TESIS DOCTORAL

Thays Helena Pereira Borges

Caracterización nutricional, físico-química y organoléptica de aceites de oliva virgen producidos en Brasil en comparación con las variedades originales españolas

DOCTORADO EN NUTRICIÓN Y CIENCIAS DE LOS ALIMENTOS

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Facultad de Farmacia
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Estación Experimental del Zaidín
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La doctoranda, Thays Helena Pereira Borges y los directores de Tesis, Isabel Seiquer Goméz- Pavón y José Alberto Cardoso Pereira, garantizamos al firmar esta Tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de los directores de Tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados cuando se han utilizado sus resultados o publicaciones.

Granada, Diciembre de 2017.

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Doctoranda

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Igualmente, han sido presentados en los siguintes congresos:

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Borges, T. H., Pereira, J. A., Cabrera–Vique, C., & Seiquer, I. (2016). Arbequina variety: comparison between quality parameters, oxidative stability and fatty acid profile of olive oils produced in Spain and Brazil. I Congreso Ibérico de olivicultura, V jornadas nacionales Del

grupo de olivicultura de la sociedad española de ciencias hortícolas (SECH), VII Simpósio nacional de olivicultura da associação portuguesa de horticultura (APH). Badajoz, España

Borges, T. H., Rueda, A., & Seiquer, I. (2016). Modificaciones de las propiedades antioxidantes del aceite de oliva virgen extra durante el proceso de digestión. Relación con el contenido de clorofilas y carotenoides XI Reunión del Grupo Español de Investigación en Radicales Libres (GEIRLI). Granada, España

Borges, T. H., Pereira, J. A., Cabrera–Vique, C., & Seiquer, I. (2017). Protective antioxidant effect of bioaccessible fraction from Arbequina extra virgin olive oils in Caco-2 cells. XVIII Simposio Científico-Técnico Internacional EXPOLIVA– Feria Internacional del Aceite de Oliva y Industria afines. Jaén, España

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Borges, T. H., Cabrera-Vique, C., & Seiquer, I. (2015). Antioxidant properties of chemical extracts and bioaccessible fractions obtained from six Spanish monovarietal extra virgin olive oils: Assays in Caco-2 cells. *Food & function*, *6* (7), 2375-2383.

Seiquer, I., Borges, T. H., & Cabrera-Vique, C. (2017). AOVE D.O.P. Estepa, Fuente de Salud. Editora: © Consejo Regulador de la Denominación de Origen Estepa - Edificio Centro de Empresas - Pol. Ind.Sierra Sur Avda. Del Mantecado s/n 41560 Estepa (Sevilla). 2017.

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Congresos

Borges, T. H., Cabrera-Vique, C., & Seiquer, I. (2014). Total Phenolic Compounds and Antioxidant Capacity of varieties of Spanish Extra Virgin Olive Oils. Special Issue: Abstracts of the 37th Congress of the Spanish Society of Physiological Sciences (SECF), Granada, Spain

Borges, T. H., Cabrera-Vique, C., & Seiquer, I. (2014). Total Phenolic Compounds and Antioxidant Capacity of varieties of Spanish Extra Virgin Olive Oils. XXXVII Congreso de la Sociedad Española de Ciencias Fisiológicas (SECF)**.**Granada, España

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Borges, T. H., Serna, A., Cabrera–Vique, C., & Seiquer, I. (2015). Polifenoles y propiedades antioxidantes del aceite de oliva virgen extra, variedad hojiblanca, antes y después de un proceso de digestión *in vitro*. I Congreso Internacional, Current trends and new challenges in olive oil sector. Murcia, España

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A mis padres

A todos que ya no están

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ABTS: Ácido 2,2.-azinobis-(3-etilbenzotiazolín-6-sulfónico).

AGMI: Ácidos grasos monoinsaturados.

AGPI: Ácidos grasos poliinsaturados.

AGS: Ácidos grasos saturados.

AO: Aceite de oliva

AOV: Aceite de oliva virgen

AOVE: Aceite de oliva virgen extra

Caco-2: Células de Adenocarcinoma de Colon humano

COI: Consejo Oleícola Internacional

DNA: Ácido desoxirribonucleico.

DPPH: 2,2-difenil-1-picrilhidrazil

EFSA: Autoridad Europea para la Seguridad de los Alimentos

FRAP: Capacidad de reducción férrica del plasma

HDL: Lipoproteínas de densidad alta

IP: Índice de peróxidos.

LDL: Lipoproteínas de densidad baja**.**

LOX: Ruta de la lipoxigenasa

MTT: Reducción metabólica del Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5difeniltetrazol

REDOX: Reacciones de oxidación-reducción

RNA: Ácido ribonucleico.

RNS: Especies reactivas de nitrogeno

ROS: Especies reactivas de oxigeno

RSS: Especies reactivas de azufre

t-BOOH: ter-butil-hidroperóxido

TROLOX: Ácido 6-hidroxi-2,5,7,8-tetrametil-cromato-2-carboxílico.

CAPÍTULO 1

Justificación y Objetivos

El aceite de oliva virgen es obtenido del fruto del olivo exclusivamente por medios mecánicos u otros procedimientos físicos y es reconocido como el proprio zumo de la aceituna. Se trata de un alimento enormemente apreciado por sus particulares características organolépticas, que lo diferencian de cualquier otro aceite comestible, así como por sus efectos beneficiosos sobre la salud. Es la grasa de elección en la dieta mediterránea, siendo responsable de algunas de las propiedades saludables que se atribuyen a dicha dieta.

El olivar y la producción de aceite de oliva están concentrados en la cuenca del Mediterráneo, donde se produce el 70% del aceite de oliva mundial, destacando España como el primer productor mundial, con una producción que supera el 40% del total.

La creciente demanda en el mercado internacional y en mercados cada vez más especializados, juntos con las nuevas prácticas de cultivo agronómicas de cultivo han permitido la expansión del cultivo del olivo en diferentes regiones del mundo como Argentina, Australia, Brasil, Chile, China, Estados Unidos y otros, así como la expansión de variedades de aceituna en regiones no autóctonas dentro de los propios países productores.

En Brasil, el cultivo del olivo se introdujo en el siglo XIX, pero actualmente la producción se mantiene en cifras muy reducidas, que ni siquiera aparecen en los datos de producción mundial de aceites de oliva del Consejo Oleícola Internacional (COI). Sin embargo, Brasil sí aparece en la lista de los países consumidores; el consumo brasileño de aceite de oliva ha ido en aumento en los últimos años y actualmente supone alrededor de un 2% del total en el mundo. Se produce por tanto una situación controvertida en Brasil, ya que no tiene producción suficiente para afrontar su creciente consumo de aceite de oliva, con lo cual las cifras de importación han alcanzado en los últimos años valores históricos. No ha sido hasta la última década cuando ha empezado la producción comercial de aceite de oliva en Brasil, motivada por el aumento de poder económico y el conocimiento de las propiedades nutricionales del aceite, factores que han favorecido el incremento del consumo.

Actualmente, el aceite brasileño producido y comercializado es predominantemente de la variedad española Arbequina, debido a sus características particulares adaptadas a los cultivos intensivo y súper-intensivo y a sus propiedades organolépticas. Sin embargo, su producción es muy incipiente comparada con las necesidades del mercado y hay escasos datos sobre su calidad y composición. Hoy día se sabe que, además del perfil en ácidos

grasos (y la especial riqueza en ácido oleico), otros componentes del aceite de oliva, especialmente los de la fracción no saponificable, son excepcionalmente importantes para definir la calidad y las propiedades de los aceites. Es el caso de los compuestos fenólicos, los tocoferoles o la coenzima Q. Se hace necesario, por otra parte, recurrir a tecnologías específicas para el adecuado análisis de los componentes minoritarios o de las características sensoriales, como los compuestos volátiles o el uso de la llamada lengua-electrónica. A este respecto, hay que señalar que no existe ninguna información científica respecto a los compuestos minoritarios, el perfil organoléptico o las propiedades antioxidantes de los aceites de oliva producidos en Brasil.

La literatura científica muestra que diferentes factores (agronómicos, climáticos, geográficos y procesos tecnológicos) juegan un papel fundamental en la calidad y composición de los aceites producidos. Existen evidencias sobre el efecto y las interacciones de los factores climáticos y geográficos con la composición de los aceites de oliva, especialmente en lo referente al perfil de ácidos grasos y a los componentes minoritarios, que a su vez repercutirán en las propiedades sensoriales y saludables del aceite. De esa forma, cada variable es importante en el proceso de obtención del aceite, ya que pueden afectar a su calidad final. Además, hay que destacar que hasta la presente fecha, hay un conocimiento científico limitado sobre las características y propiedades de los aceites monovarietales de diferentes áreas geográficas, especialmente los obtenidos en las nuevas zonas de producción.

Con los antecedentes expuestos, la presente memoria de tesis doctoral tiene por objetivo general realizar un estudio comparativo amplio de los parámetros de calidad, composición y características organolépticas de aceites de la variedad Arbequina producidos en Brasil y procedentes de diferentes regiones españolas.

Este objetivo general se ha desglosado en los siguientes objetivos parciales:

1) Caracterizar las muestras en relación a los parámetros de calidad (acidez, índice de peróxidos y coeficiente de extinción específica), parámetros físico-químicos (estabilidad oxidativa y color) y composición (contenido de pigmentos – clorofilas/carotenoides y perfil de ácidos grasos) y aplicar análisis estadísticos multivariantes para clasificar los aceites de acuerdo con las zonas de producción.
2) Analizar la fracción minoritaria de los aceites en cuanto a su contenido en tocoferoles, compuestos fenólicos y coenzima Q_{10}, evaluando además,

las posibles relaciones con los factores climáticos y geográficos de las regiones productoras.

3) Estudiar las propiedades antioxidantes de los aceites, prestando especial atención a las modificaciones debidas al proceso de digestión en dichas propiedades o en los compuestos relacionados con ellas, como el contenido en polifenoles totales. Analizar, además, las propiedades antioxidantes de los aceites a nivel celular, mediante el uso de cultivos celulares.
4) Evaluar de las propiedades organolépticas de los aceites, incluyendo, además de la evaluación sensorial mediante panel de cata, el análisis de compuestos volátiles y la aplicación del análisis potenciométrico de lengua electrónica. Como objetivo preliminar de este apartado se incluyó la optimización de las condiciones del método para la determinación de compuestos volátiles.

De esa forma, esta memoria de la tesis doctoral pretende contribuir al conocimiento de las características y propiedades de los aceites de oliva virgen, incentivando su consumo y fortaleciendo la colaboración técnico-científica entre Brasil y España.

CAPÍTULO 2

Revisión bibliográfica

2.1. Historia del origen y expansión del aceite de oliva

El olivar es uno de los cultivos más antiguos del mundo, aunque su verdadero origen no está determinado con exactitud (Vossen, 2007; Kapellakis y col., 2008). Sin embargo, parece ser que tal como es conocido actualmente, su origen corresponde a la edad de Bronce (3150-1200 a.C), en la región de Persia y Mesopotamia. Posteriormente, con la expansión marítima se extendió a Siria, Palestina y norte de África, donde concretamente en Turquía, Grecia y Egipto surgieron los primeros hallazgos sobre la obtención del del aceite de oliva (AO). Durante el imperio Romano tuvo lugar la expansión del cultivo y del uso del AO, aplicado a diversas finalidades como uso farmacéutico y como fuente de energía (Vossen, 2007; Kapellakis y col., 2008).

Durante los siglos XIX y XX, con el surgimiento de otras fuentes de energía y la obtención de los aceites de semillas, disminuyó la producción de AO y para competir en el mercado de oleaginosas se utilizaron prácticas de adulteración como las mezclas con otros aceites vegetales (Vossen, 2007; Kapellakis y col., 2008). En 1959, como respuesta a las adulteraciones generalizadas, surgió el Consejo Oleícola Internacional (COI) (Vossen, 2007), una organización internacional asociativa entre los países productores con el objetivo de fortalecer, normalizar y promocionar el sector oleícola.

Actualmente, según datos del Departamento de Agricultura de los Estados Unidos (USDA) (USDA, 2017), el AO representa 2% de la producción mundial de aceites vegetales (**Figura 1**). Las perspectivas de crecimiento son alentadoras, en parte debido a la disminución reciente de la demanda de otros aceites dominantes en el mercado alimentario como el aceite de palma y a la búsqueda de alternativas más sostenibles. Otros factores positivos son la labor de promoción del COI en los países potenciales de consumo, la popularidad de la dieta mediterránea y el creciente conocimiento de sus propiedades saludables en detrimento de otras grasas animales/vegetales y las características sensoriales únicas de los aceites de oliva (Huang y Sumpio, 2008; Barjol, 2013).

De esa forma, a día de hoy, el AO es uno de los aceites comestibles de mayor valor económico entre los aceites vegetales, moviendo una cifra importante, más de 9 billones de euros a nivel mundial con respecto al consumo de 2015/2016 (COI, 2017A; COI, 2017B).

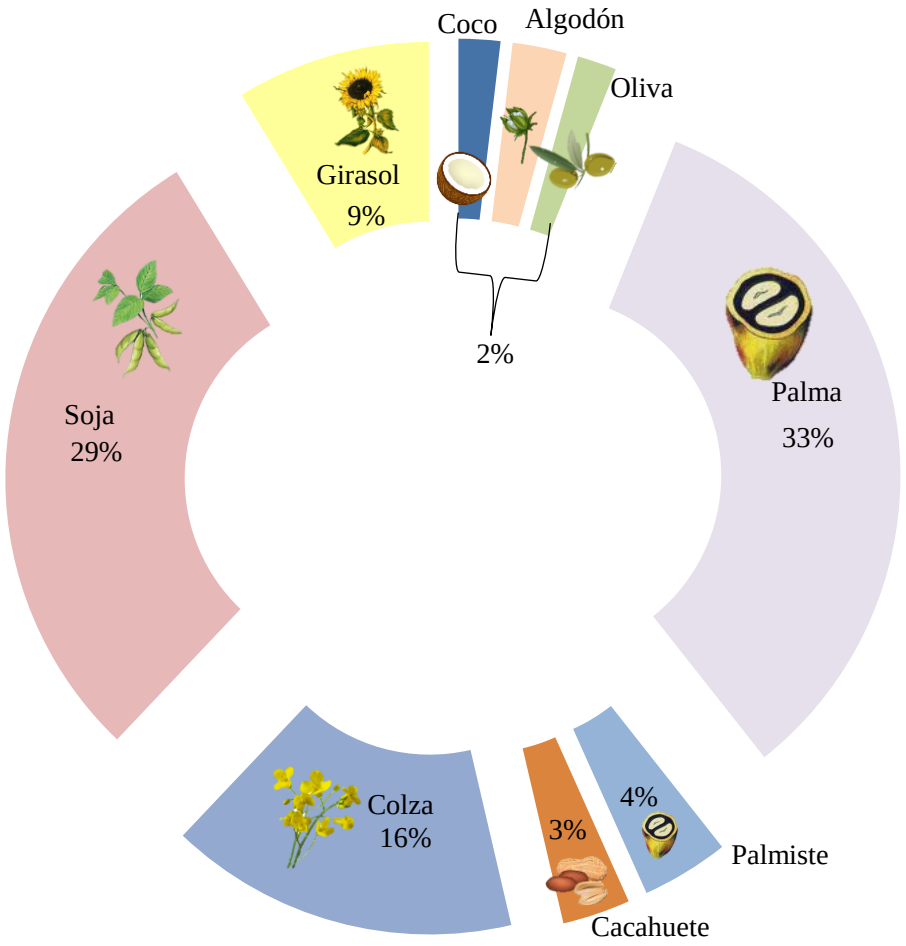

Figura 1. Producción (%) de aceites vegetales comestibles en el mundo en los años 2015-2016. Fuente: USDA, 2017.

2.2. Producción, consumo e importancia del aceite de oliva a nivel mundial

Según Vossen (2013), el olivar ocupa una superficie de más de 9 millones de hectáreas a nivel mundial, de los cuales, alrededor de 2 millones de hectáreas están situadas en España.

De acuerdo con los datos del COI de 2010-2016, detallados en la **Figura 2,** la producción a nivel mundial se concentra en los países de la Unión Europea representado aproximadamente el 70%. Destaca la Cuenca del Mediterráneo, siendo España considerada el primer productor con el 43% de la producción mundial, seguido por Italia (14%), Grecia (10%) y Portugal (3%), mientras los países extra comunitarios, representan alrededor del 30%.

Asimismo, España es el mayor exportador mundial y en los últimos años hay una aumento de las exportaciones de AO para países extra comunitarios, de aproximadamente 20% en la última campaña de 2015/2016 (COI, 2017A).

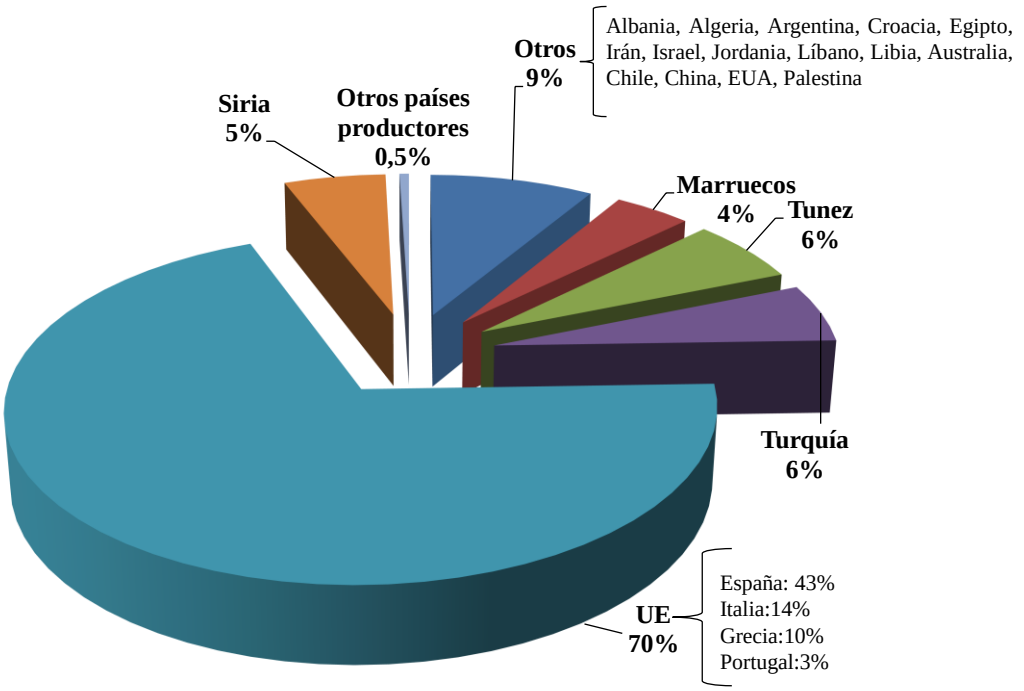

Figura 2. Producción mundial de aceite de oliva (%) en los años 2010- 2016. Fuente: COI, 2017A.

Respecto al consumo mundial de AO, los datos recientes proporcionados por el COI (2017A) (**Figura 3**), revelan que la Unión Europea (UE) es el principal consumidor (57%) y dentro de la UE el orden creciente del consumo es Italia (20%), España (17%), Grecia (5,6%), Francia (3,6) y Portugal (2,5%).

Por otro lado, se ha observado un incremento mundial del consumo (COI, 2017A) en países extra comunitarios liderando Estados Unidos con un 10%, proveniente principalmente de importación, ya que tiene producción escasa. Además, otros países presentan un gran potencial de consumo como Brasil (2%), Japón (2%), Australia (1%) y Rusia (1%) (**Figura 3**).

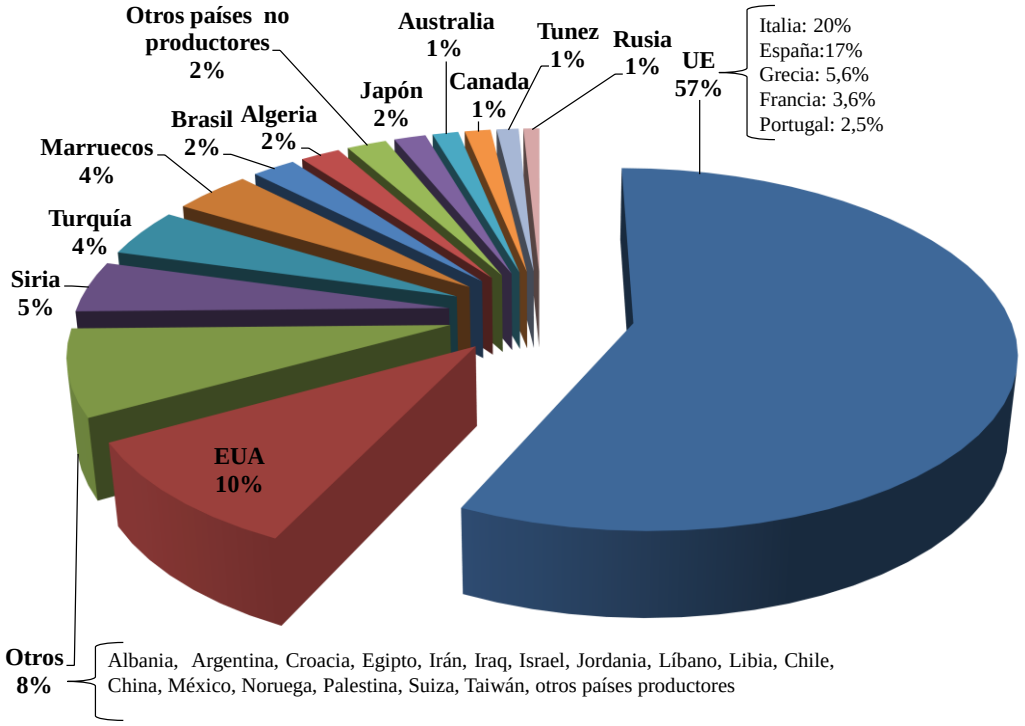

Figura 3. Consumo mundial de aceite de oliva (%) en los años de 2010- 2016. Fuente: COI, 2017A.

2.3. Producción, consumo e importancia del aceite de oliva en Brasil

En América, durante el siglo XV d.C, fueron introducidos diferentes cultivos como la vid y los olivos por los misioneros y colonizadores Europeos. No obstante, el olivo sólo se cultivó en áreas restringidas de Chile, Argentina y California (EE. UU), donde las condiciones climáticas se asemejaban a las de la Cuenca del Mediterráneo (Kapellakis y col., 2008).

En Brasil, el olivar fue introducido durante el periodo colonial y se han encontrado pruebas de ello en varias provincias, como Rio Grande do Sul, Paraná, Santa Catarina, Minas Gerais, Espirito Santo, Rio de Janeiro y São Paulo (Aued-Pimentel, 2016). Sin embargo, no ha sido hasta la última década cuando ha comenzado la producción e inversión en el sector oleícola brasileño, motivado por las altas importaciones y el creciente aumento de este mercado en Brasil. En el municipio de Maria da Fé (Minas Gerais) se obtuvo el primer AO brasileño (Aued-Pimentel, 2016; Homrich y col., 2017).

Brasil representa alrededor del 2% del consumo mundial (**Figura 3**) y las cifras de la importación de 2015/2016 (COI, 2017A), sugieren que el consumo es aún muy bajo (promedio de 0.25 kg/ habitante/año). Sin embargo, se observan perspectivas de

aumento en las importaciones (**Figura 4**), llegando a un nivel máximo de 75 mil toneladas en 2012. Durante esos años, varios factores fueron determinantes para intensificar las importaciones, principalmente la apertura comercial, aumento de la clase media brasileña y conocimiento de los beneficios para la salud del aceite de oliva (Aued-Pimentel, 2016). Por otro lado, los hábitos alimenticios brasileños y la influencia de la inmigración colaboran con el uso culinario del AO (Aued-Pimentel, 2016) y su creciente interés por el mercado oleícola.

Durante los últimos años (2015-2016), ha habido una disminución de poder económico, llevando a una inestabilidad política que justifica la caída en las tasas de importación, pero las últimas cifras del COI (octubre 2016-junio de 2017), muestran un incremento importante de las importaciones en Brasil en aproximadamente un 29% (COI, 2017E).

De esa forma, a día de hoy, el AO y el aceite de orujo consumidos en Brasil es importado de Europa (Portugal, España, Italia y Grecia) y Sudamérica (Argentina y Chile), propiciando un elevado precio del mismo y haciéndolo casi inaccesible para la mayoría de la población (Ballus y col., 2014). Con esos antecedentes, Brasil está empezando la producción del aceite con el objetivo de proporcionar un producto a precio más bajo para los consumidores en el futuro y aumentar las oportunidades en el mercado agrícola (Ballus y col., 2014).

La expansión de la olivicultura en Brasil ha comenzado en dos zonas principales: Suroeste y Sur de Brasil, correspondiente a las provincias de Minas Gerais y Rio Grande do Sul (De Oliveira da Silva y col., 2012), donde existen aproximadamente 60 productores en la región de Mantiqueira (frontera entre las provincias de São Paulo y Minas Gerais), con aproximadamente 1800 hectáreas cultivadas, el 90% centradas en la producción de aceite de oliva. La producción estimada para 2017 es de 45 toneladas de aceitunas y 50 mil litros de aceite de oliva (Homrich y col., 2017). En Rio Grande do Sul, hay alrededor de 2000 hectáreas y 160 productores, aunque muchos de los olivares considerados todavía se encuentran en fase inicial de crecimiento (SEAPI, 2017) y según los últimos datos disponibles la producción fue estimada en 400 toneladas de aceitunas y 45 mil litros de aceite (Governo do Estado do Rio Grande do Sul, 2017).

En relación a las variedades plantadas, según De Oliveira da Silva y col., (2012), el 50% de la variedad cultivada en Minas Gerais en 2012 era la variedad española Arbequina, mientras los datos más recientes revelan que el 70% de la cosecha de esta región es de la variedad Arbequina (Olive Oil Times, 2017). Sin embargo, todos los datos de

producción son incipientes todavía, existiendo solamente cinco marcas comerciales (Olive Oil Times, 2017), lo cual confirma que la producción nacional todavía es muy baja frente la demanda creciente del mercado Brasileño.

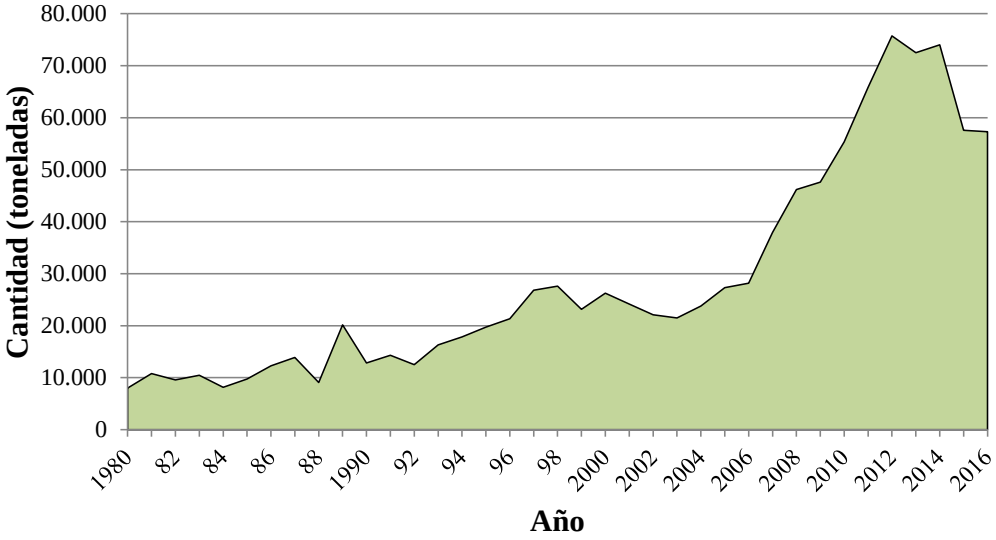

Figura 4. Importación de aceite de oliva total (incluido aceite de orujo) en Brasil en toneladas de 1980 -2016. Fuente: COI, 2017D.

2.4. Proceso de obtención y su influencia en el aceite

Para la elaboración del AO, se debe tener en cuenta que una multitud de factores juegan un papel fundamental en la calidad y composición del AO producido (**Figura 5**). Esos factores pueden clasificarse como factores agronómicos y tecnológicos. Los primeros engloban aquellos que difícilmente pueden modificase (intrínsecos), como la variedad y las condiciones geográficas y climáticas, y aquellos que pueden controlarse con relativa facilidad por el agricultor (extrínsecos), como la fertilización del suelo, recolección, transporte, etc. De esa forma, cada variable es importante en el proceso de obtención del aceite y consecuentemente pueden afectar la clasificación comercial del mismo.

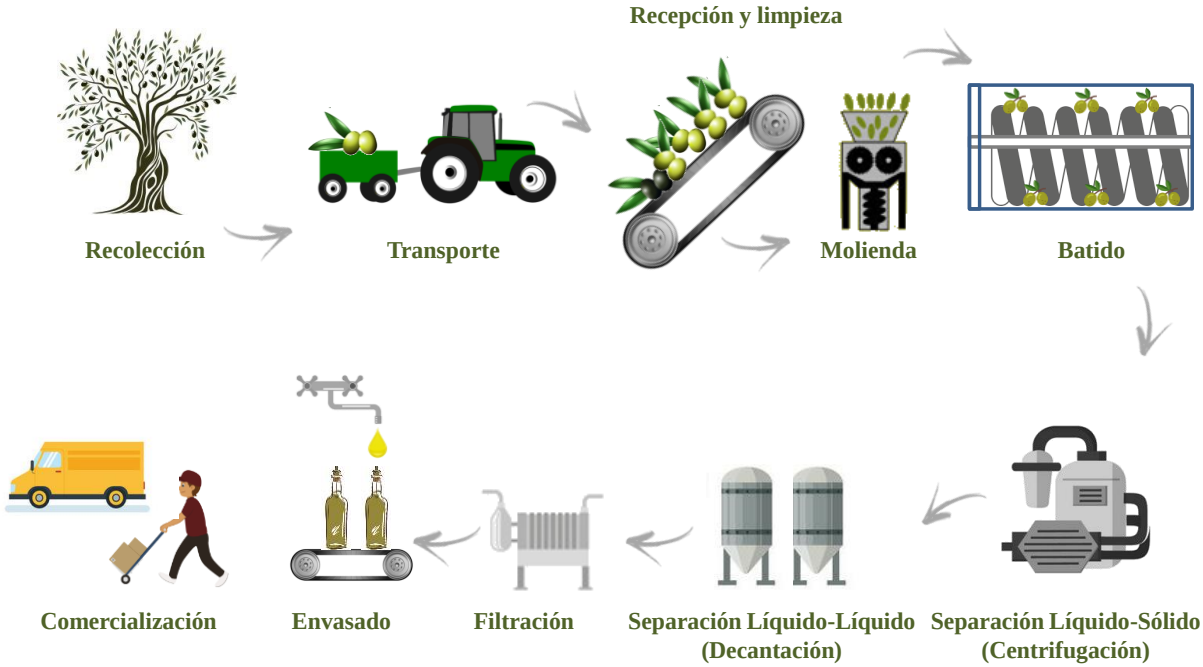

Figura 5. Etapas en la obtención del aceite de oliva.

2.4.1. Factores agronómicos

Previo a la recolección del fruto, diferentes factores agronómicos y técnicas agronómicas son fundamentales en la calidad de los AO que serán producidos, entre ellos la variedad, el sistema de cultivo, la fertilización y manejo del suelo, la poda, el control de plagas e injurias por el frio, la maduración, el régimen de agua, los sistema de recolección y el transporte.

2.4.1.1. Variedad

Uno de los primeros factores a ser considerados es la variedad. La composición y el perfil sensorial del aceite dependen de la interacción entre el potencial genético con las condiciones de cultivo, que implican una compleja interacción con el medio (clima, temperatura, precipitaciones, tasa de humedad) y con los factores agronómicos (carga de cultivo, poda, fertilización, riego, proporción del hojas/fruto y madurez del fruto), siendo todos esos factores determinantes para la expresión fenotípica consistente (Inglese y col., 2011; Vossen, 2013).

De esa forma, el contenido de componentes fundamentales del aceite, como la proporción de ácidos grasos, los pigmentos, o los compuestos fenólicos y volátiles, está ampliamente relacionado con la interacción de la variedad y las condiciones de cultivo (Angerosa y col., 2004; Inglese y col., 2011).

De acuerdo con Vossen, (2013), cuando una misma variedad es cultivada en diferentes regiones geográficas, algunas de sus características pueden mantenerse mientras que otras pueden variar significativamente. Así, la elección de una variedad es uno de los factores determinantes para los nuevos cultivos.

De las 1200 variedades cultivadas en el mundo, solamente 30 predominan en la producción del AO (Vossen, 2013), entre las que destacan las variedades españolas picual, arbequina y hojiblanca.

2.4.1.2. Sistema de cultivo

Los sistemas de cultivo no tienen una influencia directa en la calidad de los aceites, pero permiten maximizar la cantidad y calidad de las aceitunas, tieniendo influencia en la recolección (Vossen, 2007; Vossen, 2013).

Los sistemas de cultivo del olivo son:

- **Tradicional:** Es un sistema antiguo típico de la cuenca del Mediterráneo. Los espaciamientos entre árboles son de 8-18 m, poseen una densidad muy baja de alrededor de 30-173 árboles/hectáreas y rendimientos muy bajos (0,5-4 toneladas/h). La cosecha es considerada ineficaz, realizada manualmente por vareo o mecánica con uso de vibradores mecánicos.
- **Intensivo:** Sistema desarrollado en los años 80 de espaciamientos variables, pero generalmente con 7-8 m de distancia entre árboles, permitiendo que el centro esté abierto y con densidad de 250-555 árboles/hectáreas. Es adaptable a la variedad, tipo de suelo, terreno o tipo de cosecha. Las cosechas en ese sistema pueden ser manuales o mecánicas y tardan de media 6-7 años para llegar al pico productivo.
- **Súper-Intensivo:** Sistema pionero en España en los años 90, implantado inicialmente en Cataluña y que actualmente cuenta con más de 80.000 ha en todo mundo. Se utiliza sobre todo para variedades precoces como Arbequina, Arbosana y Koroneiki. La variedad Arbequina es la variedad más plantada en ese sistema debido al tamaño del fruto y vigor del árbol. Las distancias entre árboles son de 1-1,5 m dentro de la fila y de 3-5 m entre las filas, generando una alta densidad de 1655-2990 árboles/ hectáreas, de producción precoz requiriendo un manejo agronómico adecuado.

2.4.1.3. Fertilización, manejo del suelo y poda

Los fertilizantes químicos (nitrógeno, fosfato y potasio) son convencionalmente empleados en el olivar para la mejora del suelo. La concentración a la que son aplicados parece tener una estrecha relación con la calidad del AO y el rendimiento óptimo, siendo dependientes de la variedad, pero también el tipo de suelo puede influir en la composición de los aceites (Luna y Aparicio, 2002; Dag y col., 2009).

Diversos estudios muestran que la fertilización puede afectar a la composición en ácidos grasos, el contenido en compuestos fenólicos y tocoferoles (Fernández-Escobar y col., 2006; Dag y col., 2009; Romero y col., 2016). Además, Romero y col., (2016), estudiando diferentes variedades producidas en Chile han concluido que el suelo y clima tiene un gran influencia en los compuestos volátiles.

Con relación a los sistemas de manejo de suelo los principales tipos son convencional, integrado y ecológico y se diferencian en el modelo de control de plagas, tipo de fertilizantes empleados (biológico, químico) y rotación de cultivos. Ninfali y col., (2008), en un estudio comparativo entre los sistemas de manejo convencional versus ecológico durante 3 años, mostró que no hay un efecto consistente en los parámetros físico químicos, actividad antioxidante, composición en tocoferoles, compuestos fenólicos y volátiles de los AO evaluados. No obstante, datos más recientes indican que el sistema de manejo (convencional o ecológico) puede influir en el contenido de compuestos fénolicos, volátiles y en a las características sensoriales de los aceites (Jiménez y col., 2017).

Otro aspecto agronómico importante en el olivar es la poda que tiene por objetivo equilibrar la distribución de la luz dentro de la copa del árbol. Los umbrales de requerimiento de luz para un aceite de calidad no están definidos en la literatura científica, aunque se ha descrito un aumento del contenido en polifenoles y clorofilas en una variedad Italiana (Inglese y col., 2011).

2.3.1.4. Control de plagas y daños por frio

En general, las plagas tienen un efecto negativo en la calidad físico-química y organoléptica de los aceites de oliva (Angerosa y col, 2004; Inglese y col., 2011; Vossen, 2013). Una de las plagas más comunes que dañan la aceituna es la mosca del olivo (*Bactrocera oleae*) que ataca el fruto permitiendo la penetración de larvas que conlleva a la rotura de tejido y la exposición al oxígeno, pudiendo desarrollarse hongos

y bacterias, que aceleran su caída (Inglese y col., 2011). Estudios anteriores han comprobado que la infestación por la mosca del olivo afecta a los parámetros físico-químicos (acidez, índice de peróxidos y estabilidad a la oxidación), el perfil en volátiles y fenoles, teniendo un efecto variable de acuerdo con la variedad (Pereira y col., 2004; Gómez-Caravaca y col., 2008; Vossen, 2013; Malheiro y col, 2015).

Aparte de las plagas, los daños causados por el frio en las aceitunas afectan a diferentes parámetros de los aceites, como el contenido en pigmentos (clorofila, carotenoides), el color, la fracción fenólica, la estabilidad oxidativa y el perfil sensorial (Morelló y col., 2003).

2.4.1.5. Maduración

La aceituna es un fruto no climatérico, es decir, todo el proceso de maduración ocurre el árbol y una vez realizada la cosecha no hay evolución de la madurez. De esa forma, es primordial considerar, que al seleccionar el momento de la cosecha, el productor está decidiendo directamente la cantidad y la calidad de la producción anual, por lo que el punto óptimo de recolección es clave para la obtención un aceite de calidad de acuerdo con los parámetros establecidos de clasificación, las características deseables y la proporción equilibrada de grasa/humedad (Ranalli y col., 1998; Dag y col., 2011; Jiménez y col., 2013).

Durante la maduración del fruto, ocurren muchas transformaciones bioquímicas: (**Figura 6**) aumento del mesocarpio, cambio de textura (firmeza), resistencia al desprendimiento, disminución de la relación pulpa/hueso, aumento del peso, cambios de color del endocarpio/mesocarpio relacionados con disminución de los niveles de pigmentos (clorofila y carotenoides) y el aumento de las antocianinas; aumento de la proporción de aceite, cambios en la composición química (ácidos grasos, tocoferoles, polifenoles, esteroles, triglicéridos, escaleno), perfil organoléptico y actividad enzimática (Ranalli y col., 1998; Salvador y col., 2001; Morelló y col., 2004A; Baccouri y col., 2008, Gómez-Rico y col., 2008; Dag y col., 2011; Jiménez y col., 2013; Hbaieb y col., 2016B).

Además, diversos estudios revelan que los parámetros físico químicos pueden verse afectados, como el índice de peróxidos, índice de acidez, valor nutricional y estabilidad oxidativa (Ranalli y col., 1998; Salvador y col., 2001; Baccouri y col., 2008; Dag y col., 2011). La magnitud de estos cambios depende de la variedad, clima, área geográfica,

disponibilidad de agua, prácticas agronómicas y otros factores que son variables en cada cosecha anual (Morelló y col., 2004A; Dag y col., 2011).

Figura 6. Cambios durante proceso de maduración de la aceituna.

2.4.1.6. Régimen hídrico

Tradicionalmente, el régimen hídrico usado en los olivares de la cuenca del Mediterráneo es un régimen de secano (sin uso del riego), excepto por las precipitaciones del inverno (Tovar y col., 2002; Berenguer y col., 2006). Este régimen es el predominante en Andalucía, región de mayor producción mundial, en donde representa aproximadamente el 62% (Olimerca, 2017).

Sin embargo, actualmente la práctica del regadío se está ampliando, ya que la misma favorece el crecimiento vegetativo del árbol en un corto periodo de tiempo, aumentando la densidad de producción y la producción total de aceite (Tovar y col., 2002; Berenguer y col., 2006; Caruso y col., 2014).

Muchos estudios han evaluado el efecto del sistema y nivel de riego en la calidad y composición de los AO (Tovar y col., 2001; Luna y Aparicio, 2002; Tovar y col., 2002; Berenguer y col., 2006; Stefanoudaki y col., 2009; Caruso y col, 2014). Así, se ha encontrado un efecto pronunciado sobre el contenido en compuestos fenólicos, el perfil organoléptico (verde, amargo, astringente), índice de amargor y la estabilidad oxidativa de los AO, pero también algunos de los estudios relacionan que pueden influir en el contenido en tocoferoles y tener un efecto de variable dependiente en los compuestos volátiles (Tovar y col., 2001; Luna y Aparicio, 2002; Tovar y col., 2002; Baccouri y col,

2008; Stefanoudaki y col., 2009; Dabbou y col, 2011; Caruso y col., 2014; Romero y col., 2016). En contra punto, se ha descrito un efecto positivo del secano en el contenido de fenoles que puede ser atribuidos a cambios en la actividad enzimática, destacando la enzima fenilalanina amonio liasa (PAL), enzima clave en la biosíntesis de fenoles que se afecta negativamente por el regadío (Stefanoudaki y col., 2009**)**. Por otro lado, el efecto en otros parámetros de calidad y composición de ácidos grasos es controvertido (Tovar y col., 2002; Berenguer y col., 2006; Stefanoudaki y col., 2009; Caruso y col., 2014). Según Stefanoudaki y col., (2009), los efectos del régimen de agua sobre las propiedades de los AO son muy complejos y sinérgicos pudiendo variar por las condiciones agronómicas y climatológicas, debiendo tenerse en cuenta muchos factores al hacer uso del modelo hídrico empleado.

2.4.1.7. Sistemas de recolección

La recolección del olivo es un factor clave que afecta tanto a la calidad del producto como a la rentabilidad (Sola-Guirado y col., 2014), debiendo en este proceso ser evitados daños al fruto.

De acuerdo con Bernardi y col., (2016), la recolección supone cerca de 50% del valor del producto, por ser un proceso laborioso y demandar muchos trabajadores, principalmente en olivos tradicionales. Por otro lado, el sistema mecánico permite la reducción de costes y es ideal para los cultivos súper-intensivos (Jiménez y Carpio, 2008; Sola-Guirado y col., 2014). A día de hoy, los sistemas de recolección se pueden dividir en dos tipos: manual (por vareo y ordeño) y mecánico (Jiménez y Carpio, 2008; Sola-Guirado y col., 2014).

Recolección manual

- **Por Vareo:** los frutos son recogidos por operadores que usan palos largos, es conocido como método más agresivo por causar daños apreciables al árbol al desprenderse una gran cantidad de ramas y brotes.
- **Por ordeño:** forma tradicionalmente usada en que los frutos son cogidos directamente con la mano, predominante en la aceituna de mesa.

Recolección mecánica

- **Vibrador de ramas:** vibración forzada de las ramas del árbol mediante un agitador manual alimentado por un motor.

- **Vibrador de troncos:** puede ocurrir de dos formas mediante un peine agitador que hace la vibración forzada a la copa de los árboles, donde se localizaron los frutos o por un agitador de troncos acoplado a un tractor que aplica la vibración forzada a cada árbol mediante un agitador de tronco orbital, en algunos casos puede acoplarse un paraguas.

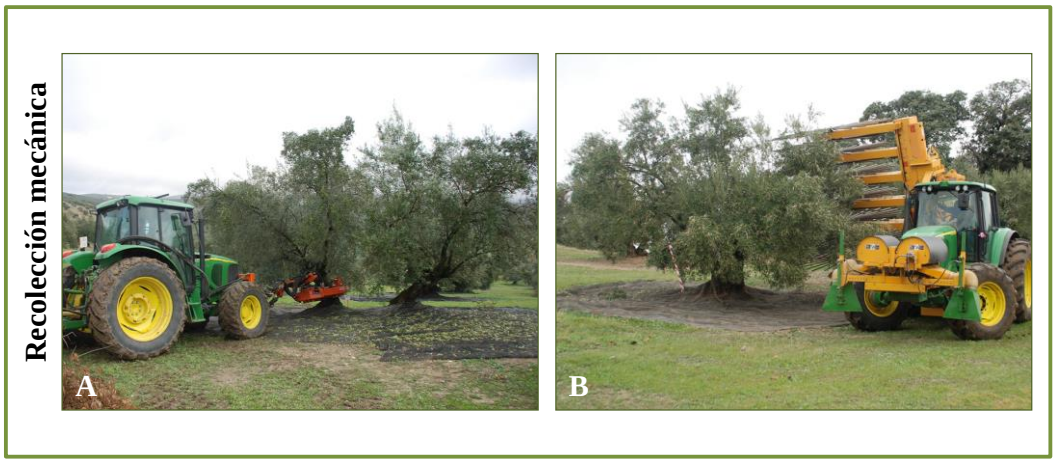

Figura 7. Recolección mecánica por agitación del tronco. **A -** Agitación por vibrador de troncos acoplado a un tractor; **B -** Agitación por peine acopado a un tractor. Fuente: Sola-Guirado y col., 2014.

2.4.1.8. Transporte

Después de la recolección, el transporte debe ser eficientemente rápido y adecuado para evitar que el fruto pueda sufrir daños y llegar al procesamiento lo más rápido posible, entre 12 - 24 horas (Vossen, 2007; Di Giovacchino, 2013). En muchos países y almazaras modernas el transporte es realizado con tractor acoplado a un remolque cubierto y las aceitunas son transportadas en cajas de plástico de capacidad de 20-25 kg o en grandes cajas con capacidad de 250-300 kg con orificios que permiten la circulación de aire, preservando la integridad del fruto y dispersando el calor de la fruta causado por su actividad catabólica (García y Yousfi, 2006; Di Giovacchino, 2013).

2.4.1.9. Almacenamiento del fruto post cosecha

El almacenamiento post cosecha debe ser controlado como uno de los puntos primordiales que pueden incidir en la calidad del aceite producido (Angerosa y col., 2004; García, y Yousfi, 2006; Yousfi y col.; 2012; Hbaieb y col., 2016A; Hbaieb y col., 2016B). Durante ese periodo deben ser evitadas ciertas prácticas, como un largo período de tiempo entre la recolección del fruto y procesamiento del AO, la molturación (el

amontonamiento del fruto para la espera del procesamiento) y las altas temperaturas de almacenamiento que conllevan a procesos degenerativos microbiológicos y enzimáticos (acción de lipasas, lipoxigenasas y liasas) en el fruto. Como consecuencia de las prácticas inadecuadas descritas, los AO pueden presentar alto índice de oxidación, compuestos volátiles indeseados y defectos sensoriales como atrojado, avinagrado y moho (Angerosa y col., 2004; García y Yousfi, 2006; Yousfi y col.; 2012; Hbaieb y col., 2016A; Hbaieb y col., 2016B).

Según Hbaieb y col., (2016B), las altas temperaturas de almacenamiento influyen en los procesos de oxidación e hidrólisis de enzimas endógenas como polifenol oxidasa, peroxidasa y β-glucosidasa, reduciendo el contenido de compuestos fenólicos.

De esa forma, datos recientes consideran que el almacenamiento en frio es una alternativa viable al gran flujo de producción en las almazaras modernas en las que la cosecha mecánica permite la recogida de una gran cantidad de frutos en poco tiempo. Así, condiciones controladas de tempo y temperatura parecen favorecer la calidad del fruto y del AO (Yousfi y col.; 2012; Hbaieb y col., 2016A).

2.4.2. Factores climáticos y factores geográficos

El efecto y las interacciones entre los factores climáticos/geográficos y la composición de los AO, especialmente en lo referente a los ácidos grasos y compuestos minoritarios es un tema de gran interés en la actualidad.

Recientemente, estos estudios han ganado importancia debido a la expansión del cultivo de variedades en los países no tradicionalmente productores y a la implantación de las nuevas prácticas de cultivo en otras zonas autóctonas como, por ejemplo, la expansión de los cultivos súper intensivos de la variedad Arbequina (Aguilera y col., 2005; Torres y col., 2009; García-González y col., 2010; Dabbou y col., 2011; Rondanini y col., 2011; Ballus y col., 2014, Farinelli y Tombesi, 2015; Romero y col., 2016).

Esos estudios son cruciales para el sector oleícola y el mercado, que requieren cada vez más información sobre la adaptación de las variedades a diversas características climáticas (precipitaciones, temperatura, humedad) y geográficas (latitud y altitud), que pueden ser muy distintas de las zonas de cultivo originales y pueden repercutir en la composición/calidad de los AO producidos en las diferentes áreas.

En general, se acepta que los parámetros climáticos y geográficos puedan tener una gran influencia en muchos aspectos, debido especialmente a su efecto sobre la actividad enzimática de los frutos que, a su vez, determina la composición química y el perfil organoléptico de los aceites, estando relacionada con la composición en ácidos grasos y tocoferoles, estabilidad oxidativa contenido de pigmentos, perfil de volátiles, compuestos fenólicos y perfil sensorial (Luna y Aparicio, 2002; Angerosa y col., 2004; Aguilera y col., 2005; Torres y col., 2009; García-González y col., 2010; Mailer y col.,2010; Rondanini y col., 2011; Romero y col., 2016).

No obstante, esos efectos son complejos y sinérgicos, no debiendo ser evaluados sin considerar factores como genotipo y otros aspectos agronómicos (Inglese y col., 2011). Según estudios previos, algunas variedades como Arbequina parecen ser especialmente afectadas por variaciones climáticas y geográficas (Tous y col., 1997; Torres y col., 2009; Rondanini y col., 2011). Esos estudios apoyan que altas temperaturas durante el periodo de síntesis de los ácidos grasos afectan negativamente la actividad enzimática resultando en alteraciones del perfil lipídico de los aceites obtenidos que presentan índices de ácido oleico muy bajos y en algunos casos no cumplen con el reglamento de calidad establecido (Torres y col., 2009; Rondanini y col., 2011). Según un estudio de Romero y col., (2016), que evaluó diferentes aceites producidos en Chile, el suelo y el clima tienen más influencia que la variedad de aceituna en la composición del aceite.

Respecto a la altitud, parece ser que los cambios en la composición de los AO pueden estar asociados a una menor susceptibilidad a los cambios de temperatura en altitud elevadas, lo cual supone una maduración del fruto más lenta (Luna y Aparicio, 2002)

El olivar es un cultivo resistente al estrés hídrico y la necesidad de agua del árbol es dependiente del clima y de la edad del árbol (Vossen, 2013).

2.4.3. Factores tecnológicos

Los factores tecnológicos son los relacionados con las etapas dentro de la almazara, desde la recepción y limpieza del fruto hasta el envasado del aceite, pudiendo cada uno de ellos tener su propia influencia en el AO producido.

2.4.3.1. Recepción y limpieza

En la recepción se realizan procesos preliminares de clasificación según la variedad, verificación de la procedencia, estado sanitario, control de peso y toma de muestras. A

continuación, las aceitunas son descargadas en una tolva de alimentación conectada por una cinta móvil donde son realizadas las operaciones de eliminación de materias extrañas (hojas, ramas, piedras y otros desechos) que generalmente se lleva a cabo con el uso de soplador de aire y/o vibración. Además algunas almazaras aplican también el lavado por circulación de agua, evitándose en este caso, el uso de agua caliente que puede afectar los compuestos volátiles (Angerosa y col., 2004; Vossen, 2007; Kapellakis y col., 2008; Di Giovacchino, 2013). Otras optan, cuando la producción proviene de aceitunas en un estadio inicial de madurez, por no hacer uso del lavado, únicamente eliminando las hojas, por interferir en el perfil sensorial (Vossen, 2007; Kapellakis y col., 2008; Di Giovacchino, 2013).

Figura 8. A - Recepción del fruto; **B -** limpieza Fuente: Di Giovacchino y col., 2013

2.4.3.2. Molienda

La molienda es el primer paso del procesamiento para la obtención del aceite y consiste en romper los diferentes tejidos celulares de la aceituna, favoreciendo la extracción del aceite que se encuentra principalmente en el mesocarpio del fruto (**Figura 9)** (Kapellakis y col., 2008; Clodoveo, 2012).

