0.3b±0.0	0.3d±0.0	0.2f±0.0	0.3e±0.0	LRI, MS, std
1005	1011	3-Carene	0.1a±0.0	0.1b±0.0	0.1a±0.0	0.1d±0.0		0.1e±0.0	LRI, MS, std
1008	1018	α-Terpinene	1.8a±0.0	1.3b±0.0	1.7a±0.0	2.0d±0.0	0.7f±0.0	1.8e±0.0	LRI, MS, std
1011	1026	p-Cymene	7.1a,b±0.1	6.6b±0.2	7.3a±0.1	8.2d±0.1	3.8f±0.1	6.8e±0.0	LRI, MS, std
1025	1029	Limonene	0.3a,b±0.0	0.3a±0.0	0.3b±0.0	0.3d±0.0	0.3f±0.0	0.3e±0.0	LRI, MS, std
1023	1031	β-Phellandrene	0.2a±0.0	0.2b±0.0	0.2a±0.0	0.3d±0.0	0.2f±0.0	0.2e±0.0	LRI, MS, std
1023	1033	1,8-Cineole	0.1a±0.0	0.1b±0.0	0.1b±0.0	0.2e±0.0	0.8d±0.0	0.1f±0.0	LRI, MS, std
1034	1044	(*E*)-β-Ocimene	0.1±0.0	tr	tr	0.1d±0.0		0.1d±0.0	LRI, MS, std
1047	1058	γ-Terpinene	7.7a±0.1	5.5c±0.1	7.0b±0.1	10.7d±0.1	2.1f±0.1	9.1e±0.0	LRI, MS, std
1078	1086	Terpinolene	0.2b±0.0	0.2a±0.0	0.2b±0.0	0.2d±0.0	0.1e±0.0	0.1e±0.0	LRI, MS, std
1103	1101	Linalool	0.9b±0.0	1.0a±0.0	0.9b±0.0	0.2e±0.0	3.8d±0.0	0.1f±0.0	LRI, MS, std
1174	1174	Borneol	0.2b±0.0	0.7a±0.0	0.2b±0.0	0.3e±0.0	0.9d±0.0	0.2f±0.0	LRI, MS, std
1175	1182	Terpinen-4-ol	0.8b±0.0	0.8a,b±0.0	0.8a±0.0	0.8f±0.0	1.0d±0.0	0.8e±0.0	LRI, MS, std
1192	1240	α-Terpineol				0.1e±0.0	0.4d±0.1		LRI, MS, std
1231	1258	Carvacrol methyl ether					0.1±0.0		LRI, MS, std
1266	1294	Thymol	2.1a±0.0	0.2b±0.0	2.2a±0.0	5.8d±0.0	2.4f±0.0	4.6e±0.0	LRI, MS, std
1275	1310	Carvacrol	69.4c±0.0	75.5a±0.4	70.4b±0.4	58.7f±0.2	77.4d±0.2	66.3e±0.2	LRI, MS, std
1421	1421	(*E*)-β-Caryophyllene	4.0a±0.2	3.8a±0.0	4.1a±0.0	4.5d±0.0	0.9f±0.0	3.0e±0.0	LRI, MS, std
1454	1459	α-Humulene	0.1a±0.0	0.1a±0.0	0.1a±0.0	0.2d±0.0		0.2e±0.0	LRI, MS, std
1492	1493	Ledene					0.1±0.0		LRI, MS, std
1500	1509	β-Bisabolene	0.1b±0.0	0.2a±0.0	0.1b±0.0	0.3e±0.0	2.0d±0.0	0.3e±0.0	LRI, MS, std
1514	1527	δ-Cadinene					0.2±0.0		LRI, MS
1581	1588	Caryophyllene oxide	0.2a,b±0.0	0.2a±0.0	0.2b±0.0		0.2±0.0		LRI, MS, std
		Monoterpene hydrocarbons	21.7	16.9	21.0	28.4	8.6	23.9	
		Oxygenated monoterpenes	73.7	78.3	74.6	66.1	86.8	72.1	
		Sesquiterpene hydrocarbons	4.2	4.1	4.2	5.0	3.2	3.5	
		Oxygenated sesquiterpenes	0.2	0.2	0.2	0.0	0.2	0.0	

LRI: Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). SD = standard deviation (0.0<0.1%). MS = tentatively identified by NIST 08 & Wiley 7. LRI (Lit) obtained from NIST08 database. LRI (Exp) obtained experimentally. tr= traces (<0.1%). std= identified by comparison with reference standards. Results are expressed as mean ± standard deviation of three determinations. a, b, c (for Tc samples), d, e, f (for Ov samples) Different letters in the same compound concentration mean statistically significant differences with $p < 0.05$.

performing triplicate analysis of the samples, to determine the components of the studied EOs (Council-of-Europe, 2011).

The three *T. capitata* samples (Tc1, Tc2 and Tc3) are shown together in Table 8.1. Some components make the difference between samples. This is the case of the high relative concentration of borneol and carvacrol in Tc2 and γ-terpinene in Tc1. These components account for the different environmental conditions and the genetic variability of the population. Results obtained about *O. vulgare* (Ov1, Ov2 and Ov3) (Table 8.1), also show some differences.

Myrcene, γ-terpinene and thymol are found in high proportion in Ov1. High concentrations of 1,8-cineole, linalool, borneol, carvacrol and β-bisabolene are found in Ov2.

Global results show six different samples having the same 9 principal molecules, *i.e.*, α-Thujene, myrcene, α-terpinene, p-cymene, γ-terpinene, linalool, thymol, carvacrol and (*E*)-β-caryophyllene. Specific molecules are found in high concentrations for each species. In all samples of *T. capitata*, α-humulene concentration is higher; whereas in *O. vulgare*, α-pinene, β-pinene, terpinen-4-ol and β-bisabolene are found in higher concentrations.

Despite the important differences found between the two species, we clustered the samples in a dendrogram representation (Figure 8.1). The dendrogram was based on agglomerative hierarchical clustering using Euclidean distance and applied to the relative area of components. The high similarity between species was unveiled. In the dendrogram, samples Tc1 and Tc3 are the most similar samples (92.5%), showing high similarity with Ov3 (79.41%). The group of all Tc samples and Ov3 has a similarity of 72.9%. The rest of the samples, Ov2 and Ov1 join the established group with 68.3% and 63.3% of similarity, respectively.

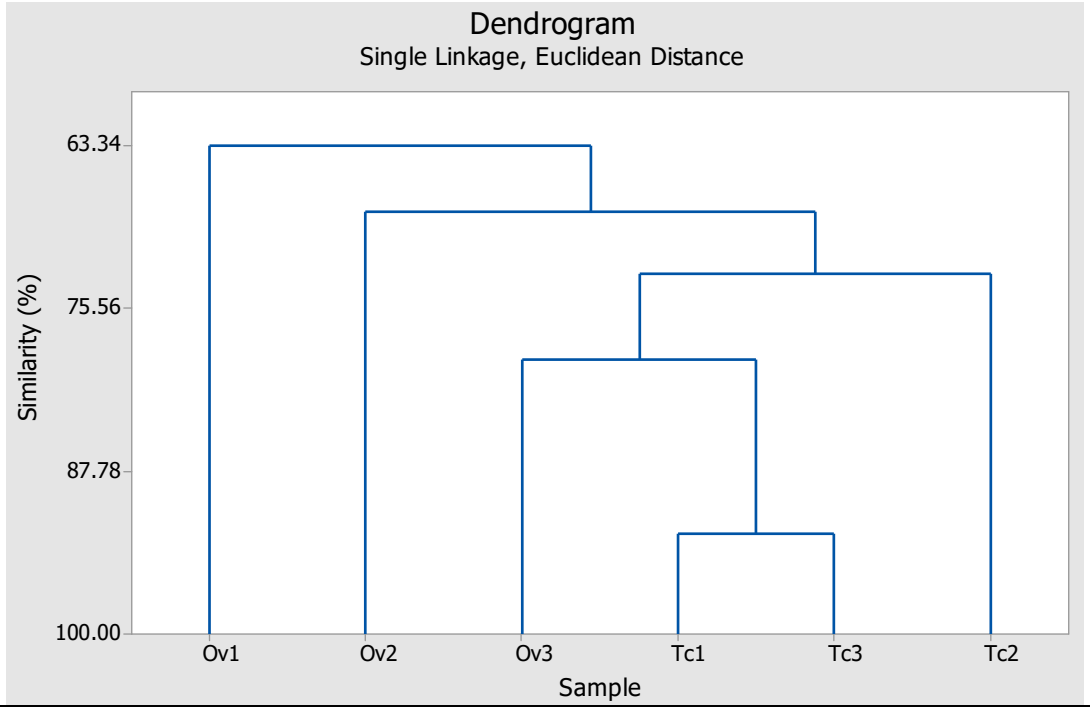

Figure 8.1
Dendrogram showing similarity of *T. capitata* and *O. vulgare* EOs samples

According to the similar concentrations of the components found between species (63.3%), both EOs can be considered the same culinary "spice" as their organoleptic properties must be derived from the chemical composition.

Oxygenated monoterpenes are highly predominant in the six samples (Table 8.1), being, at least, 66% of the total molecules. Phenol is the most abundant organic functional group, exceeding 64% of total molecules. The hydrocarbon monoterpenes represent a maximum of 28%.

Several ingredients show similar peak area percentages to those reported in literature for plant samples of *T. capitata* grown in different countries. Some main components like carvacrol, p-cymene, γ-terpinene or (*E*)-β-caryophyllene show similar concentrations to samples from Italy (Napoli et al., 2010; Russo et al., 2013), Greece (Economou et al., 2011), Portugal (Palmeira-de-Oliveira et al., 2012), Morocco (Bakhy et al., 2013) and Spain (Ballester-Costa et al., 2013). α-Thujene concentration determined is similar to the Tunisian (Amri et al., 2014; Hosni et al., 2013) and Greek (Skoula and Grayer, 2005) reports, while the case of myrcene is similar to the reports from Algeria (Tabti et al., 2014) and Tunisia (Ali et al., 2013). Molecules like thymol and α-humulene have been determined in higher concentrations in the studied samples than in any other report.

Regarding *O. vulgare*, some international reports show similar components and concentrations, this is the case of Turkey (Koldas et al., 2015), Hungary (Veres et al., 2007), Bosnia (Stoilova et al., 2008), Italy (Lukas et al., 2008) and Austria (Lukas et al., 2013). Reports from other countries show differences in the concentrations of common constituents of the EO. Thus, the Indian report (Bisht et al., 2009) shows a high concentration of thymol and the cases of Italy (Bonfanti et al., 2012) and Argentina (Farias et al., 2010) show high values of γ-terpinene. Also, the Mexican report (Hernandez-Hernandez et al., 2014) has high values for α-pinene and terpinen-4-ol. An Italian report (de Falco et al., 2013) shows the highest value of linalool, the Brazilian report (Mallet et al., 2014) shows high value of α-terpinene and the Tunisian report (Mechergui et al., 2010) shows high concentration of p-cymene. The studied samples show the highest concentration of carvacrol, followed by the Serbian report (Bozin et al., 2006) and an Italian report (de Falco et al., 2013)

8.4.2 International Standard comparative

Table 8.2. GC/MS determination of components in *T. capitata* EOs, comparative with International Standard ISO 14717:2008*.

Component	Standard*		Tc1	Tc2	Tc3
	Min (%)	Max (%)	Area (%)	Area (%)	Area (%)
α-Thujene	0.5	2.0	1.2	0.0	1.0
α-Pinene	0.5	2.0	0.8	0.8	0.8
β-Myrcene	1.0	3.0	1.8	1.2	1.8
α-Terpinene	0.5	2.5	1.8	1.3	1.8
p-Cymene	3.5	10.0	7.2	6.9	7.5
γ-Terpinene	5.5	9.0	7.8	5.7	7.3
Linalool	0.5	3.0	0.9	1.0	0.9
Terpinen-4-ol	0.5	2.0	0.8	0.8	0.9
Thymol	traces	5.0	2.2	0.2	2.3
Carvacrol	60.0	75.0	71.3	78.1	72.9
(*E*)-β-Caryophyllene	2.0	5.0	4.1	3.9	2.8

* Oil of origanum, Spanish type [*Coridothymus capitatus* (L.) Rchb.f.]