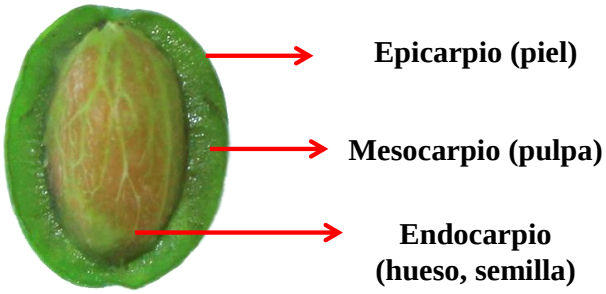

Figura 9. Partes de la aceituna.

Diferentes tipos de trituradores se han utilizado a lo largo de los años, pero a nivel industrial actualmente, entre los más comunes puede destacarse el molino de martillo (Inarejos-García y col., 2011). Las condiciones de molienda, como control de diámetros del cribo y velocidad, son establecidas dependiendo de la madurez del fruto, variedad y características deseables (Inarejos-García y col., 2011). Es conocido que la molienda puede afectar a los componentes minoritarios del aceite, principalmente los compuestos fenólicos, ya que muchos de ellos, como los secoiridoides, son originados durante este proceso por hidrólisis y catálisis, habiendo también un efecto sinérgico de las enzimas endógenas en las diferentes partes de la aceituna, como peroxidasas y polifenoloxidasa (Servili y col., 2004; Servili y col., 2007). Además, en algunos estudios el perfil de volátiles, perfil sensorial y contenido de pigmentos pueden verse afectados con los posibles cambios de temperatura durante el proceso (Angerosa y col., 2004; Inarejos-García y col., 2011).

2.4.3.3. Batido

El batido consiste en batir la pasta preparándola para la separación del aceite, proporcionando la aglutinación de las gotas de aceite de la pasta, aumentando el rendimiento de la extracción y ayudando a la posterior separación de las fases (Clodoveo y col., 2012). El proceso ocurre a través de batidoras verticales u horizontales, siendo estas usadas más comúnmente (**Figura 10**).

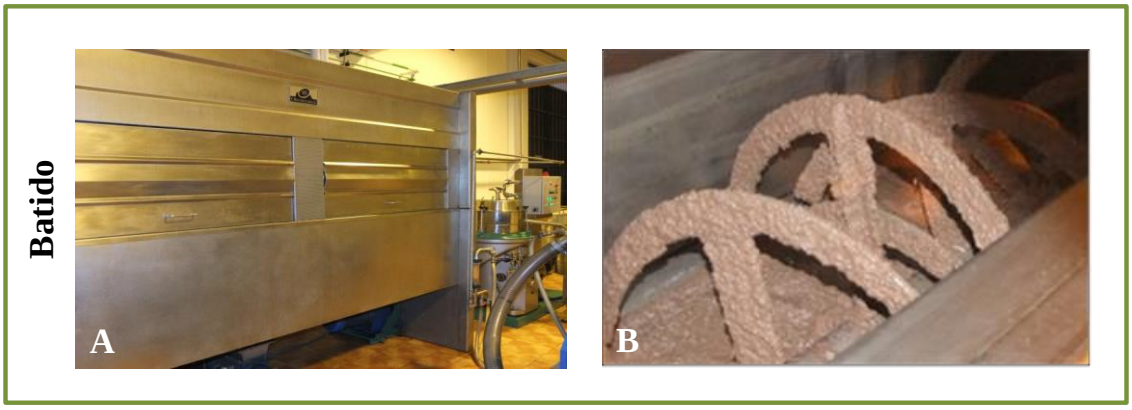

Figura 10. A - Batidora horizontal; **B -** Etapa de batido.

La influencia de las condiciones de batido como tiempo, temperatura, composición de la atmósfera de contacto con la pasta, adición de agua u otros coadyuvantes en la calidad, rendimiento y composición de los AO ha sido ampliamente estudiada en la literatura científica (Kalua y col., 2006; Servili y col., 2008; Peres y col., 2014; Reboredo-Rodríguez y col., 2014; Raffo y col., 2015). De manera que hay una fuerte relación entre las condiciones del batido con el perfil de volátiles y el perfil sensorial de los AO (Angerosa y col., 2004; Kalua y col, 2006; Reboredo-Rodríguez y col., 2014; Raffo y col., 2015), pudiendo también incidir en el contenido de fenoles (Kalua y col, 2006; Servili y col., 2008; Peres y col., 2014).

Por lo tanto, el control de las condiciones de batido, principalmente la temperatura, el tiempo y la composición del aire son cruciales, ya que pueden afectar negativamente al perfil de volátiles asociados a la inactivación enzimática en su ruta de formación, favorecer la oxidación de la pasta por la exposición del oxígeno prolongada y llevar a la pérdida de compuestos fenólicos por el efecto sobre la actividad de las enzimas endógenas (Servili y col., 2008; Reboredo-Rodríguez y col., 2014).

De este modo, las nuevas tecnologías y las condiciones ideales como reducción de nivel de oxígeno, uso de otras atmósferas y coadyuvantes, pueden ser fundamentales en la optimización de las condiciones de proceso, equilibrando un buen rendimiento y un perfil deseable del aceite.

2.4.3.4. Centrifugación

Actualmente, la separación de las fases sólido – liquido se hace comúnmente por uso de centrífugas, sobre todo por el sistema de centrifugación de dos fases, conocidas como

centrífugas horizontales o decanter (**Figura 11A**). Este sistema, fue introducido en los años 90 y es el principal sistema utilizado, representando más de 95% de las almazaras de España (Vossen, 2007; Kapellakis y col., 2008; Di Giovacchino, 2013). Su funcionamiento es sencillo, basándose en la fuerza centrífuga generada por un motor de aproximadamente 3000 *g*n que hace girar sobre un eje horizontal y permite la separación por diferencias de densidad del AO de la pasta, saliendo por un lado el aceite y por el otro el agua de vegetación (alpechín) juntamente con la pasta (orujo) o en conjunto denominado como alperujo (Di Giovacchino y col., 2001; Vossen, 2007; Kapellakis y col., 2008). Comparado a otros sistemas como el sistema de tres fases o por presión posee muchas ventajas como menor generación de aguas residuales ya que no requiere adición de agua, mayor retención de compuestos fenólicos, mayor estabilidad oxidativa y AO con perfil organoléptico deseable (más amargos y picantes), no afectando los parámetros de calidad (Di Giovacchino y col., 2001; Vossen, 2007; Kapellakis y col., 2008).

Posteriormente, la fase acuosa de aceite obtenida pasa por un proceso adicional de centrifugación con el uso de una centrifuga vertical (**Figura 11B**), que a baja velocidad separa el aceite obtenido de la fase acuosa que todavía pueda estar en contacto con el aceite (Kapellakis y col., 2008).

Figura 11. **A -** Centrífuga horizontal; **B -** Centrífuga vertical.

2.4.3.5. Almacenamiento, filtración, y envasado

Después del procesamiento, el AO es almacenado hasta la comercialización, generalmente en tanques de acero inoxidable dentro de la bodega (**Figura 12**) (Vossen, 2007; Di Giovacchino, 2013).

Durante el almacenamiento, es usual el control de materia orgánica que se deposita por decantación como forma de evitar los procesos de oxidación (Di Giovacchino, 2013). Otros factores también son controlados como la temperatura de bodega (recomendable 13-18°C), incidencia de luz, limpieza previa de los depósitos y nivel del aceite en depósitos. Recientemente, para evitar efectos no deseables de estos factores, el uso de gases inertes en los depósitos de decantación como el nitrógeno en sustitución al oxígeno se está implantando en muchas almazaras (Di Giovacchino, 2013).

Todos los factores mencionados son críticos ya que los metales, la luz, el oxígeno, el tiempo prolongado de almacenamiento y la temperatura son inductores de los procesos oxidativos del aceite e inciden directamente en los parámetros físico químicos, composición del aceite, cambios del perfil sensorial y de volátiles (Angerosa y col., 2004; Morelló y col., 2004B; Kalua y col., 2007; Sinesio y col., 2015).

Previamente a la comercialización, puede ser aplicado el proceso de filtración con la finalidad de eliminar cualquier residuo en suspensión y/o humedad, evitando de esa forma los defectos sensoriales relacionados con la fermentación de la materia orgánica y prolongando la estabilidad oxidativa de esos aceites en el mercado. La filtración, además, proporciona al aceite un color uniforme y brillante, aunque también puede promover cambios en la fracción minoritaria de los aceites, como reducción de compuestos fenólicos, compuestos volátiles (aldehídos, cetonas y esteres) y contenido de clorofila (Angerosa y col., 2004; Vossen, 2007; Lozano-Sánchez y col., 2010). Finalmente la última etapa en la almazara es el embotellado del aceite, generalmente en condiciones de anaerobiosis (Lozano-Sánchez y col., 2010).

Figura 12. **A-** Almacenamiento del aceite en depósitos de acero inoxidable, **B –** Envasado.

2.5. Composición de los aceite de oliva

El aceite de oliva está compuesto por dos fracciones principales: fracción saponificable (mayoritaria) que representa más del 98% de su composición y fracción insaponificable, aproximadamente el 2% (Lopez y col., 2014). Cada una de esas fracciones incluye diferentes compuestos (**Figura 13**).

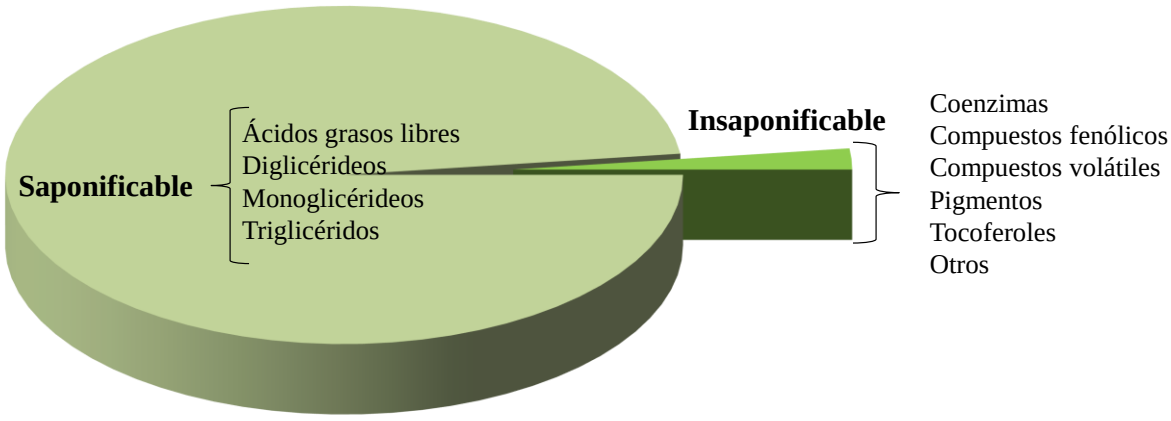

Figura 13. Composición del aceite de oliva - Fracción saponicable e insaponificable.

2.5.1. Fracción saponificable: perfil de ácidos grasos

Los ácidos grasos (AG) existen mayoritariamente como un complejo en la forma de triglicéridos (un éster derivado de glicerol asociado a tres ácidos grasos) y su contenido en el AO es responsable de su hidrofobicidad (Lopez y col., 2014).

Asimismo, los ácidos grasos simples pueden ser definidos como ácidos carboxílicos de cadena larga alifática no ramificada que presentan un grupo carboxílico en un extremo, pudiendo clasificarse según sus propiedades estructurales y químicas (**Figura 14**) como saturados (AGS), monoinsaturados (AGMI) y poliinsaturados (AGPI) (Lopez y col., 2014).

Los principales AGS encontrados en el aceite de oliva son ácido palmítico (C16:0) y ácido esteárico (C18:0), aproximadamente 7,5-20% y 0,5-5,0, respectivamente, mientras los otros AGS se encuentran en cantidades muy inferiores (≤0,3%).

Los AGMI componen la principal fracción de ácidos grasos del AO, particularmente el ácido oleico, que representa entre 55-83% de los ácidos grasos totales. El perfil mayoritario de AGMI es uno de los factores que disminuyen la susceptibilidad del aceite de oliva a la oxidación (Covas y col., 2007). A lo largo de los años, muchos estudios fueron y siguen siendo realizados en diferentes países como Grecia, Italia y España, con la finalidad de estudiar los efectos beneficiosos de la fracción saponificable del AO. El alto contenido de AGMI y bajo de AGS, en particular del aceite de oliva virgen extra (AOVE), se considera mayormente responsable de diferentes propiedades saludables, como menor riesgo y prevención de enfermedades coronarias, mejora del perfil lipídico sanguíneo asociado a la reducción de los niveles de lipoproteínas de baja densidad (LDL) e incremento de los niveles de lipoproteínas de alta densidad (HDL), y de otras evidencias como la mejora tensión arterial o el papel protector en las enfermedades neurodegenerativas (Covas y col., 2007; Huang y Sumpio, 2008; Guasch-Ferré y col., 2014; Lopez y col., 2014).

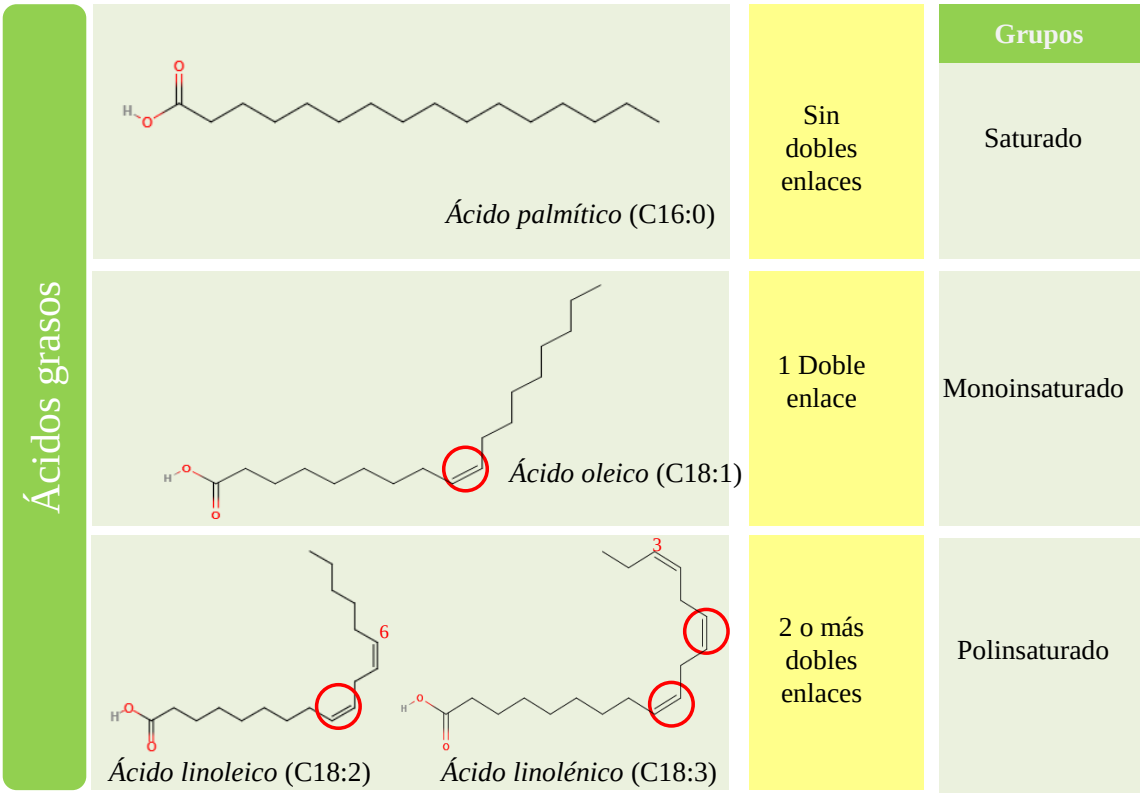

Figura 14. Clasificación de los ácidos grasos de acuerdo con las propiedades estructurales y químicas.

En relación a los AGPI, los pertenecientes a las familias n-3 (linolénico, C18:3) y n-6 (linoleico; C18:2), es decir, los ácidos grasos en que el primer doble enlace está situado entre el tercer - cuarto carbono; y entre el sexto - séptimo carbono del extremo metilo (CH_3) de la cadena, respectivamente, como muestra la **Figura 14**, tienen particular importancia en la nutrición humana, ya que el cuerpo humano no es capaz de producirlos de forma endógena, por lo que deben ser aportados por la dieta (Lopez y col., 2014; Scoditti y col., 2014).

De acuerdo con la FAO (2010), el requerimiento mínimo diario es del 1% del total de AG de la dieta para n-6 y 0,2% para n-3. En los aceites de oliva, el contenido de n-3 representa menos de 1%, mientras el de n-6 varía entre 3,5-21%. En comparación con los AGMI, los AGPI son más propensos a los procesos de oxidación debido a sus dobles enlaces. En la literatura científica se han descritos efectos positivos y negativos de los PUFA sobre la salud humana, pero los estudios recientes se enfocan a la proporción en la dieta de n-6/ n-3, más que la cantidad total (Scoditti y col., 2014; Simopoulos, 2016). Por otro lado, los AGPI desempeñan un papel esencial en el perfil

sensorial del aceite de oliva virgen (AOV), participando en la ruta de la lipoxigenasa (LOX), en la que los AGPI sufren muchas reacciones y desencadenan la generación de los compuestos volátiles (Angerosa y col., 2004; Kalua y col., 2007), información que se ampliará posteriormente (2.5.2.2).

Con respecto a las variaciones del perfil de ácidos grasos encontradas en los AO, pueden explicarse por muchos factores como factores climáticos, geográficos, variedad e índice de madurez de la aceituna (Boskou y col., 2006). Principalmente entre los factores climáticos, la temperatura es un punto de suma importancia en la regulación de las enzimas que participan activamente en la síntesis de ácidos grasos (Hernández y col., 2011).

2.5.2. Fracción insaponificable

La fracción minoritaria de los AO es compleja y extensa, presentando más de 230 compuestos menores responsables en gran medida de sus propiedades y características organolépticas y que, al contrario que en otros aceites vegetales, no han sido modificados por la extracción con solventes (Lopez y col., 2014; Servili y col., 2014). De esa forma, aliado al aporte de ácidos grasos mencionado anteriormente, los componentes menores del aceite de oliva también son responsables de la popularidad de los AO, debido a su importancia nutricional, sus propiedades saludables y sus características organolépticas que los distinguen entre los aceites vegetales. Algunos de los compuestos destacables de esa fracción son la coenzima Q_{10}, compuestos fenólicos, compuestos volátiles, pigmentos y tocoferoles.

2.5.2.1 Coenzima Q_{10}

Las coenzimas Q (CoQ) son compuestos lipófilos endógenos presentes de manera natural en las membranas celulares y las mitocondrias de mamíferos. En la naturaleza puede ser encontrados diferentes grupos (CoQ_6-Q_{10}), que diferen en su función y en relación al número de carbonos de la cadena lateral isoprenoide unida al anillo de benzoquinona, siendo la CoQ_{10} la forma predominante en humanos, principalmente en órganos que demandan más energía como el corazón, los riñones y el hígado (Thanatuksorn y col., 2009; Pravst y col., 2010; Pyo, 2010; Žmitek y col., 2014; Jankowski y col., 2016).

En el cuerpo humano, la CoQ_{10} pueden existir en dos formas oxidada (ubiquinona) o reducida (ubiquinol), habiendo una constante conversión entre ambas (**Figura 15**) (Pravast, y col., 2010; Alcázar y col., 2016).

Su principal función está en la cadena respiratoria mitocondrial y la producción de ATP, como la única enzima portadora de electrones, pero también es un reconocido antioxidante en las membranas lipídicas, sobre todo en su forma reducida, aunque no se puede descartar un papel antioxidante de la forma oxidada (Turunen, y col., 2004; Pravst y col., 2010; Jankowski y col., 2016). Además de las funciones mencionadas, la CoQ_{10} también es responsable de otras importantes funciones de modulación y regulación al nivel celular (Turunen y col., 2004).

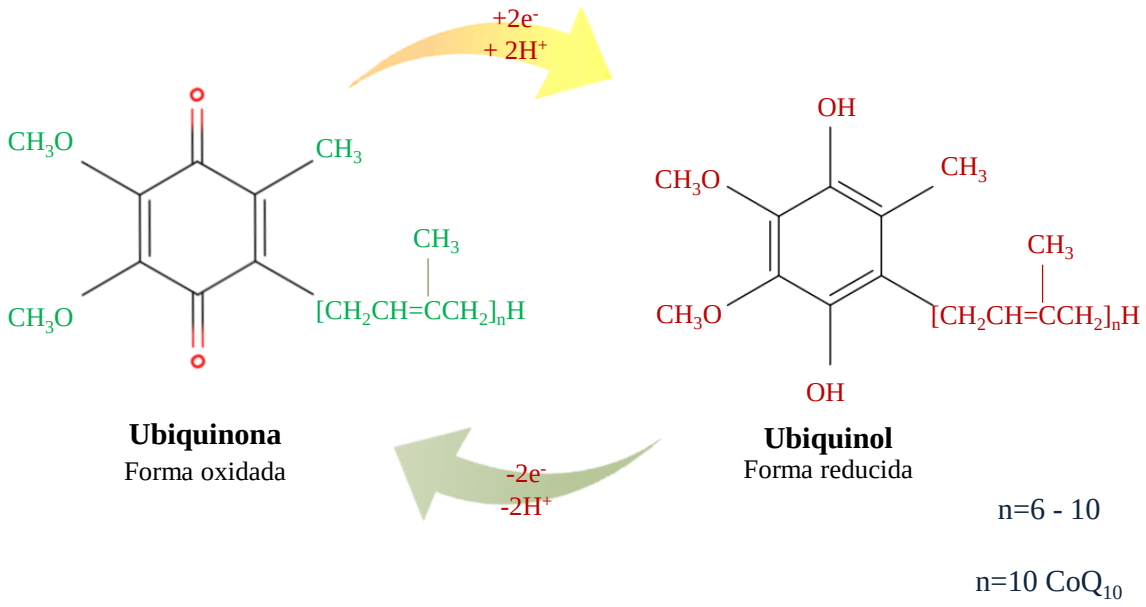

Figura 15. Reacción redox de conversión entre forma reducida y oxidada de coenzima Q. Adaptado: Alcázar- Fabra y col., 2016.

Aunque existe una síntesis endógena de CoQ, sus niveles disminuyen progresivamente con la edad, debiendo ser reemplazados diariamente por el aporte en los alimentos. En otros casos, la suplementación es aconsejada debido a sus efectos beneficiosos en diferentes enfermedades como diabetes, insuficiencia cardiaca y enfermedades genéticas (Jankowski y col., 2016). Asimismo, es importante considerar que el 25% de los niveles de CoQ_{10} son reemplazados diariamente por la vía endógena y exógena, por

lo que la ingesta de alimentos con un alto contenido en CoQ_{10} puede ser un aporte importante para la salud humana (Venegas y col., 2011).

En los alimentos la CoQ se encuentra mayoritariamente en productos de origen animal, pero también en nueces, semillas y aceites vegetales, particularmente el AO que puede ser considerado una fuente dietética importante (Pravst y col., 2010; Venegas y col., 2011; Žmitek y col., 2014). De acuerdo con Žmitek y col., (2014), el aporte de CoQ_{10} basado en 20 g de consumo diarios de AO puede variar entre 0,08 y 3,3 mg.

De acuerdo con estudios previos, el contenido variable de CoQ_{10} en aceites vegetales es explicado por diferentes factores como la variedad, el índice de madurez y parámetros tecnológicos de extracción; además, se ha sugerido que se necesitan más estudios científicos que determinen si otros parámetros pueden afectar los niveles de CoQ_{10} en aceites de oliva, como factores agronómicos, climáticos y geográficos (Venegas y col., 2010; Žmitek y col., 2014).

2.5.2.2. Compuestos fenólicos

Los compuestos fenólicos son metabolitos secundarios de las plantas e incluyen una amplia variedad de moléculas, aproximadamente 8000 compuestos, que presentan un anillo fenólico unido a un grupo hidroxilo (fenoles simples) o una estructura polifenólica (es decir, más de un anillo aromático unido a uno o más grupo hidroxilo) (Ignat y col., 2011; Lopez y col., 2014).

En las plantas, estos compuestos tienen muchas funciones biológicas, como actuar protegiendo contra la luz UV, contra patógenos, depredadores o atrayentes para polinizadores, entre otras (Ignat y col., 2011; Lopez y col., 2014).

En los aceites de oliva, esos compuestos son liberados del mesocarpio y epicarpio de la aceituna durante el proceso de extracción, siendo considerados una de las fracciones minoritarias más importantes de los AOV, ya que se relacionan con muchas propiedades biológicas, características sensoriales (amargo, picante y astringente) y capacidad antioxidante, siendo específicamente responsable de la extensión de la vida útil del producto (Servili y col., 2014; Talhaoui y col., 2016).

Por otra parte, la afinidad hidrofílica de los compuestos fenólicos facilita que se conjuguen con el agua de vegetación y al parecer, sólo un 2% presentes en la aceituna son transferidos al AO (Talhaoui y col., 2016).

El contenido total de fenoles en los AOV varía entre amplios márgenes (50-940 mg/kg), según la literatura científica (Servili y col., 2014). Sin embargo, como ya se ha

mencionado anteriormente, muchos factores influyen en su contenido como técnicas agronómicas, aspectos tecnológicos o factores climáticos y geográficos (Bakhouche y col., 2013; Kalogeropoulos y Tsimidou, 2014; Lopez y col., 2014).

Los principales grupos de compuestos fenólicos encontrados en los AOV son: ácidos fenólicos y derivados, alcoholes fenólicos, hidroxi-isocromas, flavonoides, lignanos y secoridoides (Lopez y col., 2014; Servili y col., 2014; Talhaoui y col., 2016). Algunos de sus estructuras se representan en la **Figura 16**.

Los mecanismos antioxidantes de compuestos fenólicos incluyen diferentes reacciones como la reducción de la concentración de oxígeno, la inactivación de radicales libres, descomposición de productos primarios de oxidación en especies no radicales y reducción de la sustracción de hidrógeno por parte de sustratos y quelantes de metales (Lopez y col., 2014).

De esa forma, los compuestos fenólicos del AO poseen acción antiinflamatoria y quimio-preventiva y son capaces de actuar previniendo contra los daños oxidativos en el organismo humano (ADN, lípidos y proteínas), lo cual justifica su asociación a la reducción del riesgo de desarrollo de muchas enfermedades degenerativas (Dimitrios, 2006; Martín-Peláez y col., 2013; Servili y col., 2014).

Por lo tanto, actualmente el AOV es el único alimento reconocido por la Autoridad Europea para la Seguridad de los Alimentos (EFSA) por sus niveles de hidroxitirosol y tirosol. Sin embargo, las limitaciones existentes en relación a las técnicas analíticas de extracción, separación, identificación y cuantificación de estos compuestos no permiten, a día de hoy, el etiquetado adecuado en relación a las alegaciones reconocidas, ya que no hay un método oficial establecido o patrones comerciales para todos los compuestos. Este tema es por lo tanto una prioridad para la comunidad científica, que sigue trabajando en su desarrollo (Carrasco-Pancorbo y col., 2005; Bakhouche y col., 2013; Lopez y col., 2014; Reboredo-Rodríguez y col., 2016).

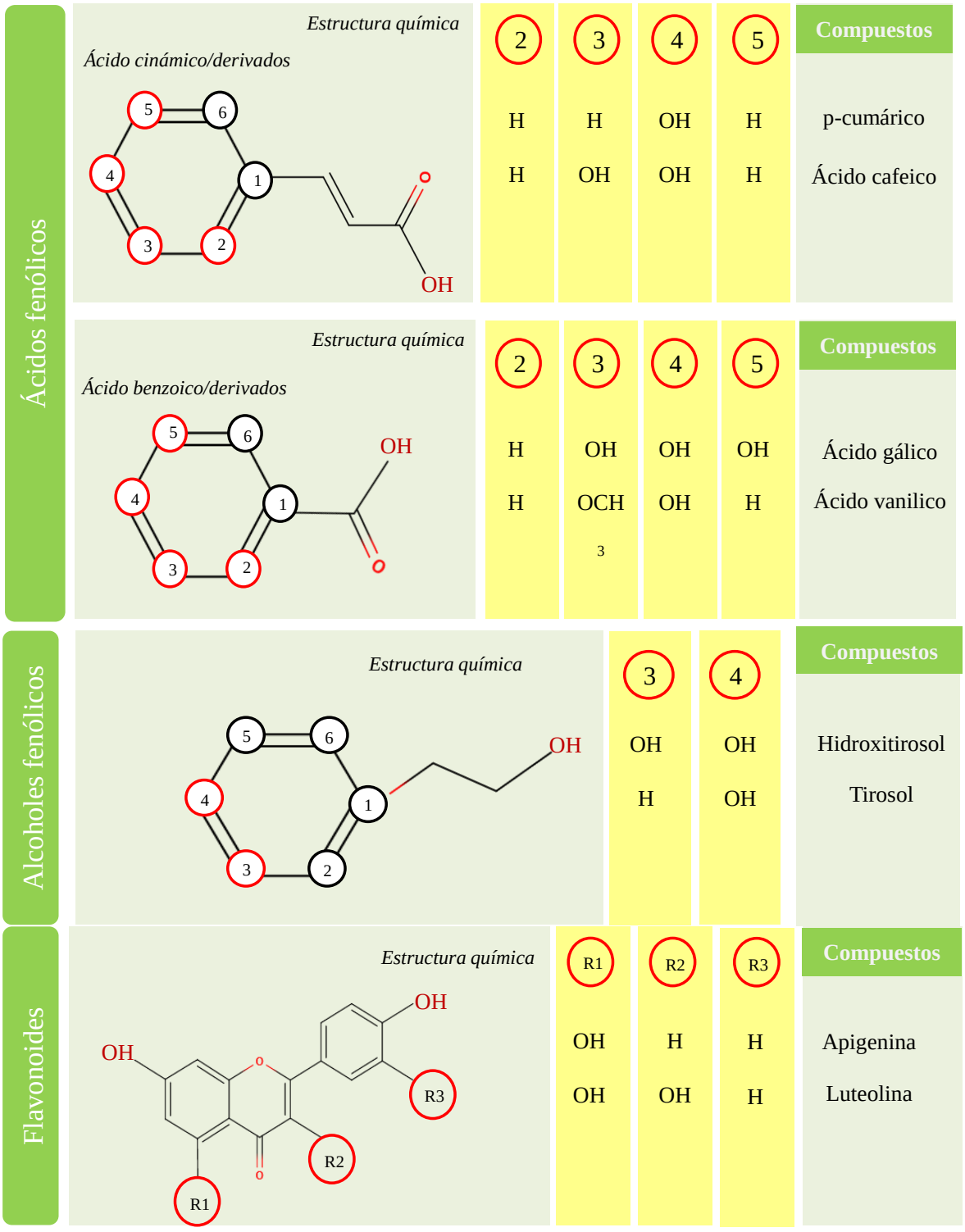

Figura 16. Estructuras químicas de las principales clases de compuestos fenólicos encontrados en los aceite de oliva vírgenes.

2.5.2.3. Compuestos volátiles

Los compuestos volátiles en el reino vegetal son considerados metabolitos provenientes de la ruta biogenética intracelular, incluyendo un amplio grupo de compuestos de bajo peso molecular (< 300 Da) susceptibles a la evaporación como aldehídos, alcoholes, cetonas, hidrocarburos y ésteres (Angerosa, 2002; Kalua y col., 2007).

Estos compuestos son directamente responsables del aroma de los alimentos, siendo capaces de estimular el epitelio olfativo, disolviéndose en la mucosa y adhiriéndose a los receptores olfativos generando percepciones por *via* orto nasal (Angerosa, 2002; Kalua y col., 2007; Barbieri y col., 2015).

El término *aroma* mantiene una relación singular con el término "*flavor*" que se refiere al efecto combinado entre la percepción orto nasal y retro nasal, o sea, olor y sabor (Barbieri y col., 2015).

En los AO, los compuestos volátiles son primordiales en la evaluación del perfil organoléptico (aromas positivos y negativos) y el grado de oxidación, por lo que influyen en la aceptabilidad del consumidor, aunque su determinación no pertenezca a los parámetros oficiales de calidad de la legislación europea para los aceites de oliva y derivados (Angerosa, 2002; Kalua y col., 2007; Barbieri y col., 2015).

Con respecto a su producción, esos compuestos son generados en dos etapas: durante el período climatérico de la aceituna y en el proceso de extracción del aceite, primordialmente durante la molienda y el batido, siendo afectada por esos dos factores, pero también por la variedad, área geográfica, clima y otros parámetros agronómicos/tecnológicos (Angerosa y col., 2004; Kalua y col., 2007).

La síntesis de los volátiles relacionados con los aromas positivos de los AOV tiene lugar a través en una serie de reacciones bioquímicas secuenciales, en las cuales los ácidos grasos linoleico (n-6) y linolénico (n-3) mediante la acción de enzimas como lipoxigenasas (LOX), hidroperoxido-liasa (HPL), alcohol deshidrogenasa (ADH), alcohol-aciltransferasa (ATT) y reacciones de isomerización, dan lugar a los principales volátiles encontrados en los aceites de oliva (**Figura 17**).

Así, los niveles de compuestos volátiles encontrados en el AO dependen de la actividad de las enzimas involucradas, siendo los asociados con aromas positivos (verde, hojas, almendra, banana) los que pertenecen a los grupos C6 y C5 y derivan de la ruta LOX, mientras que los defectos más comunes (atrojado, moho, humedad, avinagrado y rancio) están asociados con una inadecuada preservación de la aceituna que conlleva a procesos

de fermentación, desarrollo de contaminación microorganismos anaeróbicos, producción de aminoácidos o inadecuado procesamiento/almacenamiento generando productos de la oxidación del aceite (Angerosa y col., 2004, Morales y col., 2005; Kalua y col, 2007).

Otro aspecto que debe ser considerado es que los atributos sensoriales percibidos surgen de la influencia y la interacción de varios compuestos volátiles, más que de la acción de un solo compuesto y la alta concentración de volátiles no siempre resulta en una respuesta del olor, debiendo ser evaluados por cálculos del umbral como valor de la actividad odorante (Luna y Aparicio, 2002; Kalua y col., 2007; Angerosa y Campestre, 2013).

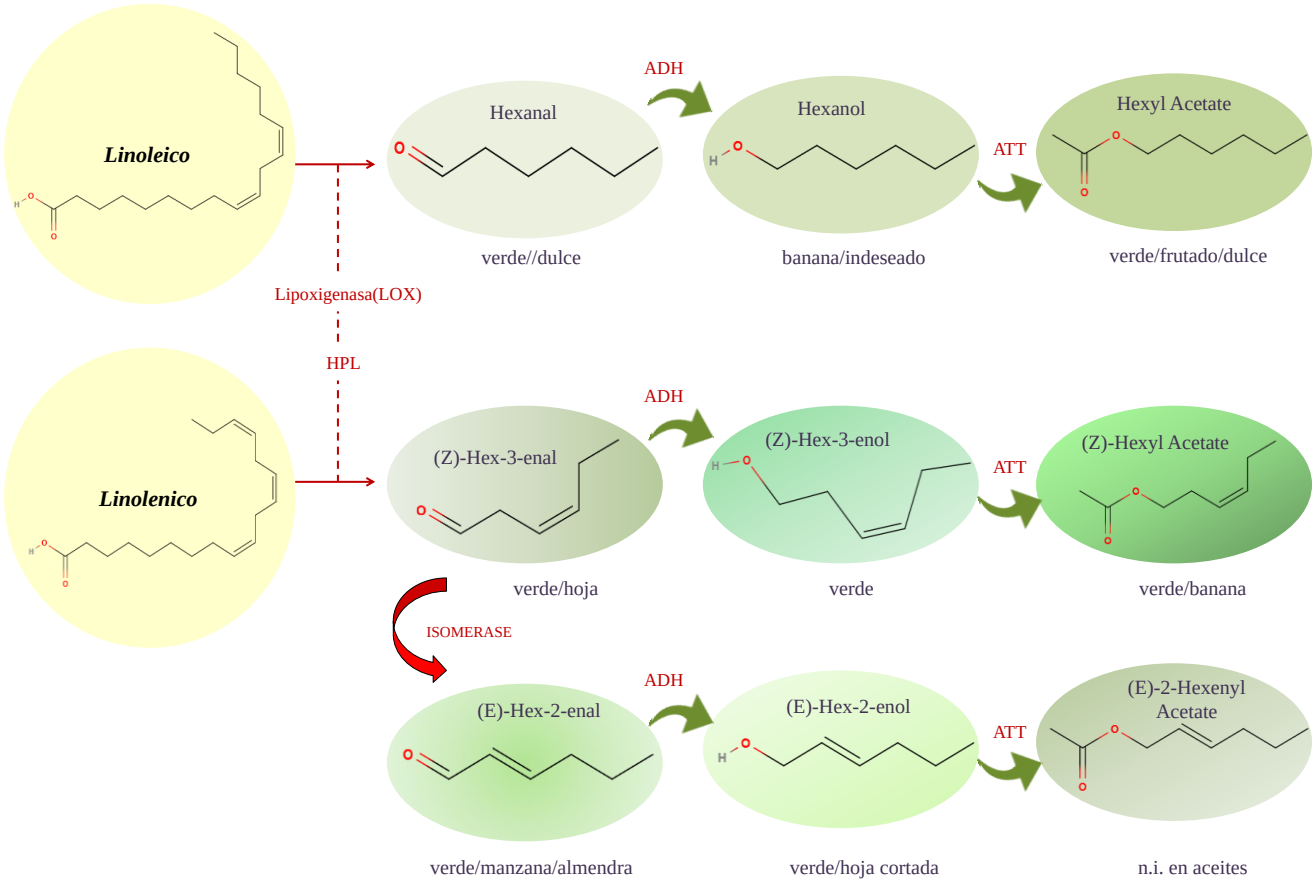

Figura 17. Ruta de formación de los principales volátiles encontrados en los aceites de oliva y sus respectivos descriptores olfativos. Adaptado: Kalua y col., 2007.

2.5.2.4. Pigmentos

Los pigmentos naturales se encuentran en diferentes organismos vivos, particularmente en plantas, donde desempeñan un papel fundamental en la fotosíntesis. Pueden ser definidos por su capacidad de absorber la luz en el rango de longitudes de onda de la región visible, produciendo un color de acuerdo con su estructura específica molecular (cromóforo), siendo esa estructura responsable por captar la energía y reflejarla, posibilitando así la percepción por el ojo humano (Delgado –Vargas y col., 2000; Tanaka y col., 2008).

Entre los grupos predominantes destacan las clorofilas y los carotenoides, que se diferencian en relación a su estructura funcional, ya que las clorofilas pertenecen al grupo de tetrapirólicos y los carotenoides se caracterizan como isoprenoides. Ambos comparten un papel importante en la estabilidad oxidativa de muchos alimentos, debido a sus propiedades antioxidantes en condiciones específicas como en la oscuridad, aunque también pueden ser pro-oxidantes en presencia de la luz y el aire (Delgado –Vargas y col., 2000; Psomiadou y Tsimidou, 2001; Criado y col., 2008; Moyano y col., 2010).

En la aceituna, los cambios de color del epicarpio y mesocarpio durante la maduración están asociados a las transformaciones bioquímicas de los pigmentos, siendo la clorofila y los carotenoides responsables del color verde/ amarillo del fruto que con la maduración progresivamente dan lugar a las antocianinas (Gandul- Rojas y col., 2013) (**Figura 18A**).

Durante la extracción del aceite solamente los pigmentos liposolubles (carotenoides y clorofilas) son transferidos desde la aceituna, confiriéndole los colores característicos del aceite de oliva, que pueden variar desde verde intenso a amarillo dorado (**Figura 18B**) (Gandul-Rojas y col., 2000; Moyano y col., 2010; Gandul- Rojas y col., 2013). Aunque, el color no es un parámetro de calidad oficial del aceite es un factor determinante en la elección del consumidor (Moyano y col., 2010) y ha sido incluido como parámetro evaluado por el departamento de agricultura de Estados Unidos en los aceites comerciales de este país para garantizar que el color sea propio del aceite de oliva (USDA ,2010).

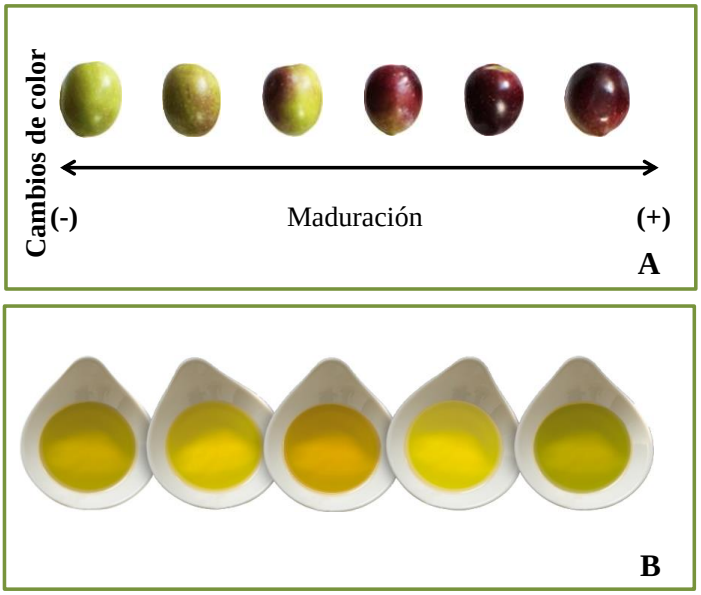

Figura 18. A - Cambios de color durante la maduración de la aceituna, **B -** Colores de aceites de oliva.

Muchos estudios se han dedicado a establecer relaciones entre el contenido de los pigmentos con parámetros de color de los AOV, incluyendo los aceites monovarietales procedentes de diferentes regiones, además, estos parámetros se han utilizado para predecir la caducidad de los aceites y determinar los diferentes fac85tores que pueden influenciar sus niveles (Gandul-Rojas y col., 2000; Psomiadou y Tsimidou, 2001; Morelló y col., 2004A; Meléndez-Martínez y col., 2007; Criado y col., 2008; Moyano y col., 2008; Peres y col.,2014).

2.5.2.5. Tocoferoles

Los tocoferoles son compuestos liposolubles y que sumados a los tocotrienoles pertenecen al grupo conocido por el término general de vitamina E, micronutriente esencial en la dieta humana, y son sintetizados por organismos fotoautótrofos, incluyendo las plantas, a través una serie de reacciones enzimáticas (Munné-Bosch y Alegre, 2002; Beltrán y col., 2010; Colombo, 2010; Georgiadou y col., 2015).

En general, los tocoferoles presentan en su estructura química un anillo central cromanol (posee un grupo fenol) al que se une una cadena lateral saturada isoprenoide denominada fitol, diferenciándose de los tocotrienoles solamente por la cadena lateral saturada (Psomiadou y col., 2000; Colombo, 2010; Lopez y col., 2014). Existen 4 isómeros de los tocoferoles (α-, β-, γ- y δ) que se distinguen en función de número y

posición de los grupos metilo del anillo cromanol, según la **Figura 19**, siendo el α-tocoferol la forma predominante y reconocida como la más activa biológicamente entre los isómeros (Beltrán y col., 2010; Lopez y col., 2014).

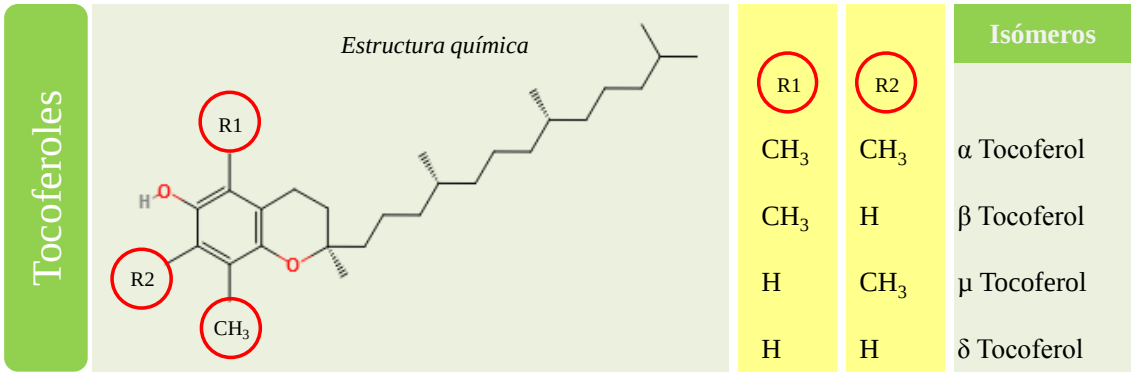

Figura 19. Estructura química de los tocoferoles. Adaptado: Lopez y col., 2014.

En relación a su ocurrencia en los alimentos, los aceites vegetales como el AOV, son considerados una de las fuentes excepcionalmente ricas en α-tocoferol, con un promedio alrededor de 90-95% de total (Kalogeropoulos y Tsimidou, 2014).

Es destacable su función como antioxidante en diversos sistemas biológicos, tanto en las propias plantas y en los alimentos como *in vivo* (Munné-Bosch y Alegre, 2002).

La capacidad antioxidante de los tocoferoles se explica por su capacidad de donar un hidrogeno del anillo fenol para los radicales libres provenientes principalmente de la oxidación lipídica, neutralizando así la formación del radical peroxil y evitando su propagación, pero también es un secuestrador del oxígeno singlete $^{1}O2$, un radical muy reactivo que oxida el ADN, los lípidos y otras sustancias (Munné-Bosch y Alegre, 2002). Otro rol desempeñado por los tocoferoles es el mantenimiento de la integridad de las membranas celulares, regulando su fluidez, así como antioxidante natural de las propias plantas, incluso como respuesta a condiciones de estrés y regulador de hormonas vegetales (Munné-Bosch y Alegre, 2002; Beltrán y col., 2010; Lopez y col., 2014).

En los AOV también actúan como antioxidantes naturales, pudiendo variar sus niveles como los otros compuestos minoritarios mencionados en función de diferentes aspectos como factores genéticos, agronómicos, geográficos, climáticos y factores tecnológicos (Psomiadou y col., 2000; Beltrán y col., 2010; Lopez y col., 2014; Kalogeropoulos y Tsimidou, 2014;Georgiadou y col., 2015).

Debido a sus propiedades antioxidantes, hay evidencias del efecto beneficioso de la vitamina E en enfermedades cardiovasculares, cáncer y enfermedades neurodegenerativas (Colombo, 2010; Lopez y col., 2014).

Además de las funciones y mecanismos descritos, los tocoferoles y especialmente el α-tocoferol, interaccionan con otros compuestos bioactivos como vitamina C, β caroteno y CoQ_{10}, posiblemente mediante mecanismos de regeneración (Munné-Bosch y Alegre, 2002; Guille y col., 2008). No se descarta su efecto pro oxidante en situaciones específicas como en presencia de luz, oxígeno, pH alcalino o trazas de iones de metales de transición (Munné-Bosch y Alegre, 2002; Guille y col., 2008; León-Camacho y col., 2013; Kalogeropoulos y Tsimidou, 2014).

2.6. Definición y clasificación del aceite de oliva

Los productos del olivar son controlados con referencia a su designación, autenticidad, calidad, pureza y otros aspectos comerciales por diferentes normativas de la Comunidad Europea, acuerdos internacionales del COI, *Codex Alimentarius* y las legislaciones propias de cada país.

De acuerdo con la definición obligatoria de comercialización del Reglamento UE n^0 1308/2013 y la definición del COI T/15 $n^0$3/2015, se pueden establecer dos grupos generales con fines comerciales: i) aceites de oliva y ii) aceites de orujo de oliva, categorías que incluyen a su vez diferentes tipos de aceite, según esquema de la **Figura 20**.

De esa forma, dentro de los aceites de oliva tenemos las siguientes denominaciones:

(a) Aceite de oliva vírgenes: aceite obtenido del fruto del olivo exclusivamente por medios mecánicos u otros procedimientos físicos aplicados en condiciones que excluyen toda alteración del producto, y que no se ha sometido a ningún otro tratamiento que no sea su lavado, decantación, centrifugado o filtración, excluidos los aceites obtenidos con el uso de disolventes o de coadyuvantes de acción química o bioquímica, por un procedimiento de re esterificación o como resultado de cualquier mezcla con aceites de otros tipos.

Este grupo engloba:

- Aptos para consumo: aceite de oliva virgen extra (AOVE) y aceite de oliva virgen (AOV).

- No aptos para consumo: aceite de oliva lampante (AOL).

(b) Aceite de oliva refinado: aceite de oliva obtenido del refinado de aceite de oliva virgen lampante.

(c) Aceite de oliva: aceite de oliva obtenido por la mezcla de aceite de oliva refinado y aceite de oliva vírgenes distinto del lampante.

Mientras que los aceites de orujo engloban los siguientes tipos:

(a) Aceite de orujo de oliva crudo: aceite que se obtiene del orujo de oliva mediante un tratamiento con disolventes o empleando medios físicos, o que corresponde, salvo en determinadas características, al aceite de oliva lampante.

(b) Aceite de orujo de oliva refinado: aceite obtenido del refino de aceite de orujo de oliva crudo.

(c) Aceite de orujo de oliva: aceite obtenido mezclando aceite de orujo de oliva refinado y aceite de oliva virgen distinto del lampante.

Además los aceites de oliva y aceites de orujo descritos en las referidas normativas tienen fijados la acidez libre expresada en ácido oleico para cada categoría.

Los parámetros de pureza y calidad de los aceites de oliva son establecidos por el Reglamento (CEE) n^0 2568/91 y sus modificaciones posteriores como el Reglamento (CEE) $n^0$1348/2013, que definen los limites en cada parámetro y los métodos de análisis que se deben aplicar (**Tabla 1**). Asimismo, es importante tener en cuenta que el aceite de oliva es el único producto entre las grasas animales y vegetales del mundo que tiene su propio acuerdo internacional y en el que la evaluación organoléptica es necesaria para la clasificación de los aceites (Aparicio y col., 2013; Vossen, 2013).

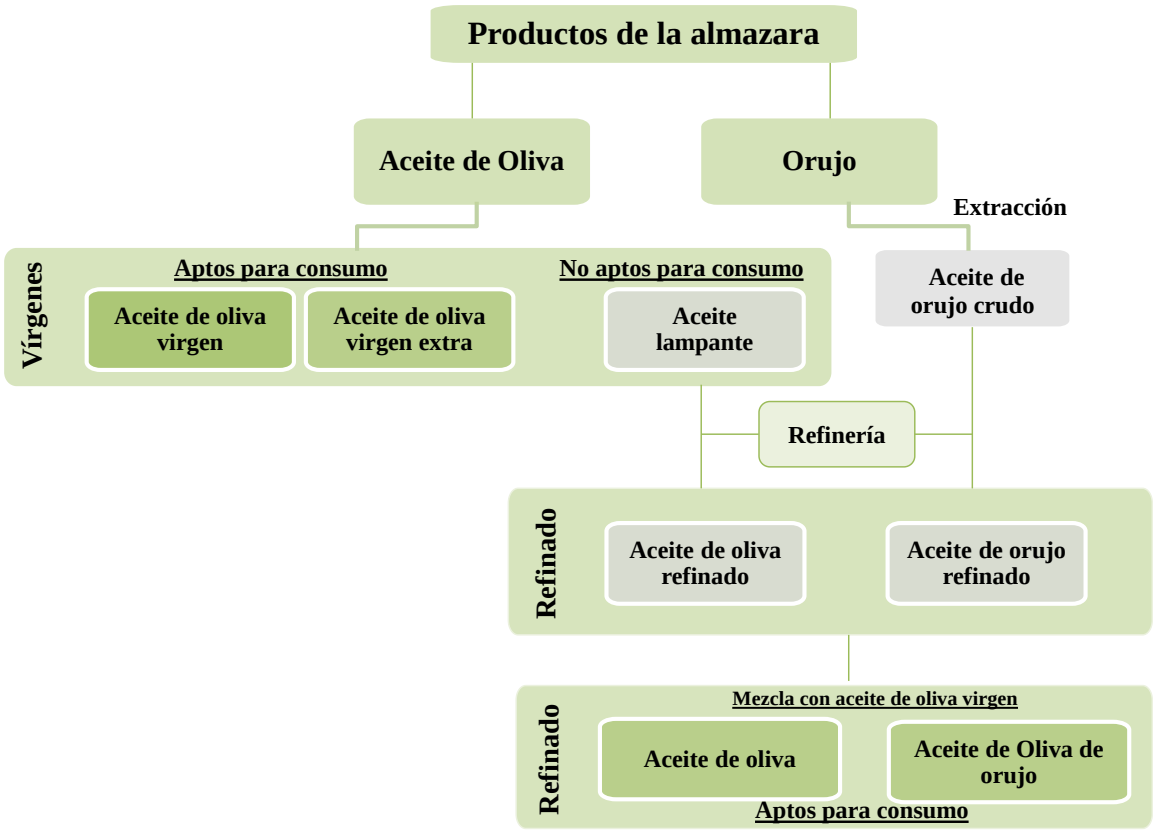

Figura 20. Tipos de aceite de oliva y orujo de Oliva comerciales. Adaptado: Mataix, 2001.

Tabla 1. Características de los aceites de oliva y de orujo establecidas por el Reglamento (CEE) n^0 2568/91 y sus modificaciones.

		Aceite de oliva virgen extra	Aceite de oliva virgen	Aceite de oliva lampante	Aceite de oliva refinado	Aceite de oliva (refinado + virgen)	Aceite de orujo de oliva crudo	Aceite de orujo de oliva refinado	Aceite de orujo de oliva
Parámetros de calidad	Acidez (%)	≤ 0,8	≤ 2,0	> 2,0	≤ 0,3	≤ 1,0	----	≤ 0,3	≤ 1,0
	Índice de peróxidos (mEq O2/kg)	≤ 20	≤ 20	---	≤ 5	≤ 15	---	≤ 5	≤ 15
	K_{232}	≤ 2,50	≤ 2,60	---					
	K_{270} o K_{268}	≤ 0,22	≤ 0,25	---	≤ 1,10	≤ 0,90	---	≤ 2,00	≤ 1,70
	ΔK	≤ 0,01		---	≤ 0,16	≤ 0,15	---	≤ 0,20	≤ 0,18
Pureza	Ceras (mg/kg)	≤ 150		≤ 300[3]	≤ 350		> 350[4]	> 350	> 350
	Monopalmitato de 2-glicerillo (%)	≤ 0,9 si C16:0 ≤ 14% ≤ 1,0 si C16:0 > 14%		≤ 0,9 si C16:0 ≤ 14% ≤ 1,1 si C16:0 > 14%		≤ 0,9 si C16:0 ≤ 14% ≤ 1,0 si C16:0 > 14%	≤ 1,4		≤ 1,2
	Estigmastadieno (mg/kg) [1]	≤ 0,05		≤ 0,50	---				
	Diferencia ECN42 (HPLC) y ECN42 (cálculo teórico)	≤ |0,2|		≤ |0,3|			≤ |0,6|	≤ |0,5|	
Sensorial	Mediana de defecto (Md)	Md = 0	Md ≤ 3,5	Md > 3,5[2]	---				
	Mediana de frutado (Mf)	Mf > 0	Mf > 0	---					
Ácidos grasos[2] (%)	Palmítico (C16:0)	7,5-20							
	Palmítoleico (C16:1)	0,3-3,5							
	Heptadecanoico (C17:0)	≤ 0,3							
	Esteárico (C18:0)	0,5-5,0							
	Oleico (C18:1)	55- 83							
	Mirístico (C14:0)	≤ 0,03							

	Linoleico (C18:2)	3,5-21					
	Linolenico (C18:3)	≤ 1,0					
	Araquídico (C20:0)	≤ 0,6					
	Eicosenoico (C20:1)	≤ 0,4					
	Behénico (C22:0)	≤ 0,2			≤ 0,3		
	Lignocérico (C24:0)	≤ 0,2					
	Σ Isómeros transoleicos	≤ 0,05	≤ 0,10	≤ 0,20		≤ 0,40	
	Σ Transoleicos + translinolenicos	≤ 0,05	≤ 0,10	≤ 0,30	≤ 0,10	≤ 0,35	
Esteroles (%)	Colesterol	≤ 0,50					
	Brasicasterol	≤ 0,10			≤ 0,20		
	Campesterol	≤ 4,0					
	Estigmatesrol	< Campesterol	---	< Campesterol	---	< Campesterol	
	β Sitosterol [5]	≥ 93,0					
	Δ-7-Estigmatenol	≤ 0,5					
Esteroles totales (mg/kg)		≥ 1000			≥ 2500	≥ 1800	≥ 1600
Eritrodiol/ Uvaol (%)		≤ 4,5	≤ 4,5[6]	≤ 4,5	> 4,5[7]	> 4,5	

Total de isómeros que han podido (o no han podido separase en columna capilar)[2] Debe estar en conformidad con el método establecido en anexo. [3] Se considera los aceites con contenido de ceras entre 300 - 350 mg/kg son aceite de oliva lampante si el contenido de alcoholes alifáticos totales es ≤ 350 mg/kg o si el contenido de eritrodiol y uvaol es ≤ 3,5%. [4] Se considera los aceites con contenido de ceras entre 300 - 350 mg/kg son aceite de orujo de oliva crudos si el contenido de alcoholes alifáticos totales es ≤ 350 mg/kg y si el contenido de eritrodiol y uvaol es ≥ 3,5. [5] Suma de: Δ-5-23-estigmastadienol + clerosterol + beta-sitosterol + sitostanol + delta-5-avanesterol + delta 5-24-estigmastadienol [6] Se considera que los aceites con contenido en ceras entre 300 y 350 mg/kg se consideran aceite de oliva lampante si el contenido en alcoholes alifáticos totales es ≤ a 350 mg/kg o si el contenido en eritrodiol y uvaol es ≤ a 3,5%. [7] Los aceites con un contenido en ceras entre 300 y 350 mg/kg se consideran aceite de orujo de oliva crudo si el contenido en alcoholes alifáticos totales es > a 350 mg/kg y si el porcentaje de eritrodiol y uvaol es superior a 3,5%. [8] Esteres etílicos de los ácidos grasos (FAEEs) mg/kg para AOVE ≤35 (campaña 2014/2015); ≤30 (campañas posteriores a 2015).
Adaptado: Reglamento (CEE) n^0 2568/91 y sus modificaciones (Reglamento (CEE) n^{0}1348/2013).

2.7. Parámetros de calidad del aceite de oliva reglamentados

Los parámetros de calidad del aceite de oliva descritos por el reglamento (CEE) n° 2568/91 y sus modificaciones, incluyen determinaciones analíticas que se refieren a los procesos hidrolíticos de degradación como acidez, procesos de oxidación primaria y secundaria determinados por los índice de peróxidos y coeficientes de extinción especifica (K_{232}, K_{20} y ΔK). Según el reglamento, se debe seguir un esquema de decisiones para la comprobación de la conformidad de una muestra de AO con la categoría declarada, empezado por los parámetros de calidad y la evaluación organoléptica, y considerando a continuación el contenido en ácidos grasos, ECN42, esteroles, eritrodiol/uvaol y ceras.

2.7.1. Acidez

Método volumétrico de titulación colorimétrico que determina los ácidos grasos libres en el aceite de oliva por la reacción de hidrólisis de los triglicéridos y diglicéridos conllevando a la ruptura de la ligación éster, conforme la reacción de la **Figura 21**. La determinación se basa en la disolución del aceite en una mezcla de disolventes y la valoración mediante una solución etanólica de hidróxido potásico, empleando como indicador de color la fenolftaleína y expresando los resultados en relación al porcentaje en ácido oleico (ácido graso mayoritario), es decir % m/m en ácido oleico.

De esa forma, el método es un indicador del grado de hidrólisis del aceite relacionado con aceites provenientes de aceitunas en una etapa avanzada de madurez y /o que experimentan degradación enzimática (lipasas) o AO proveniente de un largo tiempo de espera entre cosecha y procesamiento (Borges y col, 2015; Reboredo-Rodríguez y col., 2015).

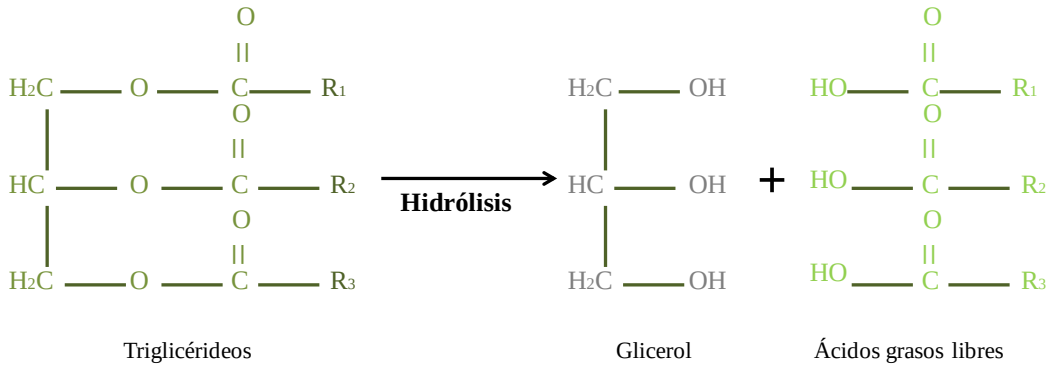

Figura 21. Reacción de oxidación por hidrólisis y formación de los ácidos grasos libres.

2.7.2. Índice de Peróxidos

Método volumétrico de titulación que determina los productos primarios de oxidación, los hidroperóxidos (**Figura 22**) expresado en mEq de O_2/kg. Su principio se basa en la disolución de la muestra en solución de ácido acético: cloroformo, tratándose con yoduro potásico y valorando con solución de tiosulfato sódico, reacción que conlleva la oxidación del yoduro potásico.

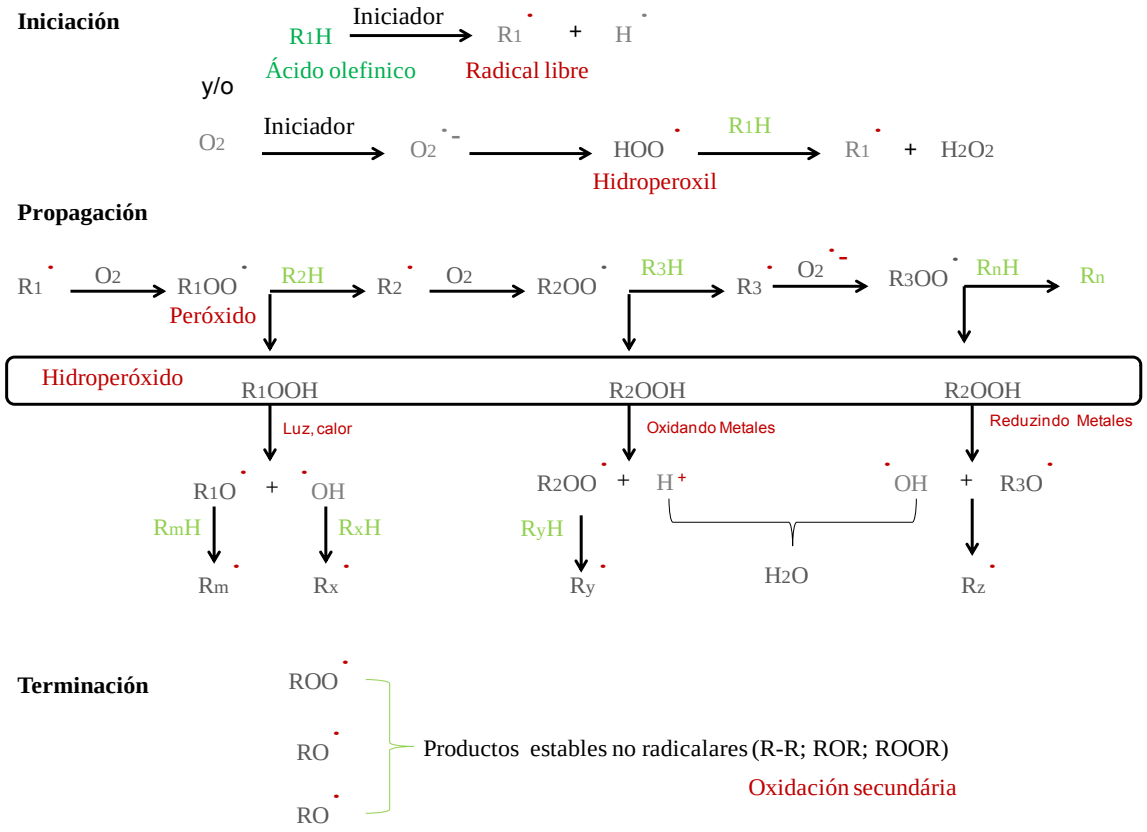

Figura 22. Esquema de peroxidación lipídica. Adaptado: Shahidi y Zhong, 2010.

2.7.3. Coeficientes de extinción específica

Prueba espectrofotométrica en el ultravioleta que indica el estado de conservación del aceite y las modificaciones inducidas por los procesos tecnológicos. Las absorciones en las longitudes de onda indicadas en el método se deben a la presencia de sistemas diénicos y triénicos conjugados, es decir los productos secundarios de oxidación (**Figura 22**). Los valores de estas absorciones se expresan en extinción específica. Su principio se basa en la disolución de la muestra en la solución requerida y se determina a las longitudes de onda prescritas.

2.8. Parámetros no reglamentados

2.8.1. Estabilidad oxidativa

El método de Rancimat es una medida acelerada de la resistencia a la oxidación empleada en diferentes tipos de aceites vegetales, muy popular por su facilidad y reproducibilidad. Este método es capaz de predecir la vida útil del aceite, basándose en la inducción de la oxidación de una cantidad fija de aceite con temperatura y flujo de aire constantes y midiendo los cambios de conductividad en el agua producidos al generar ácidos orgánicos volátiles (productos de la oxidación final) y determinando el periodo de inducción oxidativa en horas (**Figura 23**) (Farhoosh, 2007; Farhoosh y col., 2008; García-Moreno y col., 2013).

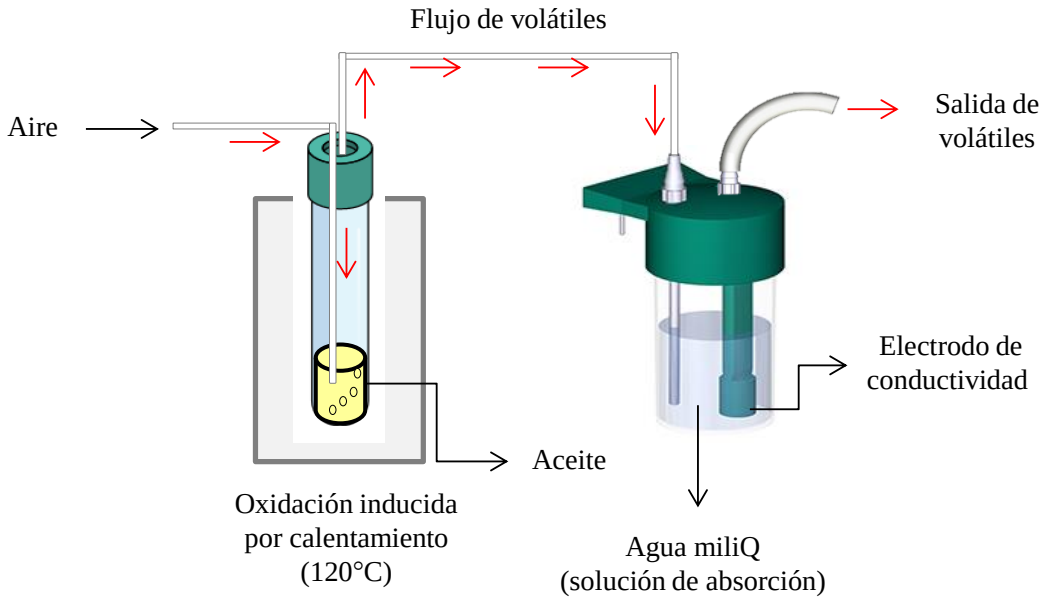

Figura 23. Esquema del principio del método de Rancimat.

2.8.2. Color

Como se ha mencionado anteriormente, el color se relaciona con el contenido en pigmentos y no es un parámetro de calidad establecido en las normativas vigentes para los AO o para la valoración organoléptica, pero puede influenciar la aceptabilidad de los consumidores (Moyano y col., 2010).

La evaluación instrumental del color se realiza mediante los sistemas CIELUV y CIELAB (Moyano y col., 2008). El CIELAB es ampliamente utilizado en alimentos, incluidos los AO, presentando una alta correlación con los pigmentos y el índice de

maduración (Melendéz-Martínez y col., 2007; Moyano y col., 2008). Las medidas de color del CIELAB, son establecidas de acuerdo con coordenadas en el espacio de cromaticidad determinado como a* (color rojo – al color verde) y b* (color amarillo- al azul), mientras la coordenada L* es considerada una estimación de luminosidad en que 0 correspondiente al negro y 100 que se refiere al blanco (**Figura 24**) (Moyano y col., 2010).

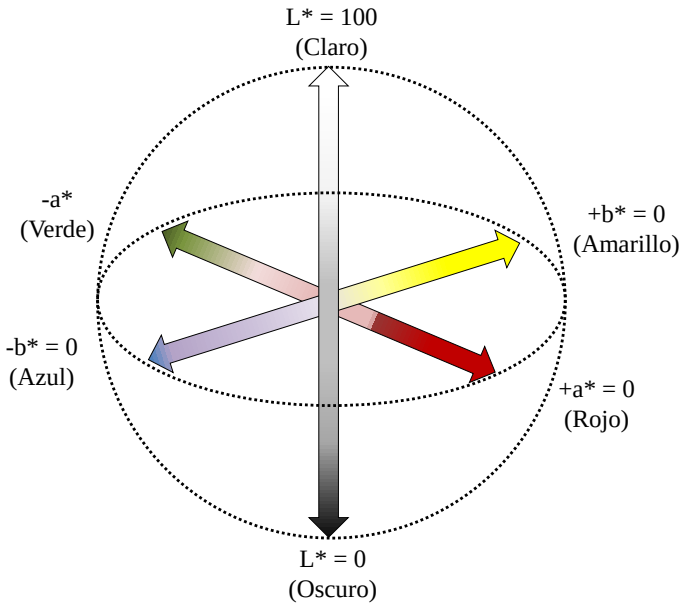

Figura 24. Coordenadas del color CIELAB.

2.9. Parámetros organolépticos

La valoración organoléptica para la clasificación de los aceites de oliva es parámetro oficial obligatorio de acuerdo con el reglamento (CEE) n^0 2568/91 y sus modificaciones posteriores vigentes establecidas en el Reglamento (UE) nº 1348/2013. Dicha reglamentación describe el procedimiento de evaluación, el método y las indicaciones para el etiquetado de los aceites.

Asimismo, establece que la clasificación de los AO debe ser realizada por un panel de catadores entrenados (8-12 personas) en una sala ambientalmente aislada entre 20°- 25° C en copas adecuadas calentadas a 28 ± 2°C cumpliendo todas condiciones para tal finalidad, siendo aplicable para los AOV los limites descritos en la **Tabla 1**, por la existencia del atributo frutado y la intensidad de defectos.

Además, la normativa hace una descripción detallada de los atributos positivos y negativos (**Tabla 2**), estableciendo también los atributos positivos en función de la intensidad y de la percepción en: intenso > 6; medio 3-6 y ligero >3.

Tabla 2. Descripción de los atributos positivos y negativos de los aceites de oliva vírgenes.

Atributos positivos	
Frutado	Conjunto de sensaciones olfativas características del aceite, dependientes de la variedad de las aceitunas, procedentes de frutos sanos y frescos, verdes o maduros, y percibidas por vía directa y/o retronasal.
Amargo	Sabor elemental característico del aceite obtenido de aceitunas verdes o en envero. Se percibe en las papilas circunvaladas de la uve lingual.
Picante	Sensación táctil de picor, característica de los aceites obtenidos al comienzo de la campaña, principalmente de aceitunas todavía verdes. Puede ser percibido en toda la cavidad bucal, especialmente en la garganta.
Atributos negativos	
Atrojado/borras	Flavor característico del aceite obtenido de aceitunas amontonadas o almacenadas en condiciones tales que han sufrido un avanzado grado de fermentación anaerobia o del aceite que ha permanecido en contacto con los lodos de decantación, que también han sufrido un proceso de fermentación anaerobia en trujales y depósitos
Moho-humedad	Flavor característico del aceite obtenido de frutos en las que se han desarrollado abundantes hongos y levaduras a causa de haber permanecido amontonadas con humedad varios días, o aceite obtenido de las aceitunas que han sido recogidas con tierra o barro y que no han sido lavadas.
Avinado – avinagrado/ Ácido-agrio	Flavor característico de algunos aceites que recuerda al vino o vinagre. Es debido fundamentalmente a un proceso fermentativo aerobio de las aceitunas o de los restos de pasta de aceitunas en capachos que no han sido limpiados adecuadamente, que da lugar a la formación de ácido acético, acetato de etilo y etanol.
Rancio	Flavor de los aceites que han sufrido un proceso oxidativo intenso.
Aceitunas congeladas (madera mojada)	Flavor característico de aceites que han sido extraídos de aceitunas que han sufrido un proceso de congelación en el árbol
Cocido o quemado	Flavor característico del aceite originado por un excesivo y/o prolongado calentamiento durante el procesado, muy particularmente durante el termo-batido de la pasta, si este se realiza en condiciones térmicas inadecuadas.
Heno-madera	Flavor característico de algunos aceites procedentes de aceitunas secas.
Basto	Sensación buco-táctil densa y pastosa producida por algunos aceites viejos.
Lubricante	Flavor del aceite que recuerda al gasóleo, a la grasa de lubricar o al aceite mineral.
Alpechín	Flavor adquirido por el aceite a causa de un contacto prolongado con alpechín que han sufrido procesos fermentativos.
Salmuera	Flavor del aceite extraído de aceitunas conservadas en salmuera.

Metálico	Flavor que recuerda a los metales. Es característico del aceite que ha permanecido en contacto, durante tiempo prolongado, con superficies metálicas, durante los procesos de molienda, batido, prensado o almacenamiento.
Esparto	Flavor característico del aceite obtenido de aceitunas prensadas en capachos nuevos de esparto. El flavor puede ser diferente si el capacho está fabricado con esparto verde o si lo está con esparto seco
Gusano	Flavor característico del aceite obtenido de aceitunas fuertemente atacadas por larvas de mosca del olivo (Bactrocera oleae).
Pepino	Flavor que se produce en el aceite cuando se mantiene en un envase hermético durante un tiempo excesivo, particularmente en hojalata, que se atribuye a la formación de 2,6-nonadienal.

Es importante destacar que las características sensoriales positivas se deben mayoritariamente a los compuestos volátiles, pero también los atributos amargo y picante se correlacionan con el contenido de compuestos fenólicos (Luna y Aparicio, 2002; Aguilera y col., 2005; Stefanoudaki y col., 2009; Jiménez y col., 2013; Barbieri y col., 2015).

Por otro lado, hay que tener en cuenta que el perfil sensorial se ve fuertemente afectado por la variedad, composición y otros aspectos como índice de maduración y condiciones de extracción (Luna y Aparicio, 2002; Angerosa y Campestre, 2013). De acuerdo con Romero y col., (2016), en variedades adaptadas en Chile el suelo y el clima tiene más influencia en las características sensoriales que la propia variedad, justo lo contrario de los aceites producidos en la Cuenca del Mediterráneo, mostrando un perfil sensorial diferente de los aceites producidos en regiones fuera del Mediterráneo.

Actualmente, a pesar de que el análisis sensorial sea aceptado como una herramienta potente para la valoración organoléptica para la clasificación de los tipos de aceite de oliva vírgenes y lampante, nuevas herramientas como los métodos basados en sensores artificiales conocidos por lengua electrónica, nariz electrónica y sistemas de visión artificial están siendo propuestas como auxiliares en la clasificación, evaluación de la calidad y diferenciación de los AO (Oliveri y col., 2009; Dias y col., 2014; Rodrigues y col., 2016; Di Rosa, 2017; Slim y col., 2017).

La lengua electrónica es definida como una herramienta analítica formada por una serie de sensores electroquímicos no específicos acoplados a un dispositivo de adquisición de señales que, a su vez, está conectado a un ordenador/portátil para análisis de las señales electroquímicas generadas (Di Rosa, 2017) (**Figura 25**).

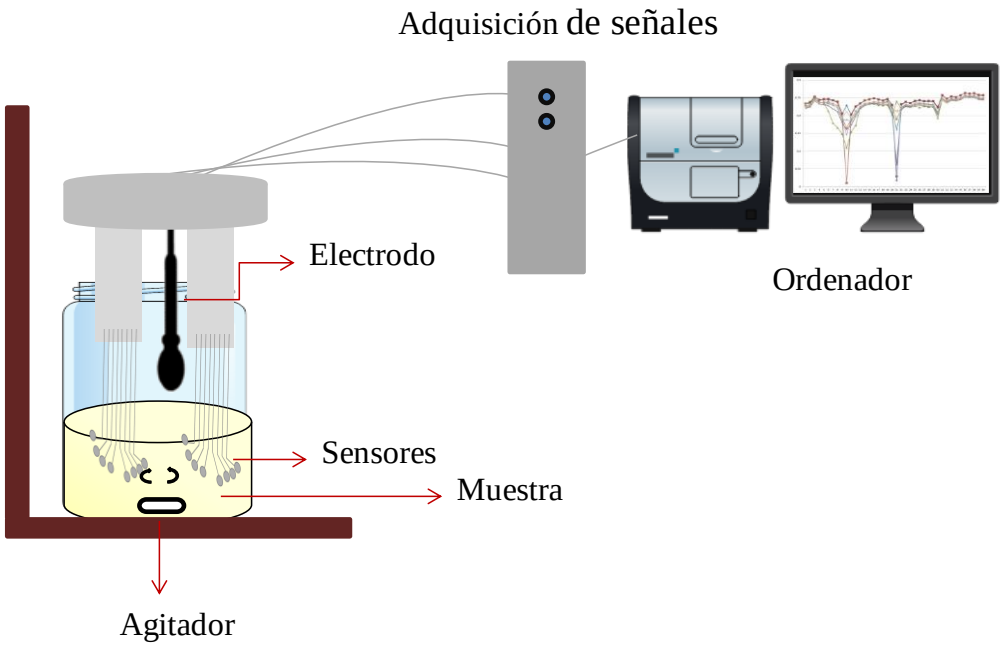

Figura 25. Componentes del análisis mediante lengua electrónica.

Diferentes sensores como electroquímicos (voltimétricos, potenciométricos, amperométricos y otros), ópticos o biosensores pueden ser utilizados, siendo más frecuentes los sensores electroquímicos (Di Rosa, 2017). Los sensores electroquímicos están constituidos por membranas poliméricas lipídicas con propiedades electroquímicas no específicas que son capaces de generar una señal a los compuestos polares en contacto con una solución hidroalcohólica sin emisión de una corriente eléctrica (Dias y col., 2014; Slim y col., 2017). Dichas soluciones son ricas en compuestos polares (esteroles, compuestos fenólicos, tocoferoles) y la señal generada por los compuestos pueden proporcionar una aproximación de las complejas funciones de los receptores gustativos humanos, mimetizando sensaciones gustativas como acidez, salinidad, dulzor, amargo y umami, pero también sensaciones táctiles como picantes (Dias y col., 2014; Di Rosa, 2017; Slim y col., 2017).

En los últimos años se ha aplicado con éxito en los aceites de oliva para distinguir los aceites función de su calidad, clasificación comercial, variedad y origen geográfico, incluyendo la discriminación entre los diferentes países (Apetrei y col., 2010, Dias et al., 2014; Olivieri et al., 2009; Rodrigues y col., 2016; Slim y col., 2017). Además, por su estrecha relación con los compuestos volátiles, se ha propuesto como una herramienta útil para complementar la evaluación organoléptica de los alimentos (Slim

y col., 2017), que se define por un efecto combinado y sinérgico de diferentes sensaciones y percepciones.

Esta metodología tiene muchas ventajas como bajo coste, rapidez, portabilidad, simplicidad y precisión (Dias y col., 2014; Di Rosa y col., 2017).

2.10. Aceites monovarietales: la variedad Arbequina

A día de hoy, el mercado del AOV está cada vez más especializado y los aceites denominados monovarietales, es decir, aceites producidos a partir de una única variedad de aceituna, están ganando un hueco en el mercado oleícola. Estos aceites son de gran interés desde un punto de vista científico y comercial por diferentes aspectos, particularmente en el mercado internacional (Japón, Brasil, Estados Unidos), donde el aceite de oliva representa todavía un producto de selección ("gourmet"). Por otra parte, el consumo especializado de los aceites monovarietales están también direccionados a productos de una determinada zona geográfica y con certificados de origen, así, el productor comparte el interés de los consumidores por distinguir su producción, destacando las peculiaridades de cada genotipo para diferentes combinaciones gastronómicas (Cecchi y Alfei, 2013). Otro aspecto destacable de los aceites monovarietales es su papel en las mezclas de aceites conocidos por "coupages" o "blends" (Luna y Aparicio, 2002).

Entre los aceites monovarietales más conocidos y cultivados mundialmente, hay que señalar los aceites provenientes de la variedad Arbequina. Esta variedad es autóctona de las regiones del noreste de España (Tarragona y Lleida), específicamente hace referencia en su nombre al pueblo de Arbeca localizado en Lleida (Vossen, 2007). Actualmente, está siendo cultivada en diferentes partes del mundo como Argentina, Australia, Brasil, Chile, Estados Unidos, Túnez y Turquía (Berenguer y col., 2006; Torres y col., 2009; García- González y col, 2010.; Mailer y col., 2010; Dabbou y col., 2011; Rondanini y col., 2011; Ballus y col., 2014, Romero y col., 2016; Uluata y col., 2016).

Su expansión mundial se explica por las características del fruto, del árbol y del aceite. Los olivos de la variedad Arbequina se caracterizan por su alta resistencia al frio, precocidad (producción entre 2-3 años), baja fuerza de desprendimiento, alta flexibilidad y buena productividad, mientras la aceituna es pequeña (alrededor de 1,9 g), por lo tanto con baja proporción pulpa/hueso y con alto rendimiento de aceite

(aproximadamente 20.5%) (Bakhouche y col., 2013; Vossen, 2013). Estos aspectos la convierten en la variedad predominante de los sistemas de cultivo intensivo y súper intensivo.

Sumados a esos factores, los aceites producidos a partir de esa variedad son altamente apreciados en el mercado internacional por sus aspectos sensoriales tradicionales establecidos como frutados y dulces, ligeramente amargos y picantes (Tous y col., 1997; Luna y Aparicio, 2002), aunque más recientemente los aceites Arbequina son más verdes, debido a su cosecha temprana, pero es relevante considerar que las nuevas plantaciones de diferentes regiones pueden producir cambios inesperados en la calidad sensorial (García-González y col., 2010).

2.11. Propiedades antioxidantes

El término "radicales libres" se refiere a átomos, moléculas o iones definidos por su inestabilidad química, ya que poseen un electrón desapareados en su órbita y consecuentemente son muy inestables y reactivos, característica que favorece las reacciones con otras moléculas para captar el electrón que les falta (Carocho y Ferreira, 2013; Sindhi y col., 2013). Son derivados de tres principales elementos, oxígeno, nitrógeno y azufre, dando lugar a las especies reactivas de oxígeno, especies reactivas de nitrógeno y especies reactivas de azufre, términos conocidos por las siglas en inglés ROS, RNS y RSS (Carocho y Ferreira, 2013)

En el cuerpo humano, los radicales libres son producidos continuamente de forma endógena dentro de las mitocondrias durante las reacciones metabólicas aérobicas, como la obtención de energía y otras reacciones de desintoxicación, señalización química y función inmune (Dimitrios, 2006; Carocho y Ferreira, 2013). Pero también pueden ser generados por vía exógena, bien por factores oxidantes externos (alcohol, drogas, contaminación ambiental, radiación UV, tabaco, ejercicios físicos intensos) y/o durante severos procesos de infección/inflamación (Mitjavila y Moreno, 2010; Carocho y Ferreira, 2013; Sindhi y col., 2013). En este sentido los ROS se destacan por su predominancia en el organismo humano, incluyendo diferentes especies como oxígeno singlete (O_2), radical anión superóxido ($O_2^{\bullet -}$), peróxido de hidrógeno (H_2O_2), radical hidroxilo (OH^-) y óxido nítrico (NO) (Carocho y Ferreira, 2013).

Cuando ocurre una sobreproducción de estas especies, es decir, un desbalance oxidativo en favor de los radicales libres sea relacionado con una incapacidad del sistema de

defensa o por la sobreexposición a los factores oxidantes, se produce una situación conocida como estrés oxidativo, que puede suponer daños en el ADN/ARN y en otras biomoléculas (lípidos, proteínas, hidratos de carbono) (Dimitrios, 2006; Sindhi y col., 2013). Hay que destacar que la actuación de estas moléculas en los lípidos conlleva a la peroxidación lipídica, proceso que se inicia por la abstracción de un átomo de hidrogeno de la cadena lateral del ácido graso (esquema representado en la **Figura 22**, apartado 2.7).

Los daños oxidativos están relacionados con el desarrollo de muchas enfermedades crónicas como el aterosclerosis, cáncer, diabetes, enfermedades cardiovasculares, neurodegenerativas (Alzheimer y Parkinson), procesos de envejecimiento y otras severas patologías (Dimitrios, 2006; Carocho y Ferreira, 2013; Sindhi y col., 2013).

Los compuestos conocidos como antioxidantes son sustancias que aún en bajas concentraciones retardan, inhiben o previenen las reacciones de oxidación de las moléculas diana, evitando el daño oxidativo y actuando en ocasiones en la regulación de las defensas antioxidantes endógenas (que incluyen las enzimas superóxido dismutasa, catalasa, glutatión peroxidasa, glutatión reductasa, glucosa-6-fosfato deshidrogenasa) y la coenzima CoQ (Pravast, y col., 2010; Carocho y Ferreira, 2013; Sindhi y col., 2013; Farzaneh y Carvalho, 2015).

De acuerdo con Carocho y Ferreira, (2013), los mecanismos de acción de los antioxidantes son los siguientes:

i) Inhibidores de las reacciones de oxidación de radicales libres, es decir como preventivos inhibiendo la formación de derivados de la oxidación lipídica.
ii) Interrumpiendo la propagación de la reacción en cadena de la peroxidación lipídica
iii) Inactivando el oxígeno singlete mediante la sinergia con otros antioxidantes
iv) Como agentes reductores conviertiendo los hidroperóxidos en compuestos estables
v) Como agentes quelantes, formando compuestos estables con los metales pro-oxidantes (hierro y cobre)
vi) Inhibidores de las enzimas pro-oxidantes, como las lipooxigenasas. (lipooxigenasas).

Así, estos compuestos desempeñan un papel fundamental para reducir los procesos oxidativos tanto el cuerpo humano como en los alimentos, en los que pueden prevenir y retardar el deterioro ayudando a mantener el sabor, la textura y el color del producto durante el almacenamiento (Sindhi y col., 2013).

Las fuentes de antioxidantes de origen natural como frutas, verduras, vino, té, especias y el aceite de oliva han sido muy estudiadas, ya que desde un punto de vista nutricional, las dietas ricas en alimentos con alto potencial antioxidante favorecen el equilibrio oxidativo y la protección endógena del organismo bajo condiciones de estrés, principio que relaciona el consumo de fuentes de antioxidantes de la dieta a la protección y/o reducción de muchas enfermedades como las mencionadas anteriormente (Dimitrios, 2006; Servili y col., 2014; Farzaneh y Carvalho, 2015).

Según la literatura científica, las principales clases de antioxidantes que se encuentran de forma natural en los alimentos son las vitaminas (A, C, E y K), los pigmentos (antocianinas y carotenoides) y los compuestos fenólicos (flavonoides, ácidos fenólicos, lignanos y estilbeno) (Farzaneh y Carvalho, 2015; Oroian y Escriche, 2015).

Numerosos estudios, tanto *in vivo* como *in vitro* o *ex vivo* han demostrado una elevada capacidad antioxidante del AOV y de muchos de sus compuestos aislados (Sanchéz, 2007; Castañer y col., 2012; Fadda y col., 2012; Franco y col., 2014; Servili y col., 2014; Chiesi y col., 2015; Incani y col., 2016). Los principales compuestos responsables de dichas propiedades en los AOV son los compuesto fenólicos, la vitamina E, los carotenoides y la $CoQ_{10,}$ entre otros (Carocho y Ferreira, 2013; Lopez y col., 2014; Servili y col., 2014) que posiblemente actúan de manera sinérgica y mediante diferentes mecanismos. Por ejemplo, se ha descrito la capacidad de la CoQ_{10} para recuperar la vitamina E, la regeneración de la vitamina E a través de los niveles de vitamina C (Carocho y Fereira, 2013), o la participación del hidroxitirosol en el aumento de los niveles endógenos de vitamina C (Lopez-Huertas y Fonollá, 2017).

Actualmente, no hay un método universal para evaluar la actividad antioxidante de los alimentos debido a la complejidad de los procesos de oxidación-reducción, y lo ideal es utilizar varios métodos que dan una idea de la actividad global, basándose en diferentes mecanismos de acción.

Entre los diversos métodos que existen, los más frecuentemente empleados en la evaluación de las propiedades antioxidantes de los alimentos son los métodos *in vitro*, que tradicionalmente comprenden una etapa previa de extracción de los compuestos

antioxidantes por su afinidad química con uso de solventes orgánicos. En la literatura científica es posible encontrar diferentes procedimientos para diferentes métodos de extracción, separación y cuantificación, con una amplia variedad de solventes y condiciones de extracción (Oroian y Escriche, 2015).

A continuación, la actividad antioxidante se puede determinar mediante reacciones colorimétricas basadas en la transferencia de electrones conocidos como captadores de radicales libres, con el uso de compuestos artificiales como son los ensayos ABTS (Ácido 2,2.-azinobis-(3-etilbenzotiazolín-6-sulfónico) y DPPH (2,2-difenil-1 picrilhidracil) o por la reducción de los iones de hierro (FRAP) (Carocho Ferreira, 2013; Farzaneh y Carvalho 2015; Oroian y Escriche, 2015). Los cambios de color consecuencia de la neutralización de los radicales libres sintéticos o de la capacidad para reducir los iones Fe^{3+} a Fe^{2+} son evaluados por espectrofotometría, obteniendo valores de absorbancia a diferentes longitudes de onda en un tiempo fijo y comparando con un derivado sintético de la vitamina E (6-hidroxi- Ácido 2,5,7,8 - tetrametilcroman - 2 – carboxílico o Trolox).

2.12. Biodisponibilidad de compuestos antioxidantes

Aunque los métodos tradicionales de extracción para la determinación de la actividad antioxidante *in vitro* sean considerados medidas relevantes, no son suficiente para la predicción de los potenciales efectos antioxidantes *in vivo* (Etcheverry y col., 2012; Carbonell-Capella y col., 2014). A este respecto, muchos estudios proponen los métodos de digestión *in vitro* como paso previo a la evaluación de las propiedades antioxidantes, con la finalidad de obtener una aproximación más fisiológica en la determinación del potencial antioxidante de los alimentos (Pastoriza y col., 2011).

La digestión *in vitro* es un método que mimetiza las transformaciones sufridas por la matriz alimentaria durante el proceso digestión, teniendo en cuenta la presencia de enzimas digestivas y sus concentraciones, pH, tiempo de digestión y concentraciones de sales, entre otros factores (Minekus y col., 2014).

En este sentido, hay dos conceptos fundamentales relacionados a la digestión de la matriz alimentaria y el estudio del potencial bioactivo de los alimentos: la bioaccesibilidad y la biodisponibilidad. La bioaccesbilidad implica que una determinada fracción del alimento o dieta para tener un función biológica, debe ser liberada de la matriz alimentaria durante la digestión, convirtiéndose en disponible para

la absorción a nivel intestinal, mientras que la biodisponibilidad se refiere a la parte del alimento que alcanza la circulación sistémica y es utilizada para las funciones fisiológicas, lo cual incluye la digestión, absorción por las células intestinales y el transporte a las células del cuerpo (Carbonell-Capella y col., 2014).

En las últimas décadas, los cultivos celulares están siendo aplicados a los estudios de biodisponibilidad con el objetivo de simular las condiciones intestinales de absorción (Seiquer y col. 2001), y hoy en día se presentan como una alternativa viable a los estudio con animales o humanos, que son más complejos, caros y de larga duración (Soler y col., 2010). Acoplados a los métodos de digestión in vitro, los cultivos celulares suponen una herramiento útil para elucidar el impacto potencial de algunos compuestos y/o de toda la matriz alimentaria en la salud humana.

En este sentido se utiliza con éxito la línea celular Caco-2 (**Figura 26**), derivada de adenocarcinoma de colon humano, cuyas células en cultivo se diferencian espontáneamente en enterócitos maduros, con la morfología y características funcionales del epitelio intestinal (**Figura 26B**) (Gonzales y col., 2015). Este modelo de línea celular acoplado a la digestión *in vitro*, ha sido utilizado como modelo de absorción intestinal de compuestos bioactivos, incluyendo los compuestos fenólicos de AOV y además, ha sido empleado para estudios de citotoxicidad y marcadores antioxidantes a nivel celular (Deiana y col., 2010; Soler y col., 2010; Rodríguez-Ramiro y col., 2011; Rubió at al., 2014; Chiesi y col., 2015; Incani y col., 2016).

No obstante, existen pocos estudios relacionados con la fracción antioxidante de los AOV que consideren las transformaciones digestivas de toda matriz alimentaria, sus metabolitos y el efecto sinérgico de los compuestos antioxidantes presentes en los aceites (Borges y col., 2015; Seiquer y col., 2015).

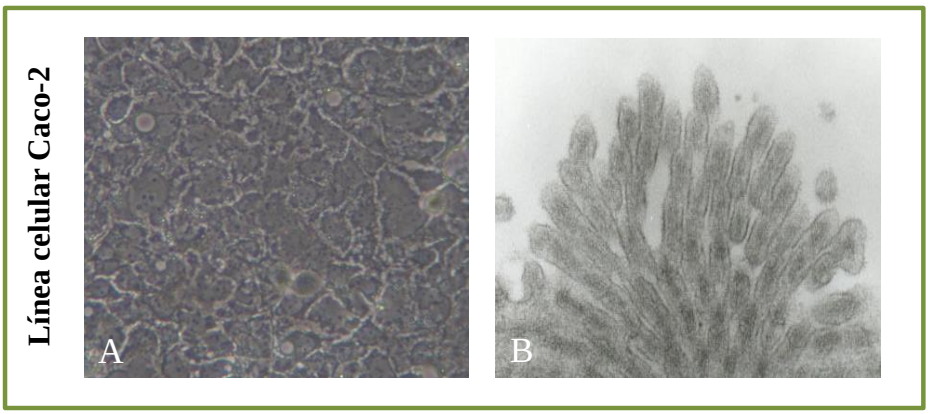

Figura 26. Células Caco-2. A- Monocapa de células. B- Microvellosidades.

2.14. Aspectos beneficiosos del aceite de oliva

Desde las primeras evidencias epidemiológicas referidas en el estudio de los siete países (Keys, 1970) hasta los datos científicos más recientes indicados por diferentes investigaciones como el estudio del efecto del consumo de aceite de oliva en poblaciones europeas (EUROLIVE), el Estudio prospectivo Europeo sobre la dieta, cáncer y salud (EPIC), el estudio Prevención con Dieta Mediterránea (PREDIMED) y otros numerosos estudios incluyendo modelos *in vivo* (animales y humanos), el consumo de aceite de oliva como fuente de grasa de la dieta mediterránea ha sido asociado a múltiples efectos beneficiosos sobre la salud humana particularmente cuando se trata de aceite de oliva virgen (Visioli y col., 2001; Covas y col., 2006; Covas y col., 2007; Barter y col., 2007; Buckland y col., 2011; Psaltopoulou y col., 2011; Castañer y col., 2012; Guasch-Ferré y col., 2015; Rodríguez-Morató y col., 2015; Toledo y col., 2015; Casas y col., 2016; García- Gavilán y col., 2017).

Estos estudios son evidencias epidemiológicas reconocidas por la comunidad científica que hacen posible el establecimiento de alegaciones nutricionales por las autoridades de seguridad alimentaria a nivel internacional. Sin embargo, las investigaciones continúan para comprobar efectos beneficiosos todavía no elucidados y establecer los mecanismos de acción de cada uno de ellos. Algunas de los aspectos beneficiosos del AOV se resumen en la **Figura 27**.

La propiedad más reconocida se relaciona con la prevención de la enfermedad cardiovascular, clásicamente atribuida al perfil de AGMI de los AO, rico en ácido oleico, pero que es un efecto complejo, que resulta de efectos sinérgicos y convergentes del perfil lipídico y de los compuestos minoritarios del AO, especialmente los compuestos fenólicos (Covas y col., 2007; González- Santiago y col., 2010; Martín-Peláez y col., 2013; Capurso y col., 2014; Buckland y col., 2015). Los estudios muestran que dietas ricas en AOV reducen los niveles y la oxidación de las LDL, manteniendo los niveles de HDL (Visioli y col., 2001; Covas y col., 2006; Barter y col., 2007; Castañer y col., 2012). De esta forma, disminuye el riesgo de las enfermedades cardiacas y vasculares, ya que el aumento de los niveles de LDL oxidadas es responsable del acumulo del colesterol y de la formación de la placa aterosclerótica en el endotelio de los vasos sanguíneos, lo cual desencadena las patologías vasculares, incluyendo infarto del miocardio y accidente cerebrovascular (Covas y col., 2007; Huang y col., 2008; Martín-Peláez y col., 2013). Además, estas propiedades están

relacionadas con sus efectos beneficiosos en la tensión arterial, propiedades anti-trombosis y funciones endoteliales (Stark y Madar, 2002; Covas y col., 2007; Servili y col., 2014).

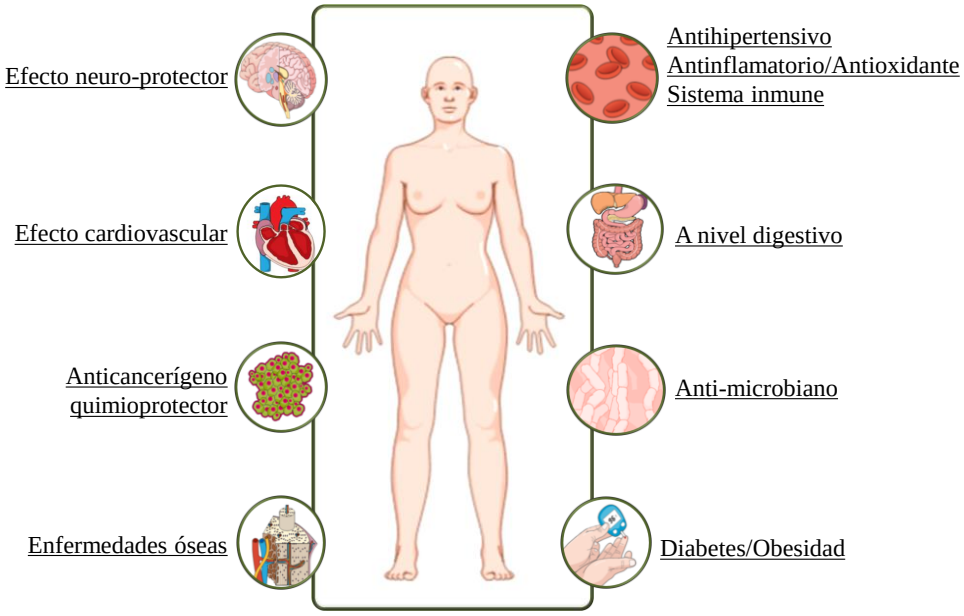

Figura 27. Efectos saludables de los aceites de oliva.

Con respecto a sus alegaciones en la prevención de las enfermedades coronarias, la agencia estadounidense *Food and Drug Administration* (FDA) recomienda el consumo de 23g de AO al día, relacionando estas propiedades al contenido de AGMI (FDA, 2004). Posteriormente, la EFSA avaló que existe una relación de causa-efecto entre el consumo de fenoles del aceite de oliva y la protección de las LDL al daño oxidativo, refiriendo esta declaración a los aceites de oliva que contengan un mínimo de 5 mg de hidroxitirosol y sus derivados (tirosol y derivados de la oleuropeina) por 20 g de AO (EFSA, 2011).

En la última década, se están conociendo datos científicos prometedores, aunque no definitivos, en relación al efecto beneficioso del consumo de aceite de oliva en la prevención de varios tipos de cáncer, particularmente sobre el cáncer de mama y del sistema digestivo (Martín-Peláez y col., 2013; Psaltopoulou y col., 2011; Servili y col., 2014; Buckland y col., 2015). Aunque no se conocen en su totalidad los compuestos responsables del efecto quimioprotector, la oleuropeína y sus derivados, compuestos fenólicos del AO perteneciente a la familia de los secoiroides, han sido propuestos como uno de los principales moduladores de los procesos cancerosos, asi como el escualeno y el ácido oleanólico (Buckland y col., 2015; Ahmad Farooqi y col., 2017).

Asimismo, se han propuesto diferentes mecanismos para explicar los efectos encontrados, siendo uno de los principales el alto potencial antioxidante de algunos compuestos como los compuestos fenólicos, es decir, menor susceptibilidad a la oxidación y estabilización de los niveles de ROS y RNS a nivel celular, como factores clave para prevenir el daño a las biomoléculas del organismo humano y el efecto mutágeno (Stark y Madar, 2002; Psaltopoulou y col., 2011; Martín-Peláez y col. 2013; Servili y col., 2014; Buckland y col., 2015).

De la misma manera, los efectos beneficiosos neuroprotectores del AO, particularmente sobre las enfermedades crónicas como el Parkinson y Alzheimer, están siendo investigados por la comunidad científica. En este sentido, estudios *in vivo* e *in vitro* han mostrado resultados positivos en cuanto a su poder antiinflamatorio (similar al ibuprofeno) implicado en estas enfermedades y han sido atribuidos a los compuestos minoritarios del aceite de oliva, especialmente los compuestos fenólicos como el oleocantal (Martín-Peláez y col. 2013; Sindhi y col., 2013; Servili y col., 2014; Rodríguez-Morató y col., 2015).

En la última década, en relación al sistema inmune se han encontrado evidencias que sugieren que el consumo de aceite de oliva puede tener un impacto en la respuesta inmune (Cárdeno y col., 2014; Aparicio-Soto y col., 2016; Medina-Remón y col., 2017), pudiendo tener un papel positivo algunas las enfermedades auto-inmunes como artritis reumatoide, lupus eritematoso sistémico, esclerosis y enfermedad inflamatoria intestinal, pero todavía son necesarios muchos esfuerzos para aclarar esos efectos y establecer la dosis adecuada, entre otros aspectos (Aparicio-Soto y col., 2016).

Además, existen pruebas del efecto antimicrobiano de los aceites de oliva y de su influencia sobre el equilibrio de la microbiota humana. Los compuestos fenólicos resultantes de la digestión del aceite no son absorbidos completamente en el intestino delgado, y parte de los mismos puede alcanzar el intestino grueso, siendo metabolizados por la microflora intestinal y pudiendo en este sentido afectar los procesos inflamatorios modulados por patógenos intestinales (Cicerale y col., 2012; Martín-Peláez y col. 2013; Servili y col., 2014).

Otros estudios relacionan la dieta mediterránea y el consumo del aceite oliva con la menor incidencia de diabetes, sobrepeso/obesidad y enfermedades óseas como la osteoporosis (Trichopoulou y col., 1997; Cicerale y col., 2012; Capurso y col., 2014; García-Martínez y col., 2014; Buckland y col., 2015; Santangelo y col., 2016).

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CAPÍTULO 3

Material y métodos

CAPÍTULO 3 Material y Métodos

3.1. Muestra

Todas las muestras de aceite evaluadas en la presente memoria corresponden a AOVE de la variedad Arbequina.

Se seleccionaron diferentes zonas geográficas de España (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid y Lérida) y dos regiones productoras de Brasil (Rio Grande do Sul y Minas Gerais). En la **Figura 28** se muestra la localización de las zonas productoras en España y Brasil y la **Tabla 3** recoge la información sobre las coordenadas geográficas, la altitud y algunas características climáticas, como nivel de precipitaciones y temperatura. Los datos climáticos se refieren a la media anual del año de producción, asimismo aportamos los datos de temperatura media mínima y máxima. Los datos han sido obtenidos de la Agencia Española de meteorología (Aemet, 2015) y el Instituto de meteorología de Brasil (INMET, 2015). Los datos de coordenadas geográficas se refieren al punto de la zona de producción (latitud, longitud and altitud) obtenidos con el programa Google Earth (Google Inc, USA).