The International Organization for Standardization (ISO) has published an International Standard for *T. capitata* EO (ISO 14717:2008). The results showed in Table 8.2 were obtained taking ISO Standards for comparison. Carvacrol concentration determined in sample Tc2 slightly exceeds the maximum allowed. Also, α-Thujene threshold is not reached by sample Tc2. Apart from these small deviations in Tc2, the rest of the components of Tc2 and the two other EOs completely meet the ISO standard.

8.4.3 Enantioselective gas chromatography/mass spectrometry (EsGC/MS study)

The enantiomeric determinations of molecules of EOs from *T. capitata* and *O. vulgare* are shown in Table 8.3. The enantiomeric distributions of the most abundant molecules, such as *(E)*-β-caryophyllene and limonene, were far from 50%, typically obtained when adulterations with synthetic racemates occur. The enantiomeric predominance is the same for the two species. The (+)-enantiomeric excess is shown in the case of: α-pinene, β-pinene, phellandrene, 3-carene, limonene, trans-sabinene hydrate and α-terpineol; while the (–)-enantiomeric excess is shown in: camphene, borneol, (*E*)-β-caryophyllene and caryophyllene oxide. The enantiomeric distributions of linalool and terpinen-4-ol show the highest variability and could be useful for their characterisation. This data could be useful to assess the origin and the authenticity of the EOs. To our knowledge, this is the widest chiral characterisation of the EOs from *T. capitata* and *O. vulgare* grown in Spain.

The enantiomeric ratios for (–)-linalool, (+)-terpinen-4-ol and (–)-borneol are similar to the ones reported from India (Bisht et al., 2009), finding differences in α-pinene, limonene and α-terpineol. The predominant enantiomers found in this study for α-pinene, camphene, β-pinene, limonene, linalool and terpinen-4-ol agree with those reported from Greece (Tateo et al., 1998), although the percentage of each enantiomer is not the same in both locations. This fact supports the use of enantiomeric distributions for location authenticity determination.

Table 8.3. EsGC/MS determination of enantiomers in *T. capitata* and *O. vulgare* EOs.

t_R (min)		Analyte	Ov1		Ov2		Ov3		Tc1		Tc2		Tc3	
(+)	(-)		(+) (%)	(-) (%)	(+) (%)	(-) (%)	(+) (%)	(-) (%)	(+) (%)	(-) (%)	(+) (%)	(-) (%)	(+) (%)	(-) (%)
7.79	7.52	α-Pinene	95	5	95	5	95	5	95	5	95	5	90	10
8.47	8.24	Camphene	10	90	10	90	5	95	5	95	5	95	5	95
8.89	9.16	β-Pinene	N/A	N/A	N/A	N/A	N/A	N/A	90	10	90	10	90	10
10.36	9.39	Phellandrene	N/A	N/A	N/A	N/A	N/A	N/A	90	10	90	10	90	10
9.46	-	3-Carene	N/A	N/A	N/A	N/A	N/A	N/A	95	5	95	5	95	5
10.52	10.00	Limonene	95	5	90	10	90	10	90	10	90	10	90	10
15.73	15.57	Linalool	5	95	5	95	5	95	48	52	45	55	51	49
18.32	18.51	Terpinen-4-ol	95	5	66	34	81	19	95	5	61	39	62	38
20.10	19.76	α-Terpineol	N/A	N/A	90	10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
20.15	19.58	Borneol	N/A	N/A	N/A	N/A	N/A	N/A	5	95	5	95	10	90
24.00	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	5	95	5	95	10	90
-	28.81	Caryophyllene oxide	5	95	10	90	10	90	5	95	5	95	10	90

N/A = Not assessed. All results show standard deviation lower than ± 5%.

8.4.4 Antioxidant activity

The ORAC antioxidant activity of each sample is expressed in TEAC (Trolox equivalent antioxidant capacity) units (µmol TE/µL EO) and resulted (Table 8.4) as follows:

$$Ov2^{ORAC} \approx Ov1^{ORAC} \approx Tc1^{ORAC} \geq Tc2^{ORAC} \approx Tc3^{ORAC} \geq Ov3^{ORAC}$$

The antioxidant activity of each EO is related to both, its composition and the intrinsic antioxidant activity of each compound. Generally, the rise in composition of oxygenated terpenes is correlated to higher ORAC antioxidant activity. In this case, carvacrol, thymol, linalool, borneol or terpinen-4-ol may be responsible for the ORAC value (Tables 1 and 4). However, the total ORAC value of the EO is determined not just by the main components but by the whole group of constituents.

The ABTS antioxidant activity of each sample is expressed in TEAC units (mmol TE/mL EO) and resulted (Table 8.4) as follows:

$$Ov2^{ABTS} \approx Tc2^{ABTS} \geq Ov1^{ABTS} \approx Ov3^{ABTS} \geq Tc1^{ABTS} \geq Tc3^{ABTS}$$

Phenols have great impact on ABTS method (Osman et al., 2006) showing a strong dependence on the addition of carvacrol and thymol concentrations, with slight deviations.

The DDPH antioxidant activity of each sample is expressed in TEAC units (µmol TE/mL EO) and resulted (Table 8.4) as follows:

$$Ov2^{DPPH} > Tc2^{DPPH} \approx Ov1^{DPPH} \approx Tc1^{DPPH} \approx Tc3^{DPPH} \approx Ov3^{DPPH}$$

The highest concentration of carvacrol is found in Ov2, thus, suggesting an influence of phenols in the results of this method.

The reducing power antioxidant activity of each sample is expressed in ascorbic acid units (mg Ascorbic acid equivalents/mL EO) and resulted (Table 8.4) as follows:

$$Tc2^{RdP} \approx Tc3^{RdP} \approx Tc1^{RdP} > Ov2^{RdP} \approx Ov3^{RdP} \approx Ov1^{RdP}$$

Phenolic compounds like thymol and carvacrol usually exhibit strong reducing power, also, some abundant alcoholic terpenoids, as the already reported linalool (Liu et al., 2012), might show a mild reducing power.

The thiobarbituric acid reactive substances generated by oxidation were measured and expressed in butylhydroxytoluene units (mg BHT/ml EO) and resulted (Table 8.4) as follows:

$$Tc2^{TBARS} \approx Ov2^{TBARS} \approx Tc3^{TBARS} \approx Tc1^{TBARS} \approx Ov3^{TBARS} \approx Ov1^{TBARS}$$

This assay may be strongly influenced by phenols like thymol and carvacrol. γ-Terpinene has been reported as an important antioxidant using this method (Ruberto and Baratta, 2000). The additions of these three component concentrations show very similar values for the six samples.

Table 8.4. Antioxidant capacity of *T. capitata* and *O. vulgare* EOs.

Sample	ORAC (µmol TE/mL EO)	ABTS (mmol TE/mL EO)	DPPH (µmol TE/mL EO)	RdP (mg AA eq/mL EO)	TBARS (mg BHT eq/mL EO)
Tc1	2.1a ± 0.2	5.2b,c ± 0.3	3.1b ± 0.2	2.8a ± 0.2	242.9a ± 27.6
Tc2	2.0a,b ± 0.2	6.1a ± 0.3	3.3b ± 0.2	3.2a ± 0.1	282.1a ± 29.9
Tc3	1.9a,b ± 0.3	4.9c ± 0.1	3.1b ± 0.4	3.1a ± 0.2	272.4a ± 36.7
Ov1	2.3a ± 0.3	5.7a,b ± 0.3	3.2b ± 0.2	1.8b ± 0.1	216.3a ± 36.0
Ov2	2.5a ± 0.2	6.2a ± 0.2	4.6a ± 0.2	2.1b ± 0.1	272.8a ± 28.1
Ov3	1.4b ± 0.1	5.6a,b ± 0.2	3.0b ± 0.2	1.8b ± 0.1	225.5a ± 22.1

AA = Ascorbic acid, TE = Trolox equivalents, BHT = butylhydroxytoluene, EO = Essential oil. a, b, c Different letters in the same antioxidant method mean statistically significant differences with $p < 0.05$.

The different *T. capitata* and *O. vulgare* EOs evaluated showed the same common 9 principal constituents, *i.e.*, α-thujene, myrcene, α-terpinene, p-cymene, γ-

terpinene, linalool, thymol, carvacrol and (*E*)-β-caryophyllene. The studied samples meet the ISO standard for *T. capitata* except in the case of Tc2 where some slight deviations were found. A new source of pure enantiomers is unveiled showing (–)-(*E*)-β-caryophyllene, (+)-α-pinene and (+)-β-pinene as abundant and enantiomerically pure compounds of typical *T. capitata* and *O. vulgare* EOs. The EOs showed moderate antioxidant activities, which may be due to carvacrol and thymol.

8.4.5 Inhibitory activity on LOX

Half maximal inhibitory concentration (IC_{50}) (µL EO/L) was measured for $Ov3^{LOX}(251.5 \pm 1.4)^{a} \approx Ov1^{LOX}(251.0 \pm 4.2)^{a} > Tc3^{LOX}(184.1 \pm 2.2)^{b} > Tc1^{LOX}(170.9 \pm 2.9)^{c} \approx Tc2^{LOX}(167.1 \pm 1.2)^{c} > Ov2^{LOX}(148.0 \pm 4.3)^{d}$. Tukey's HSD (honest significant difference) test revealed significant differences between the samples (different superscripts). For a deeper understanding of the LOX inhibitory activity of the EO, the main commercially available compounds, had their LOX inhibitory activity tested, obtaining their IC_{50} (µM) value: thymol (150 ± 11), limonene (356 ± 30), p-cymene (486 ± 32), carvacrol (2271 ± 83) and linalool (3346 ± 44). Also the nordihydroguaiaretic acid (NDGA) had its inhibitory activity tested with this method as a reference inhibitor: IC_{50}=(339 ± 9) µM.

Regarding potential anti-inflammatory properties, LOX inhibitory activity of *T. capitata* and *O. vulgare* EOs is clearly due to a combination of high inhibitory activity and abundance of carvacrol (Table 8.1). The rest of inhibitory compounds (some of them being very powerful like thymol) have modulated the inhibitory response causing small variations.

8.5 Conclusions

The biochemical compositions of *T. capitata* and *O. vulgare* are essential to understand the reported bioactivities of oregano EOs, such as antibacterial (Nabavi et al., 2015), antifungal (Tabti et al., 2014), cytotoxic and colon pathogen-adhesion inhibitor (Džamić et al., 2015), phytotoxic (Saoud et al., 2013), antioxidant in biological systems (Hortigón-Vinagre et al., 2014) and nematicidal (Oka et al., 2000). These properties, as well as the experimental results of this work, support the potential use of *T. capitata* and *O. vulgare* EOs as useful natural cosmetics and pharmaceutical ingredients.

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9. COMPARATIVA ENTRE LOS GÉNEROS *LAVANDULA* Y *THYMUS*

9.1 Resumen

Las composiciones químicas de los aceites esenciales de *Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula hybrida* cultivos Grosso y Súper, *Thymus zygis* con alta proporción de timol y linalol y *Thymus hyemalis*, de la Región de Murcia (España), se estudiaron de forma relativa (%), absoluta (mM) y quiral por GC/MS. La inhibición de hialuronidasa y la actividad antioxidante exhibidas por los aceites esenciales, se evaluaron usando los métodos $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC, capacidad quelatante, radical hidroxilo, óxido nítrico, TBARS y capacidad reductora. Los componentes más abundantes fueron, para el género *Lavandula*: linalol y acetato de linalilo, y para el género *Thymus*: timol, linalol y 1,8-cineol. La determinación quiral de los componentes principales mostró (+)-enantiómeros como terpinen-4-ol, β-pineno, borneol y α-terpineol y (–)-enantiómeros como linalol, acetato de linalilo, y canfeno para el género *Lavandula*. Para el género *Thymus* encontramos (+)-enantiómeros como α-pineno, limoneno, terpinen-4-ol y α-terpineol y (–)-enantiómeros como el borneol. Los aceites esenciales que contienen timol muestran resultados especialmente buenos en todos los ensayos excepto en capacidad quelatante, ORAC y el ensayo de captación de radicales hidroxilo. La inhibición de hialuronidasa fue especialmente efectiva en el caso del *Thymus zygis* con alta proporción de timol. Los aceites esenciales con alta concentración de timol o de la pareja linalol/acetato de linalilo tienen un uso potencial como antioxidantes. El timol muestra una fuerte inhibición de hialuronidasa.