Los aceites fueron donados directamente por los productores, y desde estas páginas queremos expresar nuestro agradecimiento al Consejo Regulador de Denominación de Origen Estepa, la Denominación de Origen Protegida Les Garrigues, Casas Hualdo, Castillo Canena, Cortijo de Jara, Quaryat Dillar, EPAMIG y a Olivas do Sul.

Se obtuvieron 3 muestras de cada una de las zonas productoras, todas correspondientes a aceites de las primeras etapas de la cosecha y obtenidos mediante el sistema de extracción de dos fases. Las muestras españolas fueron obtenidas entre Octubre y Noviembre de 2014, mientras las muestras brasileñas lo fueron entre Marzo y Abril de 2015. Los aceites fueron enviados a los laboratorios del Departamento de Fisiología y Bioquímica de la Nutrición Animal de la Estación Experimental del Zaidín-CSIC (Granada, España), donde fueron almacenados en frascos de vidrio de topacio adecuadamente para preservarlos de la luz y las altas temperaturas en refrigeración a 4°C y para preservar mejor las muestras fue inyectado nitrógeno en los frascos, las muestras han permanecido en estas condiciones hasta la realización de los análisis.

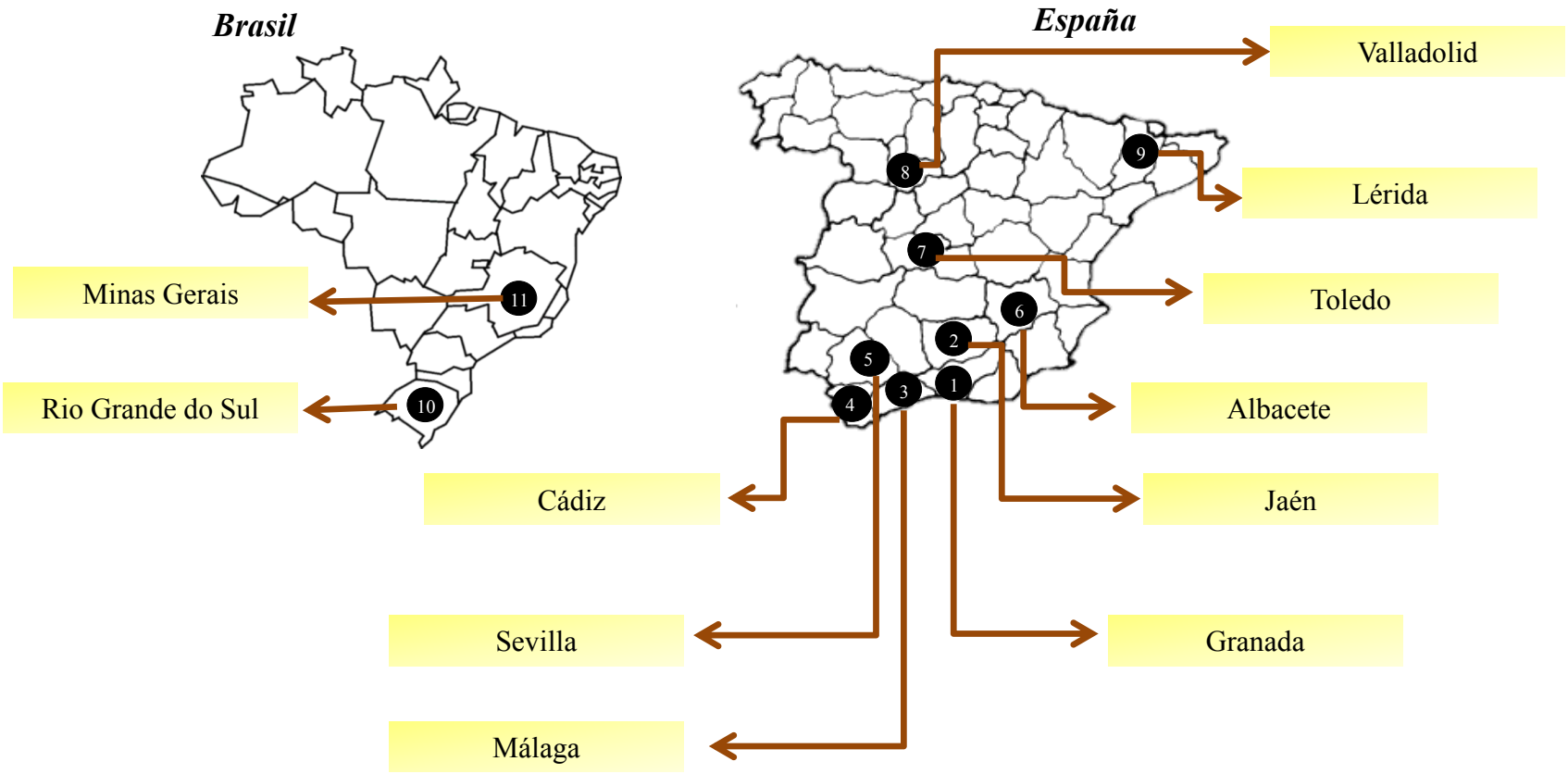

Figura 28.Zonas de producción de los diferentes aceites utilizados en este estudio: España (1-9) y Brasil (10-11).

Tabla 3. Coordenadas geográficas (latitud y longitud), altitud (m), temperatura media anual (°C), precipitaciones (mm) y temperatura media mínima y máxima anual de las diferentes zonas de producción de los aceites de España (1-9) y Brasil (10-11).

Muestra	Latitud	Longitud	Altitud	Temperatura media	Precipitaciones	Temperatura mínima	Temperatura máxima
1	37° 03’ N	3° 36’ W	905	17	385	7	26
2	38° 03’ N	3° 29’ W	580	17	422	9	26
3	37° 06’ N	4° 22’ W	883	20	411	13	27
4	36° 43’ N	6° 01’ W	47	19	636	13	24
5	37° 17’ N	4° 53’ W	416	19	598	11	27
6	39° 00’ N	1° 54’ W	677	13	293	6	25
7	39° 53’ N	4° 28’ W	459	14	391	7	26
8	41° 53’ N	5° 00’. W	845	13	394	-1	27
9	41° 36’ N	0° 35’ W	168	14	677	6	21
10	30° 00’ S	52° 52’ W	88	16	1691	3	22
11	22° 18’ S	42° 22’W	1310	17	1330	14	21

3.2. Determinaciones analíticas

Las determinaciones analíticas realizadas en la presente memoria de tesis doctoral son descritas a la continuación.

En la **Figura 28** se muestra un esquema de las determinaciones analíticas realizadas en las muestras de aceite. A continuación, se exponen de manera resumida los métodos y las técnicas analíticas utilizadas para cada una de las determinaciones realizadas, que son explicadas con más detalle en las correspondientes publicaciones que se incluyen en la presente memoria.

3.2.1. Parámetros de calidad y físico-químicos

- Acidez e índice de peróxidos: por titulación de acuerdo con los métodos estandarizados por la Unión Europea y recogidos en el reglamento (CEE) n° 2568/91.
- Coeficiente de extinción específica en el UV: por espectrofotometría de acuerdo con el reglamento (CEE) n° 2568/91.
- Estabilidad oxidativa: determinado por la estimación del tiempo de inducción a la oxidación utilizando el aparato Rancimat 743.
- Color: las coordenadas de color CIELAB (L*, a*, b*) fueron determinados por medida directa empleando un colorímetro Minolta (CR-400, Konica., Japón).

3.2.2. Composición

- Pigmentos: los pigmentos (clorofilas y carotenoides) fueron determinados por espectrofotometría según el método descrito por Minguez-Mosquera y col. (1991).
- Perfil de ácidos grasos: se determinó según el reglamento europeo (Anexos II y IX, CEE nº 2568/91), tras la obtención de los ésteres metílicos de los AG con solución metanólica de hidróxido potásico, y fueron evaluados mediante cromatografía de gases.
- Tocoferoles: determinados por HPLC (cromatografía liquida de alta resolución) de acuerdo en método descrito por Rueda y col. (2016).
- Compuestos fenólicos: esta fracción individual minoritaria fue obtenida por extracción metanol: agua (80:20v/v) (COI, 2009) y los extractos fueron analizados por UPLC (cromatografía liquida de ultra alta resolución) según Rivas y col., (2013).

- Coenzima $Q_{10:}$ los extractos fueron obtenidos de acuerdo con Venegas et al. (2011) y evaluados por HPLC.

3.2.3. Caracterización organoléptica

- Análisis sensorial: realizada según los parámetros establecidos en el reglamento (CEE) n^0 1348/13 por el panel de cata oficial del laboratorio Agroalimentario de Granada (Atarfe, Granada, España).

- Compuestos volátiles: los volátiles fueron extraídos por la técnica de espacio de cabeza empleando la microextracción en fase sólida (HS-SPME) y la fracción volátil fue determinada por cromatografía de gases masa (GC-MS) con técnica desarrollada durante la presente tesis doctoral.

- Lengua electrónica: fueron obtenidos extractos etanólicos que posteriormente fueron analizados empleando una lengua electrónica que es formada por diferentes sensores electroquímicos capaces de identificar las especies químicas presentes en las muestras, de acuerdo con la metodología descrita por Días y col., (2014).

3.1.4. Propiedades antioxidantes

La actividad antioxidante se determinó, por una parte, en los extractos químicos de los aceites y, por otra, en las fracciones residual y bioaccesible obtenidas tras la digestión in vitro, con el objeto de estudiar el efecto del proceso digestivo sobre las propiedades antioxidantes de los aceites (**Figura 28**). La fracción bioaccesible se utilizó, además, para la realización de los experimentos en las células Caco-2. Dichos experimentos consistieron en ensayos de absorción intestinal y en la evaluación de marcadores de estrés oxidativo a nivel celular. En los ensayos de absorción se analizó la actividad antioxidante absorbida a través de la monocapa de células tras la incubación con los aceites y como marcadores de estrés se estudiaron los efectos sobre la integridad celular y la generación de ROS. Estos dos últimos aspectos se analizaron en condiciones basales y ante un estrés oxidativo inducido.

En paralelo a la determinación de la actividad antioxidante (llevada a cabo en los extractos químicos, fracciones resultantes de la digestión in vitro y soluciones obtenidas en los ensayos de absorción) se analizó también el contenido de polifenoles totales, por la relación de dichos compuestos con las propiedades antioxidantes.

- **Extracción química**: los extractos de los aceites fueron obtenidos con uso de solución de metanol: agua (80:20 v/v), según Borges y col. (2015).

- **Digestión *in vitro***: La digestión gástrica e intestinal de las muestras se realizó tratando de mimetizar las condiciones fisiológicas de temperatura, pHy oscilaciones intestinales y empleando enzimas específicas para cada una de las etapas según se ha descrito en Borges y col. (2015). Tras la digestión y centrifugación de las muestras, se obtuvieron dos fracciones diferenciadas: residual (no soluble) y bioaccesible (soluble), que se conservaron a -80°C y en atmósfera de nitrógeno hasta los posteriores análisis.

- **Actividad antioxidante:** La actividad antioxidante de las muestras se evaluó mediante su capacidad para neutralizar radicales libres (ensayos ABTS y DPPH) y la determinación de su poder reductor (método FRAP). Se trata de reacciones coloreadas cuya absorbancia final se leyó en un espectrofotómetro con lector de placas multipocillo de acuerdo con la metodología descrita en Borges y col. (2015).

- **Polifenoles totales:** Se utilizó el método de Folin – Ciocalteu de acuerdo con la metodología descrita en Borges y col. (2015).

- **Ensayos en cultivos celulares (Caco-2):** las células fueron adquiridas a la Colección Europea de Cultivos Celulares (ECACC), a través del Banco de Células de la Universidad de Granada (España) y durante el mantenimiento y la realización de experimentos se mantuvieron en condiciones controladas de aire/CO_2 (95:5), humedad (90%) y temperatura (37°C). Se utilizó como medio base de cultivo el medio mínimo esencial modificado de Dulbecco (DMEM).

- **Marcadores antioxidantes a nivel celular.** La integridad celular fue evaluada por espectrofotometría empleando el método MTT (reducción metabólica del Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol), que cuantifica las células viables y metabólicamente activas. La generación de ROS se cuantificó mediante la medida de fluorescencia según el ensayo de la diclorofluorescina, descrito por Seiquer y col. (2015).

a. **Ensayos de absorción.** Para los ensayos de absorción las células se sembraron en placas bicamerales Transwell, y se aplicó la metodología desarrollada por nuestro grupo (Seiquer y col. 2015). Este ensayo supone el último paso en el estudio de la

biodisponibilidad en condiciones *ex vivo*, ya que combina la digestión in vitro y la absorción a través de células en cultivo de tipo intestinal

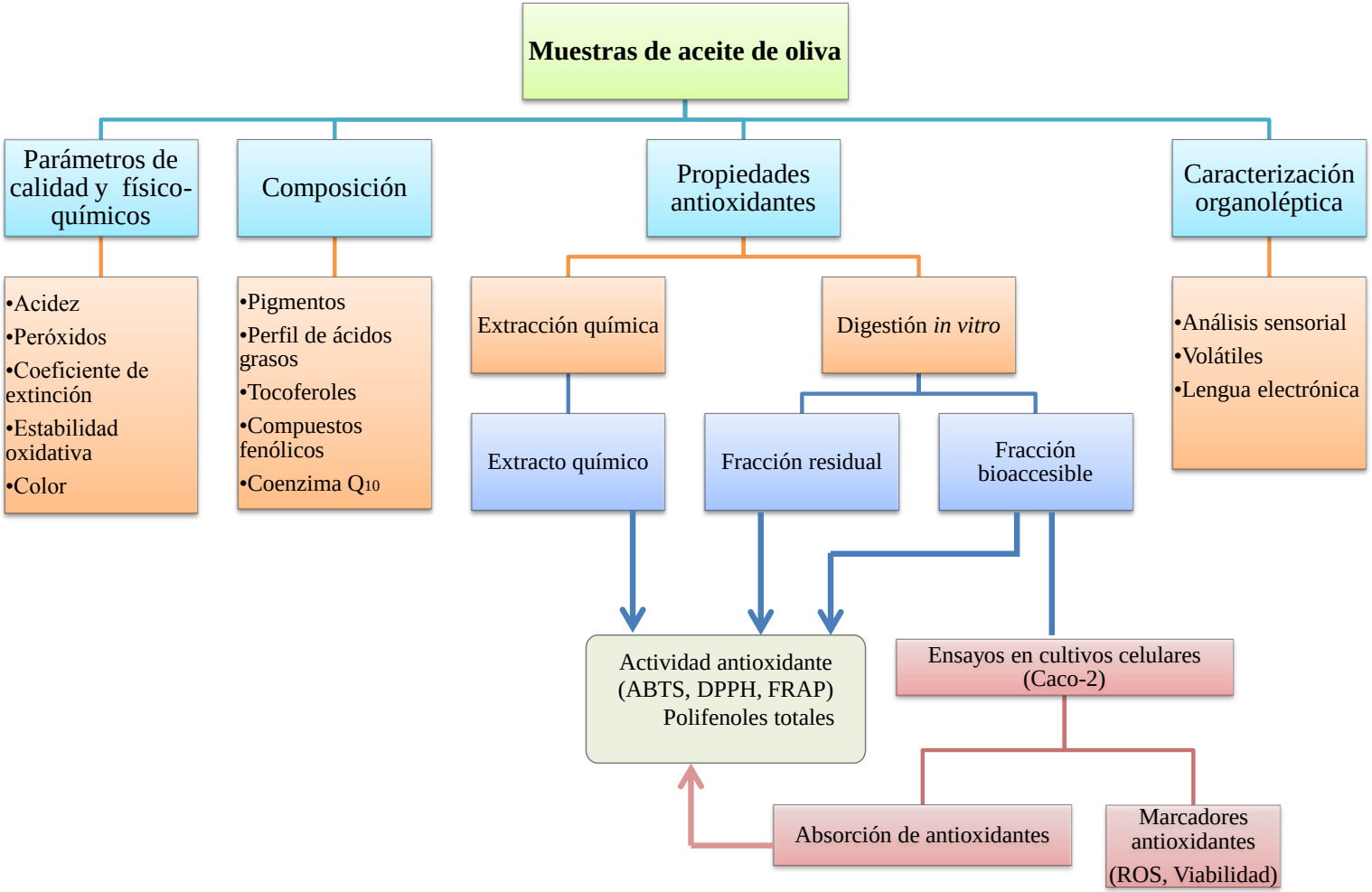

Figura 29. Esquema general de las determinaciones analíticas realizadas en las muestras de aceite.

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CAPÍTULO 4

Resultados

CAPÍTULO 4. Resultados

Artículo 1

General Paper

Characterization of Arbequina virgin olive oils produced in different regions of Brazil and Spain: Physicochemical properties, oxidative stability and fatty acid profile

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Characterization of Arbequina virgin olive oils produced in different regions of Brazil and Spain: Physicochemical properties, oxidative stability and fatty acid profile

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Abstract

Production of virgin olive oil is beginning in Brazil. This paper analyzes the characteristics of the EVOO Arbequina from Brazil in comparison with Spanish Arbequina from different regions. Quality parameters, oxidative stability, pigments, colour and fatty acid profile were assessed, and relationships with geographic and climatic conditions were studied. All the samples presented good quality and met EU standards for extra-virgin olive oil, but there were significant differences between regions and countries for many of the parameters evaluated. Major differences between Brazilian and Spanish samples were observed for free acidity and colour of the oils, as well as minor variations in the fatty acid profile. The colour differences were related to rainfall, whereas the fatty acid content was strongly influenced by altitude and temperature. These results highlight the fact that geographic area and environmental factors influence the characteristics of Arbequina oil and play an important role in newly introduced cultivars.

Keywords: Arbequina, virgin olive oil, oxidative stability, colour coordinates, fatty acids, Brazil, Spain, climatic conditions.

1. Introduction

The olive oil production has extended in recent years beyond the Mediterranean basin to non-traditional regions in the southern hemisphere (Rondanini, Castro, Searles, & Rousseaux, 2014). As an example of this trend, olive cultivation is gradually being introduced into Brazil, where small areas (around 500 ha) are currently being cultivated in the south and southeast of the country, in the regions of Minas Gerais and Rio Grande do Sul, respectively (Ballus et al., 2015). Nowadays, Brazil imports most of its olive oil and the final price is relatively high, making it inaccessible to a large part of the population (Ballus et al., 2014). Furthermore, in Brazil there is a risk of adulteration of olive oil, which further hinders access to a safe, high-quality product with nutritional benefits. Moreover, to date only very limited data have been reported about the quality and the composition of Brazilian olive oils, and from different cultivars (Ballus et al., 2014; Ballus et al., 2015).

The expansion of olive production requires the adaptation of cultivars to climate characteristics (rainfall, temperature, humidity) associated with latitudes and altitudes different from those corresponding to the olive autochthonous regions. In consequence, the oil obtained from environments outside the Mediterranean Basin could differ in quality and composition of those arising from traditional Mediterranean regions (García-González & Aparicio, 2010; Rondanini et al., 2014; Romero, Saavaedra, Tapia, Sepúlveda, & Aparicio, 2016). In addition to climate conditions and geographic area, many other factors, such as cultivar, fruit ripeness and agricultural practices, may also influence the composition and quality of olive oils (Bakhouche et al., 2013; Dabbou et al., 2009, 2010; Rondanini, Castro, Searles, & Rousseaux, 2011; Torres, et al., 2009). Among chemical parameters affecting the quality of olive oils, the fatty acid profile is

one of the most significant, and this condition may be greatly affected by environmental factors (Rondanini et al., 2014).

The Spanish Arbequina olive cultivar is one of the most widely grown and marketed in the world. This popularity arises from its easy adaptation to high density cultivation systems and new environmental conditions, small size, precocity, high oil yield, good oil quality and other agronomic characteristics such as branch flexibility and easy fruit abscission (Rondanini et al., 2011; Torres et al., 2009; Yousfi, Weiland, & García, 2012). Arbequina olive oil is highly appreciated for its soft taste. Due to the combination of these positive factors, Arbequina is acclaimed in the international market (Bakhouche et al., 2013; García-González , Tena & Aparicio, 2010; Yousfi et al., 2012) and is considered ideal for new and emerging markets, more acceptable than other oils, which can be more bitter and pungent. Thus, there are new producing countries of Arbequina cultivar over the world, such as Tunisia (Dabbou et al., 2010), Chile (García-González, Romero & Aparicio, 2010), Argentina (Torres et al., 2009) or Australia (Mailer, Ayton, & Graham, 2010). In this line, Arbequina has recently started to be cultivated in Brazil.

As yet, little is known about how geographic and climate conditions may affect the properties of the olive oil that is being to be produced in Brazil. Also, information about the similarities and differences between the newly-introduced and the autochthonous cultivars is lacking. The aims of the present study were (i) to characterise the monovarietal Arbequina olive oil produced in Brazil; (ii) to compare it with the olive oils from the same cultivar produced in different regions of Spain; (iii) to classify the oil samples according to their geographic origin, on the basis of the analysed variables. For these purposes, quality parameters (free acidity, peroxide value, specific

extinction coefficients), oxidative stability, pigments (chlorophylls and carotenoids), colour coordinates and the fatty acid profile were assessed.

2. Material and methods

2.1. Chemicals

All chemicals were analytical reagent grade or higher purity and Milli Q water (Millipore, Bedford, MA) was used throughout the assays. Methanol, cyclohexane and n-heptane were supplied by Sigma (Sigma-Aldrich, St. Louis, MO). Acetic acid, chloroform, diethyl ether, ethanol 95%, phenolphthalein, potassium hydroxide, sodium hydroxide, potassium iodate, starch and sodium thiosulphate were acquired from Panreac (Barcelona, Spain). The fatty acid methyl ester standard mixture (Supelco 37 FAME Mix) was acquired from Supelco (Bellefonte, USA).

2.2. Sampling

Extra virgin olive oil (EVOO) from Arbequina cultivar was analysed. Two regions in Brazil (Minas Gerais and Rio Grande do Sul) and nine representative regions of olive oil production in Spain (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid and Lérida) were selected to obtain the EVOO samples. Locations of the Spanish (samples 1-9) and Brazilian (samples 10 and 11) regions are shown in **Fig. 1**; geographic and climate characteristics of the production areas are shown in **Table 1**. Table 1 also classifies the areas according to the Köppen system, which divides the world's climates into major categories based on the global temperature profile and precipitations related to latitude (Kottek, Grieser, Beck, Rudol, & Rubel, 2006).

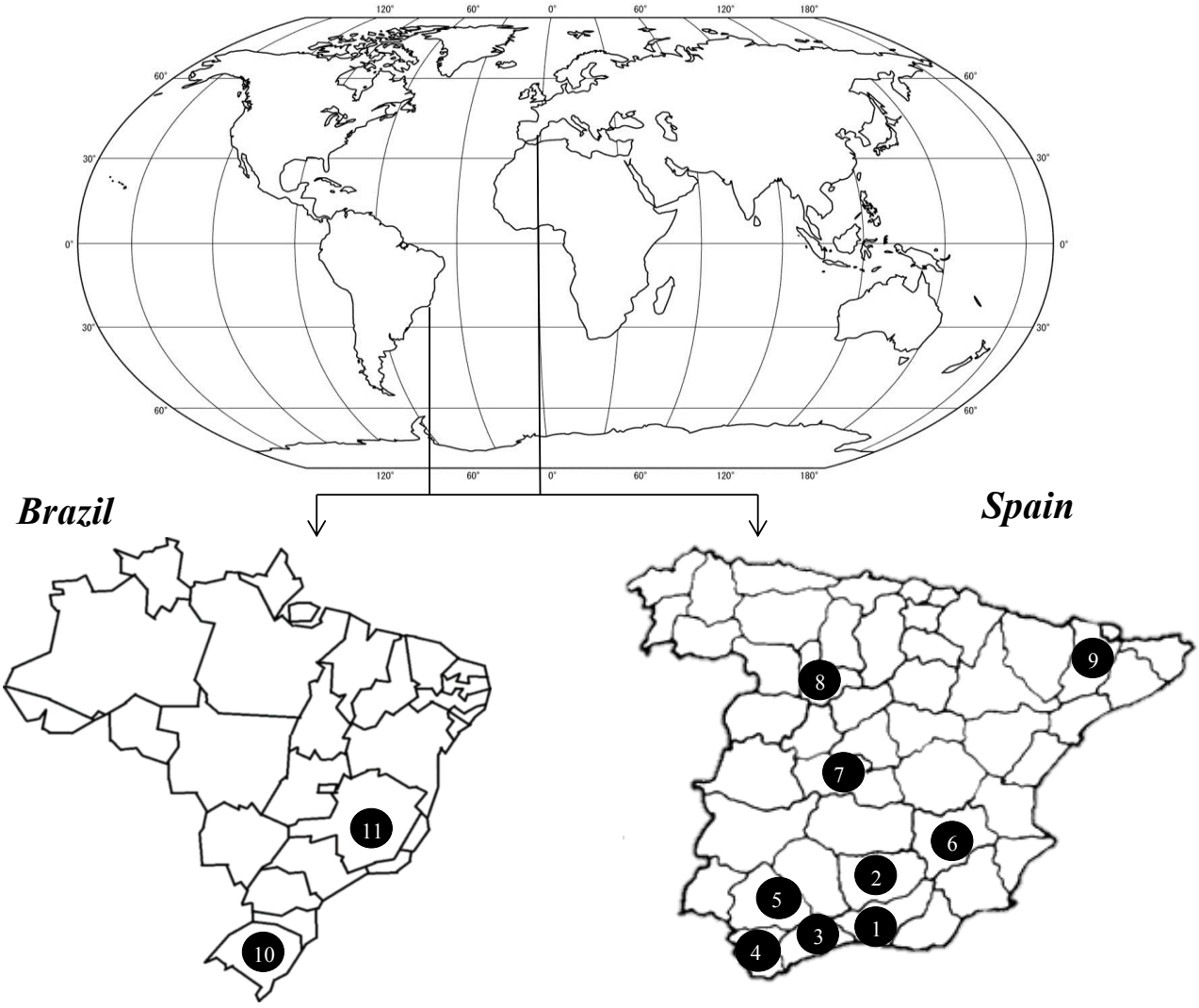

Figure 1. Geographic location of Arbequina virgin olive oils from Spain (1-9) and Brazil (10, 11). Numbers correspond to the description in Table 1 (1-Granada; 2-Jaén; 3-Málaga; 4-Cádiz; 5-Sevilla; 6-Albacete; 7-Toledo; 8-Valladolid; 9-Lérida; 10-Rio Grande do Sul; 11-Minas Gerais).

Table 1. Geographic coordinates (latitude and longitude), altitude (m), annual mean temperatures (◦C), annual rainfalls (mm) and minimum and maximum mean temperatures (◦C) of the different locations of Arbequina virgin olive oils from Spain (1-9) and Brazil (10, 11).

Oil Sample	Region	Latitude	Longitude	Altitude	Mean Temperature	Rainfall	Minimum Temperature	Maximum Temperature	Kooper Classification
1	Granada	37° 03᾽ N	3° 36᾽ W	905	17	385	7	26	Warm temperate climate with dry and hot summer (Csa)
2	Jaén	38° 03᾽ N	3° 29᾽ W	580	17	422	9	26	Warm temperate climate with dry and hot summer (Csa)
3	Málaga	37° 06᾽ N	4° 22᾽ W	883	20	411	13	27	Warm temperate climate with dry and hot summer (Csa)
4	Cádiz	36° 43᾽ N	6° 01᾽ W	47	19	636	13	24	Warm temperate climate with dry and hot summer (Csa)
5	Sevilla	37° 17᾽ N	4° 53᾽ W	416	19	598	11	27	Warm temperate climate with dry and hot summer (Csa)
6	Albacete	39° 00᾽ N	1° 54᾽ W	677	13	293	6	25	Steppe climate of mid/high altitude (Bsk)
7	Toledo	39° 53᾽ N	4° 28᾽ W	459	14	391	7	26	Steppe climate of mid/high altitude (Bsk)
8	Valladolid	41° 53᾽ N	5° 00᾽. W	845	13	394	-1	27	Warm temperate climate with dry and warm summer (Csb)
9	Lérida	41° 36᾽ N	0° 35᾽ W	168	14	677	6	21	Steppe climate of mid/high altitude (Bsk)
10	Río Grande	30° 00᾽ S	52° 52᾽ W	88	16	1691	3	22	Warm temperate climate with dry seasons and hot summer (Cfa)
11	Minas Gerais	22° 18᾽ S	42° 22᾽W	1310	17	1330	14	21	Warm temperate climate with dry winter and temperate summer (Cwb)

Geographic coordinates (latitude, longitude and altitude) proximate to olive grove were found using Google Earth program (Google Inc, USA). Temperature and rainfall values in Spain were supplied by the Spanish Meteorology Agency (Aemet, 2015). Climate values for Brazil were calculated from data provided by the National Meteorology Institute of Brazil (INMET, 2015).

All the oils, kindly provided by the producers, were obtained under a two-phase extraction system and correspond to the same crop harvest (2014/15). After extraction, the samples were sent to CSIC laboratories (Granada, Spain) for performing the analysis. Samples were adequately packaged for preserving for light and high temperatures and promptly sent by courier service. The corresponding customs permission was obtained for Brazilian samples to avoid delay at the frontier. A total of 33 olive oil samples were evaluated (n=3 from each producing region) and the analyses were carried out in triplicate. All samples were stored in dark glass bottles with nitrogen gas and maintained at 4° C until analysis.

2.3. Quality parameters

The quality parameters assessed were free acidity (FA, expressed as percentage of oleic acid), peroxide value (PV, expressed as mEq. O_2/kg of oil) and specific extinction coefficients at 232 and 270 nm (K_{232}, K_{270} and ΔK). All quality parameters were determined according to EU standard methods (Annexes II and IX of European Community Regulation EEC/2568/91).

2.4. Oxidative stability

The oxidative stability was measured by estimating the oxidation induction time, in a Rancimat 743 apparatus (Metrohm CH, Switzerland). A sample of olive oil (3g) was heated to 120 ± 1.6 °C and subjected to an inflow of air (filtered, cleaned, and dried) at 20 L/h. The resulting volatile compounds were collected in water, and the increasing water conductivity was continuously measured. The time (in hours) taken to reach conductivity inflection was recorded.

2.5. Pigments (chlorophylls and carotenoids)

The pigments (chlorophylls and carotenoids) were assessed following the method described by Minguez-Mosquera, Rejano, Gandul, Sánchez, & Garrido (1991). The oil samples were dissolved with cyclohexane (1.5:5 w/v) and absorbance was measured using a UV spectrophotometer (Pharmaspec UV 1700, Shimadzu). The chlorophyll fraction was determined at 670 nm and the carotenoid fraction at 470 nm. The results obtained are expressed as mg of pheophytin ‘‘a’’ and lutein per kg of oil, respectively.

2.6. Colour

Instrumental colour (CIE *L**, *a**, *b**) was measured directly in the olive oil samples using a Minolta Colorimeter (CR-400, Konica Minolta Corp., Japan) with illuminant D65. The colorimeter was calibrated before use with a white ceramic tile. *L** is a measure of luminance or the lightness component, which ranges from 0 to 100 (black to white). Parameters *a** and *b** are termed opponent colour axes; *a** represents red (positive) versus green (negative) colours, *b** is positive for yellow and negative for blue. Colour features were obtained as the average of three measurements performed on each oil sample.

2.7. Fatty acid profile

The fatty acid profile was determined according to EU standard methods (Annexes II and IX of European Community Regulation EEC/2568/91). The methyl esters (FAME) were obtained by cold alkaline transesterification with methanolic potassium hydroxide solution and extracted with n-heptane. The fatty acid profile was determined with a Focus GC, Thermo Scientific (Milan, Italy) chromatograph equipped with a split/splitless injector, a FID detector and a SP-2560 fused silica capillary column (100

m x 0.25 cm i.d. x 0. 0.2μm film thickness, Supelco, USA). Helium was used as carrier gas at an internal pressure of 110 kPa. The temperatures of the detector and injector were 275 ºC and 260 ºC, respectively. The oven temperature was programmed at 70 ºC during the first 4 min, increasing to 110 ºC at 8 ºC/min, then increasing to 170 ºC at 5 ºC/min and holding for 10 min, finally increasing to 250 ºC at 4 ºC/min and holding for 15 min. The split ratio was 1:50 and the injected volume was 1 μL. The results are expressed as the relative percentage of each fatty acid, calculated by internal normalisation of the chromatographic peak area eluting between myristic and lignoceric acid methyl esters. A control sample of fatty acid methyl ester standard mixture (Supelco 37 FAME Mix) was used for calibration and for the identification of the FAME by their retention times (Sigma, Spain).

2.8. Statistical analysis

The data obtained were analysed using analysis of variance (one-way ANOVA), with the geographic origin of oils (regions 1-11) as the main factor. Tukey's test was used to compare mean values between oils from the different regions, and differences were established at $P<0.05$. Moreover, the data were grouped by countries, and the overall differences between Brazilian and Spanish oils were also studied by ANOVA. The relationships of the different variables with the climate characteristics and the altitude of the producing regions were evaluated by Pearson's coefficient. In addition, a principal component analysis (PCA) was applied for reducing the number of variables analysed (oxidative stability, 2 variables corresponding to pigments, 3 to colour parameters and 18 variables of FA profile, with a total of 24 variables) to a smaller number (principal components or factors). Furthermore, using the new variables as dimensions, it allowed recognizing patterns in the data by plotting them in a

multidimensional space. All statistical calculations were carried out using SPSS version 21.0 (IBM Corporation, New York, USA).

3. Results and Discussion

Pearson's coefficient correlations between climate/geographical variables (altitude) and analytical parameters (quality parameters, pigments, colour coordinates and fatty acid profile) are presented as supplementary data in Table S1.

3.1. Quality parameters and oxidative stability

The quality parameters (FA, PV and specific extinction coefficients at K_{232}, K_{270} and ΔK) and oxidative stability are shown in **Table 2**.

FA, expressed as % of oleic acid, provides information about the state of the fruit, i.e., how the fruit was handled prior to processing and the length of time from harvest to milling. FA reports the degree of hydrolysis in the oil (Borges, Malheiro, de Souza, Casal, & Pereira, 2015; Reboredo-Rodríguez et al., 2015). According to the EU regulation (EEC 2568/91), FA content of EVOO must not exceed 0.8%. All of the samples analysed complied with this limit. The highest value was found for location 10, and differed significantly from those of the other samples ($P<0.05$). The average FA values from the Brazilian and Spanish oils were compared, and a large difference was recorded ($P<0.001$), mainly due to the high value for sample 10.

Table 2. Quality parameters - Free fatty acids - FA (% oleic acid), Peroxide value - PV(mEq O_2/ Kg), specific extinction coefficients (K_{232}, K_{270} and ΔK) - and oxidative stability (hours) of Spanish (samples 1-9) and Brazilian (samples 10-11) Arbequina EVOO (mean ± SD).

Oil sample	FA	PV	K_{232}	K_{270}	ΔK	Oxidative stability
1	0.15 ± 0.00 [a]	3 ± 0.3 [a,b]	1.50 ± 0.13 [a,b]	0.14 ± 0.01 [a]	-0.003 ± 0.001	10.02 ± 0.18 [c]
2	0.18 ± 0.02 [a]	4 ± 1.8 [b,c]	1.93 ± 0.42 [b,c]	0.20 ± 0.01 [c]	-0.013 ± 0.016	9.55 ± 2.16 [c]
3	0.25 ± 0.00 [a,b]	11 ± 0.6 [e]	2.07 ± 0.22 [b,c]	0.20 ± 0.00 [a]	-0.002 ± 0.000	15.85 ± 0.09 [d]
4	0.32 ± 0.01 [b]	11 ± 0.9 [e]	2.35 ± 0.16 [c]	0.20 ± 0.02[c]	-0.014 ± 0.004	5.32 ± 0.01 [a]
5	0.15 ± 0.05 [a]	7 ± 1.5 [d]	1.87 ± 0.28 [a,b,c]	0.12 ± 0.01 [a]	-0.002 ± 0.001	9.72 ± 1.48 [c]
6	0.24 ± 0.04 [a,b]	4 ± 0.2 [b,c]	1.52 ± 0.35 [a,b]	0.13 ± 0.03 [a]	-0.002 ± 0.000	8.07 ± 0.07 [b,c]
7	0.16 ± 0.01 [a]	3 ± 0.1 [a,b]	1.70 ± 0.17 [a,b,c]	0.14 ± 0.00 [a,b]	-0.002 ± 0.001	8.61 ± 0.02 [b,c]
8	0.16 ± 0.02 [a]	1 ± 0.1 [a]	1.18 ± 0.02 [a]	0.11 ± 0.01 [a]	-0.002 ± 0.001	19.73 ± 0.67 [e]
9	0.18 ± 0.02 [a]	3 ± 0.4 [a,b]	1.51 ± 0.08 [a,b]	0.12 ± 0.00 [a]	-0.002 ± 0.002	6.82 ± 1.19 [a,b]
10	0.75 ± 0.04 [c]	14 ± 1.4 [f]	1.94 ± 0.21 [b,c]	0.17 ± 0.01 [b,c]	-0.007 ± 0.001	9.53 ± 0.14 [c]
11	0.15 ± 0.00 [a]	4 ± 0.5 [c,d]	2.29 ± 0.29 [c]	0.13 ± 0.01 [a]	-0.002 ± 0.000	6.21± 0.22 [a,b]
p-value [a] Spain × Brazil	***	**	*	ns	ns	ns

Means within a column with different superscripts differ significantly ($P < 0.05$).
[a] ns, not significant;*P<0.05; **P < 0.01;***P< 0.001.

Another mandatory quality parameter is PV, which reflects the onset of oxidation. For EVOO, the maximum PV acceptable is 20 mEq of O_2/kg. In our study, all samples showed values below this limit, ranging from 1 mEq/kg in Spanish sample number 8 to 14 mEq/kg in Brazilian sample number 10. Significant differences were found among samples ($P<0.05$) and between countries ($P<0.01$). K_{232}, K_{270} and ΔK, were measured in the UV region at the wavelengths of 232 nm, 270 nm and 266-274 nm, respectively, corresponding to the maximum absorption of the conjugated dienes and trienes. All samples complied with the regulation that specific extinction coefficients must be lower than 2.50, 0.22 and 0.01 for K_{232}, K_{270}, and ΔK, respectively, in the EVOO category. The K_{270} and ΔK values did not differ between the countries.

Among Arbequina oils cultivated over the world, the highest values of FA (0.70 % oleic acid) and PV (11 mEq O_2/kg) have been observed in Argentina (Torres et al., 2009), whereas oil from other countries show lower values, always within quality regulations. According to Dabbou et al. (2009), the geographic origin does not significantly influence the quality parameters of oils, although they may be affected by aspects such as the health of the fruit, storage conditions, harvesting system and transport. Our findings corroborate this statement, since the global quality of the oils was basically unchanged regardless of the location, and all of the samples can be considered EVOO. However, positive correlations were found between FA, PV and K_{232} with annual temperature and rainfalls, and negative with altitude (Table S1). Supporting these relationships, oils from the areas with high rainfall, warm average temperatures and low altitude, such as number 4 (Cádiz, Spain) and number 10 (Rio Grande do Sul, Brazil), had the highest PV and FA values. The negative influence of excessive water on olive oil quality has been reported previously in comparative studies of experimental irrigation (Berenguer et al., 2006).

Rancimat is a useful method for measuring resistance to oxidation, and provides important information on the shelf life of olive oils, which could be influenced by the fatty acid profile and the presence of antioxidant compounds (Dabbou et al., 2010). In our study, Arbequina olive oils ranged between 5 to 20 hours of oxidative stability at 120°C, with significant differences between the samples. The lowest stability was found for location 4 and the highest for location 8. The average values did not differ significantly between the two countries, probably due to the large variations between samples. The oxidative stability of the Arbequina olive oils analysed in this study was similar to the values of 5-14 h of induction time reported for the same cultivar grown in Catalonia, Spain (Morelló, Motilva, Tovar, & Romero et al., 2004), and higher than those observed in other countries such as Tunisia (0.68 h) (Dabbout et al., 2010).

The Rancimat results were negatively correlated (P<0.05) with the K_{232} (r= -0.436) and K_{270} (r= -0.430) values, although no relationship was observed with the other quality markers (FA and PV), which concurs with the results of other authors (Dabbou et al., 2010). It has been reported that the oil stability measured by the Rancimat method increases in line with altitude (Aparicio & Lune, 2002); however, like Aguilera et al. (2005), we observed no such correlation. On the other hand, significant correlations were found between oxidative stability and climate factors, such as minimum and maximum temperatures (Table S1).

3.2. Pigments and colour

Table 3 shows the pigment content (chlorophylls and carotenoids) and chromatic coordinates measured by the CIELAB method (L^*, a^*, b^*) for the different oils.

Table 3. Pigments chlorophylls and carotenoids (mg kg^{-1}), and colour coordinates evaluated by the CIELAB method (L*, a*, b*) of Spanish (samples 1-9) and Brazilian (samples 10-11) Arbequina EVOO (mean ± SD).

Oil sample	Chlorophylls	Carotenoids	L*	a*	b*
1	2.89 ± 0.18 ^{a,b,c}	4.55 ± 0.38 ^{a}	24.67 ± 0.03 ^{c}	-0.550 ± 0.03 ^{g}	11.54 ± 0.02 ^{c}
2	2.07 ± 0.29 ^{a}	3.70 ±0.33 ^{a}	25.72 ± 0.52 ^{d}	-2.06 ± 0.01 ^{b,c}	12.43 ± 0.21 ^{c,d,e}
3	2.57 ± 0.17 ^{a,b}	4.78 ± 0.27 ^{a}	25.65 ± 0.02 ^{d}	-1.74 ± 0.05 ^{d}	12.56 ± 0.04 ^{d,e,f}
4	3.89 ± 1.76 ^{b,c}	9.09 ± 4.56 ^{b}	26.08 ± 0.04 ^{d,e}	-2.14 ± 0.06 ^{a,b,c}	12.28 ± 0.15 ^{c,d,e}
5	2.03 ± 0.18 ^{a}	3.76 ± 0.12 ^{a}	26.28 ± 0.06 ^{e,f}	-2.22 ± 0.06 ^{a,b}	11.75 ± 0.79 ^{c,d}
6	1.67 ± 0.06 ^{a}	2.80 ± 0.19 ^{a}	26.09 ± 0.18 ^{d,e}	-1.89 ± 0.20 ^{c,d}	13.13 ± 0.04 ^{e,f}
7	2.06 ± 0.21 ^{a}	2.50 ± 0.09 ^{a}	26.16 ± 0.02 ^{d,e,f}	-1.97 ± 0.07 ^{b,c,d}	13.39 ± 0.02 ^{f}
8	4.38 ± 0.25 ^{c}	6.26 ± 0.43 ^{a,b}	25.69 ± 0.02 ^{d}	-1.38 ± 0.03 ^{e}	12.87 ± 0.04 ^{e,f}
9	1.41 ± 0.38 ^{a}	2.42 ± 0.71 ^{a}	26.69 ± 0.27 ^{f}	-2.37 ± 0.12 ^{a}	11.82 ± 0.60 ^{c,d}
10	1.73 ± 0.16 ^{a}	3.85 ± 0.32 ^{a}	19.54 ± 0.05 ^{a}	0.06 ± 0.07 ^{h}	4.36 ± 0.04 ^{a}
11	1.39 ± 0.02 ^{a}	2.17 ± 0.05 ^{a}	24.00 ± 0.01 ^{b}	-0.84 ± 0.04 ^{f}	9.74 ± 0.00 ^{b}
p-value ^{a} Spain × Brazil	ns	*	***	***	***

Means within a column with different superscripts differ significantly ($P < 0.05$).
^{a} ns, not significant;*P<0.05; **P < 0.01;***P< 0.001.

Although colour is not included among quality standards, it plays an important role in the perceptions and preferences of consumers and may also be related to the maturation stage of the fruit. Consumers associate a dark green colour with high quality and pale yellow with refined and lower-quality olive oils (Gandul-Rojas, Gallardo-Guerrero, Roca, & Aparicio-Ruiz, 2013). According to our results, the colour of the Brazilian EVOO clearly differed from that of the Spanish samples (P<0.001). On average, the Brazilian samples were less green (higher a* values) and paler yellow (lower b* values) than the Spanish ones, which in addition had more luminosity (higher L* values). Some previous studies of Spanish Arbequina olive oils (Criado, Romero, Casanovas, & Motilva, 2008; Morelló et al., 2004) have reported higher values for L* and b* and similar a* values, in comparison with ours, while another reported similar values for a* and b* (Moyano, Meléndez-Martínez, Heredia, 2008). On the other hand, colour parameters were strongly correlated with rainfall (Table S1), i.e. high levels of rainfall (as occurring in Brazil) tend to produce oils with lower lightness and greenness intensity. To the best of our knowledge, no data have been reported about colour measured by instrumental methods of Brazilian olive oils.

The colour parameters of olive oils are mainly the result of chloroplast pigments, i.e. chlorophylls and carotenoids, which play an essential role in oxidative stability (Criado et al., 2008). The chlorophyll content in the Arbequina oils analysed ranged from 1.39 to 4.38 mg/kg, with significant differences among locations (P<0.05). Although Spanish oils showed on average higher chlorophyll content than Brazilian ones, differences between countries did not reach statistical significance (Table 3). The carotenoid content presented a wide range, from 2.42 to 9.09 mg/kg, with significant differences between areas and countries (P<0.05). Therefore, not only cultivar (Moyano et al., 2008) but also geographic and climate characteristics seem to affect the pigment

content of olive oils. However, in the present study, the only significant relationships found were those between carotenoids and maximum temperatures (Table S1).

Values of chlorophylls found in the present study are lower than those reported in Arbequina EVOO from Tunisia and Catalonia (Spain), whereas levels of carotenoids are similar than those shown in olive oils from Tunisia (Dabbou et al., 2010; Morelló et al., 2004). An association between colours and pigments of oils has been proposed in the literature (Moyano et al. 2008); on accordance, in the present study, Brazilian oils, which showed lower average values of chlorophylls and carotenoids than Spanish ones, were also less green and yellow (higher *a** and lower *b** values), although we have not found statistical correlations between these parameters. Thus, factors other than pigment content could have affected the colour variation of the oils, as colour is also associated with other chemical and physical properties, mainly related with the quality of the product (Moyano, Heredia & Melendez-Martinez, 2010).

The antioxidant properties of the pigments are known to affect oxidative stability of oils (Criado et al., 2008). Our analysis showed that Rancimat correlated with carotenoid levels (r= 0.487, $P<0.01$), but not with chlorophylls (Table S1), in accordance with findings of Dabbou et al. (2010).

3.3. Fatty acid profile

The fatty acid profile (% weight of methyl esters), the sums of total saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids and the n6/n3, MUFA/PUFA and PUFA/SFA ratios are presented in **Table 4**.

Table 4. Fatty acid profile (% weight of methyl esters) of Spanish (samples 1-9) and Brazilian (samples 10-11) Arbequina EVOO (mean ± SD).

	1	2	3	4	5	6	7	8	9	10	11	P^a
C16:0	11.99 ± 0.10^{b}	17.43 ± 0.05^{e}	14.14 ± 0.03^{c}	16.98 ± 0.68^{e}	17.34 ± 0.18^{e}	15.19 ± 0.53^{d}	19.55 ± 0.16^{f}	9.44 ± 0.20^{a}	13.97 ± 0.27^{c}	19.08 ± 0.42^{f}	14.15 ± 0.046^{c}	ns
C16:1	1.05 ± 0.01^{b}	1.89 ± 0.28^{d}	1.08 ± 0.05^{b}	1.59 ± 0.00^{c,d}	1.51 ± 0.09^{c}	1.64 ± 0.06^{c,d}	1.55 ± 0.01^{c}	0.58 ± 0.01^{a}	1.13 ± 0.11^{b}	3.16 ± 0.11^{e}	1.72 ± 0.02^{c,d}	**
C17:0	0.11 ± 0.01^{b,c}	0.10 ± 0.01^{a,b,c}	0.13 ± 0.00^{c}	0.11 ± 0.01^{b,c}	0.12 ± 0.00^{c}	0.10 ± 0.00^{a,b,c}	0.10 ± 0.03^{a,b,c}	0.10 ± 0.02^{a,b,c}	0.11 ± 0.01^{b,c}	0.07 ± 0.00^{a}	0.08 ± 0.00^{a,b}	**
C17:1	0.24 ± 0.01^{c,d}	0.21 ± 0.00^{a,b,c}	0.24 ± 0.01^{d}	0.21 ± 0.01^{a,b,c}	0.23 ± 0.01^{b,c,d}	0.23 ± 0.01^{b,c,d}	0.22 ± 0.03^{b,c,d}	0.23 ± 0.01^{b,c,d}	0.21 ± 0.01^{b,c,d}	0.17 ±0.00^{a}	0.20 ± 0.01^{a,b}	**
C18:0	1.92 ± 0.02^{d,e}	1.81 ± 0.04^{c,d}	2.02 ± 0.02^{f}	1.89 ± 0.07^{d,e}	1.82 ± 0.03^{c,d}	1.78 ± 0.01^{c}	1.61 ± 0.04^{b}	2.13 ± 0.03^{h}	1.93 ± 0.02^{e,f}	1.56 ± 0.01^{b}	1.44 ± 0.04^{a}	**
C18:1	75.22 ± 0.20^{c}	64.09 ± 1.36^{a}	71.66 ± 0.07^{c}	63.12 ± 0.72^{a}	63.85 ± 0.09^{a}	66.69 ±1.77^{b}	64.66±0.15^{a,b}	79.68 ±0.13^{d}	70.60 ± 0.04^{c}	64.29 ± 0.28^{a}	70.64 ± 0.04^{c}	ns
C18:2	8.04 ± 0.02^{b}	13.17 ±1.19^{e}	9.51 ± 0.00^{c}	14.76 ± 0.01^{f}	13.77 ± 0.13^{e,f}	13.24 ± 0.77^{e}	11.04 ± 0.08^{d}	6.56 ± 0.02^{a}	10.82 ± 0.11^{d}	10.49 ± 0.07^{c,d}	10.29 ± 0.04^{c,d}	ns
C18:3	0.58 ± 0.01^{c}	0.56 ± 0.01^{b,c}	0.46 ± 0.01^{a}	0.55 ± 0.02^{b,c}	0.56 ± 0.03^{c}	0.60 ± 0.04^{c}	0.55 ± 0.01^{b,c}	0.46 ± 0.01^{a}	0.48 ± 0.02^{a}	0.51 ± 0.00^{a,b}	0.69 ± 0.00^{d}	ns
C20:0	0.34 ± 0.01^{b,c,d}	0.34 ± 0.01^{b,c,d}	0.35 ± 0.00^{c,d}	0.36 ± 0.01^{d}	0.35 ± 0.01^{d}	0.34 ± 0.02^{b,c,d}	0.32 ± 0.01^{a,b}	0.36 ± 0.00^{d}	0.35 ± 0.00^{c,d}	0.30 ± 0.00^{a}	0.32 ± 0.01^{a,b,c}	**
C20:1	0.29 ± 0.00^{c,d}	0.25 ± 0.02^{a,b}	0.25 ± 0.01^{a,b}	0.25 ± 0.01^{a,b}	0.25 ± 0.02^{a,b}	0.26 ± 0.01^{a,b,c}	0.24 ± 0.02^{a,b}	0.27 ± 0.00^{b,c,d}	0.25 ± 0.01^{a,b}	0.23 ± 0.00^{a}	0.31 ±0.01^{d}	ns
C22:0	0.11 ± 0.00^{c,d}	0.10 ± 0.00^{a,b}	0.10 ± 0.00^{b,c}	0.11 ± 0.01^{c,d}	0.11 ± 0.00^{c,d}	0.11 ± 0.00^{b,c}	0.10 ± 0.00^{b,c}	0.12 ± 0.00^{d}	0.11 ± 0.00^{b,c}	0.09 ± 0.00^{a}	0.10 ± 0.01^{b,c}	**
C24:0	0.04 ± 0.01^{a}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.01^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.01^{a,b}	0.05 ± 0.00^{a,b}	0.05 ± 0.00^{b}	ns
SFA	14.57 ± 0.11^{b}	19.84 ± 0.10^{e}	16.80 ± 0.03^{c,d}	19.51 ± 0.74^{e}	19.81 ± 0.15^{e}	17.58 ± 0.54^{d}	21.73 ± 0.14^{f}	12.21 ± 0.17^{a}	16.51 ± 0.26^{c}	21.14 ± 0.42^{f}	16.15 ± 0.07^{c}	ns
MUFA	76.81 ± 0.09^{e}	66.43 ± 1.10^{a,b}	73.24 ± 0.03^{d}	65.17 ± 0.72^{a}	65.86 ±0.07^{a}	68.58 ±1.34^{c}	66.67 ± 0.09^{a,b}	80.76 ± 0.15^{f}	72.20 ± 0.14^{d}	67.85 ± 0.34^{b,c}	72.87 ± 0.03^{d}	ns
PUFA	8.62 ± 0.02^{b}	13.73 ± 1.20^{e}	9.97 ± 0.01^{c}	15.32 ± 0.02^{f}	14.33 ± 0.10^{e,f}	13.84 ± 0.81^{e}	11.60 ± 0.08^{d}	7.02 ± 0.03^{a}	11.29 ± 0.13^{d}	11.00 ± 0.08^{c,d}	10.99 ± 0.04^{c,d}	ns
n6/n3	13.76 ± 0.10^{a}	23.52 ± 1.74^{d}	20.74 ± 0.32^{b,c}	26.74 ± 0.99^{e}	24.43 ± 1.57^{d,e}	22.07 ± 0.61^{b,c,,d}	20.05± 0.44^{b}	14.31 ± 0.14^{a}	22.77 ± 0.62^{c,d}	20.54 ± 0.04^{b,c}	14.86 ± 0.11^{a}	ns
Index 1	8.91 ± 0.02^{f}	4.88 ± 0.51^{b}	7.35 ± 0.01^{e}	4.25 ± 0.04^{a}	4.59 ± 0.03^{a,b}	4.98 ± 0.39^{b}	5.75 ± 0.03^{c}	11.5 ± 0.02^{g}	6.40 ± 0.06^{d}	6.17 ± 0.01^{c,d}	6.63 ± 0.02^{d}	ns
Index 2	0.59 ± 0.01^{b}	0.69 ± 0.06^{c}	0.59 ± 0.00^{b}	0.79 ± 0.03^{d}	0.72 ± 0.01^{c,d}	0.79 ± 0.02^{d}	0.54 ± 0.01^{a,b}	0.57 ± 0.01^{a,b}	0.68 ± 0.02^{c}	0.52 ± 0.01^{a}	0.68 ± 0.01^{c}	ns

Index 1. MUFA/PUFA
Index 2. PUFA/SFA
Means within each file with different superscripts differ significantly ($P < 0.05$).
[a] P value for Spain × Brazil (ns, not significant;*P<0.05; **P < 0.01).

Twelve fatty acids of Arbequina olive oils were identified, 6 of them SFA, 4 MUFA and 2 PUFA. The major fatty acid found was oleic acid (C18:1), the content of which ranged from 63.12 % (sample 4) to 79.68 % (sample 8), followed by palmitic acid (9-20%), linoleic acid (7-15%), stearic acid (1-2%) and palmitoleic acid (0.6-3%). Other fatty acids were found at a concentration of less than 1%. All samples complied with European regulations for EVOO. The *trans* isomers were not detected.

A significant effect ($P<0.05$) of the geographic area was observed in the fatty acid profile. The oil from location 8 presented the highest content in MUFA and the lowest content in PUFA and, consequently, the highest MUFA/PUFA ratio. This sample had the lowest susceptibility to oxidation, as measured by the Rancimat test, thus confirming that the MUFA/PUFA ratio is associated with the oxidative stability of oils (Reboredo-Rodriguez et al., 2014; Reboredo-Rodriguez et al., 2015). In the present study, the statistical correlation observed between Rancimat and the MUFA/PUFA ratio ($r= 0.754$, $P<0.001$) supports this statement. Moreover, other relationships between Rancimat and the fatty acid profile of oils were found ($P<0.01$), such as those with SFA ($r= -0.521$), MUFA ($r= 0.650$) and PUFA ($r= -0.686$), showing that samples with a higher content of MUFA and lower PUFA and SFA values have the best oxidative stability. These findings, taken together, confirm that oxidative stability is not dependent on a single parameter but is mainly influenced by the overall fatty acid composition of the oil (Dabbou et al., 2010).

The comparison between countries revealed significant differences for some minor fatty acids but not for the major ones, such as oleic, palmitic and linoleic acids. As a consequence, the indexes and ratios calculated from the fatty acid profile did not differ between the Brazilian and Spanish oils.

The fatty acid profile has been widely examined in olive oils, and scientific evidence has shown that the quality of the fat content has a strong impact on oil quality and thus on consumer health (Rueda, Seiquer, Olalla, Gimenez, Lara & Cabrera-Vique, 2014). The fat composition of Arbequina virgin olive oils from different regions of Spain and under different agronomic and production conditions, have revealed contents of oleic acid (58-79%) and palmitic acid (12-18%) and values of SFA, MUFA and PUFA similar to those found in our study (Yousfi, Weliard, & García, 2012; Reboredo-Rodriguez et al., 2015). Studies of Brazilian Arbequina oil (Minas Gerais region) have reported values of linoleic and linolenic acids close to those found in the present assay, and different data concerning oleic acid content, ranging from 61.3% to 75% (De Oliveira, Ramos, Pio & Cardoso, 2012; Ballus et al., 2014).

According to studies of Arbequina olive oils recently produced in Australia, Argentina and Tunisia, different cultivation and environmental conditions may have a strong effect on the fatty acid composition of oils, with oleic and palmitic acids being especially affected (Dabbou et al., 2010; Mailer et al., 2010; Rondanini et al., 2011; Torres et al., 2009). Among the environmental factors considered, temperature plays an essential role in the fatty acid composition of oils, by regulating fatty acid desaturases (Hernández et al., 2011). It has been shown that low temperatures increase the polyunsaturated fatty acid content of plants, thus maintaining the fluidity of biological membranes (Los & Murata, 1998). Corroborating this relationship, in the present study a significant association ($P<0.05$) was recorded between minimum temperatures and the C18:2 and C18:3 content of the oils analysed, whereas maximum temperatures were mainly correlated with the percentages of saturated fatty acids (Table S1).

In Arbequina oils from Argentina, it has been observed that the oleic acid content is dependent on the mean temperature during fruit growth, and can decrease by up to 2%

for each °C of increased temperature (Rondanini et al., 2011). Therefore, the low oleic acid content found in those oils, compared with the Spanish, has been attributed to high temperatures (Rondanini et al., 2011; Torres et al., 2009). In our study, no relationship between mean temperature and oleic acid content in oils was found, but a significant negative correlation was observed between this fatty acid and minimum ambient temperature (Table S1).

It has been suggested that environmental factors other than temperature may play a role in the fatty acid composition of oils (Romero, Tovar, Ramo, & Motilva, 2003). In the present study, we verified significant correlations ($P<0.01$) between altitude and levels of palmitic, oleic and linoleic acid, and also with SFA, MUFA, PUFA, n6/n3 and MUFA/PUFA ratios. According to our results, altitude was positively related to the amount of oleic acid and negatively related to PUFA content, which is in apparent disagreement with the observations of previous studies (Aparicio & Luna, 2002). However, the latter authors explain that changes in the fatty acid profile due to altitude have a minimal effect on oxidative stability of oils. In agreement, in the present study no relationship was found between altitude and oxidative stability, measured by the Rancimat method, despite the effects on the FA profile (Table S1). Thus, the temperature and especially the altitude were the environmental factors mainly related to the differences in the fatty acid profile found among oils of the present study.

In addition to the geographical and climate conditions considered in the present study, other factors such as agronomic aspects (ripeness index, storage conditions, processing) or further environmental variables (light intensity, humidity, evapo-transpiration, soil) may also influence the chemical composition of olive oil (Romero et al., 2016; Aparicio & Luna, 2002; Hernandez et al.; 2011; Rondanini et al., 2014).

3.4. Principal Component Analysis (PCA)

To obtain a global discrimination of oils from different geographic areas and to reduce the number of variables, a PCA was performed including oxidative stability, pigments (chlorophylls and carotenoids), colour (coordinates *L**, *a** and *b**) and fatty acid profile.

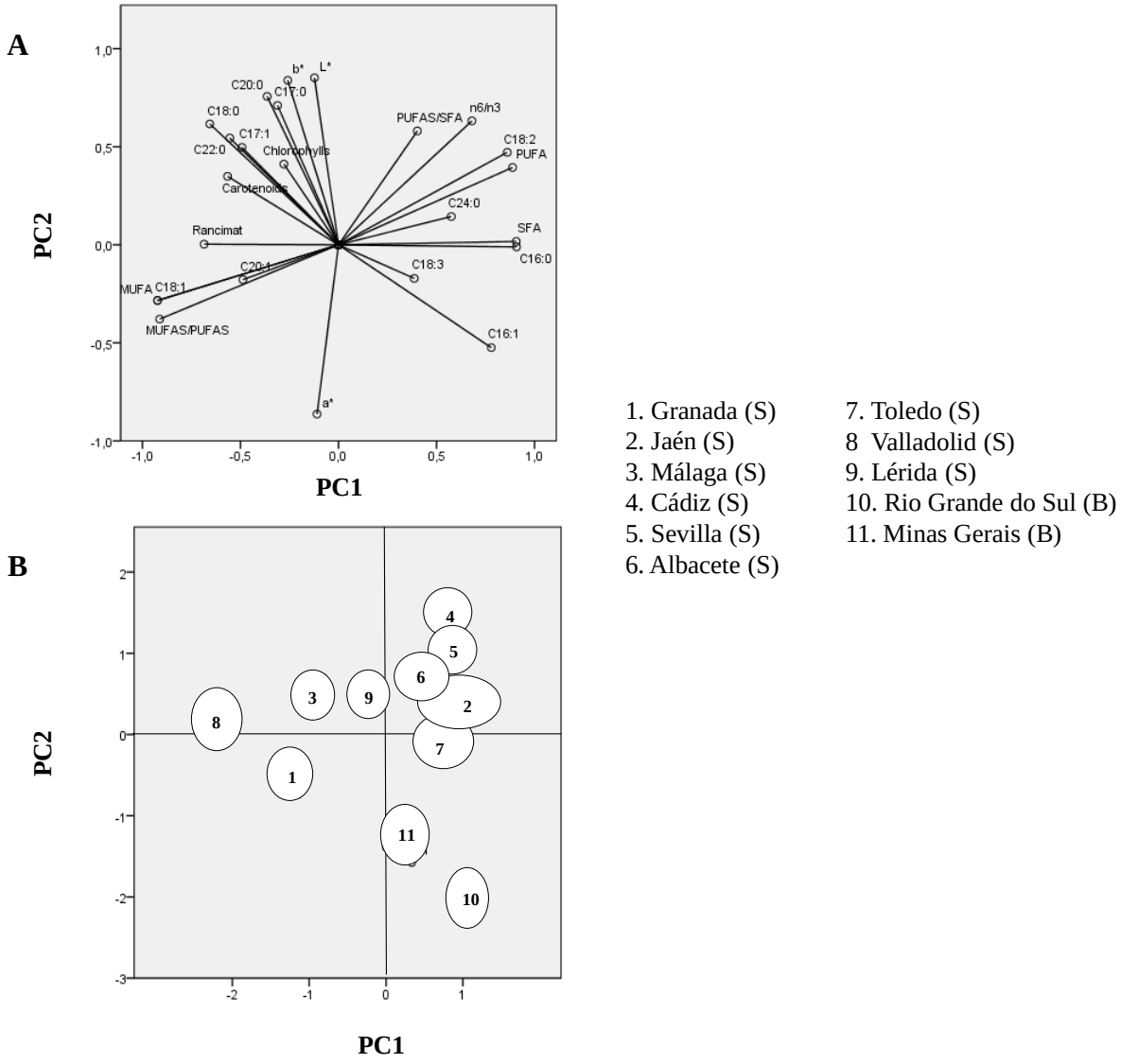

Figure 2. Graphic representation of the PCA obtained from the oxidative stability, pigments, colour and fatty acid profile. A) Vector arrows of the different variables. Identification of variables as described in the text. B) Distribution of the oils. (S) Spain, (B) Brazil.

The two main components obtained explained 66.97 % of the total variance (40.97 % PC1 and 26.00 % PC2). **Figure 2A** depicts the vector arrows of the variables, showing their contribution to PC1 and PC2. The first component was mainly explained by the fatty acid profile, with C18:1, MUFA, MUFA/PUFA, C16:0, SFA and PUFA producing the greatest influence, (loadings of -0.923, -0.923, -0.912, 0.908, 0.907 and 0.887, respectively). Rancimat values, highly related with fatty acid composition, were also an important contributor to PC1 (loading -0.685). The second component was clearly governed by colour parameters (L^* 0.852, a^* -0.863, b^* 0.839 of loading). **Figure 2B** represents the spatial distribution of the oils from the different locations according to PC1 and PC2 (the values of each oil were grouped for greater clarity). Taking into account factor 1, we can see that sample 8 is located in the extreme left-hand side of the graph, followed by samples 1, 3 and 9, while there is evident proximity among the oils from Spanish locations 2, 4, 5, 6, 7 and Brazilian locations 10 and 11, which are all located on the right-hand side of the graph. This distribution is associated with the fat composition of the oils, and particularly to the oleic acid content. According to factor 2, Brazilian samples 10 and 11 are located in the lower part of the graph, mainly due to differences in colour coordinates compared with the Spanish oils. Spanish sample 1, with similar a^* values than Brazilian oils, is also represented in the negative region of PC2. Considering both components together, the oils from Brazil are located in the lower right quadrant and separated from the other oils, but separation among the Spanish samples is also evident (i.e. samples 1 and 8 from the other Spanish oils, and the considerable distance between samples 2 and 8). Therefore, the differences between the samples, based on the parameters evaluated in the present assay, do not allow us to clearly separate the oils according to their countries of origin.

4. Conclusions

The results obtained provide useful data on the characteristics of Arbequina olive oils from Brazil and Spain and show that the geographic area of cultivation may affect the physicochemical properties and composition of olive oils. Although the parameters evaluated were within the limits established in EU regulations for EVOO, significant differences between Spanish and Brazilian oils were observed, and also among oils from the same country. Rainfall was showed as the most influential climate feature on colour of oils; thus Brazilian oils, produced in regions of heavy rainfall, had lower green intensity and were paler yellow that Spanish oils, produced in drier regions. The fatty acid composition of oils was also strongly related to environmental factors, particularly with altitude. Thus, findings of the present study reveal that geographic and climate aspects of producing areas may significantly influence the quality and composition of Arbequina olive oil.

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Supplementary data. Table S1. Pearson's correlation coefficient (r) between climate variables and altitude and analytical parameters (quality parameters, pigments, color coordinates and fatty acid profile).*p<0.05 **p<0.01

	Altitude	Temperature	Rainfalls	Minimum Temperature	Maximum Temperature
FA	-0.506**	0.070	0.683**	-0.216	-0.322
PV	-0.329	0.623**	0.602**	0.384*	-0.190
K_{232}	-0.055	0.647**	0.393*	0.726**	-0.241
K_{270}	-0.486**	0.260	0.210	0.174	-0.140
ΔK	0.341	-0.237	-0.061	-0.132	0.014
L*	0.09	-0.048	-0.836**	0.231	0.402*
a*	0.262	-0.015	0.643**	-0.280	-0.303
b*	0.225	-0.105	-0.923**	0.146	0.528**
Carotenoids	-0.006	0.079	-0.338	-0.207	0.407*
Chlorophylls	-0.237	0.315	-0.146	0.040	0.236
Rancimat	0.332	-0.091	-0.301	-0.442**	0.589**
C16:0	-0.570**	0.209	0.298	0.299	-0.085
C16:1	-0.405*	0.098	0.733**	0.040	-0.403*
C17:0	-0.023	0.337	-0.653**	0.247	0.596**
C17:1	0.361*	0.091	-0.753**	0.083	0.636**
C18:0	-0.043	0.019	-0.666**	-0.256	0.560**
C18:1	0.595**	-0.278	-0.218	-0.409*	0.090
C18:2	-0.504**	0.297	-0.021	0.508**	-0.036
C20:0	0.019	0.239	-0.553**	0.152	0.414*
C20:1	0.713**	-0.012	0.001	0.141	-0.200
C18:3	0.408*	0.085	0.217	0.434*	-0.314
C22:0	0.202	-0.064	-0.577**	-0.045	0.341
C24:0	-0.086	0.087	0.219	0.306	-0.165
ΣSFA	-0.599**	0.225	0.254	0.298	-0.041
ΣMUFA	0.594**	-0.281	-0.139	-0.434*	0.046
ΣPUFA	-0.488**	0.297	-0.015	0.515**	-0.045
n6/n3	-0.731**	0.301	-0.094	0.355*	0.069
MUFAS/PUFAS	0.488**	-0.314	-0.120	-0.565**	0.155
PUFAS/SFA	-0.110	0.162	-0.230	0.435*	-0.084

Artículo 2

General Paper

Comparative analysis of minor bioactive constituents (CoQ_{10}, tocopherols and phenolic compounds) in Arbequina extra virgin olive oils from Brazil and Spain

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Comparative analysis of minor bioactive constituents (CoQ_{10}, tocopherols and phenolic compounds) in Arbequina extra virgin olive oils from Brazil and Spain

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ABSTRACT

There is currently an emerging production of olive oil in Brazil but it is still poorly characterized. In this study, we performed a comparative analysis of minor bioactive constituents ($CoQ_{10,}$ tocopherols and phenolic compounds) in extra virgin olive oil from different regions of Brazil and Spain, of Arbequina cultivar. Significant variations ($P < 0.05$) in the concentration of the compounds analyzed were observed among oils from the different growing areas, not only between Spanish and Brazilian samples but also within zones of the same country. All the oils analyzed showed a high content of CoQ_{10}, which ranged from 48 to 85 mg/L. The α – tocopherol was the major isomer quantified and three main groups of phenolic compounds were identified: flavonoids (apigenin, luteolin), phenolic acids (naringenin, p-coumaric acid, vanillic acid) and phenolic alcohols (hydroxytyrosol). Climatic and geographic factors of the production zones greatly influenced the minor fraction composition; positive relationships between altitude and the level of CoQ_{10}, tocopherols and phenolic compounds of the oils were observed, whereas a negative correlation with rainfalls was found. Chemometric analyses demonstrated that oils were differentiated by the chemical composition and origin area and that polyphenols (particularly hydroxytyrosol) had the major weight in the oil classification.

Keywords: Food composition; Food analysis; Arbequina; Virgin olive oil; Coenzyme Q_{10}; Tocopherols; Polyphenols; Chemometric analysis.

Abbreviations

CoQ_{10}, coenzyme Q_{10}; EVOO, extra virgin olive oil; EFSA, food safety authority; HCA, hierarchical cluster analysis.

1. Introduction

It is well-known that chemical composition of olive oils consists of major (saponifiable fraction) and minor constituents (unsaponifiable fraction). The minor constituents, despite present in lower amounts (up to 2%), are a complex mixture of more than 230 compounds (Lopez et al., 2014; Servili et al., 2014). Among them, phenolic compounds and tocopherols are of great interest, mainly due to their nutritional value, antioxidant potential and health benefits.

The phenolic compounds are secondary plant metabolites that have one phenol ring (phenolic acids/ phenolic alcohol) or several aromatic rings with one or more hydroxyl groups (polyphenols) (Ignat et al., 2011; Lopez et al., 2014). Over the last few decades, multiple biological properties, providing antioxidant, anti-inflammatory, chemopreventive and anti-cancer benefits, as well as sensorial proprieties has been attributed to phenol compounds of olive oils (Servili et al., 2014). Recently, their protective effect over blood lipids from oxidative stress has been recognized by the European Food Safety Authority (EFSA,2011), thus stimulating, even more, the interest for olive oil polyphenols and allowing the use of its health claims (Reboredo-Rodriguez et al., 2016; Martín-Peláez et al., 2013). Tocopherols are known as lipophilic phenols that include 8 occurring forms: 4 tocopherols and 4 tocotrienols (α, β, γ and δ). In extra virgin olive oil (EVOO), the most predominant is the α-tocopherol (up to 90% of total), recognized as the most active form of vitamin E in mammals , although different factors such as cultivar and geographic location of the olive trees may influence its concentration (Lopez et al., 2014; Kalogeropoulos and Tsimidou 2014). These natural antioxidants not only provide nutritional value to virgin olive oils but also contribute to its stability, protecting from oxidation (Lopez et al., 2014; Kalogeropoulos and Tsimidou 2014).

Another minor compound with great value is the coenzyme Q10 (CoQ_{10}), an endogenous lipophilic compound that is involved in essential cell regulations and modulations, mainly in the mitochondrial respiratory chain (Jankowski et al., 2016; Thanatuksorn et al., 2009). In the body it exists in either oxidized (ubiquinone) or reduced form (ubiquinol); mainly in its reduced form it is recognized as an effective endogenous antioxidant, although an antioxidant role of the oxidized form cannot be discarded (Pravst et al., 2010; Jankowski et al., 2016). Additionally, it has the ability to recycle α-tocopherol by sparing or regeneration (Pyo, 2010). Due to redox reactions, continuous conversion between ubiquinone and ubiquinol takes place *in vivo* and, moreover, ubiquinone is also reduced during or following the intestinal absorption (Pravst et al., 2010). Therefore, the functions of CoQ_{10} are not affected by the form in which it is consumed (Pravst et al., 2010). Most of the CoQ_{10} in the human body is from endogenous synthesis, but levels decline progressively with increasing age and should be replaced daily by nourishment (Jankowski et al., 2016). In this sense, the EVOO consumption may be a dietary natural source for increasing intake of CoQ_{10} (Venegas et al., 2011; Žmitek et al., 2014). A wide range of possible benefits for human health has been reported for CoQ_{10} (Pravst et al., 2010; Jankowski et al., 2016; Turunen et al., 2004). The levels of these minor bioactive constituents are variable in EVOO. These variations have been attributed to different factors, including agronomic and technological practices, cultivar, ripening stage, climate conditions and geographic origin (Servili et al., 2009; Laincer et al., 2016). Nevertheless, factors influencing the CoQ_{10} content of olive oils have been scarcely investigated (Žmitek et al., 2014).

In recent years, the demand of olive oils is rising over the world, and emerging countries such as Brazil are beginning to produce it. Actually, the Arbequina cultivar is one of the most cultivated in Brazil, and data on physicochemical proprieties, oxidative

stability and fatty acid profile of Arbequina Brazilian oils have been recently published by our research group (Borges et al., 2017). However, little is known about how geographic and climate conditions may affect the minor components of the olive oils in Brazil (Ballus et al., 2014, 2015). Also, there is a lack of information about the similarities and differences between the newly-introduced and the autochthonous cultivars. Moreover, to our knowledge, nothing has been published about CoQ_{10} levels of Brazilian olive oil and very little about specific varieties in Spain (Žmitek et al., 2014). Finally, there is also a lack of information of the relationship between CoQ_{10} with other bioactive constituents such as phenolic compounds and tocopherols. With this background, the aims of this work were: i) to characterize the minor constituents CoQ_{10}, tocopherols and individual phenolic compounds of monovarietal Arbequina olive oil produced in Brazil; (ii) to compare it with the olive oils from the same cultivar produced in different regions of Spain and (iii) to classify the oil samples according to their geographic origin, on the basis of the analyzed variables and by applying chemometric analysis.

2. Materials and methods

2.1. Chemicals

All chemical products, standards and solvents for the analysis performed were analytical reagent grade or higher purity (Sigma-Aldrich, St. Louis, MO, USA) and Milli Q water (Millipore, Bedford, MA) was used throughout the assays. CoQ_{10} from Sigma-Aldrich (code: C9538) was used to prepare standard solutions of different concentrations.

2.2. Samples

EVOO from Arbequina cultivar was analyzed. Nine regions of olive oil production in Spain (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid and Lérida, samples 1 to 9) and two regions in Brazil (Rio Grande do Sul and Minas Gerais, samples 10 and 11) were selected to obtain the EVOO samples. The olives were harvested always at the early stage of harvest; the harvest date was: late October to mid-November of 2014 for Spanish samples and March to early April of 2015 for Brazilian samples. The oil was extracted within 24h, under a two-phase extraction system.The oils (n=3 from each producing region) were directly donated by the producers, adequately packaged for preserving from light and high temperatures and sent to CSIC laboratories (Granada, Spain) to perform the analysis. As was shown previously (Borges et al., 2017), samples meet quality standards established by the European Union regulation n° 2568/91 for extra virgin olive oil. The geographic coordinates (latitude and longitude), altitude (m), annual mean temperatures (°C), annual rainfalls (mm) and minimum and maximum mean temperatures (°C) of the different producing areas of Arbequina virgin olive oils are depicted in **Table 1**.

Table 1. Geographic coordinates (Latitude and Longitude), altitude (m), annual mean temperatures (◦C), annual rainfalls (mm) and minimum and maximum mean temperatures (◦C) of the different locations of Arbequina virgin olive oils from Spain (1-9) and Brazil (10, 11).

Oil Sample	Location	Latitude	Longitude	Altitude	Mean Temperature	Rainfall	Minimum Temperature	Maximum Temperature
1	Granada	37° 03’ N	3° 36’ W	905	17	385	7	26
2	Jaén	38° 03’ N	3° 29’ W	580	17	422	9	26
3	Málaga	37° 06’ N	4° 22’ W	883	20	411	13	27
4	Cádiz	36° 43’ N	6° 01’ W	47	19	636	13	24
5	Sevilla	37° 17’ N	4° 53’ W	416	19	598	11	27
6	Albacete	39° 00’ N	1° 54’ W	677	13	293	6	25
7	Toledo	39° 53’ N	4° 28’ W	459	14	391	7	26
8	Valladolid	41° 53’ N	5° 00’. W	845	13	394	-1	27
9	Lérida	41° 36’ N	0° 35’ W	168	14	677	6	21
10	Rio Grande do Sul	30° 00’ S	52° 52’ W	88	16	1691	3	22
11	Minas Gerais	22° 18’ S	42° 22’W	1310	17	1330	14	21

Geographic coordinates (latitude, longitude and altitude) proximate to olive grove were found using Google Earth program (Google Inc, USA). Climatic data of temperature and rainfall were supplied by the Spanish Meteorology Agency (Aemet, 2015) and the National Meteorology Institute of Brazil (INMET, 2015).

2.3. Determination of CoQ_{10}

The samples were analyzed according to Venegas et al. (2011). A quantity of 990 μL of 1-propanol was mixed with 10 μL of the oil, vortex and centrifuged at 11300g for 5 min at room temperature. The subsequent supernatant was diluted 1/500 in 1-propanol prior to HPLC injection. CoQ_{10} present in the oil extract were separated by reversed-phase high-performance liquid chromatography (HPLC, Gilson, WI) with a C18 symmetry column (3.5 μm, 4.6 x 150 mm) (Waters Chromatography, Barcelona, Spain) using a mobile phase consisting of methanol, ethanol, 2-propanol, acetic acid glacial (500:500:15:15), and 50 mM sodium acetate at a flow rate of 0.9 mL/min. The electrochemical detector consisted of an ESA Coulochem III with the following setting: guard cell (upstream of the injector) at +900 mV and conditioning cell at -600 mV (downstream of the column) followed by the analytical cell at +350 mV. While this method is able to detect CoQ_{10} in its reduced (ubiquinol) and oxidized (ubquinone) form, ubiquinol was not detected in our conditions of extraction and analysis. The CoQ_{10} concentrations of the oxidized form were estimated by comparison of the peak areas with those of standard solutions of known concentrations (0, 25, 100, 300, and 600 ng/mL). Values of calibration curve are reported in Supplementary Table. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.jfca.2017.07.036. The results were expressed in mg per L of sample.

2.4. Determination of Tocopherols

The tocopherols isomers were determined as described by Rueda et al. (2016). Briefly, 1 g of oil was dissolved in 25 mL n-hexane. The samples were analyzed by an HPLC system (Water Alliance 2695 separations module, Milford, MA) equipped with a silica column (4 mm × 250 mm) and eluted with hexane: isopropanol (99.25:0.75 v/v) at

a flow rate of 1 mL/min during 25 min. The tocopherols were detected by fluorescence (Water 2475) with excitation wavelength at 290 nm and emission wavelength at 330 nm. A calibration curve using external standards (α-, β-, γ-, and δ-tocopherols) was used for quantification. The results were expressed as mg per kg of sample.

2.5. Individual phenolic compounds

The individual phenolic fraction of samples was performed after an extraction with methanol/water (80:20) according to the International Olive Oil Council (IOOC, 2009). The extracts were analysed by UPLC-TOF-MS following the method validated by Rivas et al. (2013). The UPLC system consisted of a AcQuity UPLC equipped with a binary pump system (Waters, Milford, MA, USA) using a AcQuity UPLC BEH C18 column (1.7mm, 2.1 mm x 100 mm inner diameter). The column was kept at 40°C and the flow rate was 0.4 mL/min. The mobile phase was composed by eluent A, MilliQ water with formic acid (0.1%) and eluent B, methanol with formic acid (0.1%). The elution started at 5% of eluent B for 1 min, then was linearly increased to 100% of eluent B in 11 min and kept isocratic for 5 min; then, it got back to initial conditions in 0.1 min; the equilibration time was 2.9 min. The injection volume was 7µL, and all samples were filtered through 0.22 mm before chromatography. The UPLC was coupled to a Micromass/Waters LCTPremier XE benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 100 to 1000. The optimum values of the electrospray ionization MS parameters were as follows: capillary voltage 2.6 kv; drying gas temperature 200°C, desolvation gas flow 800 L/h. For optimum detection resolution, a solution was prepared of 1 mg/L leucine enkephalin in acetotrinile/H_2O (1:1, v/v) containing 0.1% formic acid continuously

infused at a flow rate of 0.05 mL/min by an external rotary pump. This solution was also used for continuous calibration of the equipment, as its perfusion is simultaneous to that of the sample and served as a reference. Mass calibration was performed using a solution of sodium formate (containing 0.05 of formic acid and 5 mM of sodium hydroxide in iso- propanol/H_2O 9:1, v/v). Accurate mass data of molecular ions were processed with MassLynxs (Waters).

Analytical parameters of the methods used are shown as supplementary file.

2.6. Statistical analysis

Results were analyzed by analysis of variance (one-way ANOVA), with the geographic origin of oils (regions 1–11) as the main factor. Tukey's test was used to compare mean values between oils from the different regions, and differences were established at $P < 0.05$. The relationships of the different variables with the climate characteristics and the altitude of the producing regions were evaluated by Pearson's coefficient. These statistical calculations were carried out using SPSS version 21.0 (IBM Corporation, New York, USA).

In addition, chemometric analysis was performed including all the minor bioactive compounds evaluated in the present study (CoQ_{10}; tocopherols - α, β, γ and phenolic compounds). In a first explorative step a hierarchical clustering analysis (*HCA)* was carried out to identify eventual similarities between the olive oils samples of different geographic origin, by calculating multidimensional squared Euclidean distances of scores applying the single linkage-clustering method. Posteriorly, to reduce the variables into a small number of factors and explore the contribution of variables to oil differentiation, a factorial analysis (FA) using a varimax rotation was applied. All

the chemometric analysis were performed using Stat Graphics Centurion XV software (Stat Point Technologies, Inc., USA, 2006).

3. Results and discussion

3.1. CoQ_{10}

The levels of CoQ_{10} are shown in **Figure 1**. According to Pravst et al (2010), foods containing over than 46 mg CoQ_{10}/L of olive oil are considered a very rich source of CoQ_{10}. Therefore, all the samples evaluated in the present study may be classified as very rich CoQ_{10} sources. As shown in **Figure 1**, statistical differences ($P < 0.01$) were found among CoQ_{10} content of the samples ranging between 85.3 ± 5.8 mg/L (region 1, corresponding to Granada, Spain) and 48.7 ± 1.6 mg/L (region 9, Lérida, Spain) (mean ± SE). Brazilian oils showed intermediate values of 49.5 ± 2.3 mg/L and 60.0 ± 1.9 mg/L, for areas 10 and 11, respectively. There is limited information about CoQ_{10} content in monovarietal EVOO. A previous study comparing different cultivars from several countries (Žmitek et al., 2014) showed that CoQ_{10} is greatly driven by genetic factors; the highest content was observed in the cultivar Hojiblanca (98 mg/L) followed by Picual (63 mg/L) and Arbequina (58 mg/L). Values of 77.5 mg/L of CoQ_{10} content have been found in Spanish EVOO of Picual cultivar (Venegas et al., 2011), similarto the highest levels detected among oils in the present assay (samples from Spanish locations 1, 2, 3 and 6). Higher values, above 90 mg/L, have been observed in commercial Italian EVOO (Cabrini et al., 2001).

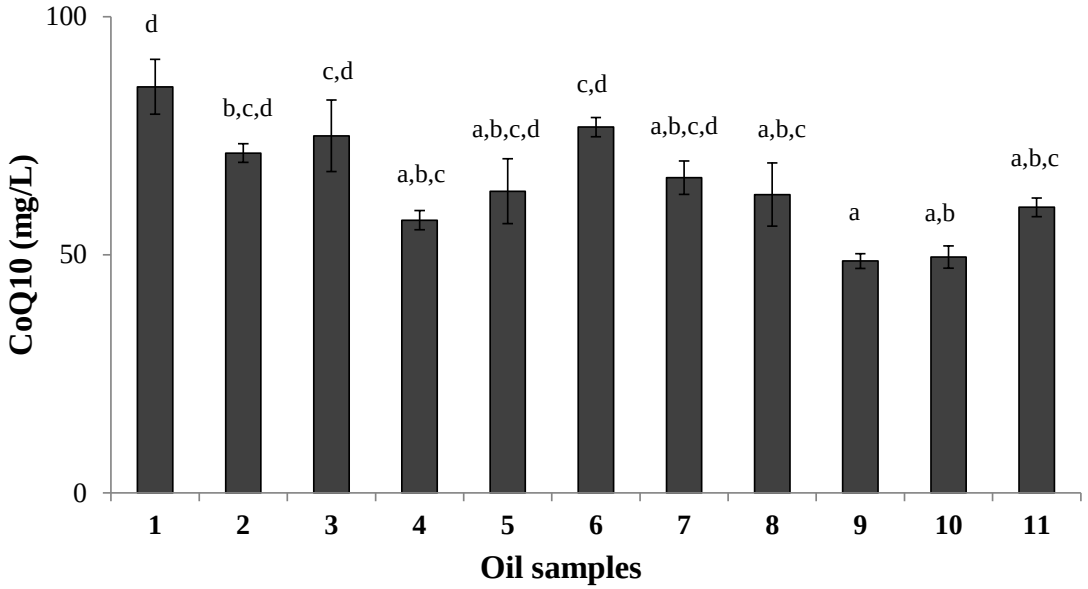

Figure 1. Coenzyme Q_{10} levels of Arbequina olive oils from Spain (1-9) and Brazil (10-11). (values are means ± SE, n=3). Different letters indicate significant differences (ANOVA and Tukey test, $P < 0.05$).

These findings support that EVOOs are one of the best natural sources of dietary CoQ_{10} (Žmitek et al., 2014). Since the current intake of CoQ_{10} in developed countries is not sufficient to compensate the age-related decline (Žmitek et al., 2014), promoting the intake of EVOO may be a good alternative to supplementation.

In addition to genetic factors, the CoQ_{10} content of oils may be affected by geographic and climate conditions, as was supported by the results of the present assay, in which significant differences were found between oils from the same cultivar. In this sense, statistical correlations ($P < 0.01$) were found between CoQ_{10} content and geo climate factors of production areas, positive with altitude (r= 0.461) and maximum temperature (r= 0.484) and negative with rainfalls (r= -0.494). Accordingly, the areas of low rainfall and high altitude (Table 1), such as Granada (region 1), produced the oil with the highest CoQ_{10} content. In the same line, high rainfall levels in Brazilian growing areas (regions 10 and 11) could have a negative effect on the CoQ_{10} content of

oils, although in the case of oil from region 11 this effect seems to be partly counteracted by the positive influence of the high altitude.

3.2. Tocopherols

Table 2 shows tocopherols content (α, β, γ and total) for Arbequina EVOO from Spain (1-9) and Brazil (10-11). The major isomer quantified was α - tocopherol, representing more than 98% of the total, ranging from 92 to 208 mg/kg of oil, followed by lower amounts of β (0.8 - 1.9 mg/kg) and γ (0.7 - 2.15 mg/kg) fractions. No detectable values of δ- tocopherol were observed.

The content of the tocopherol fractions differed between EVOO samples from the different regions ($P < 0.05$). Values of Brazilian oils were within the range found for Spanish samples, with the only exception of the γ –isomer, which was lower among Brazilian oils. Thus, it was shown that the Arbequina oils newly introduced in Brazil have similar tocopherol content that the oils from the autochthonous Spanish cultivar.

In general, α - tocopherol concentration found in the present assay is similar to values reported for Arbequina EVOO from Spain and the introduced from Argentina, Tunisia and Turkey, which vary from 150 to 300 mg/kg (Beltrán et al., 2010; Dabbou et al., 2010; López-Cortés et al., 2013; Torres et al., 2009; Uluata et al., 2016; Yousfi et al., 2012). The lowest contents of α - tocopherol among samples of the present study were found in Spanish locations 4 (Cádiz) and 9 (Lérida). The low tocopherol level in oil of location 4 could be related to the low oxidative stability (5.32 hours, measured by the Rancimat method) previously described in oils from this region (Borges et al., 2017). Large variations in α-tocopherol content have been observed in Brazilian Arbequina EVOO from Minas Gerais depending on the harvest year (62 and 201 mg/kg for crop years 2010 and 2011, respectively) (Ballus et al., 2014). Thus, values of 147

and 171 mg/kg found in Brazilian samples in the present study (zones of Rio Grande do Sul and Minas Gerais, respectively) were within this wide range.

Table 2. Tocopherols content (mg/kg) of Arbequina olive oils from Spain (1-9) and Brazil (10-11).

	α - Tocopherol	β- Tocopherol	γ - Tocopherol	Total
Regions				
1	179 e	1.00 a,b,c	1.90 e	182 e
2	202 f	1.03 b,c	0.90 a,b,c	204 f
3	157 c,d	0.85 a,b	1.15 d	159 c,d
4	92 a	0.80 a	0.75 a	93 a
5	164 d	0.90 a,b,c	0.73 a	166 d
6	198 f	1.05 b,c	1.13 c,d	200 f
7	208 f	0.85 a,b	1.00 b,c,d	210 f
8	167 d,e	0.85 a,b	2.15 f	170 d,e
9	128 b	0.75 a	1.05 c,d	130 b
10	147 c	0.85 a,b	0.70 a	148 c
11	171 d,e	0.90 a,b,c	0.80 a,b	173 d,e
SEM	0.87	0.01	0.01	0.86

Means values in each column with different letters are significantly different between regions or countries (n=3, ANOVA and Tukey test, $P < 0.05$).

Some previous data show that tocopherol content of olive oils has a genetic component, i.e., it is highly depending on cultivar, but it may also be affected by climatic conditions, mainly temperature, rainfalls and altitude (Beltrán et al, 2010; Dabbou et al., 2009; Ilyasoglu et al., 2016). Therefore, the growing location area, with different conditions of rainfall and temperature, influence the tocopherol content and composition of the oils (Aguilera et al., 2005), in agreement with present results. In addition, significant correlations were verified relating geo climate conditions with tocopherol content of the samples (**Table 4)**. In concordance with previous research reporting increased tocopherol levels in dry crop seasons (Beltrán et al., 2010; Ilyasoglu et al., 2016), our findings show a negative relationship between rainfalls and γ ($P<0.01$) and total tocopherol content ($P<0.05$). On the contrary, some authors do not

observe a consistent influence of rainfalls on tocopherol levels in Tunisian oils (Dabbou et al., 2009), since the effects of climatic conditions on tocopherols seem to be cultivar-dependent (Beltrán et al., 2010).

Relationships between the temperature of the growing area and tocopherols have been scarcely studied. In the present study, conflictive correlations among temperature and tocopherols were found, since they were negative with the mean (for α-, γ- and total) and minimum temperatures (for γ-) and positive with maximum temperatures (for α- and total tocopherols) (**Table 4**). That means that annual mean temperature of the growing zone significantly affects the tocopherol content, but peaks of cold and heat also seem to disturb this variable. Regarding the altitude effects, some authors observe increased tocopherol content in olive oils with increasing the altitude (Dabbou et al., 2009) and, thus, altitude has been proposed as an important factor to be considered in tocopherol level of oils (Kalogeropoulos and Tsimidou, 2014). In this line, positive correlations of altitude with tocopherol concentration of the oils have been found in our study (**Table 4**). However, oils from areas with the highest altitude were not those with the highest tocopherol content, which suggests that altitude has not a definitive influence and could be counteracted by effects of other geo climatic factors.

As was reported before, the climate conditions have primarily an effect on biochemical reactions during growth and ripening, mainly in some enzymatic reactions that are essential for the tocopherol synthesis (Beltrán et al., 2010; Ilyasoglu et al., 2016). In this sense, increasing the tocopherol content could be an auto-protection mechanism of plants against some stress conditions, such as water stress (Beltrán et al., 2010). However, there is a maximum level depending on the fruit development (Georgiadou et al., 2015). In this line, our results show that climate factors are linked to

tocopherols content and may impact them, but the influence of other factors providing synergic or antagonist effects, as the maturation index, cannot be discarded.

3.3. Phenolic compounds

The concentration of phenolic compounds (apigenin, luteolin, naringenin, p-coumaric acid, vanillic acid and hydroxytyrosol) of olive oil samples from different geographical areas of Spain and Brazil is shown in **Table 3**. Statistical differences between samples ($P < 0.05$) were found in all individual compounds. Strong differences were observed between Brazilian oils, being the content of all phenolic compounds always higher in sample 10 than sample 11. Three main groups of phenolic compounds were identified: flavonoids (apigenin, luteolin), phenolic acids (naringenin, p-coumaric acid, vanillic acid) and phenolic alcohols (hydroxytyrosol). Flavonoids were the major group, ranging from 93 to 36% of the total quantified, followed by phenolic acids (10-30 % of the total), with the exception of samples from location 3 (with 14 % of flavonoids and only 2% of phenolic acids). The highest levels of hydroxytyrosol were found in oils from Spanish locations 3 (1500 µg/kg, contributing 83 % to the total phenols examined) and Brazilian region 10 (1050 µg/kg, 31 % of the total). The Brazilian sample 11 showed a different profile of phenolic compounds in comparison with the other samples; with equilibrate quantities of the quantified phenolic groups.

Table 3. Phenolic content (μg/kg) of Arbequina olive oils from Spain (1-9) and Brazil (10-11).

	Phenolic content					
	Flavonoids			**Phenolic acids**		**Phenol alcohols**
	Apigenin	Luteolin	Naringenin	p-Coumaric acid	Vanillic acid	Hydroxytyrosol
Regions						
1	265 e,f	858 c,d	25.0 a,b	55.0 c	119 c	90.0 a,b
2	309 f,g	1054 d,e	51.5 c,d	59.0 c	59.5 b	287 d,e
3	26.0 a	241 a,b	30.0 b	n.d	n.d	1500 g
4	204 b,c	773 c	16.5 a	24.5 a,b	28.5 a	3.00 a
5	161 b	463 b	63.5 d	13.5 a	n.d	379 e
6	326 g	1257 f,g	50.0 c	97.0 e	124 c	251 c,d,e
7	424 h	1148 e	56.0 c,d	94.0e	121 c	161 b,c,d
8	184 b,c	748 c	16.0 a	91.0 e	n.d	204 b,c,d
9	236 d,e	1236 f,g	58.5 c,d	31.0 b	70.0 b	149 b,c
10	398 h	1482 g	133 e	228 f	n.d	1050 f
11	15.0 a	92.0 a	21.0 a,b	74.0 d	n.d	92.0 a,b
SEM	40.3	133	10.1	18.7	16.2	8.1

Means values in each column with different letters are significantly different between regions or countries (ANOVA and Tukey test, $P < 0.05$).

Current data show a large variation of phenolic content for EVOO samples, ranging between 50-940 mg/kg (Servili et al., 2014). Nevertheless, available data in the literature are very difficult to compare, since different methods have been used to separate, identify and quantify the phenolic compounds in EVOO samples around the world (Ballus et al., 2015). Besides, commercial standards are not totally available and some of these compounds are identified and quantified on the basis of other ones with similar structures (Ballus et al., 2015; Bakhouche et al., 2013). In this sense, nowadays there is not an official method able to show a complete profile of phenolic compounds and satisfy the new health claims on olive oil phenols, although the scientific community has been working on developing it (Reboredo-Rodriguez et al., 2016).

In accordance with data of the present study, flavonoids (apigenin, luteolin) and phenolic acids (p-coumaric acid, vanillic acid) have been previously found in Arbequina cultivar (Bakhouche et al., 2013, Rivas et al., 2013; Yousfi et al., 2012). However, up to our knowledge, naringenin was not reported yet in Arbequina oil, but was recently detected in oleaster, leaves, olive barks, wastewater and EVOO of Empeltre and Arauco cultivars from Argentina (Bouarroudj et al., 2016, De Fernandez et al., 2014, Leouifoudi et al., 2014; Tóth et al., 2015). On the other hand, hydroxytyrosol is one of the most important phenolic compounds in olive oils (Bakhouche et al., 2013) and it has been widely associated with the high antioxidant capacity in EVOO (Servili et al., 2014). Accordingly, Arbequina oils from region 3 (Málaga, Spain) have a high resistance to oxidation (15.85 hours of oxidative stability, by the Rancimat method) (Borges et al., 2017). The health beneficial properties of hydroxytyrosol have been recognized by the EFSA (EFSA, 2011). Concerning geographical area and climate conditions, several authors have studied the relationship of phenolic compounds with them (Bakhouche et al., 2013; Dabbou et al., 2009; Ilyasoglu et al., 2016). In this line,

positive correlations between altitude and some of the analyzed phenolic compounds such as naringenin (r=0.458), p-coumaric acid (r=0.578) and hydroxytyrosol (r=0.618) were found in our study (**Table 4**). In agreement, Dabbou et al. (2009) attribute variations of phenolic profile of Tunisian EVOO to the geographic area characteristics, particularly to altitude. Furthermore, scientific data have shown that water status also influence the phenolic profile of the olive oils, but the effect may differ depending on the compounds, since the enzymes involved in the biosynthetic pathway of phenolic compounds are affected by water stress conditions in a different way (Ilyasoglu et al., 2010; Stefanoudaki et al., 2009). Thus, correlations between climatic conditions and minor olive oil compounds may be mainly explained by the effects in enzymatic activity reactions during the growth and ripening of olive fruits (Ilyasoglu et al., 2016). Accordingly, relationships between climate variables of temperature and rainfalls and phenol composition of oils were found in the present assay (**Table 4**).

Table 4. Pearson's correlation coefficient (r) between climate variables and altitude and analytical parameters (Coenzyme $Q_{10,}$ Tocopherols and Phenolic content).

	Altitude	**Temperature**	**Rainfalls**	**Minimum temperature**	**Maximum temperature**
Coenzyme Q_{10}	0.461**	0.060	-0.494**	0.073	0.484**
α Tocopherol	0.543**	-0.361*	-0.316	-0.219	0.385*
β Tocopherol	0.376*	-0.074	-0.257	-0.020	0.307
γ Tocopherol	0.437**	-0.396*	-0.446**	-0.588**	0.309
Total	0.547**	-0.365*	-0.321*	-0.226	0.389*
Apigenin	0.314	-0.390*	-0.249	0.097	-0.179
Luteolin	0.315	-0.456**	-0.207	0.075	-0.374*
Naringenin	0.458**	0.000	0.286	0.439*	-0.440*
p-Coumaric acid	0.578**	-0.364*	0.417*	-0.021	-0.534*
Vanillic acid	-0.037	-0.470**	-0.511**	-0.191	0.066
Hydroxytyrosol	0.618**	0.466**	0.064	0.563**	0.066

Symbols indicate significant correlations (* P <0.05, ** P < 0.01).

3.4. Chemometric analyses.

A hierarchical cluster analysis (HCA) was applied as an initial approach for grouping samples that share common characteristics according to the analyzed variables. HCA was obtained using the Euclidean distance of scores as a similarity criterion and the results are shown in **Figure 2.A**. The dendogram plot defined five distinctive clusters. Firstly, three separated cluster were observed for samples from Rio Grande do Sul (10), Valladolid (8) and Málaga (3).The fourth cluster was composed by the oils from Cádiz (4), Lérida (9), Sevilla (5) and Minas Gerais (11). The last one was represented by the samples from Granada (1), Jáen (2), Albacete (6) and Toledo (7). It was observed that oil from Rio Grande do Sul showed the biggest Euclidean distance (high significance clustering), i.e the lowest similarities comparing with the other groups.

In the factorial analysis, three factors justifying 76% of total variance were obtained (F1 33%, F2 29%, F3 14%). F1 was mainly explained by phenolic compounds (apigenin 0.92; luteolin 0.92; naringenin 0.81 and p-coumaric acid 0.77), F2 was composed by CoQ_{10} (0.84) and tocopherols (α 0.83; β 0.83 and γ 0.36), while F3 was mainly characterized by hydroxytyrosol (-0.87) and vanillic acid (0.66). According to these factors, a spatial representation of the oils was obtained (**Figure 2.B**). A clear separation of sample 10 was observed mainly due to F1 and F3, and thus, related with the different content of polyphenols in this oils compared with the other samples, particularly luteolin, p-coumaric acid and hydroxytyrosol. Also according to F1 and F3, samples from locations 1, 2, 6 and 7 showed evident proximity. The remainder oils were relatively grouped but, among them, samples from Málaga (3) were slightly away from the other locations, which may be associated with its high level of hydroxytyrosol. This entirely means that F2 (governed by CoQ_{10} and tocopherols) was not able to

differentiate EVOO samples clearly, according to the content of lipophilic compounds in the samples. However, F2 and particularly F3, with a strong impact of hydroxytyrosol, could classify the samples in three separated blocks, as commented. Thus, phenolic compounds were a useful tool to delimitate EVOO samples from the different geographic area. In general terms, HCA was confirmed by the factorial analysis, which in turn showed the weight of the variables in the classification of the oil samples.

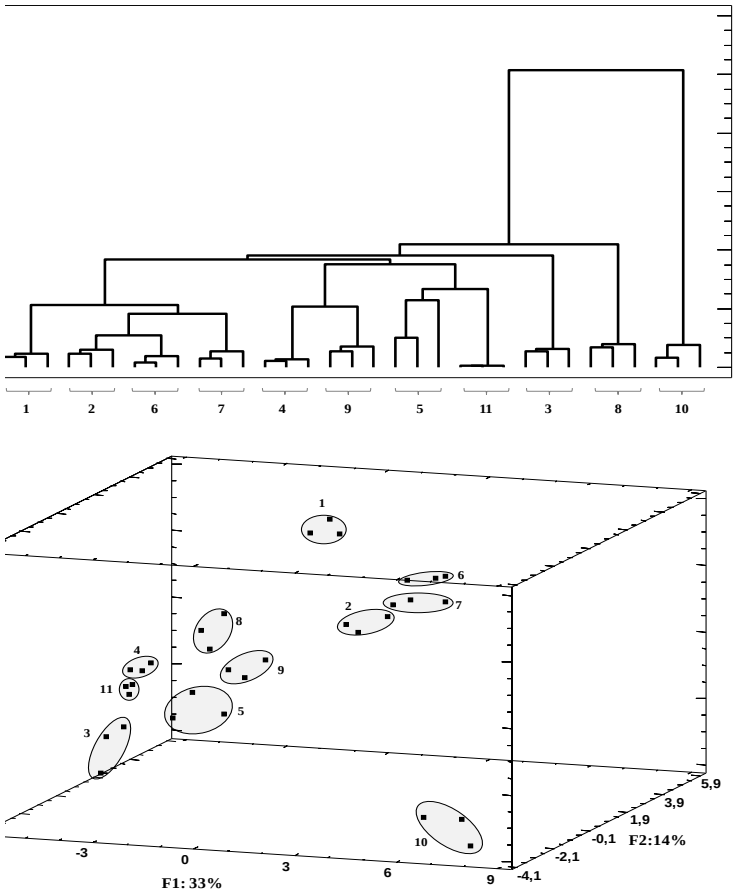

Figure 2. **A-** Dendrogram plot showing the conglomeration of olive oil samples from Spain (1-9) and Brazil (10-11) obtained by clustering of CoQ_{10}; tocopherols - α, β, γ and phenolic compounds showing. **B**- PCA 3D plot obtained from CoQ_{10}; tocopherols - α, β, γ and phenolic

compounds representing the distribution of olive oil samples from Spain (1-9) and Brazil (10-11).