9.2 Summary

The chemical compositions of essential oils of *Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula hybrida* cultivar Grosso and cultivar Super, *Thymus zygis* with high proportions of thymol and linalool and *Thymus hyemalis*, from Murcia country (Spain), were studied in relative (%), absolute (mM) and chiral concentrations by GC/MS. Hyaluronidase inhibition and antioxidant activities of the essential oils were evaluated using $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC, Chelating power, Hydroxyl radical, Nitric oxide, TBARS, and Reducing power assays. Linalool and linalyl acetate were the most abundant components in *Lavandula* genus whereas thymol, linalool and 1,8-cineole were the most abundant molecules in the respective *Thymus* species. Chiral determination of the main components showed (+)-enantiomers like terpinen-4-ol, β-pinene, borneol and α-terpineol and (–)-enantiomers like linalool, linalyl acetate and camphene in *Lavandula* sp. In the case of *Thymus* sp. (+)-enantiomers like α-pinene, limonene, terpinen-4-ol and α-terpineol and (–)-enantiomers like borneol were found. Essential oils containing thymol were found especially powerful in all assays but chelating power, ORAC and hydroxyl radical scavenging assays. The capacity for inhibiting Hyaluronidase showed that *T. zygis* with high proportion of thymol was the

most effective inhibitor. Essential oils containing Thymol and linalool/linalyl acetate have potential use as antioxidant agents. Thymol shows strong inhibition of hyaluronidase.

9.3 Introduction

The genus *Lavandula* belongs to the *Lamiaceae* family and includes 39 known species. However, there are three important species due to their terpenoid rich essential oils (EOs), which are obtained by hydrodistillation. They are *L. angustifolia* Mill. (*L. officinalis* Chaix ex. Vill, *L. spica* L, *L. vera* DC) or true lavender; *L. latifolia* Medik. or spike lavender; and the natural hybrid *L. x intermedia* Emeric ex Loisel (*L. hybrida* L.) or lavandin which is derived from a cross of *L. latifolia* x *L. angustifolia*. EOs are extensively used in perfumes, in cosmetics, in food manufacturing for flavouring beverages, ice-creams, candies, baked goods and chewing gums, and in aromatherapy as relaxants (Baser and Buchbauer, 2010; Hassiotis et al., 2014).

L. angustifolia is one of the most desired lavender oils in the cosmetic and aromatherapeutic industries, that is due to the high concentration of both linalool/linalyl acetate and low camphor concentration. However, true lavender produces this interesting EO in relatively low amounts. For higher production of this EO, hot dry climates and medium altitudes (700–1200 m) are required (Woronuk et al., 2011). The yield of EO from lavandin is three-fold higher than the one of *L. angustifolia*, albeit with much lower application in perfumery and therapy due to the undesirably high levels of camphor. Such oil is preferentially used as antiseptic, antifungal and antibacterial agent (Woronuk et al., 2011). These biological properties have also been attributed to *L. angustifolia* and *L. latifolia*. In addition, they have also been used as sedative, carminative, anti-depressive and anti-inflammatory agents, although clinical studies show inconclusive results (Dobetsberger and Buchbauer, 2011). *L. latifolia* is also believed to be effective for burns and insect bites (Munoz-Bertomeu et al., 2007).

The genus *Thymus* is one of the eight most important genera regarding the number of species included: more than three hundred, including hybrids, varieties and ecotypes (Figueiredo et al., 2008).

Thymus hyemalis Lange, winter thyme, can be found mainly in the South East of Spain (Alicante, Murcia and Almeria) (Jordan et al., 2006). Chemical variability has been reported for the essential oils of this species, the presence of at least 4 chemotypes in that region of Spain was stated beyond the seasonal variations and edaphic and climatic conditions (Jordan et al., 2006; Saez, 1995a).

Antimicrobial activity of *T. hyemalis* EO was found in 2008 (Rota et al., 2008), being thymol chemotype more active than carvacrol chemotype. Antimicrobial activity along with antioxidant activity of winter thyme EO were also reported (Tepe et al., 2011).

Thymus zygis Loefl. ex L. is a widespread endemic plant in the Iberian Peninsula. At least, eight chemotypes have been found in this region (Saez, 1995b). Seasonal variations and phenological stages are also factors which contribute to the chemical variability of *T. zygis* EOs (Moldao-Martins et al., 1999).

Antimicrobial, antigiardial, antiviral, anti-enzymatic and antioxidant activities of *T. zygis* EOs have been reported (Ballester-Costa et al., 2013; Dandlen et al., 2010;

Dandlen et al., 2011; Gonçalves et al., 2010; Machado et al., 2010; Pina-Vaz et al., 2004; Tepe et al., 2011).

Gas chromatography (GC), coupled with mass spectrometry detection, is a powerful technique used for volatile components analysis, since it provides qualitative and quantitative data for complex mixtures such as those usually present in EOs (O'Shea et al., 2012).

The quantitative composition of EOs may be estimated by means of relative percentage abundance, internal standard normalized percentage abundance and true quantitation of one or more compounds by a validated method (Bicchi et al., 2008). So far, relative percentage abundance is the most commonly method used in the EO analysis. However, it should only be used to measure relative component ratios in a single sample and not to compare compositions of a group of EOs, because relative percentage abundances are not standardized (Bicchi et al., 2008).

Furthermore, there are scarce chiral studies accounting for the main components of the studied EOs. Observing the enantiomeric distribution of the main molecules of the EOs is highly interesting, since different bioactivities and organoleptic properties depend on the enantiomeric ratios. These characteristics are useful in the formulation and authenticity assessment of drugs, fragrances and flavours (Baser and Buchbauer, 2010; Mosandl, 2004).

Antioxidant activities of the mentioned EOs have been found interesting because oxidation, induced by reactive oxygen and nitrogen species (ROS and RNS), can damage membranes, lipids and lipoproteins and can induce DNA mutation. These types of cell or tissue injuries have been associated with ageing, atherosclerosis, carcinogenesis and cardiovascular as well as Alzheimer's and other neurological diseases. Thus, preventing or minimizing these oxidation processes, by the use of antioxidant substances that scavenge hydroxyl ($HO^{\cdot}$), nitric oxide ($NO^{\cdot}$) or other free radicals, may help in the treatment of the mentioned illnesses (Angelo et al., 2014; Aprotosoaie et al., 2014; Baser and Buchbauer, 2010; Dobetsberger and Buchbauer, 2011; Figueiredo et al., 2008; Nikolić et al., 2014).

The extracellular matrix (ECM) breakdown is related with oxidative stress and degradation of proteins such as collagen and elastin. It is also related with hydrolysis, catalyzed by hyaluronidase, of proteoglycans with glycosaminoglycans such as hyaluronic acid. Hyaluronic acid is a polysaccharide composed of D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds, with a size from 5000 Da to 20 MDa *in vivo.* Hyaluronic acid imbibes water, provides resilience to cartilages, replaces fibres of degraded collagen and enhances the regeneration of collagen by the dermis and the ECM. The hyaluronic acid/hyaluronidase system participates also in many pathophysiological conditions such as envenomation, acrosome reaction / ovum fertilization, microbial pathogenesis and cancer progression. The inhibitors of hyaluronidase might serve as contraceptive, anti-venom/toxin, anti-microbial, anti-aging, anti-inflammatory and anticancer agents (McAtee et al., 2014).

The aim of this study is to determine thoroughly the chemical composition and bioactivities of the EOs of four species of *Lavandula* genus (*Lavandula angustifolia*, *Lavandula latifolia*, *Lavandula hybrida* cultivar Grosso and cultivar Super) and three of *Thymus* genus (two samples of *Thymus zygis* and one sample *Thymus hyemalis*). Source plants were grown under organic farming in the Murcia country (Spain). Their

relative and absolute concentrations, as well as the proportions of their main chiral compounds will be determined. Also, several antioxidant methods will evaluate the antioxidant capacity of EOs against different oxidant agents. Furthermore, the potential bioactivity of EOs on the important and multifunctional hyaluronic acid/hyaluronidase system, will be determined. Thus, this study is focused on increasing the diversity of available compositions and bioactivities of these EOs with samples from Murcia and comparing them with EOs from different parts of the world. Hence, this study aims to broaden the knowledge about composition, antioxidant and anti-hyaluronidase properties of *Lavandula* and *Thymus* EOs.

9.4 Results and Discussion

Seven samples have been studied in this work, namely *Lavandula angustifolia, Lavandula latifolia, Lavandula hybrida* cultivar Grosso*, Lavandula hybrida* cultivar Super*, Thymus zygis* with high proportion of thymol*, Thymus zygis* with high proportion of linalool and *Thymus hyemalis.*

The EOs were obtained by hydrodistillation in yields ranging from 0.2 to 1.5% (w/w). The chemical composition (Table 9.1) of the selected EOs is shown in Table 9.2 for *Lavandula* group and Table 9.3 for *Thymus* group. The listing order corresponds to the elution order in a non-polar SLB5-ms column.

The main components present in the studied *L. angustifolia* oil were linalool, α-terpineol, linalyl acetate and (*E*)-β-caryophyllene. Similarly, samples with plant material from Italy (Angelo et al., 2014; Maietti et al., 2013), Greece (Hassiotis et al., 2014), Bosnia-Herzegovina (Nikolić et al., 2014), Pakistan (Hussain et al., 2011), France(Dohi et al., 2009) and Spain (Santana et al., 2012) show high concentration of linalool and linalyl acetate. Just one of the above mentioned studies found 0.23% of thymol in *L. angustifolia* EO (Hussain et al., 2011) (Table 9.2). Other species of the *Lavandula* genus also show small amounts of thymol among its components like *Lavandula coronopofolia* (Aburjai et al., 2005).

In the case of *L. latifolia* oil, the main components were β-pinene, 1,8-cineole, linalool, camphor, and (*E*)-α-bisabolene. Other samples from Spain where found in the literature, while 1,8-cineole, linalool and camphor were common for all the cases, β-pinene was found among the main components only in the case of Valencia (Munoz-Bertomeu et al., 2007) and Castilla La Mancha (Santana et al., 2012). The high concentrations of linalool and (*E*)-α-bisabolene found in our sample could be a peculiarity of the growing zone. Comparatively, our sample is very similar to the ones from Zaragoza (Guillén and Cabo, 1996) and the average of Spain (Herraiz-Peñalver et al., 2013).

Lavandula hybrida samples show some differences between the two cultivars, 1,8-cineole, linalool, camphor, borneol, terpinen-4-ol and linalyl acetate being the main components of *L. hybrida* cv. Grosso oil. And the most abundant compounds present in *L. hybrida* cv. Super oil were (*Z*)-β-ocimene, 1,8-cineole, (*E*)-β-ocimene, linalool, camphor and linalyl acetate. Characteristic compounds of *L. hybrida* in the majority of samples are 1,8-cineole, linalool, camphor and linalyl acetate.