4. Conclusions

Findings of the present study contribute to increasing the knowledge of the EVOO grown in Brazil, a country with an incipient production of olive oil but with great potential for its cultivation. In addition, comparative information with the original cultivar produced in Spain is reported. Significant differences in the minor fraction composition (CoQ_{10}, tocopherols and phenolic compounds) were observed not only between Spanish and Brazilian Arbequina oils but also between oils from the different producing areas within each country. A high level of CoQ_{10} content of olive oils was observed, especially among Spanish oils. Climatic and geographic factors of the production zones seem to greatly affect the content of the parameters analyzed; positive relationships of the altitude with the level of CoQ_{10}, tocopherols and phenolics of the oils were observed, whereas negative correlation with rainfalls were also shown. Chemometric analyses demonstrated that oils were differentiated according to chemical composition and origin area and that polyphenols (particularly hydroxytyrosol) had the major weight in the oil classification.

The influence of other factors than those considered in the present study, such as the ripeness index of olives, cannot be discarded, and therefore, the lack of this information may be considered as a limitation of the study.

Conflicts

The authors declare no competing financial interest.

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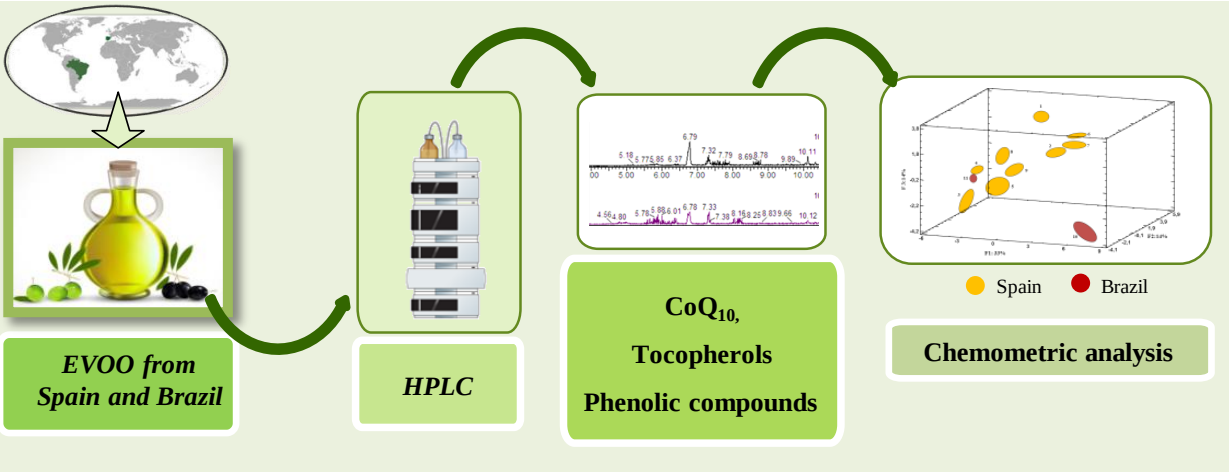
Table . Analytical parameters of methods: tocopherols, polyphenols and coenzyme Q_{10}.

Compound	Chemical Formula	RT (min)	LOQ (µg/Kg)	Calibration (mg/L)	R^2
Phenols					
Hydroxytyrosol	$C_8H_{10}O_3$	2.74	10	y=0.17x+0.99	0.999
Apigenin	$C_{15}H_{10}O_5$	7.30	10	y=0.97x+62.08	0.933
Naringenin	$C_{15}H_{12}O_5$	6.77	10	y=0.86x+22.73	0.988
Luteolin	$C_{15}H_{10}O_6$	6.84	10	y=0.68x+36.192	0.95
Syringic acid	$C_9H_{10}O_5$	4.26	10	y=0.08x+1.83	0.991
p-Coumaric acid	$C_9H_8O_3$	4.69	10	y=0.26x+0.008	1
Gallic acid	$C_7H_6O_5$	1.38	10	y=0.15x+0.45	0.999
Vanillic acid	$C_8H_8O_4$	3.93	10	y=0.05x-0.30	0.999
Ferulic acid	$C_{10}H_{10}O_4$	4.98	10	y=0.43x-0.90	0.994
Tocopherols					
Compound	**Chemical Formula**	**RT (min)**	**LOQ (mg/Kg)**	**Calibration (mg/L)**	**R^2**
α Tocopherol	$C_{29}H_{50}O_2$	5.358	50	$y=2.88\cdot10^5x+3.41\cdot10^4$	0.999
β Tocopherol	$C_{28}H_{48}O_2$	9.507	2.5	$y=3.83\cdot10^5$ x-$3.34\cdot10^3$	0.999
γ Tocopherol	$C_{28}H_{48}O_2$	9.976	5	$y=3.67\cdot10^5$ x-$5.62\cdot10^3$	0.999
δ Tocopherol	$C_{27}H_{46}O_2$	15.278	2.5	$y=4.29\cdot10^5$ x-$3.76\cdot10^3$	0.999
Coenzyme Q_{10}					
Compound	**Chemical Formula**	**RT (min)**	**LOQ (ng/mL)**	**Calibration (mg/L)**	**R^2**
Q_{10}	$C_{59}H_{90}O_4$	16.2	2.5	$y=3.27\cdot10^{-5}$x-2.59	0,996



GRAPHICAL ABSTRACT

Artículo 3

General Paper

Study of the antioxidant potential of Arbequina extra virgin olive oils from Brazil and Spain applying combined models of simulated digestion and cell culture markers

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Study of the antioxidant potential of Arbequina extra virgin olive oils from Brazil and Spain applying combined models of simulated digestion and cell culture markers

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Short title: Antioxidant potential of Arbequina extra virgin olive oil

ABSTRACT

A physiological approach to assessing the antioxidant potential of Arbequina EVOO from different zones of Brazil and Spain was performed, applying a combined model of simulated digestion and cell cultures, using the Caco-2 cell line. Our results showed an increasing of total phenolic content (TPC) and antioxidant properties promoted by the *in vitro* digestion. Preincubating Caco-2 cells with bioaccessible fractions of oils counteracted the cytotoxic effect promoted by an oxidising agent (*t*-BOOH), preserving cell viability and reducing the generation of reactive oxygen species (ROS). The protective effect on ROS production was associated with the antioxidant activity (DPPH assay), but no relation with the TPC of the digested samples was found. Differences in the parameters evaluated were observed among the samples, which were related to climatic characteristics of the production zones. It was concluded that transformations during the digestive process are important for establishing the antioxidant potential of the oils.

Keywords: Extra virgin olive oil, Antioxidant properties, Polyphenols, Digestion, Caco-2 cells, ROS.

Abbreviations: ABTS, 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid; BF, bioaccessible fraction; DMEM, Dulbecco's modified minimal essential medium; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EVOO, extra virgin olive oil; FRAP, ferric reducing power; FBS, heat-inactivated fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RF, residual fraction; ROS, reactive oxygen species; *t*-BOOH, tert –butylhydroperoxide; TPC, total polyphenol content.

1. Introduction

Extra virgin olive oil (EVOO) consumption has been intensely associated with multiple healthy properties, such as prevention of coronary and degenerative disease (Chiesi, Fernandez-Blanco, Cossignani, Font, & Ruiz, 2015; Servili et al., 2013). These beneficial effects have been mainly attributed to polyphenols, which are powerful antioxidants, and also possess other potent biological activities such as anti-inflammatory, anti-cancer, antimicrobial, antiviral, anti-atherogenic or hypoglycemic (Barbaro et al., 2014). Special attention has received the study of the antioxidants of EVOO, due to their importance from health, biological and sensory points of view (Servili et al., 2014)

Several studies have been focused on the characterisation of phenolic content and antioxidant capacity of monovarietal olive oils of different areas from the Mediterranean basin and, more recently, from newly introduced varieties worldwide (Bouarroudj, Tamendjari & Larbat, 2016; Dabbou et al., 2010; Köseoğlu, Sevim & Kadiroğlu, 2016; Uluata et al., 2016; Xiang et al., 2017). In this regard, Brazil has beginning to cultivate olives and has shown a huge potential to produce olive oil. Currently, 70% of Brazilian olive oil production is from Arbequina cultivar (Olive Oil Times, 2017); however, information on the antioxidant power of Brazilian virgin olive oil is still lacking.

Antioxidant properties of oils have been traditionally studied in chemical extracts by applying assays to evaluate radical scavenging capacity (DPPH and ABTS) and ferric-reducing power (FRAP) (Kalogeropoulos et al., 2014; Samaniego Sánchez et al., 2007). Nevertheless, the use of extraction procedures using organic solvents may not be a real physiological approach to measure the potential *in vivo* effects of the oils, owing to that the first requirement for a dietary compound to exert a biological activity

is the maintenance of its properties after the digestion process (Pastoriza, Delgado-Andrade, Haro & Rufián-Henares, 2011). This statement implies that bioactive compounds must be bioaccessible, i.e., released from the food matrix during digestion and, thus, available to be absorbed by the intestinal cells (Carbonell-Capella, Buniowska, Barba, Esteve & Frígola, 2014). The *in vitro* static methods simulating gastrointestinal digestion allow mimicking physiological conditions *in vivo*, taking into account the presence of digestive enzymes and their concentrations, pH, digestion time and salt concentrations, among other factors (Minekus et al., 2014). In addition, coupled to cell line models enable studies of antioxidant cell markers and intestinal absorption, elucidating the potential impact of these compounds on human health (Borges, Cabrera-Vique & Seiquer, 2015; Soler et al., 2010). Previously, our research group has applied successfully Caco-2 cell lines as a model to study absorption and metabolism in small intestine of minerals and trace elements, products of Maillard reaction and phenolic compounds from various foodstuff, including EVOO (Mesías , Seiquer & Navarro, 2009; Rueda, Cantarero, Seiquer, Cabrera-Vique & Olalla, 2017; Ruiz-Roca, Navarro & Seiquer, 2008; Seiquer, Rueda, Olalla, & Cabrera-Vique, 2015).

Antioxidant and chemoprotective properties of individual components of olive oil extracts and olive byproducts have been reported in cell cultures such as Caco-2, HepG2 and red blood cells, as well as the protective effects against induced oxidative stress (Chiesi et al., 2015; Deiana et al., 2010; Incani et al., 2016; Paiva- Martins et al., 2015; Rubió et al., 2014). However, there is scarce information concerning the potential antioxidant effect of all complex bioactive components of EVOO and their metabolites after the digestive process, or about the subsequent intestinal absorption.

The influence of different factors on the antioxidant properties of EVOO has been well-documented, such as cultivar, storage period, stages of olives ripening and

technologic aspects of oil production (Köseoğlu, Sevim & Kadiroğlu, 2016; Uluata et al., 2016). However, up to the date, there are limited data regarding the antioxidant activity of monovarietal EVOO from different geographical areas, after the biotransformation underwent during gastrointestinal digestion. In a previous paper we have shown that some characteristics of Arbequina EVOO, such as oxidative stability or colour, are related with geographic and climatic factors of the production zones (Borges et al., 2017). Thus, it is possible that antioxidant properties of oils could also be influenced by geoclimatic conditions.

Therefore, the aim of the current study was to perform a physiological approach of the antioxidant potential of Arbequina EVOO from different Brazilian and Spanish producing regions. With this purpose, a combined model of in vitro digestion and cell cultures was applied. The antioxidant activities of oils after digestion have been studied by in vitro assays (DPPH, ABTS and FRAP) and cell antioxidant markers (cell proliferation and reactive oxygen species production), testing, moreover, the protective effect against an induced oxidative stress. Finally, the absorption of polyphenols and antioxidant activity across Caco-2 monolayers was also performed.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical reagent grade or high purity. Bidistilled deionized water was obtained from a Milli-Q purification system (Millipore, Bedford, MA). Folin – Ciocalteau reagent, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), pepsin, pancreatin, bile salts, HEPES and tert - butylhydroperoxide (*t*-BOOH), as were all cell culture media and cell culture-grade

chemicals were provided by Sigma (Sigma–Aldrich, St. Louis, MO). 2,2-azinobis-(3-ethylbensothiazoline)-6-sulfonic acid (ABTS) was obtained from Amresco (Solon, USA). Ethanol and methanol were purchased from VWR (Barcelona, Spain). Sodium bicarbonate, acetate sodium, sodium carbonate, hydrochloric acid (37%), caffeic acid, hydrochloric acid, anhydrous sodium carbonate and potassium hexacyanoferrate(III) were obtained from *Merck* (Darmstad, Germany). 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) and iron (III) chloride for the ferric reducing power (FRAP) method was obtained from Fluka Chemicals (Fluka Chemicals, Madrid, Spain).

2.2. Samples

A total of 33 samples of Arbequina EVOO from 11 different geographic zones (n = 3 from each producing region) were studied. Nine regions of olive oil production in Spain (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid and Lérida, samples 1 to 9) and two regions in Brazil (Rio Grande do Sul and Minas Gerais, samples 10 and 11) were selected. The olives were harvested always at the early stage of harvest; the harvest dates were: late October to mid-November of 2014 for Spanish samples and March to early April of 2015 for Brazilian samples. The oil was extracted within 24h, under a two-phase extraction system. The oils were directly donated by the producers, adequately packaged for preserving from light and high temperatures and sent to CSIC laboratories (Granada, Spain) to perform the analysis. Information on the geographic coordinates and geo climate characteristics of the different locations of the oils is shown as supplementary material (**S1**). All the samples were according with the n

ormative established by the European Union regulation n° 2568/91 for extra virgin olive oil, as was showed previously (Borges et al., 2017). The scheme of the general procedure applied on the EVOO samples in the present assay can be observed in **Fig. 1**.

All the determinations were done in triplicate, unless otherwise stated.

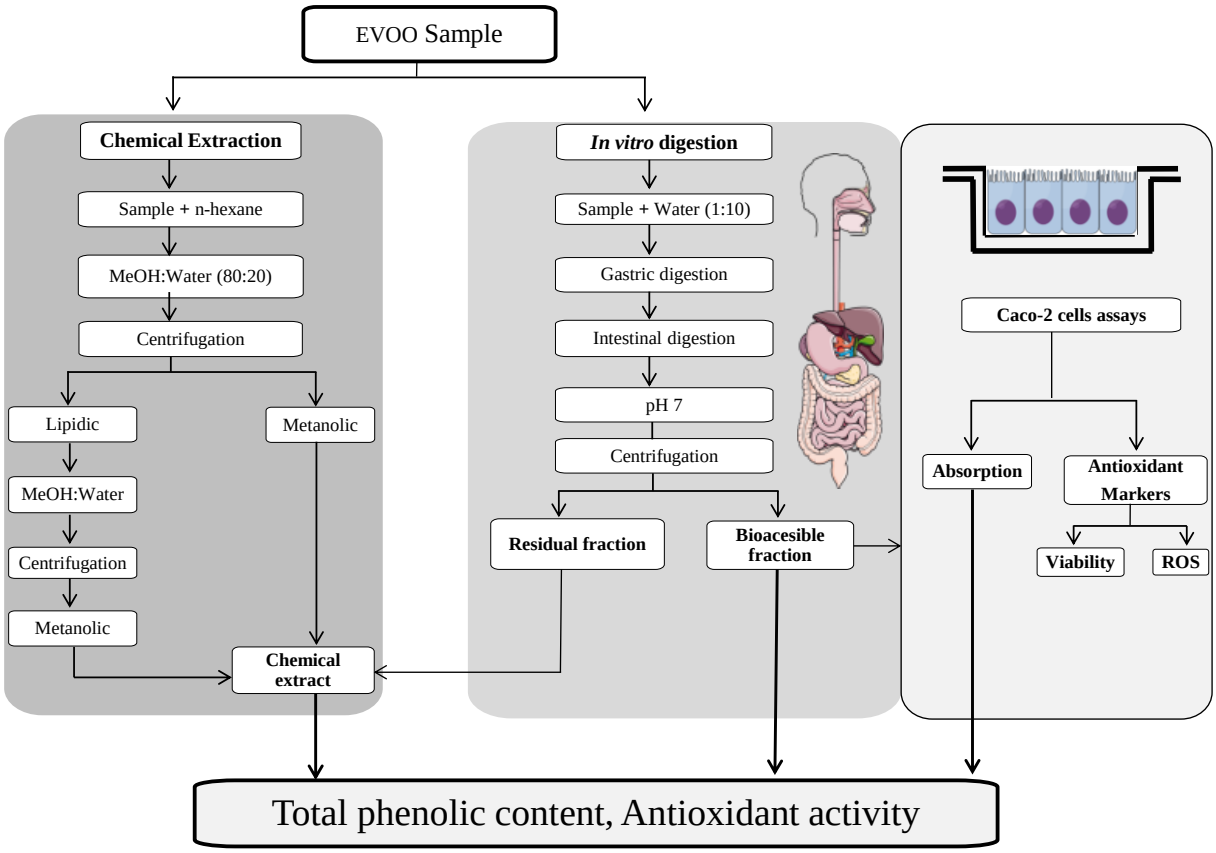

Fig. 1. Scheme of the general procedure applied to the EVOO samples.

2.3. In vitro digestion

The *in vitro* digestion was performed as described by Borges et al (2015), including sequential steps of gastric and intestinal digestion. Briefly, the oil samples were mixed with Mili-Q water (1:10, w/v) and subjected to gastric (pH 2, pepsin solution) and intestinal (pH 7, pancreatin/bile salts solution) phases (110 oscillations/ min; 37 °C, 2

h). The samples were then centrifuged at 10000 rpm for 30 min at 4°C (Sorvall RC 6 Plus centrifuge) to separate the soluble or bioaccessible fraction (BF) and the residual fraction (RF). The samples were protected from light all over the process and submitted to sonication previous to each step. Blanks with no sample were run in parallel and analysed to discard interferences due to the reagents of the digestion process.

The BF and RF were stored at -80 °C in bottles protected from the light under a nitrogen blanket until used to determine the total polyphenol content (TPC) and antioxidant activity. Aliquots of the BF were also used for Caco-2 cell experiments.

2.4. Chemical extraction

The chemical extraction was carried out previous to analysis of TPC and antioxidant assays, in oil samples and in the RF obtained after the in vitro digestion. Two grams of oil, or the total RF, were mixed with 1 mL of n-hexane and the mixture was vigorously shaken. Then, 2 mL of methanol/water (80: 20 v/v) were added in order to assay the polar fraction. The solution was centrifuged at 4000 rpm for 5 min (Sorvall RC 6 Plus centrifuge, Thermo Scientific, Madrid, Spain), the extraction was repeated in the lipophilic part and the methanolic extracts were combined to obtain the chemical extract.

2.5. Total phenolic content and antioxidant activity

The methodologies previously described by Borges et al (2015) were followed. TPC was determined using the Folin – Ciocalteu reactive and measuring the absorbance at 750 nm in a Victor X3 multilabel plate reader (Waltham, Massachusetts, USA). The results were expressed in mg of caffeic acid equivalents (CAE) per kg of sample.

To study the antioxidant capacity of the samples the ABTS and DPPH assays (for measuring the free radical scavenger activity) and the FRAP method (for assessing the reducing power) were performed. The final absorbance was measured at 750, 520 and 595 nm, for ABTS, DPPH and FRAP assays, respectively, using the Victor multilabel plate reader. A calibration curve of Trolox was performed in each method and the results were expressed in mM of Trolox equivalents per kg of sample.

2.6. Cell culture assays

Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) through the Cell Bank of Granada University (Spain). Culture flasks were purchased from Corning Costar (Cambridge, MA, USA). The cells were maintained by serial passage in 75 cm^2 plastic flasks containing high-glucose Dulbecco's modified minimal essential medium (DMEM), with heat-inactivated fetal bovine serum (FBS) (10%), $NaHCO_3$ (3.7 g/L), nonessential amino acids (1%), HEPES (15 mM), bovine insulin (0.1 UI/mL), and 1% antibiotic-antimycotic solution. The cells were grown under atmosphere of air/CO_2 (95:5) at 90% humidity and 37°C and given fresh medium every 3 days.

2.6.1 Antioxidant cell markers

The antioxidant potential of the digested oils (BF) was also assessed at the cell level, performing two assays: modifications on cell proliferation and reactive oxygen species (ROS) generation. Determinations were carried out both at basal conditions and against an induced oxidative stress.

The viability and quantification of cell proliferation of the Caco-2 cells was assessed by the colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide , Roche, Mannheim, Germany). Cells were seeded in 96-well microtiter plates at a density of 25×10^4 cells/mL (100 µL/well) and incubated for 24 hours at 37° C to allow adherence. Basal effect of the samples on cell viability was studied at different concentrations BF: DMEM FBS-free (1:2 and 1:3, v/v). Spent medium was removed and the diluted samples (100 µL/well) were added to the cells and incubated during 2h. The control wells received DMEM FBS-free. Immediately, 10 µL of MTT were incorporated followed by 4h of incubation. Finally, 100 µL of solubilisation solution were added to each well and the plates were stood overnight in incubation, prior to measure cell viability. To study the protective effect against oxidative damage, after 2 hours of initial exposure with the diluted BF, samples solutions were removed and 100 µL of tert-butyl hydroperoxide (*t-BOOH*) at different concentrations (3 mM and 5 mM) were added as oxidising agent, and incubated for 1h. Then, the same procedure as above was applied. After overnight incubation, the plates were mixed during 1 hour in a plate stirrer until complete solubilisation of the purple formazan crystals and the absorbance was measured at 595 nm using a Victor X3 multilabel plate reader. The cell viability was expressed as a percentage of the data obtained from control wells. Data are means from at least two independent experiments (n = 6 per experiment).

For determination of ROS generation we used the dichlorofluorescin (DCFH) assay as described by Seiquer Rueda et al (2015). In presence of free radicals (ROS), the DCFH become dichlorofluorescein (DCF) and emit fluorescence; thus, quantifying fluorescence under different situations (basal conditions and oxidizing conditions with *t*-BOOH) the ROS production may be estimated. The data were expressed as fluorescence units, from at least two independent experiments (n = 3 per experiment).

2.6.2. Absorption assays

Trypsinization, seeding into permeable supports (Transwell, 24 mm diameter, 4.7 cm^2 area, 3 μm pore size, Costar) and absorption assays across Caco-2 cell monolayers were performed as described elsewhere (Seiquer et al., 2015). BF were used for the Caco-2 experiments diluted 1:3 (v:v) with DMEM FBS-free, since cell viability after 2 h of exposure to the samples in such conditions, assessed by MTT, was never < 90%. After the incubation period, the buffer from the basolateral chamber was removed and used to determinate total polyphenols and antioxidant activity (DPPH and FRAP), according to the methods described. Absorption across the cell monolayers was expressed as the percentage (%) transported/well from the initial solution added to the apical chamber.

2.7. *Statistical analysis*

The data obtained were analysed applying analysis of variance (one-way ANOVA), with the geographic origin of oils (regions 1–11) as the main factor. Tukey's test was used to compare mean values between oils from the different regions, and differences were established at $P < 0.05$. Moreover, the data were grouped by countries, and the overall differences between Brazilian and Spanish oils were also studied by ANOVA. The relationships between the different variables and with the climate characteristics and the altitude of the producing regions were evaluated by Pearson's coefficient. All statistical calculations were carried out using SPSS version 21.0 (IBM Corporation, New York, USA).

3. Results and Discussion

3.1. TPC and antioxidant capacity before and after gastrointestinal digestion

Total phenolic content and antioxidant activity of oils were measured before and after the *in vitro* digestion, i.e. in the chemical extracts and in the fractions obtained from the digestive process, both bioaccessible and residual (BF and RF).

3.1.1. Chemical extracts

Results of TPC and antioxidant activity (ABTS, DPPH and FRAP) in chemical extracts are shown in **Table 1**. TPC of the Arbequina EVOO analysed presented a large range of variation between 75 and 302 mg CAE/ kg oil, concerning samples 11 (Minas Gerais, Brazil) and 3 (Málaga, Spain) respectively ($P < 0.05$). Likewise, a wide range of phenolic compound content in EVOO (from 50 to more than 1000 mg/kg oil) and different chemical structures have been reported in literature, linked to numerous factors such as cultivar, geo-climatic conditions, degree of fruit ripeness and processing methods (Samaniego Sánchez et al., 2007; Servili et al., 2014). Previous data on Arbequina virgin olive oil show TPC values comparable with those of the present assay (Borges et al., 2015; Uluata et al., 2016).

The antioxidant activity measured in the chemical extracts of the oils was significantly affected by growing regions ($P < 0.05$). The lowest values were observed for samples 4 (Cádiz, Spain) and 11, whereas Spanish oils 3 (Málaga) and 8 (Valladolid) presented the highest antioxidant capacity. It has been shown than geographic and climatic conditions of production areas may affect the quality and composition of olive oils (Mailer, Ayton, & Graham, 2010). Therefore, relationships of TPC and antioxidant properties with altitude, maximum and minimum temperature and rainfalls of the different region were analysed in the current assay. It was found that the maximum temperature of the growing zone was positively related ($P<0.001$) with the TPC ($r= 0.801$) and with antioxidant properties of the oil extracts (ABTS $r=0.624$;

DPPH r=0.715 and FRAP r=0.664), whereas rainfalls correlated negatively with TPC (P<0.01, r = -0.520). On accordance, Brazilian oils, produced in regions with high levels of rainfalls (Table S1), showed on average lower values of TPC than Spanish oils. No global differences of antioxidant activity were found between oils from the different countries.

The antioxidant effect of EVOO has been mainly ascribed to polyphenols (Samaniego-Sánchez et al, 2007) and, in agreement, strong relationships (P<0.001) were found in chemical extracts between TPC and ABTS (r= 0.673), DPPH (r=0.709) and FRAP (r=0.786) assays.

3.1.2. Bioaccessible fractions

Measuring TPC and antioxidant properties in the BF may be indicative of the impact of the digestive process and provides complementary information comparing with chemical analysis of foods (Soler et al., 2010).

The present study found a positive effect of the digestive process in TPC and antioxidant properties of all the samples (**Table 2**), showing increased values of these parameters in the BF compared with the chemical extracts. These results agree with previous studies that found a rise of TPC and antioxidant capacity after digestion of oils (Borges et al., 2015; Seiquer et al., 2015) and other food matrices (Arques, Pastoriza, Delgado-Andrade, Clemente, & Rufián-Henares, 2016; Pastoriza et al., 2011; Szawara-Nowak, Bączek & Zieliński, 2016), although negative effects have been also shown (Dinnella, Minichino, D'Andrea & Monteleone, 2007). Thus, our results support that *in vitro* digestion is a crucial step that releases a high amount of phenolic and antioxidant compounds, which seem difficult to extract from the food matrix with the solvents traditionally used (Szawara-Nowak et al., 2016).

Significant variations were found in bioaccessible TPC between oils, but no relationships with altitude or climatic factors of the regions were detected, contrary to those observed in the chemical extracts. Thus, the negative effect of high rainfall in EVOO TPC (Inglese et al. 2010) was only found in the chemical extracts, but no in the BF of the oils.

Table 1
Total phenolic content (TPC, mg of CAE per kilogram of oil) and antioxidant activity (ABTS, DPPH and FRAP, mmol of Trolox per kilogram of oil) determined in the chemical extract of Arbequina EVOO from Spain (samples 1-9) and Brazil (samples 10 and 11).

Oil sample	TPC	ABTS	DPPH	FRAP
1	$168 \pm 1.14^{b,c}$	$0.41 \pm 0.04^{b,c}$	$0.93 \pm 0.05^{b,c,d}$	$0.93 \pm 0.02^{a,b}$
2	$163 \pm 13.519^{b,c}$	$0.59 \pm 0.14^{c,d,e}$	1.45 ± 0.18^{e}	$1.35 \pm 0.40^{b,c}$
3	302 ± 29.45^{e}	0.73 ± 0.01^{e}	1.58 ± 0.01^{e}	2.22 ± 0.23^{e}
4	$196 \pm 13.5^{c,d}$	0.20 ± 0.01^{a}	0.52 ± 0.02^{a}	0.54 ± 0.03^{a}
5	227 ± 9.27^{d}	$0.60 \pm 0.13^{d,e}$	$1.22 \pm 0.30^{d,e}$	$1.35 \pm 0.38^{b,c}$
6	$197 \pm 13.5^{c,d}$	$0.46 \pm 0.05^{b,c,d}$	$0.74 \pm 0.05^{a,b,c}$	$0.83 \pm 0.05^{a,b}$
7	$174 \pm 2.85^{b,c}$	$0.46 \pm 0.03^{b,c,d}$	$0.81 \pm 0.03^{a,b,c}$	$0.90 \pm 0.09^{a,b}$
8	290 ± 15.61^{e}	0.73 ± 0.01^{e}	1.52 ± 0.04^{e}	$1.64 \pm 0.14^{c,d}$
9	104 ± 21.3^{a}	$0.34 \pm 0.01^{a,b}$	$0.65 \pm 0.20^{a,b,c}$	0.70 ± 0.24^{a}
10	151 ± 4.44^{b}	$0.56 \pm 0.02^{c,d,e}$	$0.97 \pm 0.01^{c,d}$	$1.06 \pm 0.02^{a,b,c}$
11	75.0 ± 2.18^{a}	$0.33 \pm 0.01^{a,b}$	$0.56 \pm 0.06^{a,b}$	0.57 ± 0.01^{a}
P-value Spain × Brazil	**	n.s	n.s	n.s

Values are expressed as mean ± SE. Means values in each column with different letters are significantly different ($p < 0.05$). Comparing countries: ns, not significant $P>0.05$; * $P < 0.05$; ** $P < 0.01$.

Polyphenols are strongly affected during the digestive process, mainly due to changes of pH and interactions with other compounds, which differ depending on their chemical structure (Dinnella, Minichino, D'Andrea & Monteleone, 2007). Some of the major olive oil phenols (tyrosol, hydroxytyrosol and lignans) are relatively stable after gastrointestinal digestion and are able to generate derivate compounds owing to digestion, whereas other (flavonoids and secoiridoids) are unstable (Soler et al., 2010). Hydroxytyrosol seems to be decisive for phenolic bioaccessibility, as during digestion it is released from its precursors, the secoiridois, and its bioaccessibility may be over 100% (Rubió et al., 2014). A limitation of this study is that individual phenols were not characterised, but, according to previous bibliography, phenol profile within genotype may be affected by environmental conditions of oil producing regions (Inglese et al., 2010). Therefore, probably due to the different composition, the phenolic fraction of the oils of the current assay underwent different bio-transformation rates during digestion. As a result, TPC and antioxidant activity after digestion of the oils were different comparing with values found in the chemical extracts, and statistical differences between samples also varied.

Average values of antioxidant activity after digestion of Spanish oils differed significantly from Brazilian samples, being DPPH values higher and FRAP values lower in Brazilian BF than in Spanish samples. This suggests that in biological systems compounds than scavenge free radicals are not necessarily capable of reducing oxidants (Frankel & Meyer, 2000) and that transformations during the digestive process are decisive for establishing the antioxidant potential of the oils.

Relationships of antioxidant activity in the BF with climatic factors were also different than those observed in the chemical extracts, since bioaccessible DPPH was negatively correlated with maximum temperature ($P<0.01$, $r= -0.697$) and positively

with rainfalls ($P<0.01$, $r= 0.583$). Moreover, whereas antioxidant properties were strongly related with TPC in oil extracts, no relationships was found in the BF after the digestive process, suggesting that after digestion other compounds than polyphenols could also be responsible for antioxidant properties. In agreement, other authors have found that correlations between TPC and antioxidant activity (measured by ABTS, DPPH and FRAP assays) are reduced after the digestion of fruits, compared with the initial values in chemical extracts (Chen et al., 2014).

3.3.3. Residual fractions

After *in vitro* digestion of Arbequina EVOO significant amounts of TPC and antioxidant activity were detected in the RF (**Table 2**). This fraction accounted a 20% of TPC, 19% ABTS, 7% DPPH and 39% FRAP from the total recovered after digestion (**Fig. 2**).

Traditionally, the residual fraction after digestion is not considered when studying phenolic bioavailability, but certain absorption cannot be discarded as it has been described that polyphenols that remain in the large intestine after digestion may be transformed by the intestinal microbiota into a series of low molecular- structures potentially absorbable (Cardona, Andres-Lacuerva, Tulipania, Tinahonesb & Queipo-Ortuno, 2013). In fact, certain polyphenols are very poorly absorbed in the small intestine in their intact forms, but are extensively catabolized buy the gut microbiota and the products are efficiently absorbed through the colonic epithelium (Williamson & Clifford, 2017). Moreover, these residues deserve to be considered in the global antioxidant power of the digested oils, especially as a source of reducing capacity agents (Seiquer et al., 2015), as it has been suggested by others after digestion of different food matrices (Pastoriza et al., 2011; Szawara-Nowak, Bączek & Zieliński, 2016). It has also been shown that unabsorbed polyphenols may exert beneficial local effects at the

intestinal tract, interacting with the microbiota, mucosal cells and dendritic projections in the lumen (Martin & Bolling, 2015). Therefore, both fractions obtained after the *in vitro* digestion process may be responsible for health effects derived from polyphenol-rich foods.

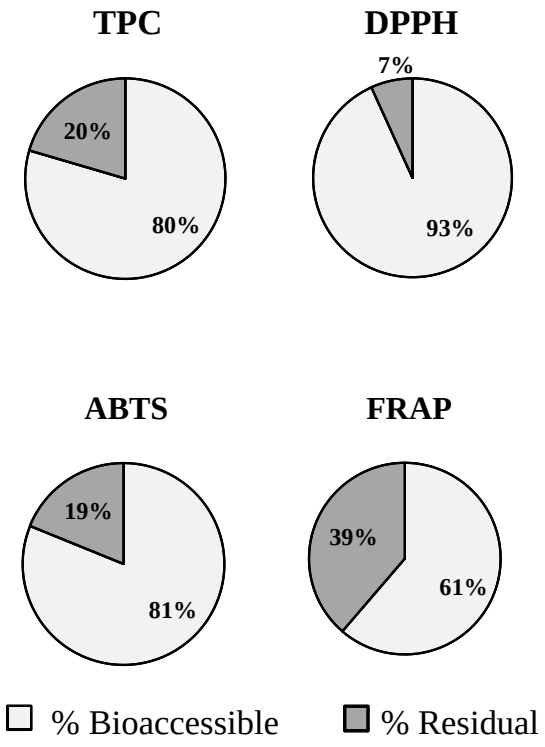

Fig. 2. Average distribution of TPC and antioxidant activity recovered after digestion corresponding to bioaccessible (%) and residual (%) fractions.

Table 2
Total phenolic content (TPC, mg of CAE per kilogram of oil) and antioxidant activity (ABTS, DPPH and FRAP, mmol of Trolox per kilogram of oil) determined in bioaccessible fraction and residual fraction after *in vitro* digestion of Arbequina EVOO from Spain (samples 1-9) and Brazil (samples 10 and 11).

	TPC		ABTS		DPPH		FRAP	
Oil sample	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction	Bioaccessible fraction	Residual fraction
1	451 ± 29.8^{a,b,c,d}	110 ± 12.7^{a,b}	4.70 ± 0.31	1.35 ± 0.10^{d,e}	2.58 ± 0.31^{b,c}	0.38 ± 0.07^{c}	4.04 ± 0.42^{d,e}	1.53 ± 0.13^{a,b}
2	538 ± 53.1^{c,d}	101 ± 25.1^{a,b}	4.53 ± 0.12	0.92 ± 0.17^{a,b,c}	2.27 ± 0.45^{b}	0.42 ± 0.02^{c}	3.58 ± 0.79^{c,d,e}	1.51 ± 0.22^{a,b}
3	506 ± 3.06^{a,b,c,d}	140 ± 16.2^{b,c}	4.42 ± 0.13	1.01 ± 0.14^{a,b,c,d}	0.99 ± 0.08^{a}	-	2.54 ± 0.40^{b,c}	2.14 ± 0.17^{b,c,d}
4	392 ± 66.1^{a}	67.7 ± 24.8^{a}	4.59 ± 0.09	1.03 ± 0.02^{a,b,c,d,e}	2.54 ± 0.13^{b,c}	0.41 ± 0.04 ^{c}	3.08 ± 0.44^{c,d}	1.39 ± 0.21^{a}
5	411 ± 48.0^{a,b}	194 ± 33.6^{c}	4.28 ± 0.10	0.71 ± 0.20^{a}	2.08 ± 0.27^{b}	-	3.25 ± 0.10^{c,d}	2.26 ± 0.24^{c,d}
6	566 ± 38.9^{d}	127 ± 41.2^{a,b}	4.45 ± 0.10	0.99 ± 0.18^{a,b,c}	2.56 ± 0.19^{b,c}	0.18 ± 0.18^{a,b}	3.85 ± 0.39^{d,e}	2.47 ± 0.25^{d}
7	436 ± 4.02^{a,b,c}	109 ± 12.2^{a,b}	4.53 ± 0.19	1.37 ± 0.06^{e}	1.91 ± 0.24^{b}	0.29 ± 0.04^{b,c}	3.68 ± 0.52^{c,d,e}	2.14 ± 0.41^{b,c,d}
8	432 ± 18.9^{a,b,c}	136 ± 15.7^{b,c}	4.26 ± 0.12	1.27 ± 0.03^{c,d,e}	2.33 ± 0.12^{b,c}	-	4.62 ± 0.35^{e}	2.19 ± 0.24^{c,d}
9	391 ± 81.1^{a}	96.8 ± 16.1 ^{a,b}	4.56 ± 0.05	1.19 ± 0.11^{b,c,d,e}	2.51 ± 0.07^{b,c}	0.35 ± 0.04^{b,c}	3.51 ± 0.15^{c,d,e}	1.73 ± 0.05^{a,b,c}
10	531 ± 12.1^{b,c,d}	121 ± 9.21^{a,b}	4.29 ± 0.16	0.80 ± 0.08^{a}	2.97 ± 0.26^{c,d}	-	1.40 ± 0.18^{a,b}	1.63 ± 0.18^{a,b,c}
11	473 ± 41.6^{a,b,c,d}	92.6 ± 4.83^{a,b}	4.39 ± 0.15	0.85 ± 0.04^{a,b}	3.30 ± 0.12^{d}	-	1.24 ± 0.18^{a}	1.52 ± 0.16^{a,b}
P-value Spain × Brazil	n.s	n.s	n.s	*	**	n.s	**	n.s

Values are expressed as mean ± SE. Means values in each column with different letters are significantly different ($p < 0.05$). Comparing countries: ns, not significant $P>0.05$; * $P < 0.05$; ** $P < 0.01$.

3.2. Antioxidant cell markers

In order to assess the effects of the BF of the oils at the cellular level, two antioxidant markers on Caco-2 cells were studied: cell viability and ROS generation, both at basal conditions and against induced oxidative stress.

Firstly, BF was mixed with increasing proportions of DMEM FBS-free to evaluate the effect of the sample concentration in the viability of Caco-2 cells. **Fig. 3. A** shows the viability obtained, expressed as percentage in relation to the control value (DMEM FBS-free), after 2 h of incubation with dilutions 1:2 and 1:3 (v/v) BF: DMEM FBS-free (differences are not indicated to avoid overlapping). When samples were diluted 1:2 (v/v), statistical differences of cell proliferation were observed after exposure to the different samples ($P < 0.01$), ranging from 70% (corresponding to Brazilian sample 11) to 130% (in Spanish sample 3). Treatment with BF from samples 2 (Jaén), 6 (Albacete), 7 (Toledo), 9 (Lérida), 10 (Rio Grande do Sul) and 11 (Minas Gerais) led to cell viability values lower than 85%, showing that monovarietal oils from different producing areas had different effects on cell integrity. When dilution of BF was increased to 1:3, high values of viable cells were observed after exposure to all samples (≥90%) and, therefore, dilution 1:3 was selected for further assays, as it was shown not damage cellular integrity in any case.

To evaluate the protective effect on cell viability, an induced oxidative stress was promoted by *t*-BOOH at different concentrations, 3 mM and 5 mM (**Fig. 3.B**). When a slight damage was caused (*t*-BOOH 3 mM), control cells showed a drastic reduction in cell viability (67%), while cells preincubated with the samples were able to preserve the viability (>80%), thereby preventing cell damage due to the oxidising agent, without significant differences among oils. At stronger oxidising conditions (*t*-BOOH 5 mM) the proportion of viable cells was reduced to approximately 62%, and

pretreatment for 2h with the digested oils also neutralised the oxidative damage and improved the cell proliferation. However, in such conditions, certain differences were observed between samples, and some of them (samples 6 and 7) did not reach significant protective effects compared to control oxidized cells. Similar protective effect of cell viability has been shown with digested samples of Picual EVOO and also, although to a lesser extent, of extra virgin argan oil (Seiquer et al., 2015). Other studies have confirmed a protective effect in Caco-2 cells viability of EVOO extracts and phenolic compounds such as oleuropein and tyrosol, against the cytotoxic damage promoted by the mycotoxin alternariol or a pro-oxidant agent (Chiesi et al., 2015; Incani et al., 2016). It has been suggested that ingested polyphenols have a direct protective effect in the gastrointestinal tract by scavenging reactive species and/or preventing their formation (Halliwell, Rafter & Jenner, 2005). However, in the present assay no relationships were found among bioaccessible TPC of the samples and cell proliferation under stressed conditions.

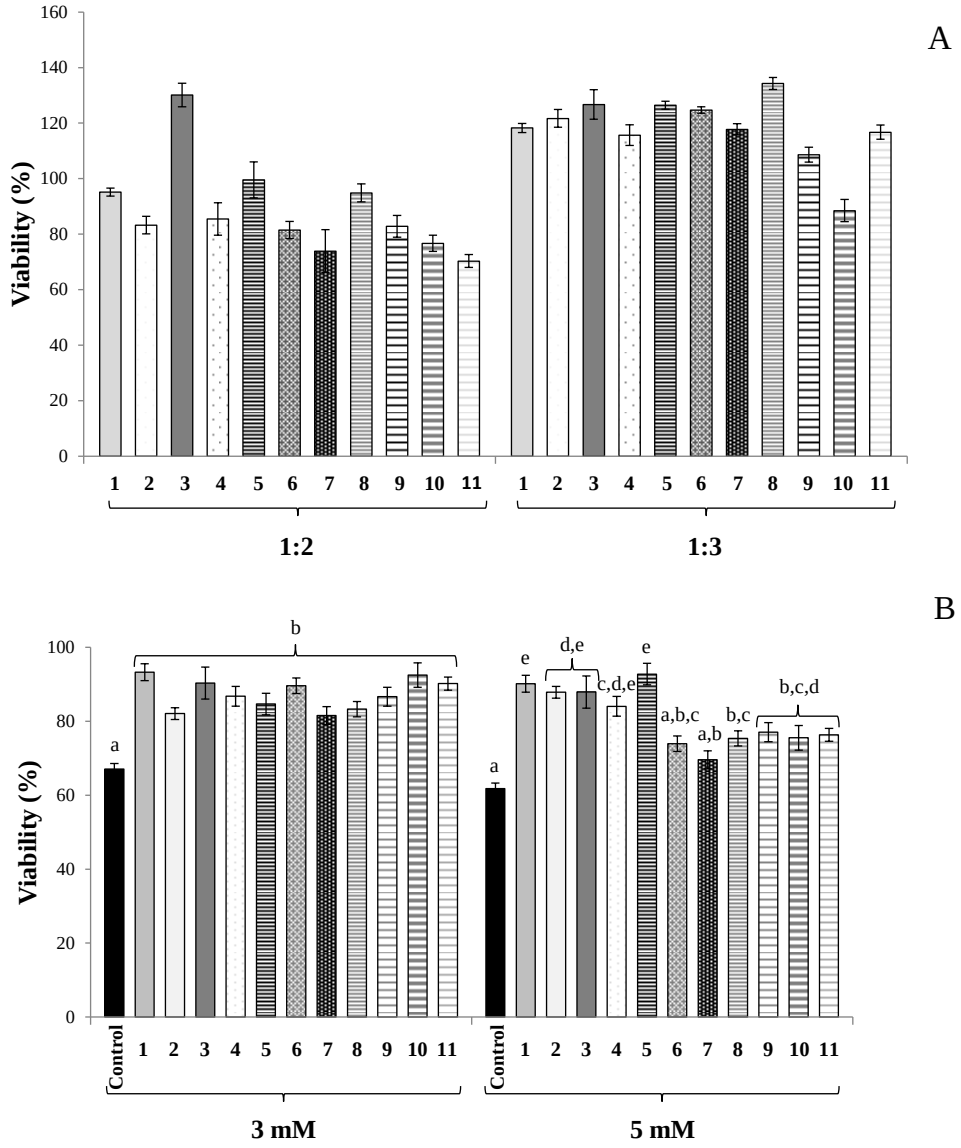

Fig. 3. A: Cell viability (%) in Caco-2 cells after 2 hours of incubation with bioaccessible fractions of Arbequina EVOO from Spain (samples 1-9) and Brazil (samples 10 and 11), diluted 1:2 and 1:3 (v/v) with DMEM FBS-free. B: Cell viability in Caco-2 cells preincubated with the different samples and oxidized with *t*-BOOH 3 mM and 5 mM. (mean ± SE, n = 6). Bars with different minor letters differ significantly within 3 mM or 5 mM *t*-BOOH effect ($P < 0.05$).

The ROS generation by Caco-2 cells after exposure to the different Arbequina samples is shown in **Fig. 4**. Under basal conditions, a slight ROS cell production was detected, as result of the normal cell functionality. In such conditions, a reduction of the ROS levels was obtained after exposure to BF of oils compared to control wells,

ranging from 16 to 47%. Statistically significant reductions were observed for samples 4 (Cádiz), 5 (Sevilla), 8 (Valladolid), 9 (Lérida), 10 (Rio Grande do Sul) and 11 (Minas Gerais) in relation to control ($P<0.05$), showing that digested oils are able to modify the redox status of intestinal cells, even under non-stressed conditions. The intestinal tract is continually attacked by luminal microbes and by oxidized compounds from the diet, exposing it to frequent oxidative changes and, thus, there is a need of maintenance of the redox intestinal homeostasis (Biasi et al., 2014). Although homeostatic mechanisms is one of the uncontrolled aspects in cell cultures, decreasing intracellular levels of ROS can act as biological signal molecules to regulate the redox homeostasis *in vivo* and should be considered positive in the antioxidant cell response.

In order to induce an oxidative injury, Caco-2 cells were treated with *t*-BOOH (5 mM for 2 h). The damage produced by *t*-BOOH caused a drastic increasing of ROS generation in the cells (**Fig. 4**) and, thus, the possible protective effect of oils was evaluated. Preincubating the cells with BF of oils prevented the free radical production due to the pro-oxidant agent from 16 % (sample 6, Seville) to 53% (sample 10, Rio Grande do Sul), although in some cases (oils 5 and 6) differences comparing with oxidised cells were not significant. Our results are in line with previous data that relate a protective effect on induced ROS production of digested samples from different monovarietal EVOO (Borges et al., 2015). The beneficial effect of EVOO at intestinal level has been directly related to the presence of phenolic compounds, able to exert antioxidant actions (Biasi , Astegiano, Maina, Leonarduzzi & Poli, 2011) or preserving the cellular antioxidant defences (Di Benedetto et al., 2007). Thus, several studies have been mainly focused on exploring the role of individual polyphenols (Chiesi et al., 2015; Di Benedetto et al., 2007; Rodríguez-Ramiro, Martín, Ramos, Bravo & Goya, 2011) or methanolic extracts from EVOO (Incani et al., 2016) and olive leaves (Difonzo

et al., 2017) in preventing oxidative damage in cell cultures. A clear difference between the referred studies and the present assay is that the complete bioaccessible fraction obtained after digestion of EVOO, instead of chemical extracts o isolated compounds, was used, as a more physiological approach to probe the protective role of oils at intestinal level. In the current study, no relationship was found between the positive role of the samples on preventing ROS generation and the phenolic content in the BF. However, a correlation was observed with the DPPH assay (P <0.01; r= -0.450), i.e., the higher the DPPH value, the lower the ROS production in stressed conditions (see samples 10 and 11- **Table 2** and **Figure 4**). Thus, compounds originated from the EVOO digestion may prevent or delay the progression of intestinal diseases characterized by oxidative stress, as it has been shown that diet components, together with antioxidant enzymes, are involved in the intestinal mucosa response aimed at preventing oxidative damage (Biasi et al., 2014)

When data were grouped by countries, it was observed that cells treated with Brazilian oils were on average more protected against *t*-BOOH- induced ROS generation than those exposed to Spanish oils (P<0.001), according to the highest DPPH values found in Brazilian BF.

Our data suggest that, after biotransformations undergone during the digestive process, the resulting phenolic fraction of oils could not be the only responsible for protecting cells from oxidation, which was also supported by the lack of correlation between TPC and antioxidant activity found in the BF of oils (commented in section **3.1.2**). Further studies on the phenolic profile of the oils of the present study could help to elucidate the possible role of individual compounds in the protective effect against oxidation.

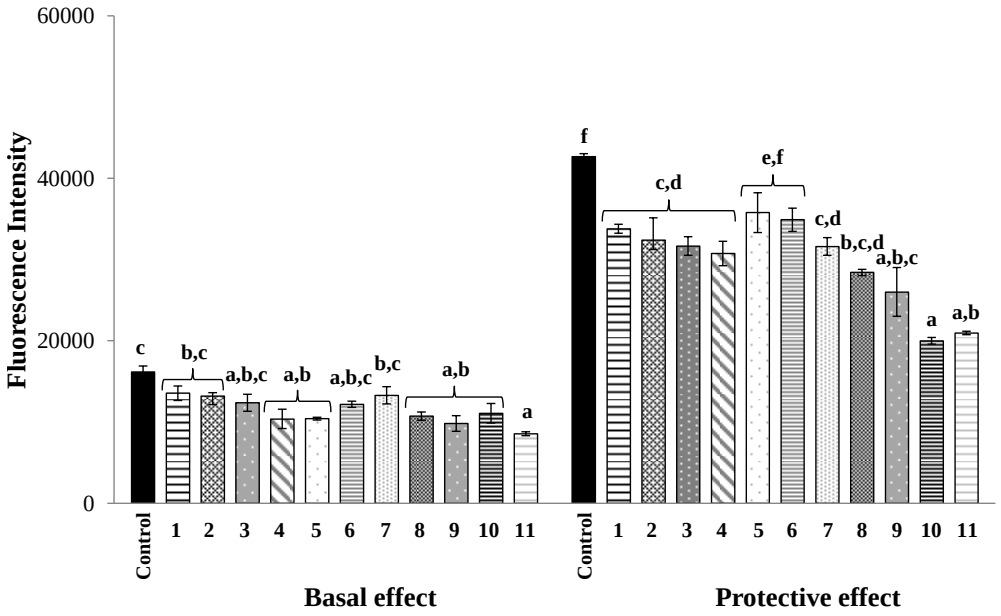

Fig. 4. ROS generation in Caco-2 cells expressed as fluorescence intensity at 90 minutes after incubation (basal effect) with bioaccessible fractions of Arbequina EVOO from Spain (samples 1-9) and Brazil (samples 10 and 11) or preincubated with samples and oxidized with *t*-BOOH 5 mM (protective effect). Control cells received culture medium. (Mean ± SE, n = 3). Bars with different minor letters differ significantly within basal or protective effect ($P < 0.05$).

3.3. Absorption across Caco-2 cells

The Caco2 cell lines derived from human intestinal epithelial adenocarcinoma is a suitable model system for the evaluation of intestinal functions and nutrient absorption (Mesías et al., 2009; Seiquer et al., 2015). In this study, the absorption of TPC and antioxidant properties of digested oils across Caco2 cell monolayers was studied. For the scavenging ability the DPPH assay was selected, due to DPPH activity was mainly located in the bioaccessible fraction (93%) and also due to its positive relationships with the protecting effect from oxidative damage. Non-significant amounts of FRAP activity (less than 0.1% in all the cases) were recovered in basolateral chambers after incubation (data not shown), probably due to that significant proportions of reducing activity

(nearly 40 %) remains in the residual fractions after digestion or to a short time of exposure at experimental conditions (2h).

Results are presented in **Table 3**. Total TPC and DPPH activity recovered in basal chambers after incubation was measured and absorption was calculated as the percentage of the total initial content exposed to cell monolayers.

Absorption of TPC ranged from 32.5 to 110%, corresponding to samples 6 and 8, respectively with statistically significant differences between oils ($P < 0.05$). Average absorption of phenolic compounds was higher ($P<0.05$) from Brazilian oils than from Spanish oils (97% *vs* 67%, respectively). Low proportions of TPC absorption (32-55%) were observed for samples 1, 2, 3, 4 and 6, despite the high bioaccessibility previously detected (**Table 1**). However, it must be taken into account that phenolic compounds could continue the absorption process in the lower digestive tract, as it has been recently suggested (Pereira- Caro et al., 2015). On the other hand, incubation with samples 5 and 8 surpassed 100% of TPC absorption. This finding could be explained by two hypotheses. Firstly, Folin - Ciocalteu method is a not a selective assay, determining all kinds of phenolic molecules and, thus, a variety of compounds may interfere with the reactive to give apparently elevated phenolic concentrations (Cerretini & Bendini, 2010). Secondly, the metabolites present in the basal medium could have been produced intracellularly and excreted to the exterior or produced directly by secreted enzymes (Soler et al., 2010). In this sense, different mechanism has been described in the Caco-2 cell model to evaluated the permeability of phenolic compounds (passive paracellular transport or active efflux process) and potential interference by either efflux or metabolic process have been described for some compounds, causing deviation from the predicted permeability (Farrell, Poquet, Dew, Barber & Williamson, 2012). Previous studies on the transport of polyphenols from olive oil extracts through Caco-2 cells have

shown that compounds present a high conjugation rate (including methylation, sulphation and glucuronidation) that, in some cases, such as hydroxytyrosol, led to almost complete metabolic conversion (Rubió et al., 2014).

The present findings support that olive oil phenolics could be well absorbed at the intestinal level (Tuck, Freeman, Hayball, Stretch & Stupans, 2001). Moreover, depending on the phenolic profile, conjugation may affect the efflux of phenols from inside the enterocyte (Soler et al., 2010). The majority of previous studies have been performed with individual phenolic compounds or chemical extracts, although absorption through intestinal cells after digestion of oils has been scarcely investigated. In an earlier study, we have found a percentage of phenolic absorption of 25 % from digested samples of Picual EVOO (Seiquer et al., 2015), lower than reported in the current study with oils from Arbequina cultivar.

Antioxidant compounds were well absorbed across cell monolayers, leading to 30-52% of DPPH activity recovered in the basal chambers. Statistical differences ($P > 0.05$) were found between individual samples and between countries (51 % *vs* 43% for Brazilian and Spanish oils, respectively). The results suggest a good bioavailability of the antioxidant properties of oils, which were maintained and absorbed after digestion. Although this assay does not identify the compounds responsible for the activity, other than polyphenols should be implicated, as no correlation was observed between absorption of DPPH and TPC.

In general, the data concerning absorption of antioxidant compounds from digested foods are very limited. Additionally, the knowledge about which compounds could be responsible for the potential biological effects *in vivo* is controversial. Several authors related that one of major compounds linked to beneficial effects *in vitro*, hydroxytyrosol, in spite to be well absorbed in the intestinal tract, has really a poor

bioavailability in its free form due to an important presystemic metabolism in gut, leading to the formation of sulfate and glucuronide conjugates (Miro - Casas et al., 2003; Pastor et al., 2016; Pérez - Mañá et al., 2015;). According to Pastor et al. (2016), the concentration of hydroxytyrosol in body fluids is too low to explain the observed biological activities in *in vitro* and *in vivo* models at higher doses/concentrations. Thus, without discarding the activity of secondary phenolic metabolites, it may be postulated that antioxidant properties recovered at the intestinal level are also due to compounds other than polyphenols, with could be able to cross the intestinal barrier in an effective manner. In this regard, all the bioactive compounds (tocopherols, polyphenols, carotenoids, coenzyme Q_{10}) could be linked to the antioxidant activity found in the present study.

Results of the current assay support the use of in vitro gastrointestinal digestion coupled to Caco-2 cells as a reliable tool for investigating antioxidant properties of the oils at the digestive level. However, aspects others than those controlled in these experiments, such as homeostatic modulation of redox status, presence of oxidative stress conditions or the impact of the intestinal microflora in the digestive process, could affect the *in vivo* produced metabolites as well as the resulting antioxidant effect of the EVOO studied.

Table 3
Total initial content, total recovered and absorption of TPC and DPPH (% from the initial solution) across Caco-2 monolayers after 2 h of incubation with the BF of Arbequina EVOO from Spain (samples 1-9) and Brazil (samples 10 and 11).

	TPC			DPPH		
Oil sample	Total initial (mg/per well)	Total recovered (mg/ per well)	Absorption (%)	Total initial (mg/per well)	Total recovered (mg/ per well)	Absorption (%)
1	0.227	$0.111 \pm 0.01^{a,b,c,d}$	$49.1 \pm 4.74^{a,b}$	0.109	$0.055 \pm 0.01^{a.b,c}$	50.1 ± 2.21^{b}
2	0.212	$0.118 \pm 0.01^{b,c,d,e}$	$55.9 \pm 5.88^{a,b,c}$	0.115	$0.050 \pm 0.01^{a.b}$	$43.0 \pm 0.70^{a,b}$
3	0.189	$0.070 \pm 0.01^{a,b}$	37.9 ± 7.00^{a}	0.126	$0.066 \pm 0.02^{a.b,c}$	52.4 ± 12.8^{b}
4	0.198	$0.080 \pm 0.01^{a,b,c}$	44.5 ± 3.04^{a}	0.160	$0.049 \pm 0.01^{a.b}$	30.7 ± 0.10^{a}
5	0.149	$0.169 \pm 0.01^{g,h}$	102 ± 19.4^{d}	0.154	$0.061 \pm 0.01^{a.b,c}$	$40.0 \pm 2.22^{a,b}$
6	0.209	0.068 ± 0.01^{a}	32.5 ± 3.57^{a}	0.119	0.047 ± 0.02^{a}	$39.2 \pm 5.98^{a,b}$
7	0.214	0.188 ± 0.02^{h}	$87.7 \pm 7.86^{c,d}$	0.133	$0.069 \pm 0.02^{b,c}$	52.2 ± 4.54^{b}
8	0.146	$0.161 \pm 0.02^{e,f,g}$	110 ± 17.4^{d}	0.178	$0.069 \pm 0.01^{b,c}$	$38.9 \pm 4.76^{a,b}$
9	0.140	$0.116 \pm 0.01^{b,c,d,e}$	$83.4 \pm 9.73^{b,c,d}$	0.134	$0.060 \pm 0.01^{a.b,c}$	$44.4 \pm 6.74^{a,b}$
10	0.134	$0.130 \pm 0.02^{d,e,f}$	97.0 ± 15.4^{d}	0.140	0.072 ± 0.01^{c}	51.5 ± 5.56^{b}
11	0.127	$0.124 \pm 0.03^{c,d,e,f}$	97.6 ± 21.0^{d}	0.132	$0.066 \pm 0.01^{a.b,c}$	50.0 ± 3.64^{b}
P-value Spain × Brazil	-	n.s	*	-	*	*

Absorption was calculated as the percentage absorbed in the basal chamber from the total initial quantity in the apical chamber. Values are means ± SD. Values with different superscript letters within each file are significant different at $P < 0.05$. Comparing countries: ns, not significant $P>0.05$; * $P < 0.05$; ** $P < 0.01$.

4. Conclusions

For the first time, antioxidant activity performed by different methods and combined with cellular models of Brazilian EVOO was evaluated. At the same time, the current study contributes to the database of antioxidant proprieties of Spanish Arbequina EVOO. The *in vitro* digestion promoted the release of bioactive compounds leading to an increase of TPC and antioxidant potential in all samples, after the biotransformations undergone by the digestive process of EVOO. Thus, the bioaccessible fractions obtained after the *in vitro* digestion of oils protected cell integrity from oxidative damage, and were able to reduce the ROS generation by the cells. On average, Arbequina Brazilian oils showed higher values of antioxidant activity (measured by the DPPH assay) after digestion, and better protective effects against induced ROS production, than Spanish oils.

Findings of the present study support that modifications during digestion are decisive to establish the antioxidant potential of the oils, which is linked, among other biological activities, to the health properties of the EVOO. The results also show that climatic conditions, especially rainfalls, affected the antioxidant behavior of oils produced in different producing zones. The influence of other factors than those considered in the present study, such as the ripeness index and further environmental variables (light intensity, humidity, evapo-transpiration, soil) cannot be discarded.

The lack of information of phenolic profile of the oils is a limitation of this study. Current studies of our group are aimed at determining the composition of the minor fraction of the oils, including, phenolic compounds, tocopherols and coenzyme Q_{10} Thus, further papers could give additional information on the compounds responsible for the antioxidant properties examined in the present assay.

Conflicts

The authors declare no competing financial interest.

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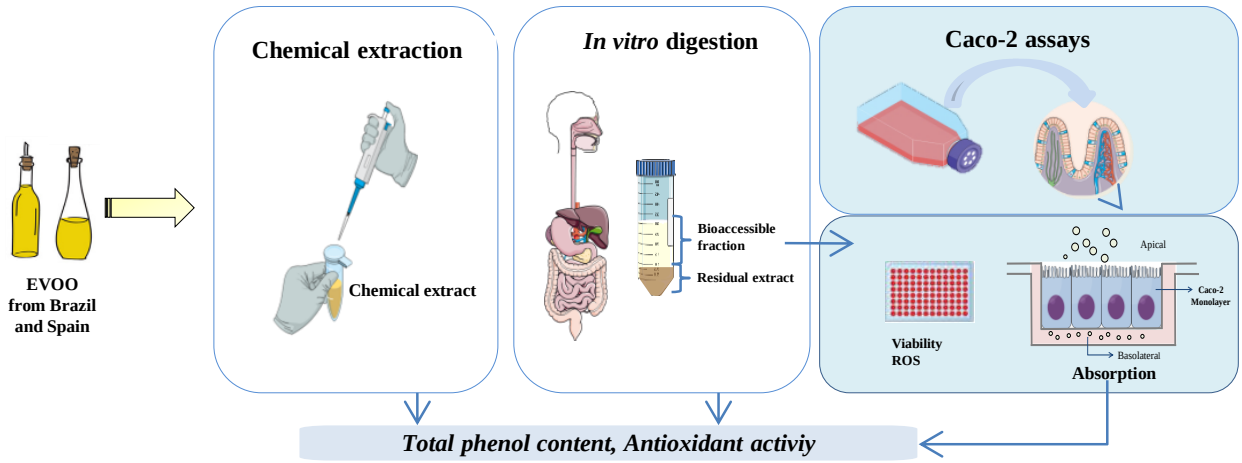

GRAPHICAL ABSTRACT

Artículo 4

General Paper

Use of Response Surface methodology (RSM) for the identification of the best extraction conditions by headspace solid phase micro extraction (HS-SPME) of the volatile profile of cv. Arbequina extra-virgin olive oil

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Use of Response Surface methodology (RSM) for the identification of the best extraction conditions by headspace solid phase micro extraction (HS-SPME) of the volatile profile of cv. Arbequina extra-virgin olive oil

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Abbreviations: **CCD**, Central Composite Design; **DVB/CAR/PDMS,** divinylbenzene/carboxen/polydimethylsiloxane; **EVOO**, Extra virgin olive oil; **HS-SPME**,headspace solid-phase microextraction; **RSM**, Response surface methodology.

Abstract

The effect of the experimental conditions for assessing the global volatile profile of extra virgin olive oils (EVOO) by headspace solid phase microextraction/gas chromatography - mass spectrometry (HS-SPME/GC–MS) was studied, in order to obtain the maximisation of total peak areas of the compounds. Response surface methodology (RSM) was applied to Arbequina EVOO and influence of oil quantity, extraction time and extraction temperature on total area and extraction of the major desirable compounds was analysed. The experimental data were adequately fitted into second-order polynomial models with non-significant lacks of fit ($p>0.05$), coefficients of determination (R^2) higher to 0.88 and R^2-adjusted >0.78. A strong similarity was found between the predicted values and the experimental ones. Furthermore, the surface plots showed that when increasing the extraction temperature, time and oil quantity, the extraction of the volatile compounds was favoured. The conditions to obtain the maximum response in the extraction of "green" volatile compounds of Arbequina olive oil by HS-SPME placed in 50 ml vials were 4.6 g of oil, 43 min and 59 °C.

Practical applications: Findings of the present work show that specific conditions of HS-SPME/GC–MS affect the extraction of volatile compounds of cv. Arbequina olive oil and establish the optimum extraction parameters to determine more efficiently the global profile of this fraction, taking into account the organoleptic characteristics of the cultivar. The results also contribute to the knowledge of the volatile profile of monovarietal olive oil cv. Arbequina, which is one of most cultivated and consumed worldwide.

Keywords: Headspace solid phase microextractio/ Gas chromatography-mass spectrometry/Aroma/Extraction conditions/ Monovarietal Olive oil.

1 Introduction

The aroma of olive oils is attributed to a complex mixture of volatile compounds, mainly aldehydes, alcohols, ketones, hydrocarbons and esters. Compounds of six and five carbon atoms represent the most important fraction and are generated from polyunsaturated fatty acids by the lipoxygenase pathway (LOX) [1]. The production of volatiles occurs in low amounts during the climacteric period of olives and at a high level in the oil extraction process, particularly during the crushing and malaxation steps [1-2].

Sensory properties play an important role to discriminate different types of edible oils, categories and monovarietal olive oils. In this sense, volatile compounds are decisive to check olive oil quality and to assess their degree of oxidation [2]. Consequently, these compounds affect the sensory perception and acceptability of consumers [2-4].

The headspace solid phase microextraction (HS-SPME) technique consists in extracting volatile substances by exposing a fibre coated with a stationary phase (adsorbent), to the vapour phase (headspace) in equilibrium with the olive oil contained in a thermostated vial, sealed with a perforable septum. The analysis is performed by thermal desorption by inserting the fibre directly into the GC injector at a suitable temperature [1].

Nevertheless, only an adequate SPME sampling condition enables a high efficiency and sensitivity of the method of analysis [5]. In this way, optimal microextraction conditions are linked to sample quantity, temperature, time and type of fibre [5].

Several studies have been published using HS-SPME to characterize the profile of volatile compounds of olive oils [6-13]. However, a wide range of conditions have been used, concerning quantity (1 - 20 g) [14-15], time (10 minutes - 6 hours) [8-14] and temperature of extraction (25^0C - 100^0C) [5-14]. For this reason, comparison between bibliographic data and the definition of a sensorial profile of olive oils implies great difficulty. Nowadays, a complete validation of the current methodologies is still lacking and none of these methods based on SPME–GC can be considered fully established; therefore, quantification of volatiles may be subjected to significant errors that hinder the sensory interpretation from the chemical data [13]. Thus, standardizing the extraction conditions, taking into account the specificities of each monovarietal olive oil, is an interesting challenge that has not been reported before.

The response surface methodology (RSM) is a mathematical and statistical tool that has been applied to study the effects of multiple factors and their interactions on one or

more response variables, being very effective to reduce the number of experiments and with high reproducibility [16]. Even though this methodology has been successfully applied to different types of foods such as lettuce, olive paste and beef [17- 19], few studies exist on olive oil or mixture of olive oil with other food ingredients [16, 20-21]. However, of our knowledge, until now RSM has not been applied to extraction conditions of volatiles of olive oil.

Concerning monovarietal olive oils, cv. Arbequina is cultivated in different regions and is popular worldwide [15,22]. Moreover, it is very appreciated due to organoleptic characteristics such as: fresh, herbal and green; tomato flavour, notes of apple and banana; sweet almond and artichoke undertones, in general with a low-medium intensity of pungency and bitterness [3, 9, 12, 15, 23].

Thus, the present study aimed to model and to study the effect of extraction conditions of HS-SPME, i.e. the quantity of sample, time and temperature, in order to obtain the maximisation of total peak areas from the major volatile compounds of the monovarietal Arbequina olive oil. For this purpose, the RSM was used.

1 Materials and methods

2.1 Sample

Monovarietal Arbequina extra virgin olive oil (EVOO) was used in the present study. The oil was directly obtained from producers in the 2014/2015 harvest and was stored protected from light at 4 °C until analysis. The Arbequina orchard was located in the Valladolid province (Spain), and the harvest of the fruits occurred on 2.5 ripening index. The olive oil was classified as extra virgin olive oil according to legal requirements (European Regulation 2568/91) performed in our laboratory previously, showing free acidity of 0.2% oleic acid, peroxide value of 3.7 mEq O_2/kg, K_{232} = 1.50, K_{270} = 0.16, ΔK = -0.01, median intensity of sensory defects = 0 and median intensity of fruity positive attribute > 0 on sensory analysis.

2.2 Volatile headspace solid-phase microextraction (HS-SPME)

For the headspace solid-phase microextraction (HS-SPME) a fibre of 2 cm coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 μm) from Supelco (Bellefonte, USA) was used. This type of fibre has been widely used for

determination of volatiles in olive oils, showing good effectiveness and reproducibility and high affinity with compounds of low and medium molecular weight [5,24,29]. The olive oil sample was placed in 50 ml vials to avoid any contact of samples with fibre and to provide efficient extraction, according to previous studies in a different food matrix [30]. Then, 4-methyl-2-pentanol was added as internal standard (100 ppm in methanol; 10 μl) and the vials were sealed with a polypropylene cap with silicon septum. The volatiles were released at the selected extraction temperature in a water bath and vigorously stirred with a stir bar (350 rpm) during the time required to allow the equilibration in the headspace. After the equilibrium time (5 min), the DVB/CAR/PDMS fibre was exposed during a precise extraction time for volatiles adsorption and immediately inserted into the injection port of the GC system for thermal desorption and reconditioning (10 min at 280 °C). Experiments were performed at different conditions of quantity of sample, extraction temperature and extraction time (see below).

2.3 Gas chromatography-mass spectrometry (GC-MS) conditions

Chromatographic analysis was performed on a Shimadzu GC-2010 Plus equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. A TRB-5MS (30 m × 0.25 mm × 0.25 μm) column (Teknokroma, Spain) was used. The injector was set at 220 °C and the manual injections were made in splitless mode. The mobile phase consisted in helium (Praxair, Portugal) at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min. The oven temperatures were the following: 40 °C during1 min; up to 220 °C with a rate of 2°C/min during 30 min. The ionization source was maintained at 250 °C with ionization energy of 70 eV, and with an ionization current of 0.1 kV. All mass spectra were acquired by electron ionization. The ionization was left off during the first 2 minutes. The MS spectra fragments were compared with those obtained from a database (NIST 11), and also with comparison of GC retention index. Furthermore, retention indices were obtained using commercial n-alkanes C_7-C_{30} (Sigma-Aldrich, St. Louis, U.S.A.) by direct splitless liquid injection (1 μL) while all further conditions of GC and MS as settled for the volatile analysis. The identification was also performed considering for tentative of identification at least 90-95% of match spectra and for identification at least 98%. For semi-quantification purposes, the areas of the chromatographic peaks were determined integrating the re-constructed chromatogram

from the full scan chromatogram using for each compound the ion base (m/z intensity 100%). For optimisation purposes, each individual ion peak area and the total area were calculated.

2.4. Experimental design and data analysis

The response surface methodology (RSM) of Minitab® software (version 16, Coventry, England) was used to determine the best extraction conditions for the major volatiles of the cv. Arbequina extra virgin olive oil. A Central Composite Design (CCD), full-factorial (α=1,682), with three independent factors, namely, quantity of oil (X_1), time (X_2) and temperature (X_3) was applied. The response variables were the total area of the peaks and the main compounds peaks, namely, Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, 1-hexanol, Z-3-hexen-1-ol acetate, hexyl acetate and E-2-hexen-1-ol acetate. **Table 1** shows the range and centre point values of the three independent variables that have been coded to 5 levels: -1.682, -1, 0, +1 and + 1.682. The experimental design consists of six axial points at a distance of ±1.682 from the centre, six replicates of the central point that are used to determine the experimental error for data reproducibility, and eight cube points (**Table 2**). The experimental runs were randomized in order to minimize the effects of unexpected variability in the observed responses. Furthermore, each point of the CCD was carried out in duplicate as described previously to optimise extraction antioxidant properties [18].

The experimental data from the CCD was analyzed using response surface regression and fitted to a second-order polynomial model, as indicated in Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted dependent variable; β_0 is the model constant that fixes the response at the central point of the experiment (intercept); β_i is the regression coefficient for the linear effect terms; β_{ii} is the quadratic effect term; β_{ij} is the interaction effect term of variables i and j; X_i and X_j are the independent variables. The adequacy of the model was predicted through the analysis of variance (ANOVA) including the F-ratio, which established the lack-of-fit of the model and the determination coefficients (R^2) and adjusted R^2. Additionally, the experimental and predicted values for each dependent variable were compared. The significant factors affecting each dependent variable were selected according to the Student t-test at 95% confidence level. On the basis of the

responses obtained previously, the optimum point was determined by applying the "Response Optimizer" tool of Minitab® with the goal of obtaining simultaneously all the maximal responses of peak areas considering the compounds identified and the total area. Moreover, the desirability function was applied for optimization of response and establish the best condition for all the peaks simultaneously, i.e, applying this function is possible the transformation of each response into a dimensionless individual desirability (di) scale [25].In this sense, the scale of the individual desirability function ranges between d = 0, for a completely undesirable response, and d = 1, for a fully desired response [25].

Table 1. Coded and uncoded (true) values of variables of CCD model.

	Coded units				
	-1.682	-1	0	1	+1.682
	True values				
Oil, X_1 (g)	1.3	2.0	3.0	4.0	4.7
Time, X_2 (min)	13.18	20.00	30.00	40.00	46.82
Temperature, X_3 (ºC)	33.18	40.00	50.00	60.00	66.82

3 Results and discussion

3.1 Effects of SPME conditions on the volatile composition of cv. Arbequina olive oil

In olive oil the six straight-chain carbon compounds (C6) are among the most important ones for aroma, being aldehydes and alcohols mainly linked to green-fruity notes and esters to fruity and aromatic flavour [24,26 27].

In the present work, the main volatiles found were C6 compounds as aldehydes (Z-3-hexenal, E-2-hexenal), alcohols (1-hexanol, Z-3-hexen-1-ol) and the corresponding esters (Z-3-hexen-1-ol acetate, hexyl Acetate and E-2-hexen-1-ol acetate). Our results are in concordance with previous studies showing a high content of C6 compounds in cv. Arbequina olive oils from the Mediterranean basin [9-10, 12, 15, 22]. According to Pérez et al., [26], the content of C6 is on average 2–160 times higher than that of the rest of the groups of volatile compounds in the oils. All the volatile compounds identified in the present work were related with positive sensorial characteristics such as: almonds, apple, astringent, banana, bitter, fresh, green-fruity, green leaves, pungent, sweet and tomato [1, 3, 28]. Besides, E-2-hexenal is also considered as an index of freshness of olive oils [4, 29].

In the analyzed cv. Arbequina olive oil, the seven most representative identified volatile compounds were Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, 1-hexanol, Z-3-hexen-1-ol acetate, hexyl acetate and E-2-hexen-1-ol acetate (**Table 2**).

Regarding experimental values of total area, it was observed the highest peak area for condition 9 (1.32g/30 min/50ºC) and slightly lower values were found for the conditions 7 (4g/40 min/60ºC) and 18 (2g/40min/60ºC). In all cases, high temperatures (50-60 °C) and long times of extraction (30-40 min) were effective in providing high total peak areas.

Moreover, the condition 7 (4g/40 min/60ºC) showed the highest peak values for E-2-hexenal, Z-3-hexen-1-ol acetate, hexyl Acetate and E-2-hexen-1-ol Acetate. On the other hand, the best condition for extracting Z-3-hexenal, Z-3-hexen-1-ol and 1-hexanol compounds, was the 17 (4.68g/30 min/50ºC). In this sense, the results obtained indicated that the peaks area increased with increasing temperature and time.

Furthermore, Nonanal was detected in all conditions tested (data not shown) in between 0.09-2.6 ng of internal standard.g^{-1} of olive oil. This compound, when detected in higher quantities than in the present assay, has been associated with signs of rancidity [2,1]. Other studies also reported low values of nonanal previously for Arbequina olive oils [10,15]. At the same time, the temperature conditions seem not to be the responsible for the low content of nonanal found due to the fact that we did not observe a marked effect or any tendency linked to temperature conditions.

In addition, hexanal was not detected in our analytical conditions as well as recent study that didn't detected in commercial olive oils from Spain, Italia and France [32]. Possible reasons may be: a very low level of this compound (traces) exists in the sample, in a similar way than described for olive oil from Tunisian cultivars [31] or associated with the time of retention close to the internal standard in our experimental conditions hinder the identification.

Table 2. Observed responses and predicted values for total area and major volatile peaks compounds in cv. Arbequina olive oil.

Condition	Oil (g)	Time (min)	T (°C)	Total area ($x10^8$)		Z-3-hexenal ($x10^7$)		E-2-hexenal ($x10^7$)		Z-3-hexen-1-ol ($x10^7$)		1-hexanol ($x10^6$)		Z-3-hexen-1-ol Acetate ($x10^7$)		hexyl acetate ($x10^6$)		E-2 hexen-1-ol acetate ($x10^6$)	
				E	***P***	***E***	***P***	***E***	***P***	***E***	***P***	***E***	***P***	***E***	***P***	***E***	***P***	***E***	***P***
1	3.0	30.	50.00	6.48	6.28	1.10	1.19	3.32	3.27	1.13	1.06	2.55	2.39	3.05	2.92	1.63	1.58	0.99	0.95
2	2.0	40.00	40.00	6.38	5.70	1.03	1.03	2.92	3.27	0.91	0.90	2.01	2.00	2.25	2.59	1.19	1.44	0.71	0.61
3	4.0	20.00	40.00	4.93	5.51	1.16	1.19	2.35	2.64	0.84	0.78	1.82	1.71	1.33	1.68	0.61	0.65	0.45	0.52
4	2.0	20.00	40.00	5.09	5.70	0.92	1.03	2.02	2.37	0.68	0.67	1.51	1.45	1.18	1.68	0.55	0.56	0.40	0.49
5	3.0	30.00	50.00	6.31	6.28	1.27	1.19	3.32	3.27	1.06	1.06	2.45	2.39	2.84	2.92	1.50	1.58	0.93	0.95
6	3.0	30.00	33.18	5.11	5.04	1.22	1.26	2.36	3.22	0.78	0.77	1.65	1.66	1.28	1.60	0.62	0.76	0.38	0.44
7	4.0	40.00	60.00	6.81	6.07	1.17	1.19	3.59	3.49	1.09	1.08	2.42	2.39	4.62	4.69	2.69	2.59	1.56	1.69
8	4.0	20.00	60.00	5.76	6.07	1.17	1.19	3.06	3.09	1.03	1.04	2.29	2.33	2.97	2.71	1.65	1.72	0.96	0.83
9	1.3	30.00	50.00	6.92	6.60	0.87	0.88	2.70	2.73	0.82	0.85	1.83	1.89	2.58	2.92	1.47	1.42	0.81	0.72
10	2.0	20.00	60.00	6.02	6.45	0.94	0.95	2.76	2.69	0.90	0.87	2.01	1.93	1.51	2.71	0.84	1.53	0.52	0.86
11	3.0	13.18	50.00	4.83	5.77	1.01	1.07	2.28	2.31	0.82	0.83	1.81	1.85	1.46	1.70	0.69	0.84	0.52	0.54
12	3.0	30.00	66.82	6.24	6.31	0.96	1.12	3.08	3.32	0.93	0.94	2.04	2.05	4.04	4.24	2.20	0.24	1.52	1.46
13	3.0	46.82	50.00	6.71	5.77	1.15	1.07	3.44	3.40	1.06	1.05	2.39	2.36	3.74	4.14	2.08	0.23	1.28	1.36
14	3.0	30.00	50.00	6.02	6.28	1.14	1.19	3.13	3.27	1.01	1.06	2.28	2.39	2.69	2.92	1.40	1.58	0.92	0.95
15	3.0	30.00	50.00	6.09	6.28	1.18	1.19	3.18	3.27	1.03	1.06	2.25	2.39	2.77	2.92	1.49	1.58	0.87	0.95
16	4.0	40.00	40.00	5.76	5.32	1.23	1.27	3.18	3.67	1.05	1.07	2.36	2.40	2.22	2.59	1.15	1.62	0.69	0.86
17	4.7	30.00	50.00	5.96	5.96	1.30	1.28	3.44	3.41	1.14	1.13	2.60	2.56	2.77	2.92	1.48	1.73	0.89	0.91
18	2.0	40.00	60.00	6.78	6.45	0.95	0.95	2.97	3.08	0.90	9.06	1.93	1.99	3.95	4.69	2.29	2.41	1.41	1.44
19	3.0	30.00	50.00	6.29	6.28	1.18	1.19	3.29	3.27	1.04	1.06	2.36	2.39	3.08	2.92	1.75	1.58	0.97	0.95
20	3.0	30.00	50.00	6.47	6.28	1.26	1.19	3.37	3.27	1.08	1.06	2.49	2.39	3.07	2.92	1.68	1.58	1.03	0.95

Average of duplicate extractions. E- Experimental values; P - Predicted values

3.2 Model fitting using Response Surface Methodology (RSM)

The experimental and predictive values for total area and major volatile compounds peaks at each extraction conditions are shown in **Table 2**. The predicted values were determined according to the quadratic models obtained; based on the independent factors, namely, quantity of oil (X_1), time (X_2) and temperature (X_3). Thus, the predicted values (Y) (Z-3-hexenal, E-2-hexenal, Z-3-hexen-1-ol, 1-hexanol, Z-3-hexen-1-ol acetate, hexyl acetate and E-2-hexen-1-ol acetate) were assessed as a function of the significant linear, quadratic and interaction effects ($p<0.05$), as shown in **Table 3**. The response surface models obtained with a 0.05 level of significance were the following (Eqs. 2-9):

$$Y_{Total\ area} = 62.80 - 1.90X_1 + 5.18X_2 + 3.75X_3 - 1.80X_2^2 - 2.13X_3^2 \quad (2)$$

$$Y_{Z-3-Hexenal} = 119 + 11.8X_1 - 4.01X_3 - 3.81X_1^2 - 4.00X_2^2 \quad (3)$$

$$Y_{E-2-Hexenal} = 32.7 + 2.02X_1 + 3.23X_2 + 2.27X_3 - 0.705X_1^2 - 1.46X_2^2 - 1.95X_3^2 - 1.25X_2X_3 \quad (4)$$

$$Y_{Z-3-Hexen-1-ol} = 106 + 8.52X_1 + 6.60X_2 + 5.04X_3 - 2.46X_1^2 - 4.13X_2^2 - 7.10X_3^2 - 4.75X_2X_3 \quad (5)$$

$$Y_{1-Hexanol} = 23.9 + 1.99X_1 + 1.52X_2 + 1.17X_3 - 0.601X_1^2 - 1.02X_2^2 - 1.91X_3^2 - 1.23X_2X_3 \quad (6)$$

$$Y_{Z-3-Hexen-1-ol\ Acetate} = 29.2 + 7.24X_2 + 7.83X_3 + 2.67X_2X_3 \quad (7)$$

$$Y_{Hexyl\ Acetate} = 158 + 9.21X_1 + 43.9X_2 + 48.6X_3 + 14.9X_1X_3 + 16.4X_2X_3 \quad (8)$$

$$Y_{E-2-Hexen-1-ol\ Acetate} = 95.2 + 5.50X_1 + 24.4X_2 + 30.1X_3 - 4.79X_1^2 + 7.00X_1X_3 + 11.6X_2X_3 \quad (9)$$

The values obtained experimentally for all response variables were similar to the predicted values (**Table 2**), indicating that values fitted the models in a satisfactory way. The quality of the models for total area and major volatile compounds was evaluated by the lack-of-fit, R^2 and adjusted R^2 values (**Table 3)**. The *p*-values of lack-of-fit higher

than 0.05 indicate that the variation between samples is mainly due to the factors selected for the model and the pure error [18]. Furthermore, R^2 and adjusted R^2 values closer to unity indicate that the empirical models fit satisfactorily the real data. All models showed non-significant lack-of-fit values (p>0.05). Also, high values for R^2 and adjusted R^2 were observed, ranging from 0.882 to 0.981 and 0.777 to 0.964, respectively.

In general, the three factors studied had a significant role on the response variables studied, with the exceptions of oil quantity (X_1) on Z-3-hexen-1-ol acetate and extraction time (X_2) on Z-3-hexenal. Furthermore, in almost all situations, oil quantity, extraction time and temperature presented a positive linear effect on the responses analyzed. Individually, the extraction temperature (X_3) had the most significant impact on Z-3-hexen-1-ol acetate, hexyl acetate and E-2-hexen-1-ol-acetate confirmed for the higher coefficient values observed in Table 3, although the quantity of oil showed the highest effect on Z-3-hexen-1-ol and 1-hexanol. On the other hand, the extraction time had the highest effect on total area and E-2-hexenal.

Some response surface plots of total area and major volatiles present in cv. Arbequina olive oil are shown in **Figures 1 and 2**. The application of high times and temperatures increased the total area, while the effect of oil quantity was less evident.

The major volatile compounds detected were Z-3-hexen-1-ol acetate and E-2-hexenal. These compounds have been reported previously in some studies of cv. Arbequina olive oils [9-10, 12, 15, 22, 28]. In earlies studies, oils from this cultivar were associated with ripe fruit aroma, slight oily odour and sweet taste [33], but, according to García-González, Romero and Aparicio [22], the market tendency with a high demand of greener and bitterer oils has favoured the production of monocultivar Arbequina oils from low ripened olives and, as a results, increasing sensorial descriptors as cut-green, tomato and a medium bitter taste, sensory traits associated to the predominance of Z-3-hexen-1-ol acetate and E-2-hexenal. For these reasons, we selected the surface plots of Z-3-hexen-1-ol acetate and E-2-hexenal to illustrate the behaviour of the major compounds (**Fig 2.A, Fig 2.B)**. For the former compound, it was stated by the quadratic model (**Eq. 7**) that only time and temperature influenced the extraction of this compound. On the contrary, all the factors influenced E-2-hexanal extraction, the highest linear coefficients being obtained for time and temperature. Nevertheless, in the case of E-2-hexenal, a slight decrease on the total area was observed at 60 °C and 40 min of extraction time onwards.

Concerning Z-3-hexen-1-ol, (**Fig 2.C)**, after fixing the quantity of olive oil in 3 g , a similar behaviour to E-2-hexenal was observed. Regarding the oil quantity (**Fig 2.D**), when it increased, the area of the peak of this compound also increased, showing that the region of higher oil quantity and extraction time would promote the extraction of this compound. Furthermore, we found that all the other compounds presented similar behaviours to those described above (data not shown).

Thereby, our results suggest that high values of each one of the three analysed variables is individually favourable for a better extraction; however, for some compounds, the simultaneous application of higher temperature and longer time of extraction might favour their degradation, decreasing the peak area. These results are in concordance with Beltran, Aguilera and Gordon [6], who showed a significantly increase of volatile compounds in the headspace of olive oils emulsions with increasing temperature. The higher concentration is mainly attributed to the rise of vapour pressure due to temperature increase that enhances the mass transference of analytes, increasing their concentration in the gas phase [5-6].

Table 3. ANOVA for the quadratic models developed to describe the influence of oil quantity (X_1), time (X_2) and temperature (X_3) on total area and major volatile compounds peaks from cv. Arbequina olive oil.

Term constant	**Total area (x10^7)**		**Z-3-hexenal (x10^5)**		**E-2-hexenal (x10^6)**		**Z-3-hexen-1-ol (x10^5)**		**1-hexanol (x10^5)**		**Z-3-hexen-1-ol acetate (x10^6)**		**hexyl acetate (x10^4)**		**E-2-hexen-1-ol acetate (x10^4)**	
	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*	Coefficient	*p*
Constant	62.80	0.000	119	0.000	32.7	0.000	106	0.000	23.9	0.000	29.2	0.000	158	0.000	95.2	0.000
X_1	-1.90	0.004	11.8	0.000	2.02	0.000	8.52	0.000	1.99	0.000	1.87	0.148	9.21	0.035	5.50	0.025
X_2	5.18	0.000	3.28	0.076	3.23	0.000	6.60	0.000	1.52	0.000	7.24	0.000	43.9	0.000	24.4	0.000
X_3	3.75	0.000	-4.01	0.036	2.27	0.000	5.04	0.000	1.17	0.001	7.83	0.000	48.6	0.000	30.1	0.000
X_1^2	0.51	0.286	-3.81	0.040	-0.705	0.010	-2.46	0.019	-0.601	0.038	-1.11	0.651	-4.65	0.236	-4.79	0.040
X_2^2	-1.80	0.005	-4.00	0.033	-1.46	0.000	-4.13	0.001	-1.02	0.002	-1.38	0.095	-7.60	0.066	-3.11	0.158
X_3^2	-2.13	0.002	-3.56	0.052	-1.95	0.000	-7.10	0.000	-1.91	0.000	-1.17	0.284	-6.78	0.095	-1.22	0.561
$X_1 . X_2$	-0.21	0.767	-0.738	0.740	0.324	0.300	0.558	0.647	0.317	0.369	-1.21	0.692	-6.43	0.223	-4.80	0.109
$X_1 . X_3$	0.70	0.330	0.078	0.972	0.416	0.191	0.106	0.930	0.127	0.714	2.51	0.592	14.9	0.013	7.00	0.028
$X_2 . X_3$	-0.39	0.580	-2.00	0.378	-1.25	0.020	-4.75	0.002	-1.23	0.004	2.67	0.009	16.4	0.008	11.6	0.002
Lack-of-fit		0.504		0.637		0.687		0.919		0.831		0.229		0.428		0.154
R^2	0.952		0.882		0.981		0.965		0.952		0.976		0.970		0.975	
R^2 ***adjusted***	0.908		0.777		0.964		0.933		0.910		0.954		0.940		0.952	

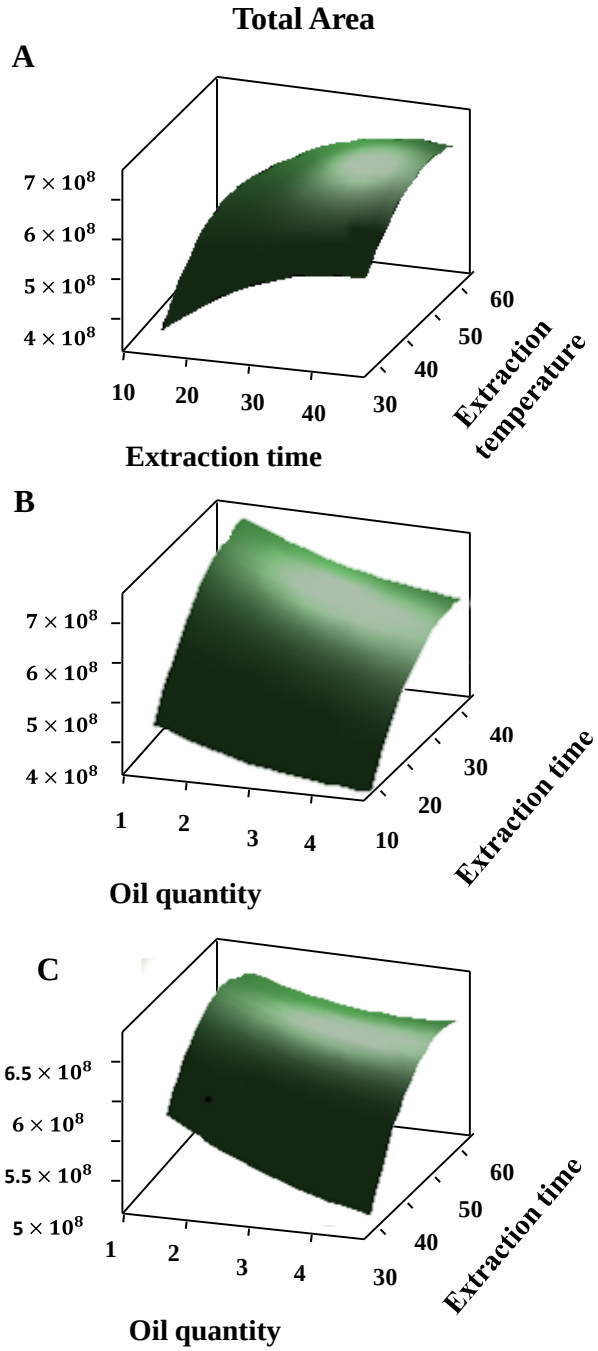

Figure 1. Response surface plots of total area considering middle values for oil quantity (3 g) (**Fig 1-A**), temperature (50°C) (**Fig 1.B**) and time (30 min) (**Fig 1.C**).

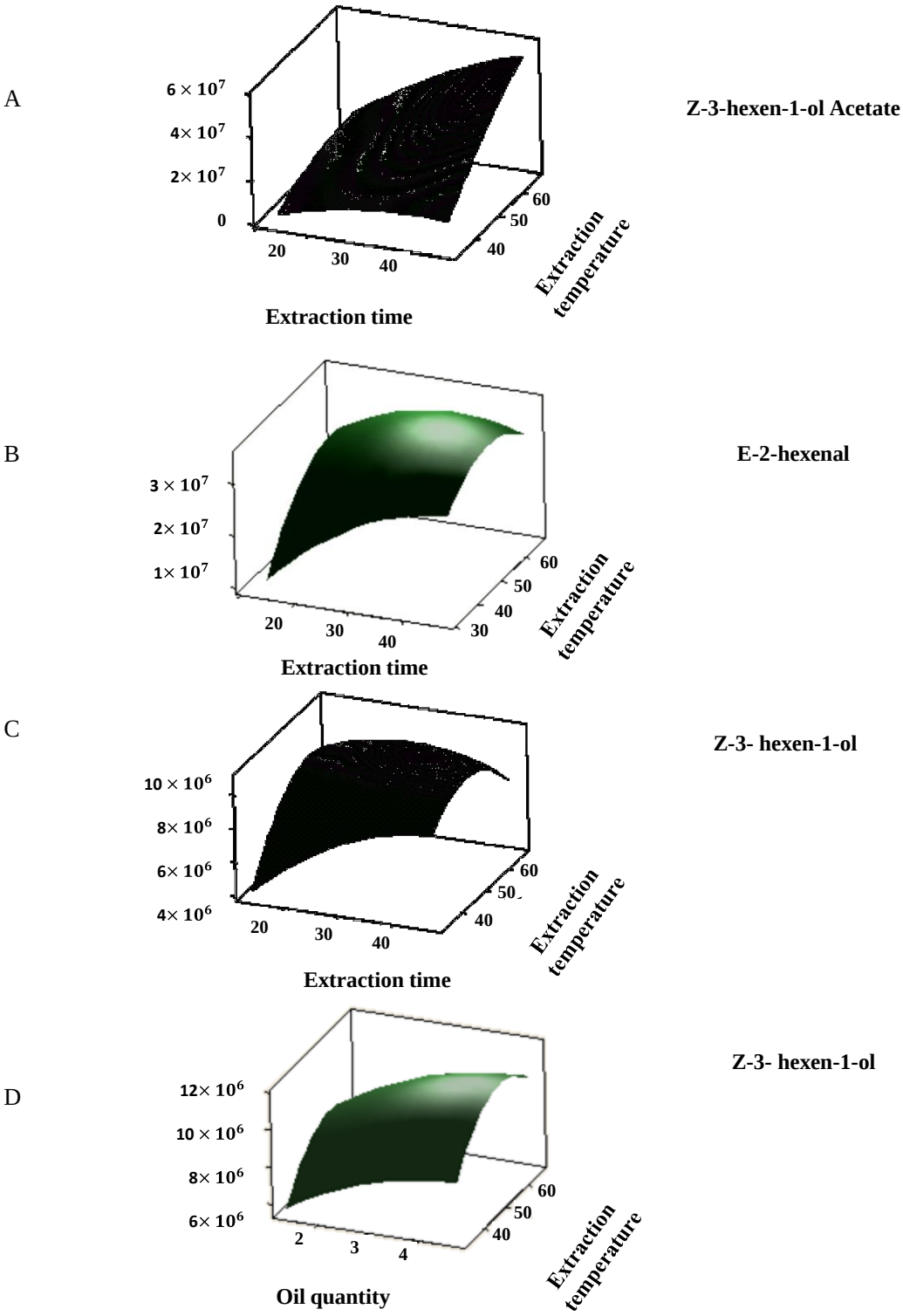

Figure 2. Response surface plots of Z-3-hex-1-ol Acetate (**Fig 2-A**), E-2-hexenal (**Fig 2.B**) and Z-3-hexan-1-ol (**Fig 2.C**) fixing the middle value for oil quantity (3 g). Response surface plot of Z-3-hexan-1-ol fixing temperature (50°C) (**Fig 2.D**)

3.3 Optimisation of volatiles extraction and verification of the models

The optimal extraction point was 4.6 g of oil, 43 min and 59 °C with 0.88 of desirability (D). The predicted and experimental values of each compound peak, the calculated desirability (D) and the percentage of error are depicted in **Table 4.** The scale of the individual desirability function ranges between 0 for a completely undesirable response, and 1 for a fully desired response. Hence, a global D value of 0.88 predicted in the present work, is quite acceptable to target the ideal extraction conditions to define the volatile profile of cv. Arbequina olive oils. The experimental values were obtained by conducting assays in triplicate under the recommended optimum conditions. **Table 4** shows close similarity between the predicted values of the responses and the experimental models, with low errors (<15%) and good desirability (0.65-1.00). The high values of desirability showed that our goal to maximise the extraction of the volatile compounds studied in the present work was successfully achieved.

Table 4. Validation of the optimal point of extraction.

	D[1]	Y Predicted	Y Experimental	Error (%)[2]
Total area (x10^8)	0.72	6.63	6.73	1.51
Z-3-hexenal (x10^7)	0.65	1.16	1.21	4.31
E-2-hexenal (x10^7)	1.00	3.62	3.54	2.21
Z-3-hexen-1-ol (x10^7)	0.88	1.10	1.06	3.63
1-hexanol (x10^6)	0.86	2.49	2.42	2.81
Z-3-hexen-1-ol acetate (x10^6)	1.00	4.62	4.82	4.33
hexyl acetate (x10^6)	0.97	2.67	2.80	4.87
E-2 hexen-1-ol acetate (x10^6)	1.00	1.56	1.77	13.46

[1] D refers to desirability.
[2] Error (%) was calculated by the equation: $\frac{Yexperimental - Ypredicted}{Ypredicted} 100$

4 Conclusions

The temperature was the extraction condition of HS-SPME/GC–MS which mainly affected the volatile profile of cv. Arbequina olive oils; however, the time also presented a strong influence on the extraction of aroma compounds. Furthermore, the control of the proposed parameters allows an optimal extraction of desirable volatile compounds improving the total areas of the peaks. The maximum response for all volatile compounds analysed was obtained using 4.6 g of oil; 43min and 59 °C. The results obtained through the developed quadratic models show that that RSM was a good and adequate tool to study the behaviour of volatiles depending on the factors considered, to maximise the aroma profile analysis in monovarietal olive oil. However, the extraction conditions must be optimised for each kind of varietal olive oil, since each one could present specific volatile compounds. In a future work, we aim to evaluate the different profile of Arbequina olive oils to study the possible differences of global volatile profiles applying the current parameters.

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Artículo 5

General Paper

Application of electronic tongue for assessing the sensory profile of Brazilian and Spanish EVOO based on estimation of volatile and phenolic compounds

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Application of electronic tongue for assessing the sensory profile of Brazilian and Spanish EVOO based on estimation of volatile and phenolic compounds

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ABSTRACT

It is well-known that the global organoleptic characteristics of olive oil are based upon the combined effect of odour, taste and chemical responses. In this sense, E-tongue is a new powerful tool that combined with chemometric analysis has been proposed to improve the quality assessment and the adequate classification of olive oils. Nevertheless, the E-tongues have been scarcely applied for the indirect assessment of volatile or phenolic compounds. In this work, we propose to use the E-tongue for assessing parameters related to the global organoleptic profile of Arbequina EVOO based on the potentiometric fingerprints of the olive oil's hydroethanolic extracts from different regions of Brazil and Spain. Among volatile compounds detected, E-2-hexanal and Z-3-hexenyl acetate, which are linked to desirable odour descriptors such as green, green astringent, bitter, apple-like, banana-like and fruity, showed the highest levels. The multiple linear regression (MLR) models based on 8-15 sensor signals were able to assess the phenolic contents with good accuracy ($R^2 \geq 0.930$ - repeated K-fold-CV) and also showed a promising potential for the evaluation of the volatile profiles of Arbequina EVOO ($R^2 \geq 0.818$ - repeated K-fold-CV). The findings of this study showed that the E- tongue is a helpful analytical tool and enabled preliminary insights regarding the organoleptic profile of the Arbequina olive oil.

Keywords: Electronic tongue; Volatiles; Phenols; Arbequina.

1. Introduction

Olive oil is a food product with recognized healthy and nutritional properties, mainly due to their composition in phenolic compounds such as flavonoids, phenolic acids and phenol alcohols (Lopez et al., 2014; Servili et al., 2014). These chemical compounds are not only responsible for the olive oil antioxidant capacity but also by several gustatory positive sensations, including bitterness, pungency and astringency. In addition, the volatile compounds usually found in olive oil are responsible for the positive olfactory attributes (Fortini et al., 2017; Kalua et al., 2007). The overall organoleptic quality assessment of olive oil is based upon the perception of flavour, which is the combined effect of odour (perceived via ortho-nasal and retronasal routes), taste and chemical responses (pungency) (Barbieri et al., 2015). The unique organoleptic characteristics make extra virgin olive oils (EVOO) distinguishable from other vegetable oils (Barbieri et al., 2015; Procida et al, 2016), in this sense, a sensory evaluation carried out according to a trained panel is required as part of the legal control classification of the olive oils according to established by the European regulation (ECC, 2013). However, although being the only homologated method for the organoleptic evaluation of olive oils, the use of trained panellists may present several disadvantages namely, the cost price of the formation and training of a panel, the impossibility to evaluate large number of samples and the delay of results for several days, and the certain degree of subjectivity (Apetrei et al., 2010). Usually, the determination of phenolic compounds in olive oils is carried out by ultra-performance chromatographic and mass spectrometry methods (Rivas et al., 2013); and the volatile profile is evaluated by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) (Fortini et al., 2017). These analytical approaches, as well as the sensory analysis by trained panellists, are time-demanding and expensive. Thus, electronic sensor devices have been proposed to directly or indirectly evaluate olive oil odour and taste, namely electronic noses and tongues (E-noses and E-tongues, respectively).

Some electrochemical devices combined with chemometric tools have been proposed to improve the quality assessment and authentication of foods, namely voltammetric or potentiometric electronic tongues (E-tongues). E-tongues are powerful instruments that allow the extraction of representative chemical fingerprints of liquid food matrices based on the electrochemical signals recorded (Di Rosa et al., 2017). The E-tongue employment has many advantages such as low-cost, simple, portable, fast, accurate,

reliable and robust (Dias et al., 2014; Di Rosa et al., 2017). In regard to olive oils, it has been successfully applied for their classification according to the olive cultivar, geographical origin (within different regions of the same country or different countries), physicochemical quality (EVOO, VOO or LOO) and/or sensory attributes (level of positive sensations such as degree of bitterness and fruitiness, or the presence and intensity of organoleptic defects such as rancid, winey-vinegary, musty and fusty), providing an additional support for trained sensory panels (Apetrei et al., 2010; Dias et al., 2014, 2016; Oliveri et al., 2009; Slim et al., 2017, Souayah et al., 2017; Veloso et al., 2016, 2018). Thus, the E-tongue could also be envisaged as a taste sensor device to complement the phenolic and volatile profile characterization of olive oil, since the organoleptic profile is defined by a combined and synergic effect of different olfactory gustatory–retronasal positive sensations and perceptions, to which phenolic and volatile chemical species are related. Indeed, voltammetric E-tongues have been successfully applied to evaluate the bitterness index of EVOO as well as to quantify the total polyphenolic contents and the concentrations of phenolic compounds and phenols detected in EVOO, using partial least square (PLS1 and PLS2) models (Apetrei et al., 2007, 2010; Apetrei & Apetrei, 2013; Rodríguez-Méndez et al., 2008). In contrast, to our best knowledge, potentiometric E-tongues have been scarcely applied as a quantitative analytical tool for assessing EVOO's phenolic contents, although their capability for monitoring the contents of quality physicochemical data and oxidative stability during EVOO storage has been previously demonstrated (Rodrigues et al., 2016). Also, it has been reported the feasibility of applying potentiometric E-tongues, comprising lipid polymeric membranes, to determine the concentration of standard solutions that mimic sensory positive attributes usually perceived in olive oils namely, bitterness, fruitiness and green sensations (Slim et al., 2017;Veloso et al., 2016). On the other hand, no attempt has been made to evaluate the possibility of using E-tongue devices (potentiometric or voltammetric) for the indirect assessment of volatile compounds levels (e.g., alcohols, aldehydes, hydrocarbons, esters and terpenes), which are related to the olfactory positive sensations usually perceived in olive oils.