Reports from Spain (Castilla La Mancha) (Santana et al., 2012) show similar concentration of borneol to the studied sample, as well as the French report (Bombarda et al., 2008) which also shows terpinen-4-ol among the main components. The report from Turkey (Andoğan et al., 2002) shows ocimene like the studied samples, but the

Table 9.1
Standards used in GC/MS and calibration curves for EOs analysis

Analyte	Calibration curve*	R^2	Calibration range (mM)	RSD (%)	LOD (mM)	LOQ (mM)	Standard source	Product Reference
Nonane	*Internal standard*						SAFC	442694
α-Thujene	y = 0.566x - 0.045	0.997	0.50 - 10.09	6.0	0.16	0.47	EML	
(–)-α-Pinene	y = 0.471x - 0.018	0.997	0.25 - 10.06	4.5	0.08	0.25	Fluka	80599
(+)-Camphene	y = 0.272x - 0.008	0.995	0.66 - 10.51	0.4	0.14	0.44	SAFC	w222909
Sabinene	y = 0.460x - 0.026	0.998	0.24 - 9.78	0.9	0.07	0.21	Extrasynthese	5062 S
(–)-β-Pinene	y = 0.400x - 0.016	0.995	0.26 - 10.29	3.7	0.09	0.26	Fluka	80609
3-Octanone	y = 0.309x - 0.031	0.996	2.56 - 10.24	4.1	0.64	1.95	SAFC	w280305
Myrcene	y = 0.188x - 0.024	0.993	2.24 - 8.97	3.5	0.56	1.70	Fluka	64643
Hexyl acetate	y = 0.452x - 0.057	0.992	2.40 - 9.59	1.7	0.32	0.96	Fluka	25539
α-Phellandrene	y = 0.430x - 0.012	0.998	0.50 - 9.52	0.9	0.16	0.49	Aldrich	77429
α-Terpinene	y = 0.425x - 0.035	0.997	0.45 - 9.45	2.8	0.15	0.44	Aldrich	86473
p-Cymene	y = 0.830x - 0.020	0.995	0.25 - 9.95	4.9	0.02	0.05	Aldrich	c121452
(+)-Limonene	y = 0.246x - 0.008	0.995	0.60 - 9.55	0.9	0.12	0.36	Fluka	62118
(*Z*)-β-Ocimene	y = 0.218x - 0.011	0.996	0.70 - 6.97	2.2	0.16	0.48	SAFC	w353901
1,8-Cineole	y = 0.233x - 0.008	0.995	2.39 - 9.55	8.7	0.60	1.82	SAFC	w246506
γ-Terpinene	y = 0.366x - 0.017	0.995	0.62 - 9.87	2.8	0.12	0.37	Aldrich	223190
(+)-*trans*-Sabinene hydrate	y = 0.268x - 0.007	0.996	0.63 - 10.00	2.1	0.18	0.56	Fluka	96573
(–)-Linalool	y = 0.214x - 0.008	0.994	0.88 - 8.80	4.9	0.22	0.67	Fluka	74856
1-Octen-3-yl acetate	y = 0.279x - 0.015	0.995	2.05 - 8.21	1.4	0.38	1.16	SAFC	w358207
(+)-Camphor	y = 0.181x - 0.024	0.994	0.99 - 9.85	1.5	0.25	0.76	Alfa Aesar	A10708
(–)-Borneol	y = 0.266x - 0.020	0.998	0.57 - 9.06	4.4	0.19	0.57	Alfa Aesar	A12684
(–)-Terpinen-4-ol	y = 0.241x - 0.003	0.997	0.60 - 9.57	4.3	0.20	0.60	Aldrich	11584
Hexyl butyrate	y = 0.332x - 0.034	0.991	4.84 - 7.74	0.6	0.77	2.34	SAFC	w256811
(+)-α-Terpineol	y = 0.175x - 0.003	0.998	1.02 - 10.23	4.5	0.26	0.79	Fluka	83073
Tetradecane	*Internal standard*						SAFC	442708
Verbenone	y = 0.183x - 0.032	0.994	1.30 - 9.88	1.8	0.42	1.28	Aldrich	218251
Citronellol	y = 0.137x - 0.014	0.998	0.90 - 8.69	9.0	0.29	0.87	SAFC	S60330
Methyl ether of carvacrol	y = 0.239x - 0.013	0.999	0.75 - 9.06	5.8	0.23	0.70	Fluka	43778
(–)-Linalyl acetate	y = 0.217x - 0.006	0.998	0.72 - 7.18	0.2	0.18	0.55	SAFC	w263605
Geraniol	y = 0.338x - 0.018	0.999	0.70 - 7.18	3.8	0.22	0.66	SAFC	w250716
Geranial	y = 0.372x - 0.022	0.994	0.85 - 5.59	11.7	0.28	0.85	SAFC	w230316
Bornyl acetate	y = 0.317x - 0.029	0.997	0.65 - 8.16	5.5	0.20	0.61	Fluka	45855
Thymol	y = 0.313x - 0.029	0.998	0.25 - 10.03	4.8	0.08	0.25	Sigma	T0501
Carvacrol	y = 1.069x - 0.118	0.997	0.65 - 10.35	1.3	0.21	0.65	SAFC	w224502
Neryl acetate	y = 0.242x - 0.007	0.999	0.73 - 7.31	3.8	0.18	0.55	SAFC	w277304
Geranyl acetate	y = 0.249x - 0.009	0.999	0.46 - 7.42	2.9	0.15	0.46	Aldrich	173495
Hexadecane	*Internal standard*						Fluka	52209
(–)-(*E*)-β-Caryophyllene	y = 0.079x - 0.002	0.998	4.41 - 7.05	6.6	0.71	2.16	Sigma	22075
α-Humulene	y = 0.541x - 0.019	0.999	0.40 - 6.35	2.2	0.13	0.40	Aldrich	53675
(*E*)-α-Bisabolene	y = 0.111x - 0.043	0.999	1.74 - 6.97	3.4	0.31	0.94	Alfa Aesar	A18724
(–)-Caryophyllene oxide	y = 0.105x - 0.004	0.999	2.54 - 10.17	2.7	0.46	1.40	SAFC	w509647

*Response ratio vs. Concentration ratio, internal standard correction applied.
Each internal standard is reference compound for the analytes that follow.

reports from Italy (Maietti et al., 2013) and Greece (Papachristos et al., 2004) show different main compounds from the samples studied.

The main components of the *Lavandula* group were oxygenated monoterpenes, mainly alcohol (linalool), ester (linalyl acetate) and ether (1,8-cineole). *L. angustifolia* showed the best yield in linalool production whereas both cultivars of *L. hybrida*

showed the highest yields for linalyl acetate and terpinen-4-ol. The lowest yield of linalyl acetate and the highest of camphor and 1,8-cineole among the *Lavandula* group was found in *L. latifolia*. The high content of camphor found in *L. latifolia* and *L. hybrida* seems related to the content of sesquiterpenes of caryophyllene-type when compared to the low content of camphor shown in *L. angustifolia*, *i.e.* high concentration of (*E*)-β-caryophyllene shows relation to low concentrations of camphor (Cavanagh and Wilkinson, 2002; Santana et al., 2012).

T. zygis chem. thymol EO is the most studied case in the literature. In the studied sample, the main components were myrcene, α-terpinene, p-cymene, γ-terpinene, linalool, thymol and carvacrol. p-Cymene was found in all reported cases. Thymol and γ-terpinene were found among the main components in all reported cases except one from Portugal (Dandlen et al., 2010) and another from Almería (Spain) (Saez, 1995b). Portuguese reports (Gonçalves et al., 2010; Pina-Vaz et al., 2004) are the only ones showing similar concentrations to the studied sample regarding myrcene and α-terpinene. The report from Jaén (Spain) (Ballester-Costa et al., 2013) has similar concentration of thymol. The studied sample is very similar to the ones from Jaén (Spain) (Ballester-Costa et al., 2013), Córdoba (Spain) (Penalver et al., 2005), Mirandela (Portugal) (Pina-Vaz et al., 2004), and central Portugal (Gonçalves et al., 2010), and similar to the ones from the north of Portugal (Moldão-Martins et al., 2002; Moldao-Martins et al., 1999).

In the case of the *T. hyemalis* chem. cineole EO, the main compounds were α-pinene, camphene, β-pinene, p-cymene, 1,8-cineole, linalool, camphor, borneol, terpinen-4-ol, α-terpineol and geraniol. p-Cymene and borneol are present in all studied literature, whereas β-pinene, terpinen-4-ol, α-terpineol and geraniol are just present among the main components in the studied sample. Just Spanish experimental crops samples (Rota et al., 2008) and the studied sample show linalool as a main component of the EO. Regarding Spanish samples, the one from Almería (Saez, 1995a) is the most similar to the studied sample, and the ones from designed experimental crops (Jordan et al., 2006; Rota et al., 2008) are also similar to the studied sample. The Turkish sample (Tepe et al., 2011) is the most different sample from the ones reported here.

The main components present in *T. zygis* with high proportion of linalool EO were α-pinene, myrcene, α-terpinene, p-cymene, limonene, γ-terpinene, *trans*-sabinene hydrate, linalool and terpinen-4-ol. Myrcene, γ-terpinene, linalool and terpinen-4-ol were present in most of the reported samples in the literature, establishing, thus, the main common components. High similitude was found between the studied sample, the Spanish sample from Jaén (Ballester-Costa et al., 2013) and the experimental crops sample (Rota et al., 2008), higher difference can be found to the Spanish sample from Almería (Saez, 1995a) and to the central Portugal sample (Gonçalves et al., 2010).

The main components in the case of *Thymus* group (Table 9.3) were oxygenated monoterpenes, mainly alcohols (thymol, linalool, terpinen-4-ol) and ether (1,8-cineole), and monoterpene hydrocarbons, γ-terpinene, p-cymene, α-pinene and β-myrcene showing the highest concentrations.

Table 9.2. GC/MS determination and quantification of *Lavandula* sp. EOs.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	L. angustifolia Concentration (mM ± SD)	L. angustifolia Area (% ± SD)	L. latifolia Concentration (mM ± SD)	L. latifolia Area (% ± SD)	L. hybrida cv. Grosso Concentration (mM ± SD)	L. hybrida cv. Grosso Area (% ± SD)	L. hybrida cv. Super Concentration (mM ± SD)	L. hybrida cv. Super Area (% ± SD)	Identification methods
852	874	n-Hexanol	43, 56, 69, 82				0.1 ± 0.0		0.2 ± 0.0			LRI, MS
922	929	Tricyclene	79, 93, 121, 136		0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0			LRI, MS
930	939	α-Pinene	39, 91, **93**, 77	3.2 ± 0.4	0.1 ± 0.0	96.7 ± 5.4	1.7 ± 0.0	14.5 ± 0.7	0.3 ± 0.0	31.9 ± 2.7	0.6 ± 0.0	LRI, MS, std
943	957	Camphene	67, 79, **93**, 121	17.4 ± 2.0	0.3 ± 0.0	38.8 ± 1.4	0.5 ± 0.0	12.7 ± 0.5	0.2 ± 0.0	20.9 ± 1.6	0.4 ± 0.0	LRI, MS, std
964	978	Sabinene	41, 77, 91, **93**			33.3 ± 1.7	0.6 ± 0.0				0.1 ± 0.0	LRI, MS, std
972	985	β-Pinene	41, 69, 79, **93**	5.0 ± 0.1	0.2 ± 0.0	120.5 ± 6.3	2.1 ± 0.0	12.0 ± 0.3	0.5 ± 0.0	27.3 ± 1.4	0.4 ± 0.0	LRI, MS, std
963	989	3-Octanone	**43**, 57, 71, 99	74.1 ± 3.1	0.5 ± 0.0					41.2 ± 2.0	0.3 ± 0.0	LRI, MS, std
981	992	Myrcene	39, **41**, 69, 93	92.4 ± 2.7	1.1 ± 0.0	61.9 ± 2.4	0.5 ± 0.0	56.3 ± 3.1	0.5 ± 0.0	77.5 ± 7.7	1.1 ± 0.0	LRI, MS, std
985	1002	3-Octanol	41, 59, 83, 101		0.1 ± 0.0						0.1 ± 0.0	LRI, MS
1008	1011	Hexyl acetate	**43**, 56, 84, 61	12.5 ± 0.1	0.2 ± 0.0			14.2 ± 0.8	0.1 ± 0.0	44.1 ± 1.4	0.5 ± 0.0	LRI, MS, std
1005	1014	3-Carene	77, 79, 91, **93**		0.1 ± 0.0						0.1 ± 0.0	LRI, MS
1011	1022	p-Cymene	77, 91, **119**, 134	4.0 ± 0.5	0.2 ± 0.0	6.9 ± 0.2	0.1 ± 0.0	6.0 ± 0.1	0.1 ± 0.0		0.1 ± 0.0	LRI, MS, std
1025	1026	Limonene	67, **68**, 79, 93	11.8 ± 1.7	0.5 ± 0.0	66.5 ± 7.7	1.1 ± 0.0	38.1 ± 1.0	0.6 ± 0.0	72.0 ± 1.7	1.0 ± 0.0	LRI, MS, std
1023	1030	1,8-Cineole	**43**, 67, 81, 93		0.0	1369.9 ± 25.5	29.7 ± 0.2	167.3 ± 5.6	3.6 ± 0.0	455.6 ± 22.9	7.9 ± 0.2	LRI, MS, std
1024	1034	(*Z*)-β-Ocimene	41, 79, 91, **93**	105.5 ± 2.7	0.9 ± 0.0			52.3 ± 2.8	0.6 ± 0.0	220.0 ± 6.7	2.5 ± 0.0	LRI, MS, std
1034	1045	(*E*)-β-Ocimene	41, 79, 93, 121		0.9 ± 0.0		0.1 ± 0.0		0.3 ± 0.0		2.6 ± 0.0	LRI, MS
1047	1050	γ-Terpinene	77, 91, **93**, 136			16.3 ± 0.9	0.2 ± 0.0	11.1 ± 0.1	0.2 ± 0.0	12.2 ± 0.2	0.1 ± 0.0	LRI, MS, std
1054	1055	*trans*-Sabinene hydrate	77, 91, **93**, 121		tr	13.7 ± 0.8	0.2 ± 0.0	8.5 ± 0.2	0.3 ± 0.0		0.1 ± 0.0	LRI, MS, std
1072	1070	*cis*-Linalool oxide (furanoid)	43, 59, 68, 111		0.4 ± 0.0							LRI, MS
1078	1083	Terpinolene	93, 105, 121, 136		0.2 ± 0.0		0.2 ± 0.0		0.2 ± 0.0		0.4 ± 0.0	LRI, MS
1087	1088	*trans*-Linalool oxide (furanoid)	43, 59, 68, 111		0.3 ± 0.0							LRI, MS
1103	1108	Linalool	41, 55, 69, **93**	5788.9 ± 69.0	55.6 ± 0.2	2421.1 ± 19.4	41.5 ± 0.1	2368.3 ± 41.7	41.7 ± 0.2	3205.1 ± 0.7	37.3 ± 0.1	LRI, MS, std
1097	1111	1-Octen-3-yl acetate	43, **54**, 67, 81	124.9 ± 0.2	0.4 ± 0.0							LRI, MS, std
1130	1133	allo-Ocimene	91, 105, 121, 136								0.2 ± 0.0	LRI, MS
1132	1152	Hexyl isobutyrate	43, 56, 71, 89				0.1 ± 0.0		0.1 ± 0.0		0.2 ± 0.0	LRI, MS
1142	1159	Camphor	41, 81, **95**, 108	91.6 ± 4.4	0.5 ± 0.0	881.2 ± 5.6	11.6 ± 0.2	683.1 ± 10.2	6.2 ± 0.1	385.9 ± 9.7	5.9 ± 0.0	LRI, MS, std

Table 9.2. Continued.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	L. angustifolia Concentration (mM ± SD)	L. angustifolia Area (% ± SD)	L. latifolia Concentration (mM ± SD)	L. latifolia Area (% ± SD)	L. hybrida cv. Grosso Concentration (mM ± SD)	L. hybrida cv. Grosso Area (% ± SD)	L. hybrida cv. Super Concentration (mM ± SD)	L. hybrida cv. Super Area (% ± SD)	Identification methods
1170	1172	Lavandulol	41, 69, 111, 123		0.1 ± 0.0				0.7 ± 0.0		0.1 ± 0.0	LRI, MS
1174	1186	Borneol	41, 93, **95**, 121	181.8 ± 12.5	1.7 ± 0.0	74.0 ± 0.7	0.8 ± 0.0	396.8 ± 8.4	2.7 ± 0.0	115.3 ± 2.7	1.7 ± 0.0	LRI, MS, std
1175	1189	Terpinen-4-ol	71, 77, 91, **93**	27.6 ± 0.1	0.1 ± 0.0	23.9 ± 2.8	0.3 ± 0.0	359.7 ± 12.7	5.3 ± 0.0	60.0 ± 5.1	1.6 ± 0.0	LRI, MS, std
1176	1193	Hexyl butyrate	**43**, 56, 71, 89		0.2 ± 0.0	11.9 ± 0.4	0.1 ± 0.0	27.6 ± 1.5	0.5 ± 0.0	49.0 ± 1.2	0.9 ± 0.0	LRI, MS, std
1192	1206	α-Terpineol	59, 68, 79, **93**	194.0 ± 2.7	2.7 ± 0.0	70.1 ± 1.1	1.0 ± 0.0	64.1 ± 0.9	1.0 ± 0.0	80.6 ± 2.1	1.4 ± 0.0	LRI, MS, std
1232	1234	Hexyl 2-methylbutyrate	41, 57, 85, 103				0.1 ± 0.0				0.2 ± 0.0	LRI, MS
1235	1239	Hexyl valerate	43, 56, 85, 103				0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1232	1249	Geraniol	41, 69, 93, 121						0.5 ± 0.0		0.8 ± 0.1	LRI, MS
1236	1255	Linalyl acetate	41, 69, **93**, 121	1239.0 ± 2.9	22.8 ± 0.1	6.4 ± 0.1	0.1 ± 0.0	1569.9 ± 30.3	25.8 ± 0.1	1501.2 ± 22.2	25.7 ± 0.1	LRI, MS, std
1266	1284	Thymol	91, 115, **135**, 150		tr							LRI, MS, std
1272	1309	Lavandulyl acetate	43, 69, 93, 121		0.9 ± 0.0				1.9 ± 0.0		1.0 ± 0.0	LRI, MS
1342	1362	Neryl acetate	41, 69, 79, **93**	45.9 ± 0.1	0.6 ± 0.0			14.3 ± 0.1	0.3 ± 0.0	20.4 ± 0.0	0.4 ± 0.0	LRI, MS, std
1360	1380	Geranyl acetate	41, **69**, 79, 93	53.5 ± 0.1	1.1 ± 0.0			25.0 ± 0.7	0.5 ± 0.0	40.0 ± 0.5	0.7 ± 0.1	LRI, MS, std
1386	1387	β-Bourbonene	81, 123, 161, 189				0.1 ± 0.0					LRI, MS
1405	1405	α-Cedrene	93, 105, 119, 204				0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1412	1414	α-Gurjunene	105, 119, 189, 204				0.0 ± 0.0					LRI, MS
1421	1430	(*E*)-β-Caryophyllene	41, 69, 79, **93**	68.2 ± 2.4	3.3 ± 0.0	274.9 ± 3.7	1.5 ± 0.0	99.0 ± 1.5	1.2 ± 0.0	81.3 ± 0.2	1.3 ± 0.0	LRI, MS, std
1427	1441	α-Bergamotene	41, 69, 93, 119		0.7 ± 0.0		0.2 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1448	1461	β-Farnesene	41, 69, 93, 133		1.4 ± 0.1		0.3 ± 0.0		1.2 ± 0.0		0.6 ± 0.0	LRI, MS
1454	1469	α-Humulene	41, 67, 80, **93**			4.9 ± 0.0	0.1 ± 0.0	5.2 ± 0.2	0.1 ± 0.0	3.6 ± 0.0	0.1 ± 0.0	LRI, MS, std
1471	1489	γ-Muurolene	105, 119, 161, 204						0.5 ± 0.0		0.3 ± 0.0	LRI, MS
1490	1490	Germacrene D	105, 119, 161, 204		0.1 ± 0.0		0.7 ± 0.0					LRI, MS
1494	1504	(*Z*)-α-Bisabolene	41, 79, 93, 161				0.1 ± 0.0		0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1500	1507	α-Selinene	161, 175, 189, 204				0.1 ± 0.0		0.2 ± 0.0		0.1 ± 0.0	LRI, MS

Table 9.2. Continued.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	L. angustifolia Concentration (mM ± SD)	L. angustifolia Area (% ± SD)	L. latifolia Concentration (mM ± SD)	L. latifolia Area (% ± SD)	L. hybrida cv. Grosso Concentration (mM ± SD)	L. hybrida cv. Grosso Area (% ± SD)	L. hybrida cv. Super Concentration (mM ± SD)	L. hybrida cv. Super Area (% ± SD)	Identification methods
1500	1514	β-Bisabolene	41, 69, 93, 204				0.2 ± 0.0		0.1 ± 0.0			LRI, MS
1516	1532	β-Sesquiphellandrene	41, 69, 93, 161						0.1 ± 0.0			LRI, MS
1534	1549	(*E*)-α-Bisabolene	41, 79, **93**, 119			385.4 ± 26.2	2.3 ± 0.1					LRI, MS, std
1581	1597	Caryophyllene oxide	41, 79, **91**, 105	21.5 ± 0.1	0.3 ± 0.0	9.5 ± 0.1	0.1 ± 0.0					LRI, MS, std
1646	1658	δ-Cadinol	105, 119, 161, 204				0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1665	1695	α-Bisabolol/epi-α-bisabolol	41, 69, 93, 119						0.3 ± 0.0		0.3 ± 0.0	LRI, MS
		Monoterpene hydrocarbons			4.6		7.2		3.6		9.6	
		Oxygenated monoterpenes			86.7		85.2		90.5		84.6	
		Sesquiterpene hydrocarbons			5.4		5.7		3.7		2.6	
		Oxygenated sesquiterpenes			0.3		0.2		0.4		0.3	

LRI = Linear Retention Index obtained from the homologous series of n-alkanes (C7-C30). MS = tentatively identified by NIST 08 & Wiley 7. std = identified by comparison with reference standards. tr = traces (< 0,1%). SD = standard deviation. Results are expressed as mean ± standard deviation of three determinations. *Quantitation ions are shown in bold.
LRI (Lit.) obtained from NIST08 database. LRI (Exp.) obtained experimentally.

Table 9.3. GC/MS determination and quantification of *Thymus* sp. EOs.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	T. zygis high thymol Concentration (mM ± SD)	T. zygis high thymol Area (% ± SD)	T. hyemalis Concentration (mM ± SD)	T. hyemalis Area (% ± SD)	T. zygis high linalool Concentration (mM ± SD)	T. zygis high linalool Area (% ± SD)	Identification methods
928	929	α-Thujene	39, 77, **93**, 136	109.2 ± 6.0	1.7 ± 0.0	53.1 ± 2.8	0.9 ± 0.0	22.0 ± 1.3	0.3 ± 0.0	LRI, MS, std
930	938	α-Pinene	77, **93**, 105, 121	73.7 ± 5.2	1.3 ± 0.1	315.4 ± 16.1	7.7 ± 0.0	167.4 ± 10.1	3.9 ± 0.0	LRI, MS, std
943	955	Camphene	79, **93**, 107, 121	52.1 ± 3.3	0.6 ± 0.0	266.8 ± 11.1	4.3 ± 0.0	82.8 ± 3.6	1.0 ± 0.0	LRI, MS, std
964	976	Sabinene	41, 77, 91, 93				1.5 ± 0.0		0.9 ± 0.0	LRI, MS
972	982	β-Pinene	69, 77, **93**, 121	12.3 ± 0.8	0.3 ± 0.0	114.9 ± 3.4	2.8 ± 0.0	16.7 ± 0.7	0.4 ± 0.0	LRI, MS, std
963	985	3-Octanone	43, 57, 71, 99		0.1 ± 0.0				0.1 ± 0.0	LRI, MS
981	989	Myrcene	41, 69, 79, **93**	195.0 ± 10.6	2.3 ± 0.1	68.0 ± 2.0	0.9 ± 0.0	556.0 ± 9.8	6.5 ± 0.0	LRI, MS, std
985	1002	3-Octanol	41, 59, 83, 101				0.1 ± 0.0		0.1 ± 0.0	LRI, MS
997	1010	α-Phellandrene	77, **93**, 119, 136	23.1 ± 0.5	0.3 ± 0.0			33.3 ± 1.4	0.4 ± 0.0	LRI, MS, std
1005	1012	3-Carene	77, 79, 91, 93		0.1 ± 0.0					LRI, MS
1008	1019	α-Terpinene	77, **93**, 121, 136	106.4 ± 5.6	2.1 ± 0.1	33.6 ± 1.3	0.7 ± 0.0	410.4 ± 13.0	3.7 ± 0.0	LRI, MS, std
1011	1022	p-Cymene	91, 117, **119**, 134	834.5 ± 14.9	18.7 ± 0.5	252.5 ± 6.2	6.0 ± 0.1	120.1 ± 5.2	2.4 ± 0.0	LRI, MS, std
1025	1026	Limonene	**68**, 79, 93, 121	39.6 ± 0.9	0.5 ± 0.0	87.5 ± 6.6	1.2 ± 0.2	234.6 ± 11.6	3.0 ± 0.0	LRI, MS, std
1023	1030	1,8-Cineole	**43**, 81, 93, 108	12.1 ± 0.5	0.3 ± 0.0	1267.4 ± 34.0	35.8 ± 0.2	6.7 ± 0.3	0.2 ± 0.0	LRI, MS, std
1024	1034	(*Z*)-β-Ocimene	41, 79, **93**, 105	33.2 ± 0.9	0.2 ± 0.0	70.7 ± 0.6	1.1 ± 0.1	94.9 ± 4.6	0.7 ± 0.0	LRI, MS, std
1034	1045	(*E*)-β-Ocimene	41, 79, 93, 105		0.1 ± 0.0	40.4 ± 0.8	0.5 ± 0.0		0.1 ± 0.0	LRI, MS
1047	1051	γ-Terpinene	77, **93**, 121, 136	594.6 ± 4.3	11.4 ± 0.4	76.0 ± 2. 4	1.4 ± 0.0	394.2 ± 15.4	6.9 ± 0.1	LRI, MS, std
1054	1055	*trans*-Sabinene hydrate	77, **93**, 121, 136	29.8 ± 0.5	0.4 ± 0.0	76.8 ± 1.1	1.2 ± 0.0	128.6 ± 5.0	2.1 ± 0.0	LRI, MS, std
1072	1070	*cis*-Lilalool oxide (furanoid)	43, 59, 68, 111						0.1 ± 0.0	LRI, MS
1078	1083	Terpinolene	93, 105, 121, 136		0.1 ± 0.0		0.3 ± 0.0		1.4 ± 0.0	LRI, MS
1087	1088	*trans*-Lilalool oxide (furanoid)	43, 59, 68, 111						0.3 ± 0.0	LRI, MS
1103	1105	Linalool	41, 69, **93**, 121	207.9 ± 6.9	3.7 ± 0.0	297.2 ± 6.9	3.8 ± 0.0	2001.8 ± 44.9	44.4 ± 0.1	LRI, MS, std
1071	1110	β-Terpinene	77, 93, 121, 136		0.1 ± 0.0		0.8 ± 0.0		0.4 ± 0.0	LRI, MS
1132	1152	Hexyl isobutyrate	43, 56, 71, 89				0.5 ± 0.0		0.3 ± 0.0	LRI, MS

Table 9.3. Continued.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	T. zygis high thymol Concentration (mM ± SD)	T. zygis high thymol Area (% ± SD)	T. hyemalis Concentration (mM ± SD)	T. hyemalis Area (% ± SD)	T. zygis high linalool Concentration (mM ± SD)	T. zygis high linalool Area (% ± SD)	Identification methods
1142	1157	Camphor	81, **95**, 108, 152	27.2 ± 0.8	0.2 ± 0.0	185.0 ± 4.5	3.3 ± 0.0	20.6 ± 0.3	0.2 ± 0.0	LRI, MS, std
1170	1179	Lavandulol	41, 69, 111, 123				0.6 ± 0.0			LRI, MS
1174	1181	Borneol	41, **95**, 110, 121	66.8 ± 1.4	0.9 ± 0.0	484.8 ± 16.8	4.7 ± 0.2	140.3 ± 5.0	1.9 ± 0.0	LRI, MS, std
1175	1187	Terpinen-4-ol	71, **93**, 121, 136	26.6 ± 0.7	0.8 ± 0.0	99.0 ± 1.9	3.8 ± 0.0	381.5 ± 17.2	12.2 ± 0.0	LRI, MS, std
1192	1202	α-Terpineol	68, **93**, 121, 136	7.0 ± 0.4	0.1 ± 0.0	280.7 ± 22.9	3.7 ± 0.0	111.0 ± 6.8	1.6 ± 0.1	LRI, MS, std
1206	1210	*trans*-Dihydrocarvone	67, 95, 109, 152						0.2 ± 0.0	LRI, MS
1204	1214	Verbenone	91, **107**, 135, 150			50.8 ± 1.3	0.7 ± 0.0	12.3 ± 0.2	0.1 ± 0.0	LRI, MS, std
1206	1224	*trans*-Carveol	91, 105, 119, 134						0.1 ± 0.0	LRI, MS
1208	1230	Citronellol	41, **69**, 81, 95			32.0 ± 1.4	0.3 ± 0.0	8.0 ± 0.4	0.1 ± 0.0	LRI, MS, std
1231	1239	Methyl ether of carvacrol	77, **91**, 117, 134	13.6 ± 0.4	0.2 ± 0.0	42.8 ± 1.5	0.7 ± 0.0	6.3 ± 0.9	0.1 ± 0.0	LRI, MS, std
1232	1246	Geraniol	41, 69, 79, **93**			130.1 ± 2.7	3.0 ± 0.0	8.3 ± 0.1	0.1 ± 0.0	LRI, MS, std
1236	1256	Linalyl acetate	**41**, 69, 93, 121			15.3 ± 0.8	0.2 ± 0.0	44.1 ± 1.1	0.7 ± 0.0	LRI, MS, std
1245	1271	Geranial	41, **69**, 77, 91			64.7 ± 2.7	0.8 ± 0.0		tr	LRI, MS, std
1266	1283	Thymol	91, 115, **135**, 150	3148.8 ± 10.5	48.2 ± 0.9	13.8 ± 0.2	0.1 ± 0.0	16.8 ± 0.9	0.1 ± 0.0	LRI, MS, std
1272	1288	Lavandulyl acetate	43, 69, 93, 121				0.1 ± 0.0		0.1 ± 0.0	LRI, MS
1275	1309	Carvacrol	77, 91, **135**, 150	136.2 ± 5.6	3.2 ± 0.2	72.3 ± 3.0	1.9 ± 0.1		tr	LRI, MS, std
1285	1315	Bornyl acetate	43, **95**, 121, 136			36.1 ± 1.6	0.8 ± 0.0	8.1 ± 0.8	0.1 ± 0.0	LRI, MS, std
1350	1350	α-Cubebene	105, 119, 161, 204				0.2 ± 0.0			LRI, MS
1360	1379	Geranyl Acetate	41, 69, **93**, 121			53.4 ± 1.1	1.2 ± 0.0		tr	LRI, MS, std
1421	1427	(*E*)-β-Caryophyllene	41, 79, **93**, 133	37.2 ± 1.1	1.6 ± 0.0	17.8 ± 0.4	0.8 ± 0.0	23.2 ± 1.2	1.0 ± 0.0	LRI, MS, std
1445	1447	Aromadendrene	91, 133, 161, 204		0.1 ± 0.0					LRI, MS
1454	1465	α-Humulene	80, 93, 121, 147		0.1 ± 0.0		0.1 ± 0.0		tr	LRI, MS, std
1492	1496	Ledene	107, 135, 171, 204		0.1 ± 0.0		0.2 ± 0.0			LRI, MS

Table 9.3. Continued.

LRI (Lit.)	LRI (Exp.)	Analyte (X)	Qualifying and quantitation ions* (m/z)	T. zygis high thymol Concentration (mM ± SD)	T. zygis high thymol Area (% ± SD)	T. hyemalis Concentration (mM ± SD)	T. hyemalis Area (% ± SD)	T. zygis high linalool Concentration (mM ± SD)	T. zygis high linalool Area (% ± SD)	Identification methods
1514	1527	δ-Cadinene	134, 161, 189, 204		0.1 ± 0.0		0.1 ± 0.0			LRI, MS
1579	1562	Sesquisabinene hydrate	69, 119, 161, 207				0.1 ± 0.0			LRI, MS
1581	1594	Caryophyllene oxide	41, 79, **91**, 105	15.0 ± 1.9	0.1 ± 0.0	22.8 ± 0.7	0.2 ± 0.0	10.9 ± 1.2	tr	LRI, MS, std
1624	1647	Z-α-Copaene-8-ol	119, 132, 145, 159				0.1 ± 0.0			LRI, MS
		Monoterpene hydrocarbons			39.8		30.1		32.0	
		Oxygenated monoterpenes			58.0		66.7		64.7	
		Sesquiterpene hydrocarbons			2.0		1.4		1.0	
		Oxygenated sesquiterpenes			0.1		0.4		0.0	

LRI = Linear Retention Index obtained using the homologous series of n-alkanes (C7-C30). SD = standard deviation. MS = tentatively identified by NIST 08 & Wiley 7.
std = identified by comparison with reference standards. tr = traces (< 0,1%). Results are expressed as mean ± standard deviation of three determinations.
*Quantitation ions are shown in bold. LRI (Lit.) obtained from NIST08 database. LRI (Exp.) obtained experimentally.

Table 9.4. Chiral determination of *Lavandula* and *Thymus* sp. EOs.

t_R (min)			*L. angustifolia*		*L. latifolia*		*L. hybrida cv.* Grosso		*L. hybrida cv.* Super		*T. zygis* high thymol		*T. hyemalis*		*T. zygis* high linalool	
			(+) - [X]	(-) - [X]	(+) - [X]	(-) - [X]	(+) - [X]	(-) - [X]	(+) - [X]	(-) - [X]	(+) – [X]	(-) – [X]	(+) – [X]	(-) – [X]	(+) – [X]	(-) – [X]
(+) - X	(-) - X	Analyte (X)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)	(%)
6.20	6.13	α-Thujene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	39	61	N/A	N/A	N/A	N/A
7.79	7.52	α-Pinene	95	5	95	5	95	5	95	5	95	5	81	19	82	18
8.47	8.24	Camphene	5	95	27	73	21	79	5	95	N/A	N/A	5	95	N/A	N/A
8.89	9.16	β-Pinene	95	5	65	35	95	5	95	5	N/A	N/A	47	53	N/A	N/A
10.36	9.39	α-Phellandrene	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5	95	N/A	N/A	N/A	N/A
10.52	10.00	Limonene	95	5	95	5	95	5	95	5	95	5	95	5	95	5
14.28	14.51	*trans*-Sabinene hydrate	95	5	95	5	95	5	95	5	95	5	95	5	95	5
15.73	15.57	Linalool	4	96	5	95	5	95	4	96	5	95	5	95	1	99
16.72	16.46	Camphor	95	5	95	5	95	5	95	5	5	95	5	95	5	95
-	17.25	Linalyl acetate	5	95	5	95	5	95	5	95	N/A	N/A	N/A	N/A	N/A	N/A
18.02	18.18	Bornyl acetate	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	5	95	N/A	N/A
18.32	18.51	Terpinen-4-ol	95	5	95	5	95	5	95	5	95	5	62	38	73	27
20.10	19.76	α-Terpineol	95	5	76	24	87	13	63	37	N/A	N/A	62	38	95	5
20.15	19.58	Borneol	61	39	60	40	95	5	95	5	5	95	5	95	5	95
23.92	22.81	(*E*)-β-Caryophyllene	5	95	5	95	5	95	5	95	5	95	5	95	5	95
-	28.81	Caryophyllene oxide	5	95	5	95	N/A	N/A	5	95	N/A	N/A	5	95	N/A	N/A

N/A = Not assessed

Table 9.5. Results of the antioxidant and the hyaluronidase inhibition assays for EOs.

Sample	IC_{50} (mg/ml)				TEAC (µmol TE/g EO)			Inhibition degree (%)*
	Chelating power	Hydroxyl	Nitric oxide	TBARS	ABTS	DPPH	ORAC	Hyaluronidase
Lavandula angustifolia	0.44b ± 0.09	0.206c ± 0.003	11b ± 2	0.29c ± 0.02	103.0b ± 0.7	1.35b ± 0.02	1984a ± 106	11c ± 3
Lavandula latifolia	0.62a ± 0.04	0.27c ± 0.05	24a ± 4	1.50b ± 0.06	1.51d ± 0.01	0.42c ± 0.01	1272d,e ± 74	6c,d ± 2
Lavandula hybrida cv. Grosso	0.08d ± 0.01	0.30b,c ± 0.02	N/A	2.5a ± 0.3	1.13d ± 0.03	0.04d ± 0.01	1407c,d ± 90	0d ± 0
Lavandula hybrida cv. Super	0.07d ± 0.01	0.27c ± 0.04	19a ± 4	1.3b ± 0.2	1.62d ± 0.03	0.216c,d ± 0.003	1388c,d ± 60	0d ± 0
Thymus zygis high thymol	N/A	0.44b ± 0.08	1.3c ± 0.1	0.074c ± 0.002	1061a ± 10	27.7a ± 0.3	1636b ± 54	100a ± 0
Thymus hyemalis	0.239c ± 0.001	0.23c ± 0.01	1.6c ± 0.1	0.4c ± 0.1	22.9c ± 0.4	0.46c ± 0.02	1087e ± 55	32b ± 7
Thymus zygis high linalool	0.35b ± 0.01	0.85a ± 0.07	9.6b ± 0.1	0.08c ± 0.03	10.6d ± 0.9	N/A	1546b,c ± 76	22b ± 6
Standard†	0.061d ± 0.001	0.001d ± 0.15	0.06c ± 0.05	0.06c ± 0.01				

*at 0,781 µlEO/ml; †Standards used: EDTA for chelating power, Mannitol for hydroxyl radical, Rutin for nitric oxide, BHT for TBARS; a, b, c, d, e different letters in the same antioxidant column mean statistically significant differences with $p < 0.05$. N/A = Not assessed.

Thymus zygis with high proportion of thymol was found richer in thymol, carvacrol and their biosynthetic precursors: γ-terpinene and p-cymene (Poulose and Croteau, 1978). *T. hyemalis* shows a high concentration of 1,8-cineole and remarkable concentration of some of the products obtained from *ad latere* geranyl pyrophosphate reactions not leading to 1,8-cineole (Croteau et al., 1994; Croteau et al., 1989). Those reactions are: geranyl pyrophosphate → geraniol → α-terpineol → terpinen-4-ol and geranyl pyrophosphate → α-pinene ↔ β-pinene → camphene → camphor → borneol. *T. zygis* with high proportion of linalool is rich in linalool and terpinen-4-ol, and it has an interesting concentration of γ-terpinene not further developed into p-cymene and thymol.

Regarding the chiral determination (Table 9.4), (R)-(–)-linalool is shown in all determinations, as reported from Turkish EOs (Aprotosoaie et al., 2014; Özek et al., 2010). The same case happens with (R)-(–)-linalyl acetate or (S)-(+)-terpinen-4-ol reported in samples from Germany (Mosandl, 2004). However, in the studied sample, all commercially available chiral compounds were examined, finding that (S)-(–)-camphene had lower concentration in the samples of *L. latifolia* and *L. hybrida* cv. Grosso among *Lavandula* group. Interestingly, (R)-(+)-β-pinene and (1R,2S)-(+)-borneol are found in lower concentration in *L. latifolia* and *L. angustifolia* respectively, *L. hybrida* being a good source of pure (1R,2S)-(+)-borneol as reported in French and Swiss samples.(Ravid et al., 1996) High variation is found in (R)-(+)-α-terpineol throughout the *Lavandula* group.

In the case of *Thymus* samples (Table 9.4), just two of the determined enantiomers, *i.e.* (S)-(+)-terpinen-4-ol and (R)-(+)-α-terpineol, show some variation in concentration. According to the reported data from Israel (Ravid et al., 1996), (1S,2R)-(–)-borneol has high purity in these *Thymus* species. Variations were found in some worldwide studies about enantiomers of *Thymus* sp. EOs (Stahl-Biskup and Sáez, 2003), in the studied samples (R)-(+)-α-pinene and (R)-(+)-limonene were found.

Thymus zygis with high proportion of thymol sample obtained the best results for almost all the antioxidant assays (Table 9.5), just three assays showed better performance for other samples, *i.e.*, both cultivars of *L. hybrida* showed the highest chelating power, and *L. angustifolia* provided the highest $OH^{\bullet}$ and $ROO^{\bullet}$ scavenging activity.

The order of the tested samples in the reducing power assay (Figure 9.1) is: *T. zygis* high thymol > *T. zygis* high linalool ≈ *T. hyemalis* > *L. hybrida* cv. Super > *L. hybrida* cv. Grosso ≈ *L. latifolia* ≈ *L. angustifolia*. Thymol may have the best reducing activity because it was determined as the main component of *T. zygis* high thymol. Positive results also agree with that reported for *T. hyemalis* from Turkey (Tepe et al., 2011).

High performance of *L. hybrida* followed by *T. hyemalis* in chelating power (Table 9.5) can be explained by the high contribution of ester and ether groups to the general composition of the EOs. The case of *L. angustifolia,* showing high values in the hydroxyl and peroxyl radical scavenging assays, is explained by the high concentration of alcohol and ester groups, *i.e.* mainly linalool and linalyl acetate, respectively.

The results for DPPH assay (Table 9.5), reveal that *L. angustifolia* has a better performance than *L. hybrida* cv. Super, in accordance with that reported for EOs from Italy.(Maietti et al., 2013) The best performances showed by *T. zygis* high thymol and *T. hyemalis* in some antioxidant methods (Table 9.5), *i.e.* nitric oxide, ABTS and DPPH

scavenging capacity, may be due to the concentrations of the common molecules thymol and carvacrol existing in those two samples.

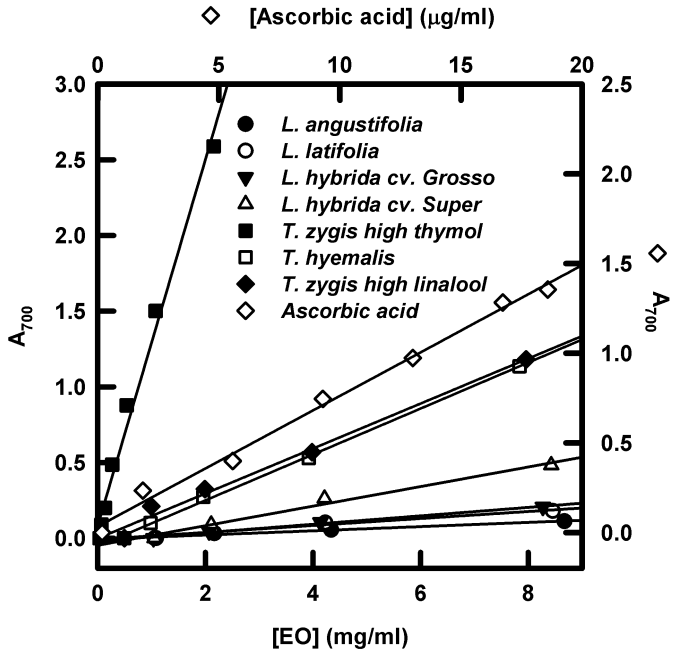

Figure 9.1
Reducing power assay
Comparative for the assayed essential oils (EOs) (bottom and left axes), and ascorbic acid as standard (top and right axes)

The phenolic molecule thymol is the main component of *T. zygis* high thymol, thus, we can attribute the antioxidant activity shown in the rest of the assays to the concentration of thymol, as already reported for TBARS in EOs from Portugal (Dandlen et al., 2010). Nevertheless, in the case of TBARS (Table 9.5), *T. zygis* high linalool, not containing thymol or carvacrol, shows a low IC_{50} as well as *L. angustifolia*, showing a good performance of the pair: linalool-linalyl acetate, in this test.

The good performance obtained using *T. zygis* high thymol in the hyaluronidase inhibition assay (Table 9.5), mainly due to thymol concentration (Table 9.3), agreed with the reported results for *Thymus* sp. EOs from Japan (Ippoushi et al., 2000). The relevant anti-hyaluronidase activity of EOs of *T. hyemalis* and *T. zygis* high linalool, as well as the weak inhibitory activity of *L. angustifolia* and *L. latifolia* EOs, could be related with their respective contents in α-pinene, camphene and α-terpineol (Tables 9.2, 9.3 and 9.5).

Therefore, the EO of *T. zygis* with high proportion of thymol could be used as hyaluronidase inhibitor to prevent the hyaluronic acid fragmentation, which has dual effects, generation of oligosaccharides with angiogenic pro-inflammatory and immunostimulatory properties; and impairment in the reservoir capacity of ECM that holds oligoelement cations, growth factors, cytokines and several enzymes for signal transduction. Thus, the EO of *T. zygis* with high proportion of thymol could aid to overcome the high number of diseases, related with the imbalance of the hyaluronic acid homeostasis(McAtee et al., 2014).

9.5 Conclusions

A sound and quantitative study of EOs of *Lavandula* sp. and *Thymus* sp. from Murcia country has been carried out. The main components of the *Lavandula* group were oxygenated monoterpenes, mainly alcohol (linalool), ester (linalyl acetate) and ether (1,8-cineole), whereas the main components in the case of *Thymus* group were oxygenated monoterpenes, mainly alcohols (thymol, linalool, terpinen-4-ol) and ether (1,8-cineole) and monoterpene hydrocarbons, with γ-terpinene, p-cymene, α-pinene and β-myrcene as the most abundant.

In *Lavandula* sp. EOs, there are high proportions of eight (+)-enantiomers, terpinen-4-ol, β-pinene, borneol and α-terpineol among them, and five (–)-enantiomers, linalool, linalyl acetate and camphene among them, those mentioned are specially relevant for their variability or purity as commented in Results and Discussion section.

In *Thymus* sp. EOs there are high proportions of five (+)-enantiomers, α-pinene, limonene, terpinen-4-ol and α-terpineol among them showing variations useful for species differentiation of the samples, and ten (–)-enantiomers borneol among them, unveiling new sources of enantiomeric pure compounds.

Linalool-linalyl acetate combination is deduced to be effective. It is found in high concentration in *Lavandula angustifolia* EO, and it showed good results when tested against hydroxyl radical, peroxyl radical and azo radicals like DPPH and $ABTS^{\bullet+}$.

Thymus zygis with high proportion of thymol obtained the best results from the antioxidant assays. The phenolic molecule thymol is the main component of *Thymus zygis* high thymol, thus it is acceptable to attribute the antioxidant activity shown in the assays to the high concentration of thymol.

The anti-hyaluronidase activity of EOs is weak in *L. angustifolia* and *L. latifolia*, relevant in *T. hyemalis* and *T. zygis* with high proportion of linalool, and high in *T. zygis* with high proportion of thymol.

The EOs of *Lavandula* sp. and *Thymus* sp., obtained from plants grown in Murcia country, have potential applications in the development of fragrances, flavours, cosmetics and drugs, they can be especially useful for treatment of diseases related with oxidative stress, extracellular matrix breakdown and hyaluronic acid homeostasis.

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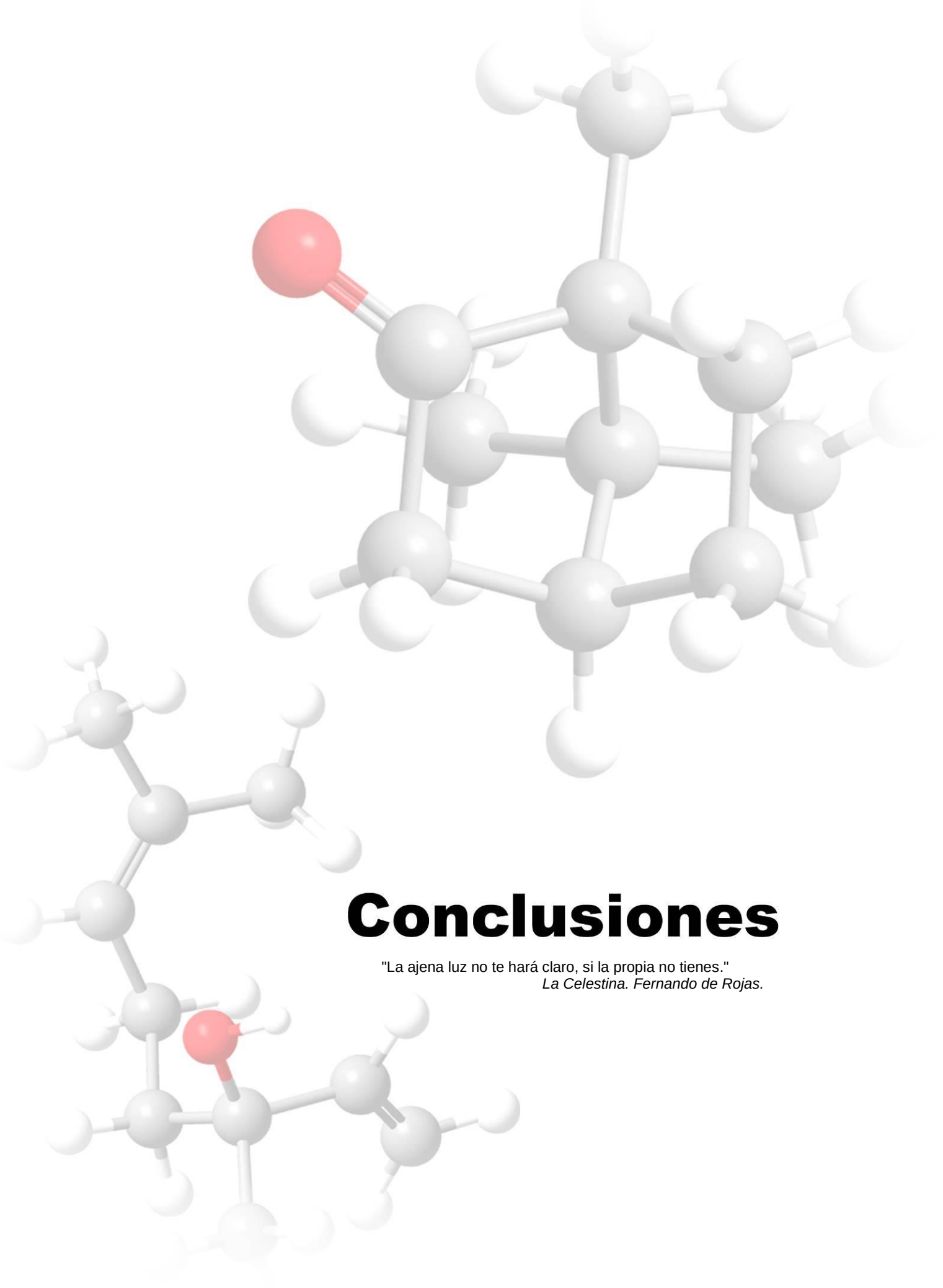


Conclusiones

"La ajena luz no te hará claro, si la propia no tienes."
La Celestina. Fernando de Rojas.

10. CONCLUSIONES

10.0 Conclusions

Apart from the conclusions already shown in each chapter of this thesis, the general and comparative conclusions can be found here:

- A method using fast gas chromatography coupled to mass spectrometry (FGC-MSD), has been optimized to analyze essential oils from different aromatic plants. The method has been successfully tested in plant from different *genus* (*Lavandula, Thymus, Thymbra, Origanum*), all belonging to *Lamiaceae* family.

- The main constituents of the essential oils from Murcia have been identified, based on retention time of commercial standards and their mass spectra, which are built in an in-lab library, and according to the spectrum available in NIST and Wiley spectral databases.

- The quantitative concentrations of the essential oil main ingredients have been determined, and their relative concentrations have been compared to worldwide literature reports, and international commercial ISO standards. The main components found were fenchone and camphor in topped lavender; linalool and linalyl acetate in lavandin and lavender; camphor, 1,8-cineole and linalool in spike lavender; thymol, γ-terpinene and p-cymene in red thyme with high thymol; γ-terpinene, terpinen-4-ol and linalool in red thyme with high linalool; 1,8-cineole in winter thyme and p-cymene, γ-terpinene and carvacrol in Spanish and Italian oreganos. The essential oils here investigated, obtained from aromatic plants grown in Murcia, have excellent compositions that support their international commerce.

- The enantiomeric distributions of the essential oil main components have been determined. The main enantiomers found are: (+)-camphor and (+)-fenchone in topped lavender; (+)-camphor, (-)-linalool and (-)-linalyl acetate in lavandin, lavender and spike lavender; (-)-linalool, (+)-limonene and (-)-borneol in red and winter thyme and (-)-linalool, (+)-limonene and (+)-terpinen-4-ol in Spanish and Italian oreganos. These enantiomeric distributions support the natural character and the absence of

adulterations, of the essential oils from Murcia here obtained and analyzed, that increase their possible commerce.

- The positive results of the different assays performed, for antioxidant capacity evaluation, encourage the potential use of essential oils as antioxidants. It has been observed that red thyme shows very high antioxidant capacity in ABTS, DPPH, RdP, TBARS and nitric oxide assay, while *Lavandula genus* shows better chelating power and better capacity to scavenge some oxygenated radicals, like the ones present in ORAC and hydroxyl assays. Both oregano species show a similar behaviour to red thyme, due to its main component, carvacrol, which is a thymol isomer. Despite the structural similarity of both isomers, thymol shows better antioxidant power.

- Important inhibitory activities on lipoxygenase and hyaluronidase have been observed in the samples. Red thyme essential oils have shown the best inhibitory results in both enzymes, mainly due to their high content of thymol. These positive *in vitro* results highlight a wide range of new uses of the essential oils, like potential application to new therapies for inflammatory diseases, or to anti-arthritic and anti-aging treatments to preserve hyaluronic acid from degradation, as well as in treatments against other diseases such as the progression of cancer.

10.1 Conclusiones

Además de las conclusiones ya presentadas en cada capítulo de este trabajo, aquí se muestran las conclusiones generales y comparativas de todas las especies tratadas en este estudio:

- Se ha optimizado un método de cromatografía de gases rápida con detección de espectrometría de masas (FGC-MSD), para el análisis de aceites esenciales de distintas plantas aromáticas, comprobando su satisfactoria aplicabilidad en plantas de diferentes géneros (*Lavandula, Thymus, Thymbra, Origanum*), de la familia *Lamiaceae*.

- Se han identificado las principales biomoléculas constituyentes de los aceites esenciales estudiados de Murcia, basándose en los tiempos de retención de patrones comerciales, y en sus respectivos espectros de masas constituyentes de la biblioteca espectral del laboratorio, concordantes con los disponibles en las bases de datos espectrales NIST y Wiley.

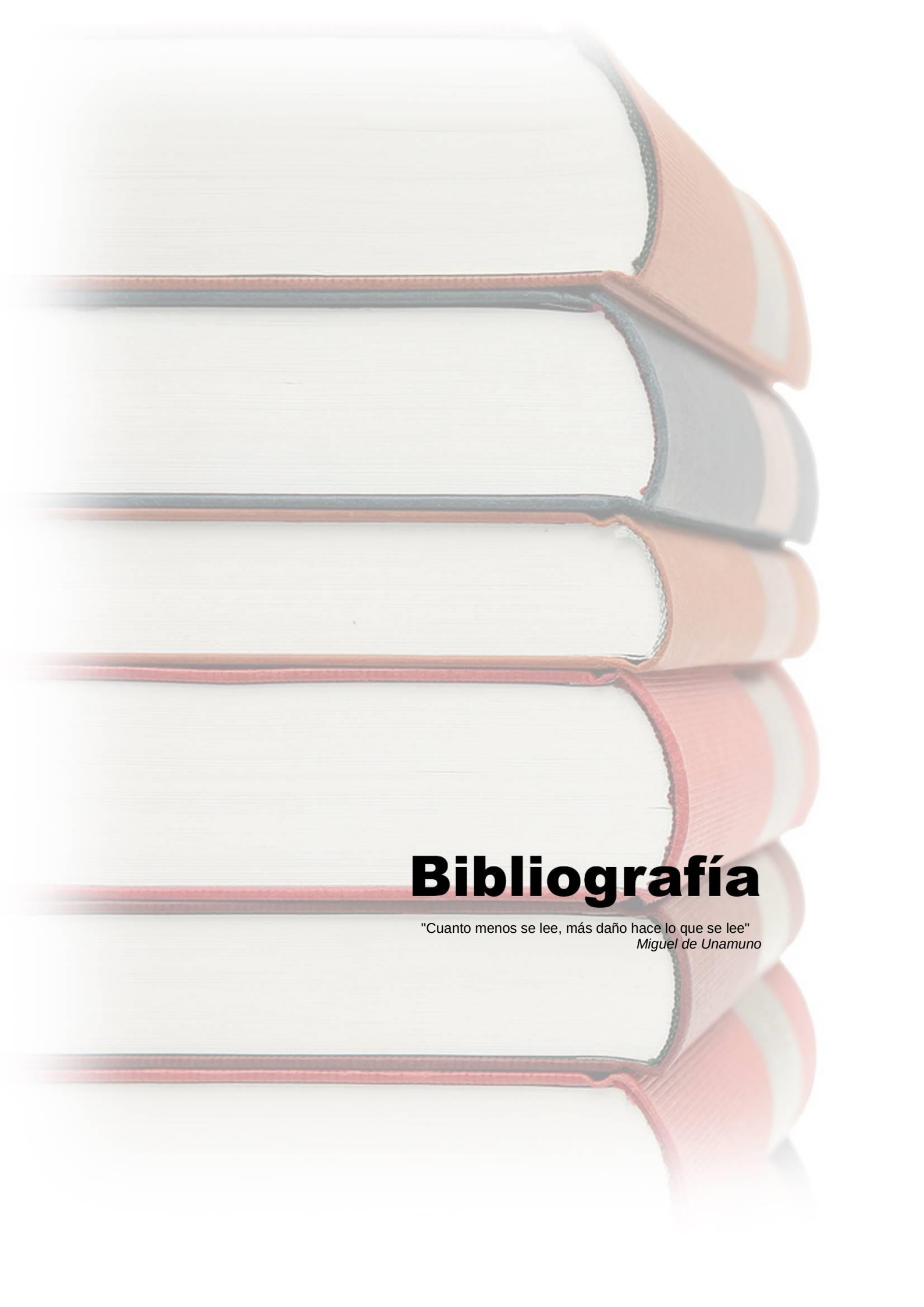
- Se han determinado cuantitativamente las concentraciones de los principales ingredientes de los aceites esenciales estudiados, contrastando sus cantidades relativas con las establecidas en las correspondientes normas comerciales internacionales ISO. Los compuestos mayoritarios determinados fueron fenchona y alcanfor en cantueso; linalol y acetato de linalilo en lavandín y lavanda; alcanfor, 1,8-cineol y linalol en espliego; timol, γ-terpineno y p-cimeno en tomillo rojo con alto timol; γ-terpineno, terpinen-4-ol y linalol en tomillo rojo con alto linalol; 1,8-cineol en tomillo de invierno, y p-cimeno, γ-terpineno y carvacrol en los orégenos español e italiano. Los aceites esenciales investigados, obtenidos a partir de plantas cultivadas en la Región de Murcia, tienen excelentes composiciones que apoyan su comercio internacional.

- Se han determinado las distribuciones enantioméricas de los principales ingredientes de los aceites esenciales estudiados. Siendo mayoritarios (+)-alcanfor y (+)-fenchona en cantueso; (+)-alcanfor, (-)-linalol y (-)-acetato de linalilo en lavandín, lavanda y espliego; (-)-linalol, (+)-limoneno y (-)-borneol en los tomillos rojo y de invierno y (-)-linalol, (+)-limoneno y (+)-terpinen-4-ol en los orégenos español e italiano. Estas distribuciones enantioméricas apoyan el carácter natural y

la ausencia de adulteraciones, de los aceites esenciales de Murcia obtenidos y analizados en este trabajo, lo cual potencia su posible comercialización.

- La evaluación de la capacidad antioxidante global, indica que las muestras de aceites esenciales son especialmente efectivas en los distintos métodos ensayados, animando a un mayor uso de los aceites esenciales estudiados como potenciales antioxidantes para distintas aplicaciones. Así observamos que el tomillo rojo presenta alta capacidad antioxidante en los ensayos ABTS, DPPH, RdP, TBARS y óxido nítrico, mientras que el género *Lavandula* muestra mayor capacidad de quelatación, y de neutralizar ciertos radicales oxigenados como los presentes en los métodos ORAC e hidroxilo. Los oréganos presentan un comportamiento cercano al del tomillo rojo, ya que su componente principal, el carvacrol, es isómero del timol. Pese a la similitud estructural de estos dos isómeros, el timol presenta mejores características antioxidantes.

- Se han observado importantes capacidades inhibidoras de lipoxigenasa y hialuronidasa en las muestras de aceites esenciales aquí estudiados. Los aceites de tomillos rojos, predominantemente, son los que mejor resultado han mostrado en la inhibición de ambas enzimas, principalmente debido al timol que contienen. Estos resultados *in vitro* sugieren una posible aplicación de los aceites esenciales en tratamientos de enfermedades de base inflamatoria, en tratamientos antiartríticos y antienvejecimiento evitando la degradación del ácido hialurónico, así como en tratamientos frente a otras enfermedades como la progresión del cáncer.



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"Cuanto menos se lee, más daño hace lo que se lee"
Miguel de Unamuno

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