Thus, the aim of the present study was to apply the E-tongue for assessing parameters related to the global organoleptic profile of Arbequina EVOO produced in different regions of Spain and Brazil, such as levels of phenolic and volatile compounds. The E-tongue was equipped with lipid polymeric sensor membranes with cross-sensibility towards chemical species that mimic positive and negative sensory sensations usually

perceived in olive oils, and its performance against the experimental phenolic and volatile data determined using chromatographic techniques (i.e., liquid chromatography coupled to time-of-flight mass spectrometry and gas chromatography-mass spectrometry with headspace solid-phase microextraction, respectively) was checked.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical reagent grade or higher purity. Bidistilled deionized water was obtained from a Milli-Q purification system (Millipore, Bedford, MA).). The internal standard 4-methyl-2-pentanol was provided by Sigma (Sigma-Aldrich, St. Louis, MO). For E-tongue analysis the chemicals (plasticizers (bis(1-butylpentyl) adipate, dibutyl sebacate, 2-nitrophenyl octylether, tris(2-ethylhexyl)phosphate and dioctyl phenylphosphonate) and additives (octadecylamine, oleyl alcohol, methyltrioctylammonium chloride and oleic acid) were obtained from Fluka with high purity ≥97%. A polyvinyl chloride with high molecular was used to support the polymer.

2.2 Samples

A total of 33 samples of Arbequina EVOO from 11 different geographic zones (n = 3 from each producing region) were studied. Nine regions of olive oil production in Spain (Granada, Jaén, Málaga, Cádiz, Sevilla, Albacete, Toledo, Valladolid and Lérida, samples 1 to 9) and two regions in Brazil (Rio Grande do Sul and Minas Gerais, samples 10 and 11) were selected. The olives were harvested always at the early stage of harvest; the harvest date was: late October to mid-November of 2014 for Spanish samples and mid-March to early Abril of 2015 for Brazilian samples. The oil was extracted within 24h, under a two-phase extraction system. The oils were directly donated by the producers, adequately packaged for preserving from light and high temperatures and sent to CSIC laboratories (Granada, Spain) to perform the analysis. The samples were transported in adequately conditions for Polytechnic Institute of Bragança to perform the volatile and E-tongue analysis. All the samples were according to the regulation established by the European Union regulation n° 2568/91 for extra virgin olive oil, as was showed previously (Borges et al., 2017a). Furthermore, to confirm the classification as EVOO a sensory analysis was performed by the Official

Panel of Laboratorio Agroalimentario de Granada (Atarfe, Granada, Spain) according to European Regulation (2013). The samples showed values for fruit sensation, bitterness and pungency ranging between 4.4-6.5, 1.8-3.3 and 2.3-3.7, respectively, and none of the oils presented any sensory defect.

2.3. Phenolic compounds determination

Total phenol content was assessed by the Folin-Ciocalteau method, as reported by Borges et al. (2017b). The final results were expressed as mg of caffeic acid equivalents per kg of olive oil (mg CAE/kg). The determination of the individual phenolic fraction of samples was performed after an extraction with methanol/water (80:20) according to the International Olive Oil Council (IOOC, 2009). The extracts were analysed by UPLC-TOF-MS following the method validated by Rivas et al. (2013) and described by Borges et al. (2017c). Briefly, the UPLC system consisted of a AcQuity UPLC equipped with a binary pump system (Waters, Milford, MA, USA) using a AcQuity UPLC BEH C18 column (1.7mm, 2.1 mm x 100 mm inner diameter). The system was coupled to a Micromass/Waters LCTPremier XE benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer equipped with an ESI interface. A calibration curve using a solution of sodium formate (containing 0.05 of formic acid and 5 mM of sodium hydroxide in isopropanol/H2O 9:1, v/v) was used for quantification and accurate mass data of molecular ions were processed with MassLynxs (Waters).

2.3.1. Volatile headspace solid-phase microextraction (HS-SPME) and Gas chromatography-mass spectrometry (GC-MS) conditions

For the headspace solid-phase microextraction (HS-SPME) a fibre of 2 cm coated with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS; 50/30 μm) from Supelco (Bellefonte, USA) was used. This type of fibre has been widely used for determination of volatiles in olive oils, showing good effectiveness and reproducibility and high affinity with compounds of low and medium molecular weight (Cavalli et al., 2003; Vichi et al., 2005, 2007). The olive oil sample was weight (4.6g) placed in 50 ml vials to avoid any contact of samples with fibre and to provide efficient extraction, all the conditions for cv. Arbequina olive oils were studied previously. Then, 4-methyl-2-pentanol was added as internal standard (100 ppm in methanol; 10 μl) and the vials were sealed with a polypropylene cap with silicon septum. The volatiles were released

at the selected extraction temperature in a water bath and vigorously stirred with a stir bar (350 rpm) during the time required to allow the equilibration in the headspace. After the equilibrium time (5 min), the DVB/CAR/PDMS fibre was exposed during a precise extraction time for volatiles adsorption and immediately inserted into the injection port of the GC system for thermal desorption and reconditioning (10 min at 280 °C). All the samples extractions were carried out in triplicate. The chromatographic analysis was performed on a Shimadzu GC-2010 Plus equipped with a mass spectrometer Shimadzu GC/MS-QP2010 SE detector. A TRB-5MS (30 m × 0.25 mm × 0.25 μm) column (Teknokroma, Spain) was used. The injector was set at 220 °C and the manual injections were made in splitless mode. The mobile phase consisted in helium (Praxair, Portugal) at a linear velocity of 30 cm/s and a total flow of 24.4 mL/min. The oven temperatures were the following: 40 °C/1 min; 2 °C/min until 220 °C; 220 ºC during 30 min. The ionization source was maintained at 250 °C with ionization energy of 70 eV, and with an ionization current of 0.1 kV. All mass spectra were acquired by electron ionization. The ionization was left off during the first 2 minutes. The MS spectra fragments were compared with those obtained from a database (NIST 11), and also with comparison of GC retention index. Furthermore, retention indices were obtained using commercial n-alkanes C_7-C_{30} (Sigma-Aldrich, St. Louis, U.S.A.) by direct splitless liquid injection (1 μL) while all further conditions of GC and MS as settled for the volatile analysis. The identification was also performed, considering for tentative of identification at least 95% of match spectra and for identification at least 98% according with Cecchi & Alfei 2013. The quantification purposes were carried out in relation to the internal standard method, i.e.; the area of each compound was divided by the area of the internal standard. All the content is show as ng of internal standard. g^{-1} of olive oil.

2.2. E-tongue analysis

The samples were extracted using a hydroethanolic solution and electrochemically analyzed in a random order, as previously described (Dias et al., 2014). In each assay, 5 g of olive oil were mixed to 50 mL of ethanol–water solutions (80:20 v/v) during 5–10 min under strong agitation. This process allowed extracting polar compounds. The mixture was left at ambient temperature during 60 min, after which, 40 mL of the supernatant solution was removed and immediately analyzed with the E-tongue. The E-tongue included two print-screen potentiometric arrays containing 40 sensors (diameter:

3.6 mm; thickness: 0.3 mm) obtained from the combination of 4 lipid additives (octadecylamine, oleyl alcohol, methyltrioctylammonium chloride and oleic acid; ≈3%); 5 plasticizers (bis (1-butylpentyl) adipate, dibutyl sebacate, 2-nitrophenyl-octylether, tris(2-ethylhexyl)phosphate and dioctyl phenylphosphonate; ≈65%) and high molecular weight polyvinyl chloride (PVC; ≈32%) (Dias et al., 2014). The type of sensors and polymeric membrane compositions (relative percentage of additive, plasticizer and PVC) were selected based on previous works considering the satisfactory signal stability over time (%RSD < 5%, for 5 min signal record) and intra- and inter-day repeatability (0.5% < %RSD < 15%) and the capability to provide qualitative and quantitative responses towards basic taste attributes (sweet, acid, bitter, salty and umami) (Dias et al., 2009; Marx et al., 2017) as well as to positive and negative sensory sensations usually perceived in olive oil (Slim et al., 2017; Veloso et al., 2018). Each sensor is identified with a letter S (for sensor) followed by a code for the sensor array (1: or 2) and the number of the membrane (1 to 20, corresponding to different combinations of plasticizer and additive used). The electrochemical analysis took 5 min enabling to record several electrochemical scans, being the last one used assuming that it corresponded to a pseudo-equilibrium state. Electrochemical assays were performed in duplicate for each olive oil sample, being a third assay carried out if the coefficients of variation of the potentiometric signals generated by the E-tongue sensors were greater than 20% (Rodrigues et al., 2016). To minimize the risk of overoptimistic results, for data split (training and internal-validation sets) and modeling purposes, only one electrochemical "average" signal profile per sample was used, avoiding that results from duplicate assays of the same olive oil sample could be included into both training and validation sets.

2.3. Statistical analysis

Since the potentiometric E-tongue profiles may led to complex chemical fingerprints of the olive oils under study, it is required the use of qualitative and/or quantitative multivariate statistical techniques coupled with variable selection algorithms to avoid the inclusion of collinear sensor signals in the final multivariate predictive models. Thus, in this work, multiple linear regression (MLR) models were used to estimate and/or predict the contents of phenolic and volatile compounds of olive oil (determined by conventional chromatography techniques), which are known to influence the sensory

quality of EVOO. The MLR models were only established for assessing the content of a specific chemical compound if it was chromatographically detected and quantified in the majority of the olive oil samples evaluated. Furthermore, the most representative and non-collinear sub-sets of sensor signal profiles among those generated by the 40 lipid sensor membranes comprised in the E-tongue were selected, in each case, by applying the simulated annealing (SA) meta-heuristic algorithm, which has been previously successfully applied as a powerful variable selection algorithm, for both qualitative (Dias et al., 2014, 2016; Slim et al., 2017; Souayah et al., 2017; Veloso et al., 2016, 2018) and quantitative (Rodrigues et al., 2016) evaluation of olive oil using potentiometric taste sensor devices. The selection was performed using as a quality criterion the maximization of the coefficient of determination (R^2) and the minimization of the root-mean-square error (RMSE) for the lowest number of sensors with non-collinear potentiometric signals responses, established for the leave-one-out (LOO) cross-validation (CV) procedure (Cadima et al. 2004,2012; Cortez, 2014).

Furthermore, a repeated K-fold-CV procedure was also applied (which is a suitable CV variant when the dataset does not allow the establishment of a representative external data sub-set) for comparison purposes with the LOO-CV technique results, and to infer about possible overfitting issues that could occur for the latter CV variant. So, in this study 4 folds with 10 repeats were chosen (K=4; repeats=10), resulting into 40 independent internal validation evaluations for each sub-set of sensors previously established using the SA algorithm. In a run, the data is randomly divided into K folds (set equal to 4 in this study), being K-1 folds used, at each time, for training purposes (to establish the best E-tongue-MLR-SA model) and the data of the remaining fold used for test purposes (internal validation). This process is repeated until all folds were used for internal validation. The procedure is then randomly repeated (10 times in this work) leading to the formation of other data folds containing different sets of olive oil samples. The E-tongue has been scarcely applied for the estimation of polyphenols in olive oils (Apretei & Apretei, 2013) and, therefore, the possibility of using the selected E-tongue-MLR-SA models (for both LOO-CV and repeated K-fold-CV procedures) as complementary tools for the quantification of the phenolic and volatile composition of Arbequina EVOO from Spain and Brazil, was checked in the present work. The checking technique involved the establishment of the 95% intervals of confidence (IC) for the slope and intercept values of the single linear regression (LR) obtained by plotting the chemical contents predicted by the E-tongue-MLR-SA models versus the

respective experimental data determined by chromatographic techniques. The proposed E-tongue based approach could be foreseen as a satisfactory tool, since the 95% IC contained the theoretic values of "zero" and "one" for the intercept and slope values, respectively (Roig & Thomas 2003a, b).

All statistical analysis were performed using the Subselect (Cadima et al., 2004, 2012) and MASS (Venables & Ripley 2002) packages of the open source statistical program R (version 2.15.1), at a 5% significance level.

3. Results and discussion

3.1. Phenolic profile

As reported and discussed by Borges et al. (2017b, 2017c), the total phenolic content in the 33 Arbequina EVOO produced in Spain and Brazil ranged between 74 and 335 mg CAE/kg oil, and it was possible to chromatographically detect and quantify individual phenolic fractions namely flavonoids (apigenin, 14-453 μg/kg; luteolin, 84-1615 μg/kg; and naringenin, 15-143 μg/kg), phenolic acids (p-coumaric acid, 0-239 μg/kg; and vanillic acid, 0-133 μg/kg) and phenols alcohols (hydroxytyrosol, 3-1620 μg/kg). **Figure 1** shows the relative proportion of the three phenolic classes of the Arbequina EVOO from each of the 11 different geographical production areas (9 from Spain, number 1 to 9; and 2 from Brazil, number 10 and 11). The results pointed out the quite different phenolic composition profiles of the Arbequina olive oil according to the production region, which may contribute to different sensory sensations, requiring their fast and cost-effective assessment using portable and in-situ measuring devices.

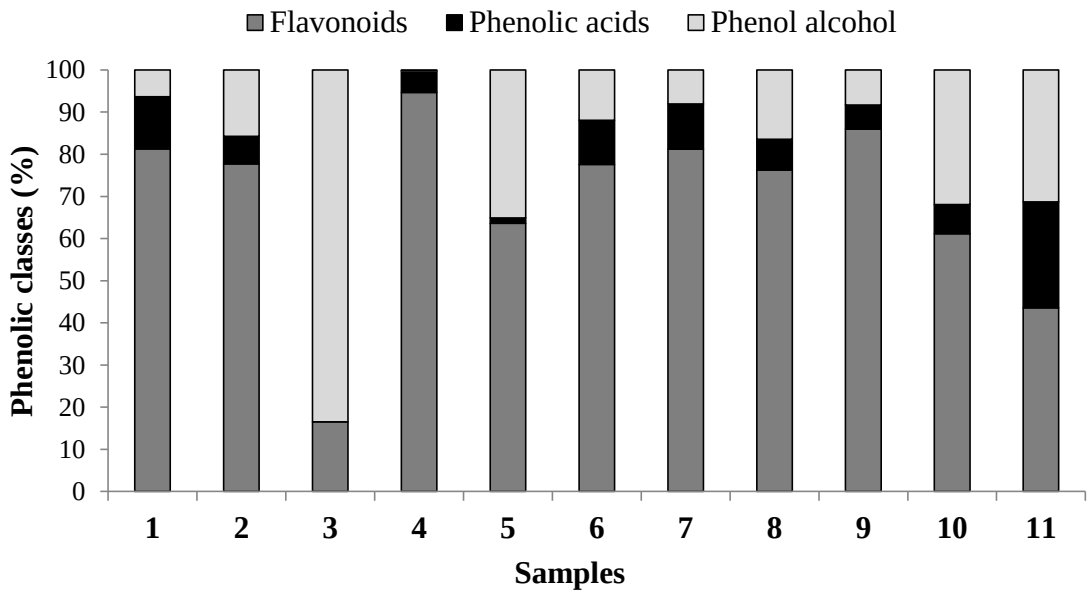

Figure 1. Amount (%) of the phenolic classes of Arbequina extra virgin olive oils obtained from different regions of Spain (samples 1-9) and Brazil (samples 10 and 11).

3.2. Volatile profile

Nineteen volatile compounds were identified in the EVOO samples (**Table 1**), being grouped according to six chemical classes: alcohols, aldehydes, hydrocarbons, esters, terpenes and phenols (**Figure 2**).

In general, the highest concentrations values were obtained for aldehydes and esters, mainly from the LOX pathway, with E-2-hexanal (48-380 μg/kg) and Z-3-hexenyl acetate (9-250 μg/kg) having higher levels. The high concentrations of these individual compounds are linked to desirable odour descriptors such as green, green astringent; bitter, apple-like, banana-like and fruity (Aparicio & Morales, 1998; García-González et al., 2010; Luna & Aparicio, 2002; Luna, Morales & Aparicio, 2006). Moreover, the predominance of these individual volatile compounds agrees with previous studies that showed them as more remarkable in Arbequina olive oils from Spain and Tunisia (García- González et al., 2010; Reboredo- Rodríguez et al., 2015). Particularly, E-2-hexanal seems to be one of the main contributors of the aroma in olive oils from different cultivars due to the low odour threshold of this compound (Luna, Morales & Aparicio, 2006; Pérez et al, 2016). In the Brazilian olive oil 11, which showed high vales of E-2-hexanal, the alcohol E-2-hexen-1-ol was also quantified (**Table 1**). A

similar behaviour was observed in Arbequina oils from Chile and Italia which showed mainly E-2-hexanal and others alcohols such as hexanol and ethanol as major volatile of olive oils (García- González et al., 2010; Procida et al., 2016). The E-2-hexen-1-ol has been linked to undesirable attributes described as green, grassy and sweet (Aparicio & Morales,1998), in spite of the fact that E-2-hexen-1-ol is a stable compound that came from the LOX pathway and depend on the level of the specific enzymes such as hydroperoxide lyase and alcohol dehydrogenase (Procida et al., 2016).

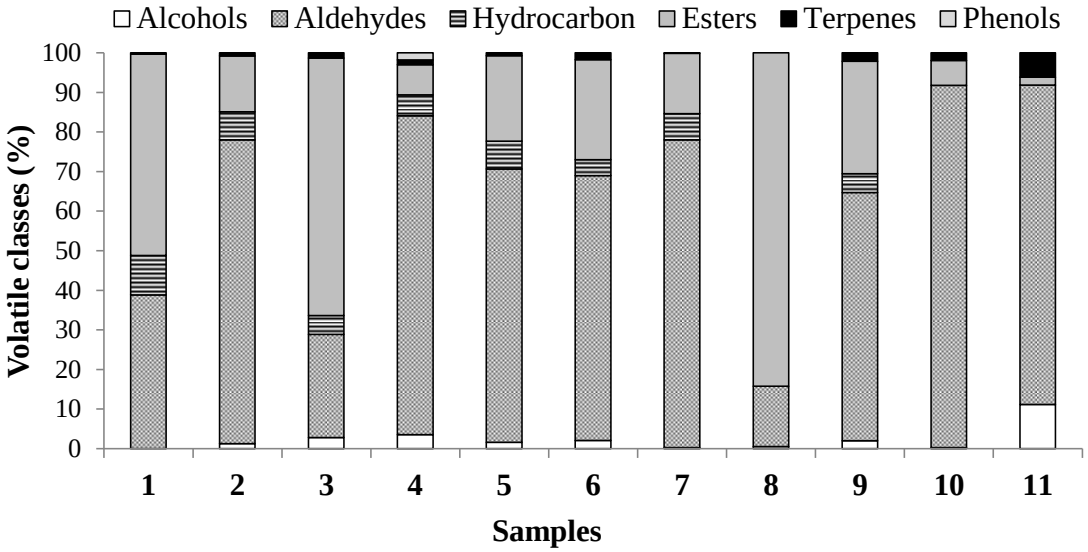

Figure 2. Amount (%) of the volatile classes of Arbequina extra virgin olive oils obtained from different regions of Spain (samples 1-9) and Brazil (samples 10 and 11).

Table 1. Volatile compounds content (µg/kg of olive oil) in Spanish (samples 1-9) and Brazilian (samples 10-11) Arbequina olive oils (mean±SD).

	OO 1	OO 2	OO 3	OO 4	OO 5	OO 6	OO 7	OO 8	OO9	OO 10	OO 11
Alcohols											
1-heptanol	-	-	-	0.77 ± 0.77	-	-	-	-	-	-	-
1-octanol	-	0.47 ± 0.18	1.38 ± 0.11[c]	0.66 ± 0.24	0.40 ± 0.04	1.48 ± 0.45	0.76 ± 0.06	-	1.37 ± 0.18	0.54 ± 0.05	0.67 ± 0.04
1-nonanol	-	0.28 ± 0.28	0.43 ± 0.08	0.49 ± 0.49[b]	0.15 ± 0.14	0.57 ± 0.12	0.32 ± 0.02	-	0.97 ± 0.25	-	-
E-2-hexen-1-ol	-	-	-	-	-	-	-	-	-	-	44.0 ± 0.41
phenylethyl alcohol	-	1.99 ± 1.98	3.22 ± 0.21	4.76 ± 2.29	4.65 ± 1.67	3.32 ± 0.06	-	1.76± 0.16	4.46 ± 0.73	-	8.72 ± 0.39
Aldehydes											
E-2-hexanal	55.5 ± 10.6	165 ± 30.1	37.1 ± 2.84	149 ± 3.10	222 ± 79.5	163 ± 3.62	288 ± 2.11	48.0 ± 4.40	209 ± 15.5	188 ± 0.70	380 ± 4.65
oxohex-2-enal	37.8 ± 0.84	-	-	-	-	-	-	-	-	-	-
nonanal	1.61± 0.92	2.90 ± 0.70	6.86 ± 0.58	2.63 ± 0.66	3.33 ± 0.52	7.59 ± 2.62	5.48 ± 0.02	1.69 ± 0.21	6.23 ± 0.99	3.26 ± 0.41	6.46 ± 0.32
octanal	-	-	2.80 ± 0.15	-	-	-	-	-	-	-	-
Hydrocarbon											
3- ethyl-1,5-octadiene	24.2 ±3.95	15.5 ± 8.00	8.49 ± 0.10	9.94 ± 4.27	22.8 ± 1.76	10.37 ± 1.31	-	-	16.4 ± 4.51	-	-
Nonane	-	-	-	-	-	-	3.74 ± 0.09	-	-	-	-
Heptane	-	-	-	-	-	-	21.1 ± 0.35	-	-	-	-
Esters											
Z-3 hexenyl acetate	107 ± 9.90	21.3 ± 5.96	95.1 ± 4.28	6.75 ± 6.51	53.2 ± 19.9	48.4 ± 3.78	57.66 ± 0.14	250 ± 10.2	68.8 ± 23.6	-	-
hexyl acetate	17.4 ± 1.22	8.82 ± 0.15	21.6 ± 1.06	7.70 ± 0.44	16.0 ±8.53	16.0 ±1.06	-	14.0±0.32	26.5 ± 1.37	8.85 ± 0.34	9.78 ±0.21
E-2 hexenyl acetate	-	-	-	-	-	-	-	9.67 ± 0.89	-	-	-
Methyl ester benzoate	-	0.81 ± 0.7	-	-	1.26 ± 1.2	-	-	-	2.11 ± 0.18	4.32 ± 0.56	-
Terpenes											
B-ocimene	-	1.20 ± 1.21	0.99 ± 0.07	1.16 ± 0.35	1.62 ± 0.22	2.84 ± 0.32	-	-	6.10 ± 1.30	3.07 ± 0.23	24.2 ± 0.81

copaeno	0.75 ± 0.12	-	1.07 ± 0.15	-	-	0.26 ± 0.26	-	-	-	-	-
farneseno	-	0.53 ± 0.53	0.38 ± 0.11	1.02 ± 0.48	0.78 ± 0.07	1.32 ± 0.19	0.49 ± 0.02	-	1.31 ± 0.56	0.97 ± 0.11	5.29 ± 0.38
Phenols											
Guaiacol	-	-	-	3.42 ± 3.40	-	-	-	-	-	-	-

3.3. Assessment of olive oil phenolic and volatile profiles using a potentiometric E-tongue-MLR-SA approach

The predictive capability of the established E-tongue-MLR-SA models (based on 8 to 15 sensor signals from the 40 potentiometric profiles recorded during the analysis of the olive oil hydroethanolic extracts) to quantify the contents of phenolic and volatile compounds found in Arbequina EVOO was evaluated using LOO-CV and repeated K-fold-CV procedures. The latter CV variant has been implemented with 4 folds and for 10 repetitions, allowing minimizing the risk of overfitting, which could result in overoptimistic results due to the use of unrealistic prediction models. The last procedure, randomly splits the initial dataset into 4 data subsets (4 folds containing each 25 % of the data) being each one used at a time for internal-validation purposes of the model established with the other 75% of the data. Since, the process is repeated 10 times (value set in this work); the internal-validation process is carried out 40 times (i.e., 4 folds × 10 repeats). The predictive performances of the selected E-tongue-MLR-SA models are shown in **Table 2**, as well as detailed information (number and type of sensors included in the MLR models) concerning the predictors used for assessing chemical compound.

Regarding the assessment of the phenolic contents the overall R^2 and RMSE values obtained for LOO-CV ($0.967 \leq R^2 \leq 0.995$ and $6.2 \leq$ RMSE ≤ 109.0 μg/kg of olive oil) and repeated K-fold-CV ($0.930 \pm 0.031 \leq R^2 \leq 0.983 \pm 0.017$ and $7.2 \pm 2.4 \leq$ RMSE $\leq 126.0 \pm 26.6$ μg/kg of olive oil) show the practical possibility of applying the proposed E-tongue-chemometric approach as a successful tool for the quantification of total polyphenols content, flavonoids, phenolic acids and phenol alcohols found in Arbequina EVOO, which are related to the positive olive oil gustatory sensations. Moreover, the results also show that the potentiometric E-tongue is able to quantify the olive oil polyphenols or total polyphenolic contents and other phenolic families with similar or greater accuracy when compared to the performance of voltammetric E-tongues or combined electrochemical devices, fusing voltammetric E-tongues and E-noses. Indeed, the complex voltammograms gathered by the E-tongues allowed a satisfactory quantification of the polyphenols (Apetrei et al., 2007, 2010; Apetrei & Apetrei, 2013; Rodríguez-Méndez et al., 2008) and, in combination with an E-nose, allow the estimation of polyphenols, flavonoids, phenolic acids and other phenolic fractions

(Apetrei et al., 2010), extracted from EVOO ($0.773 \leq R^2 \leq 0.977$ and $0.1 \leq$ RMSE $\leq$ 111.0 μg/kg of olive oil; for full cross-validation, *i.e.*, LOO-CV).

Finally, the potential of applying the E-tongue-MLR-SA models as a complementary tool of the conventional chromatographic techniques, for quantifying the phenolic contents of olive oil, was further investigated (Apetrei & Apetrei, 2013). For that, it was evaluated if the slope and intercept values of the single linear regression model established between the contents predicted by the E-tongue-MLR-SA, for LOO-CV or repeated K-fold-CV, and the total polyphenols content, determined by Folin-Ciocalteau method, and flavonoids, phenolic acids and phenol alcohols contents determined by chromatography were statistically equal to one and zero (as expected theoretically for a perfect linear fit), respectively. The parameters of the single linear regressions including the determination coefficients (R^2), the slope and intercept values and the respective 95% confidence intervals, for LOO-CV and repeated K-fold-CV, are given in **Table 3**. The results show that, at 5% significance level, the slope and intercept values were statistically equal to the expected theoretic values, since the confidence intervals contain the values one and zero, respectively. These results pointed out that the potentiometric fingerprints gathered by the E-tongue together with the MLR-SA modelling could accurately assess the phenolic contents of olive oil, with a similar quantitative performance as that of the chromatographic conventional technique.

On the other hand, and from the results shown in **Table 2**, the proposed E-tongue-MLR-SA approach, based on the potentiometric fingerprints of the olive oil's hydroethanolic extracts, showed a less promising potential for the evaluation of the volatile profiles of Arbequina EVOO (LOO-CV: $0.895 \leq R^2 \leq 0.993$ and $0.2 \leq$ RMSE ≤ 33.7 μg/kg of olive oil; repeated K-fold-CV: $0.818 \pm 0.118 \leq R^2 \leq 0.941 \pm 0.075$ and $0.2 \pm 0.1 \leq$ RMSE $\leq 34.4 \pm 9.7$ μg/kg of olive oil). Furthermore, the results presented in **Table 3** allow verifying that, the potentiometric device used is not an alternative to the chromatographic analysis, since for the majority of the volatile compounds evaluated; the slope and the intercept values were not statistically equal to the expected theoretic values. This less satisfactory performance can be partially explained since, contrary to the assessment of the phenolic compounds, which contents are directly related to the concentration found in the liquid extracts, the volatile levels are indirectly evaluated through the analysis of the same liquid extract, which may not be an accurate representation of the olive oil volatile fractions. Even so, the possibility obtaining a

preliminary insight of both phenolic and volatile profiles of olive oil with a single potentiometric assay, still is of practical interest.

Table 2. Predictive capability of the E-tongue-MLR-SA models established to quantify the concentration of the major volatile and phenolic compounds detected in the 11 Arbequina olive oils evaluated, 9 from Spain and 2 from Brazil (n=33: 11 olive oils × 3 independent samples).

Chemical compound		**Concentration range (µg/kg of olive oil)[a]**	**E-tongue-MLR-SA models[b]**				
			Nº of sensors[c]	**Determination coefficient (R^2)**		**Root-mean-square errors (*RMSE*, µg/kg of olive oil)**	
				LOO-CV[d]	Repeated *K*-fold-CV[e]	LOO-CV[d]	Repeated *K*-fold-CV[e]
Phenolics							
Total content		[74, 335] (×10^3)	13[f]	0.967	0 954 ± 0 028	17.6	17.7 ± 5.4
Flavonoids	Apigenin	[14, 453]	14[g]	0.981	0.962 ± 0.019	25.9	28.6 ± 5.9
	Luteolin	[84, 1615]	13[h]	0.969	0.930 ± 0.031	109.0	126.0 ± 26.6
	Naringenin	[15, 143]	15[i]	0.982	0.958 ±0.035	6.2	7.2 ± 2.4
Phenolic acids	p-Coumaric acid	[0, 239]	14[j]	0.995	0.974 ± 0.05	6.3	8.6 ± 6.6
	Vanillic acid	[0, 133]	13[k]	0.992	0.982 ± 0.014	6.8	8.5 ± 3.1
Phenol alcohols	Hydroxytyrosol	[3, 1620]	12[l]	0.988	0.983 ± 0.017	70.1	73.9 ± 21.7
Volatile compounds							
Alcohols	1-heptanol				*Only quantified in one olive oil*		
	1-octanol	[0, 2]	13[z]	0.932	0.881 ±0.071	0.192	0.20 ± 0.10
	1-nonanol				*Only quantified in few olive oils*		
	E-2-hexen-1-ol				*Only quantified in one olive oil*		
	Phenylethyl alcohol	[0, 9]	10[y]	0.903		1.19	
	Total	[0, 54]	13[q]	0.993	0.860 ± 0.218	1.80	1.9 ± 0.6
Aldehydes	E-2-hexanal	[45, 386]	13[x]	0.947	0.909 ± 0.072	33.7	31.7 ± 15.9
	Oxohex-2-enal				*Only quantified in one olive oil*		
	Nonanal	[0.6, 10]	13[w]	0.895	0.818 ± 0.118	1.0	1.1 ± 0.4
	Octanal				*Only quantified in one olive oil*		
	Total	[45, 392]	8[p]	0.943	0.897 ± 0.079	29.4	34.4 ± 9.7
Hydrocarbons	3- ethyl-1,5-octadiene	[0, 27]	10[v]	0.934	0.871 ±0.103	3.4	3.6 ±1.2
	Nonane				*Only quantified in one olive oil*		
	Heptane				*Only quantified in one olive oil*		

	Total	[0, 27]	10[o]	0.929	0.865 ± 0.105	3.6	3.7 ± 1.3
Esters	Z-3 hexenyl acetate	[0, 262]	14[u]	0.980	0.935 ± 0.068	10.6	15.8 ± 4.3
	Hexyl acetate	[0, 28]	11[r]	0.914	0.857 ± 0.110	3.1	3.2 ± 1.5
	E-2 hexenyl acetate				*Only quantified in one olive oil*		
	Methyl ester benzoate				*Only quantified in four olive oils*		
	Total	[7, 286]	12[m]	0.976	0.941 ±0.05	16.6	17.6 ± 3.4
Terpenes	β-ocimene	[0, 25]	14[t]	0.993	0.941 ± 0.075	0.8	0.9 ± 0.5
	Copaeno				*Only quantified in three olive oils*		
	Farnesene	[0, 6]	10[s]	0.975	0.862 ± 0.172	0.3	0.3 ± 0.2
	Total	[0, 31]	12[n]	0.990	0.905 ± 0.136	1.2	1.4 ± 0.6
Phenols	Guaiacol				*Only quantified in one olive oil*		

[a]Experimental concentration range levels found in the Arbequina olive oil (Borges et al., 2017)
[b]Multivariate linear regression (MLR) model based on sub-sets of potentiometric sensors, established using the simulated annealing (SA) algorithm, selected among the 40 possible signal profiles obtained with the electronic tongue (E-tongue) during the analysis of the olive oil hydroethanolic extracts
[c]Number of signals included in the E-tongue-MLR-SA model, selected from the 40 electrochemical signals recorded by E-tongue during analysis of each olive oil hydroethanolic extract
[d]LOO-CV: leave-one-out cross validation procedure
[e]Repeated *K*-fold-CV: cross-validation procedure with 4 folds, ensuring that at least 25% of the original data are used for internal validation, and 10 repetitions
[f]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:2, S1:8, S1:9, S1:11, S1:12, S1:13, S1:14, S1:20, S2:2, S2:5, S2:7 and S2:15
[g]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:2, S1:4, S1:7, S1:8, S1:11, S1:13, S1:14, S1:20, S2:1, S2:5, S2:9, S2:17, S2:18 and S2:20
[h]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:2, S1:3, S1:4, S1:6, S1:12, S2:1, S2:2, S2:8, S2:9, S2:11, S2:14, S2:15 and S2:18
[i]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:6, S1:7, S1:8, S1:10, S1:11, S1:14, S1:16, S1:17, S1:18, S2:2, S2:8, S2:9, S2:13 and S2:14
[j]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:4, S1:7, S1:12, S1:16, S1:17, S1:20, S2:2, S2:4, S2:9, S2:10, S2:17, S2:18 and S2:20
[k]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:3, S1:7, S1:10, S1:11, S1:15, S1:20, S2:1, S2:2, S2:6, S2:7, S2:14, S2:17 and S2:19
[l]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:4, S1:5, S1:9, S1:13, S1:15, S1:19, S1:20, S2:2, S2:3, S2:4, S:7 and S2:17
[m]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:2, S1:3, S1:6, S1:9, S1:10, S1:11, S1:20, S2:5, S2:13, S2:15 and S2:19
[n]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:2, S1:6, S1:16, S1:19, S2:2, S2:3, S2:5, S2:10, S2:12, S2:15 and S2:17
[o]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:3, S1:8, S1:13, S1:15, S1:19, S1:20, S2:2, S2:5 and S2:11
[p]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:2, S1:12, S1:19, S1:20, S2:2, S2:6, S2:14 and S2:20
[q]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:4, S1:9, S1:10, S1:12, S1:14, S1:15, S1:17, S2:3, S2:5, S2:10, S2:14, S2:16 and S2:19
[r]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:2, S1:3, S1:5, S1:7, S1:8, S1:9, S1:20, S2:2, S2:5,S2:6 and S2:12
[s]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:6, S1:9, S1:11, S1:17, S2:3, S2:5, S2:10, S2:16, S2:19 and S2:20
[t]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:2, S1:6, S1:7, S1:10, S1:16, S2:2, S2:3, S2:5, S2:8, S2:10, S2:12, S2:14 and S2:15
[u]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:5, S1:9, S1:12, S1:15, S1:17, S2:1, S2:4, S2:8, S2:9, S2:11, S2:13, S2:17, S2:18 and S2:19
[v]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:6, S1:9, S1:12, S1:15, S2:2, S2:5, S2:8, S2:10, S2:18 and S2:19
[w]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:4, S1:8, S1:13, S1:14, S1:15, S1:16, S2:5, S2:8, S2:9, S2:10, S2:11, S2:17 and S2:19
[x]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:5, S1:7, S1:8, S1:13, S1:14, S2:1, S2:5, S2:10, S2:15, S2:17, S2:18 and S2:20
[y]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:6, S1:8, S1:10, S1:18, S1:19, S1:20, S2:5, S2:8, S2:11 and S2:13
[z]E-tongue sensor signals included in the E-tongue-MLR-SA model: S1:1, S1:2, S1:3, S1:5, S1:6, S1:8, S1:12, S1:18, S1:20, S2:4, S2:7, S2:15 and S2:19

Table 3. Parameters of the single linear regression established between the values of concentration levels predicted by E-tongue-MLR-AS models (LOO-CV and repeated *K*-fold-CV) and the respective experimental concentration values quantified by the conventional chromatographic techniques samples: coefficient of determination (R^2); slopes, intercept values and respective confidence intervals (CI) at 95%.

Chemical compound		**LOO-CV[a]**					**Repeated K-fold-CV[b]**				
		R^2	Slope	Slope CI[a]	Intercept	Intercept CI[b]	R^2	Slope	Slope CI[a]	Intercept	Intercept CI[b]
Phenolics											
Total content		0.967	0.980	[0.885, 1.076]	3.26	[-15.57, 22.08]	0.926	0.978	[0.948, 1.008]	3.88	[-2.07, 9.84]
Flavonoids	Apigenin	0.981	0.951	[0.881, 1.020]	4.51	[-13.90, 22.92]	0.951	0.953	[0.929, 0.977]	4.30	[-1.93, 10.53]
	Luteolin	0.969	0.962	[0.872, 1.052]	13.50	[-72.04, 99.04]	0.914	0.962	[0.930, 0.994]	12.44	[-18.04, 42.93]
	Naringenin	0.982	0.942	[0.876, 1.008]	1.00	[-2.79, 4.79]	0.946	0.949	[0.924, 0.973]	0.66	[-0.74, 2.07]
Phenolic acids	p-Coumaric acid	0.995	0.979	[0.942, 1.016]	0.17	[-3.21, 3.55]	0.967	0.971	[0.951, 0.990]	0.41	[-1.37, 2.20]
	Vanillic acid	0.992	0.970	[0.924, 1.016]	0.08	[-3.14, 3.31]	0.971	0.958	[0.940, 0.976]	0.09	[-1.17, 1.35]
Phenol alcohols	Hydroxytyrosol	0.988	0.960	[0.906, 1.014]	5.72	[-26.00, 37.43]	0.971	0.962	[0.944, 0.981]	5.39	[-5.25, 16.03]
Volatile compounds											
Alcohols	1-octanol	0.932	0.892	[0.765, 1.020]	0.073	[-0.038, 0.184]	0.841	0.905	[0.862, 0.947]	0.057	[0.020, 0.094
	phenylethyl alcohol	0.903	0.867	[0.716, 1.019]	0.47	[-0.14, 1.07]	0.752	0.870	[0.816, 0.924]	0.31	[0.094, 0.529]
	Total	0.993	0.977	[0.934, 1.020]	0.30	[-0.42, 1.02]	0.982	0.991	[0.976, 1.010]	0.241	[-0.002, 0.484]
Aldehydes	E-2-hexanal	0.947	0.956	[0.838, 1.075]	9.17	[-14.65, 32,98]	0.887	0.969	[0.931, 1.006]	6.78	[-0.74, 14.31]
	nonanal	0.895	0.861	[0.704, 1.018]	0.61	[-0.16, 0.70]	0.734	0.878	[0.820, 0.935]	0.6	[0.27, 0.83]
	Total	0.943	0.909	[0.791, 1.027]	16.09	[-8.24, 40.42]	0.871	0.905	[0.867, 0.943]	17.0	[9.17, 24.77]
Hydrocarbons	3- ethyl-1,5-octadiene	0.934	0.912	[0.784, 1.040]	0.80	[-0.91, 2.51]	0.840	0.924	[0.880, 0.968]	0.6	[-0.04, 1.14]]
	Total	0.929	0.902	[0.770, 1.034]	0.898	[-1.13, 2.92]	0.836	0.907	[0.863, 0.951]	0.61	[-0.06, 1.28]
Esters	Z-3 hexenyl acetate	0.980	0.995	[0.920, 1.070]	2.542	[-4.50, 9.58]	0.946	0.989	[0.964, 1.015]	2.77	[0.36, 5.18]
	hexyl acetate	0.914	0.903	[0.756, 1.051]	1.563	[-0.68, 3.81]	0.792	0.919	[0.868, 0.970]	1.56	[0.78, 2.34]
	Total	0.976	0.994	[0.912, 1.076]	2.08	[-6.79, 10.96]	0.945	1.001	[0.980, 1.032]	1.26	[-1.58, 4.11]
Terpenes	β-ocimene	0.993	0.968	[0.924, 1.011]	0.165	[-0.17, 0.50]	0.974	0.970	[0.953, 0.987]	0.12	[-0.01, 0.25]
	farnesene	0.975	0.934	[0.856, 1.012]	0.08	[-0.06, 0.22]	0.934	0.927	[0.901, 0.954]	0.07	[0.03, 0.12]
	Total	0.990	0.959	[0.910, 1.008]	0.23	[-0.24, 0.69]	0.965	0.951	[0.931, 0.971]	0.18	[-0.01, 0.37]

[a]LOO-CV (leave-one-out cross-validation); [b]Repeated *K*-fold-CV (4 folds × 10 repeats); [c]95% slope confidence interval; [d]95% intercept confidence interval

Conclusions

The study allowed verifying the successful combination of a potentiometric electronic tongue with multiple linear regression models for assessing the contents of phenolic and volatile compounds found in olive oil, which are responsible for positive gustatory and olfactory sensations. The work showed that the potentiometric fingerprints of olive oil hydroethanolic extracts could be satisfactorily used to quantify the phenolic levels (total polyphenols content, flavonoids, phenolic acids and phenol alcohol) found in Arbequina olive oil, produced in different countries (Spain and Brazil). Moreover, the accuracy of the lab-made potentiometric device was similar to that achieved with ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry as well as to those previously reported for voltammetric electronic tongues. Furthermore, it was also verified the possibility of using this potentiometric device, as an indirect tool, to assess the levels of olive oil volatile compounds, based on the signals profiles recorded during the analysis of the olive oil liquid extracts. Although, the accuracy achieved was lower compared to the gas chromatography-mass spectrometry with headspace solid-phase microextraction, the information gathered by the electronic tongue enabled extracting preliminary insights regarding the volatile composition of the Arbequina olive oil. Thus, considering the low-cost, the analysis time and the simplicity of the electrochemical-chemometric procedure, together with the versatility of evaluating in a single assay the levels of both phenolic and volatile compounds, the proposed approach can be envisaged as a helpful analytical tool for olive oil analysis.

Conflicts

The authors declare no competing financial interest.

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CAPÍTULO 5

Discussión general

CAPÍTULO 5: Discusión general

Durante los últimos años, Brasil ha experimentado un aumento creciente en el consumo de aceite de oliva virgen, promovido por el interés del consumidor hacia un producto saludable y con unas excelentes características organolépticas. Si bien hoy día la mayor parte del AOV consumido en Brasil es importado, el incremento del consumo ha estimulado una producción propia, actualmente incipiente pero que empieza a desarrollarse a gran velocidad. Hay que tener en cuenta, por otra parte, que las especiales condiciones climáticas y geográficas de Brasil, un país de considerable extensión y clima diverso, podrían influir en las características de los aceites producidos, hecho que ya se ha observado en estudios anteriores con la expansión del cultivo del olivo en otros países (Torres y col., 2009; Rondanini y col., 2011). Todas estas circunstancias hacen necesario un estudio completo del AOV brasileño, con objeto de ofrecer al consumidor un producto de características conocidas, que además, sea competitivo en el mercado oleícola internacional. Las exigencias del sector hacen que actualmente, además de establecer los parámetros de calidad reglamentados para los aceites de oliva, sean convenientes estudios más amplios que analicen los componentes minoritarios o las propiedades antioxidantes, que van a repercutir en los efectos saludables del aceite y a su vez van a suponer un estímulo más para su consumo. Bajo estas premisas surgió el presente trabajo, que ha pretendido llenar en parte un vacío existente en la literatura científica.

Los aceites brasileños analizados en esta memoria, corresponden a dos zonas de producción, Río Grande do Sul y Minas Gerais, situadas respectivamente al sur y al suroeste del país, sometidas a condiciones climáticas y situaciones geográficas distintas. Los aceites españoles proceden de 9 de las regiones productoras del país y están distribuidas prácticamente por toda la geografía española. Aunque España sea un país relativamente pequeño, se dan en él diferentes climatologías y topografías. Por otra parte, ambos países están enormemente distanciados y localizados en diferentes hemisferios. Con este escenario, la presente memoria de tesis doctoral pretendió realizar un estudio comparativo amplio de los parámetros de calidad, composición y características organolépticas de aceites de la variedad Arbequina producidos en Brasil y procedentes de diferentes regiones españolas. Se intentaron unificar otros factores esenciales en la calidad y composición de los aceites, tales como la variedad

(Arbequina) etapa de recogida de la aceituna (fase temprana de recolección) y sistema de extracción del aceite (extracción en dos fases).

Todos los aceites analizados en el presente estudio, tanto españoles como brasileños, cumplieron los requisitos establecidos por la legislación europea (CEE/2568/91) para su clasificación como AOVE (artículo 1, Tabla 2) en cuanto a acidez (no superior a 0.8% expresado en ácido oleico), índice de peróxidos (inferior a 20 mEq O2/kg), coeficientes de extinción específica y perfil de ácidos grasos (el contenido en oleico varió entre 63 y 80%). Además, el panel oficial de cata confirmó la misma categoría para todas las muestras, según la evaluación sensorial (mediana de defectos igual a 0 y mediana de frutado superior a 0) (artículo 5). Por tanto, de acuerdo con nuestros resultados, el origen geográfico de las muestras no perjudicó la calidad global de los aceites, como ya había sido sugerido por otros autores (Dabbou y col., 2009), ya que todos fueron clasificados como virgen extra. Ahora bien, aunque dentro de los límites establecidos, se observaron diferencias significativas entre los distintos aceites en los parámetros mencionados de grado de acidez, índice de peróxidos y coeficientes de extinción K_{232} y $K_{270.}$ Además, se mostró que dichas diferencias tenían relación con las condiciones geo-climáticas de las áreas de producción, ya que se encontraron correlaciones significativas positivas de las variables mencionadas con el nivel de precipitaciones y la temperatura media anual, y negativas con la altitud.

La composición en ácidos grasos de los aceites, de gran impacto en la calidad de los mismos y en sus propiedades saludables (Rueda y col., 2014), presentó ciertas diferencias significativas entre las muestras de las distintas zonas geográficas. Ahora bien, cuando las diferencias eran estudiadas agrupando los aceites según el país de procedencia, el perfil de los ácidos grasos mayoritarios no se vio afectado (artículo 1, Tabla 4). Es de destacar que se confirmó la relación entre la composición en AG de las muestras y su estabilidad oxidativa (Reboredo-Rodríguez y col., 2014; Reboredo-Rodríguez y col., 2015), ya que se observó que los aceites con mayor contenido en AGMI y menores valores de AGPI y AGS eran más resistentes a la oxidación, medida por el método Rancimat.

Además, se detectó que la altitud y la temperatura de las zonas productoras podrían afectar al perfil lipídico de las muestras, lo cual puede estar asociado a que la temperatura es capaz de regular la actividad de las enzimas desaturasas y por tanto juega

un papel esencial en el perfil lipídico de los aceites (Torres y col., 2009; Hernández y col., 2011; Rondanini y col., 2011).

El color de los aceites, aunque no se incluye entre los parámetros de calidad, juega un papel importante en las preferencias y la elección por parte del consumidor (Moyano y col., 2010) y es el resultado de dos pigmentos principales: clorofilas y carotenoides, que a su vez están relacionados con la estabilidad oxidativa del aceite (Criado y col., 2008). El presente trabajo puso de manifiesto que el color de los AOVE brasileños y españoles (medido por las coordenadas CIELAB) era significativamente distinto, ya que las muestras procedentes de Brasil tuvieron menos luminosidad (L*) y tonalidades más alejadas del verde (valores a* superiores) y del amarillo (valores de b* inferiores) que los aceites españoles. Precisamente el nivel de precipitaciones, mucho más elevado en las zonas de Brasil, resultó estar estrechamente asociado a las tres coordenadas de color. Es conocido que el consumidor asocia el tono verdoso y amarillo-dorado con los aceites de calidad, y el color amarillo más claro con los aceites refinados y de menor calidad (Gandul-Rojas y col., 2013). Por tanto, la cantidad de lluvia es una circunstancia importante a tener en cuenta en la expansión del cultivo del olivo.

El análisis multivariante de las variables analizadas hasta ese momento, mostró que los aceites podían ser clasificados en base a dos componentes principales: el primero, claramente definido por la composición en AG, principalmente la proporción de ácido oleico y la relación AGMI/AGPI, y el segundo, por los parámetros de color. En base a ambos componentes, se obtuvo una representación gráfica en la que los aceites brasileños estaban separados de los españoles, pero también se observó una separación evidente entre los aceites españoles. Es decir, las diferencias en los parámetros estudiados, asociadas en muchos casos a factores climáticos y geográficos, permitieron la clasificación de las muestras según la zona de producción, independientemente del país de origen.

Además de la fracción mayoritaria saponificable, existe en el aceite de oliva una fracción insaponificable formada por componentes minoritarios que, aunque solo supone aproximadamente un 2%, contiene multitud de compuestos, muchos de ellos esenciales para la calidad y las propiedades de los aceites (Lopez et al., 2014). Por su especial importancia, han sido investigados en este trabajo los compuestos fenólicos, los tocoferoles y la CoQ_{10}.

Los aceites analizados mostraron cantidades de CoQ_{10} entre 48 y 85 mg/L, cifras que permiten que sean clasificados como "alimentos ricos en CoQ_{10}" (Pravst y col., 2010). La importancia de este hecho radica en que la CoQ_{10} es un compuesto esencial para la cadena respiratoria mitocondrial y al que se le han atribuido muchas propiedades saludables (Pravst y col., 2010; Jankowski y col., 2016), pero cuya síntesis endógena disminuye progresivamente con la edad, hecho que ha de ser compensado con la ingesta en la dieta (Žmitek y col., 2014). Por tanto, los aceites Arbequina evaluados pueden ser propuestos como buenas fuentes alimentarias para mantener los niveles de CoQ_{10} en el organismo.

El contenido en tocoferoles mostró cifras similares a las observadas en estudios previos con aceites Arbequina españoles y de otros países, como Argentina, Túnez y Turquía (Beltrán y col., 2010; Dabbou y col., 2010; López-Cortés y col., 2013; Torres y col., 2009; Uluata y col., 2016; Yousfi y col., 2012), entre 92 – 208 mg/kg para la fracción α-tocoferol; y, al igual que en el caso del CoQ_{10}, los aceites brasileños tuvieron valores intermedios entre los de aceites españoles. Según nuestros datos, el aceite que presentó los valores inferiores de α-tocoferol tuvo también una estabilidad oxidativa menor (artículo 1, artículo 2, muestra 4 - Cádiz), lo cual confirmó que el α-tocoferol, fracción mayoritaria de los tocoferoles en los AOV, no solo proporciona un enorme valor nutricional al aceite, sino que contribuye a la estabilidad del mismo, protegiéndolo de la oxidación.

Los compuestos fenólicos del AOV han recibido en las últimas décadas una gran atención ya que, además de estar estrechamente relacionados con las características sensoriales, se consideran responsables de muchas propiedades beneficiosas sobre la salud, hasta el punto de que su efecto protector sobre el estrés oxidativo ha sido reconocido oficialmente por la Agencia Europea de seguridad Alimentaria (EFSA, 2011). El análisis de compuestos fenólicos sigue siendo hoy día un desafío entre los investigadores, ya que no existe un método estandarizado de forma oficial, y las diversas metodologías empleadas para separar, identificar y cuantificar estos componentes suelen mostrar datos variables que hacen difíciles las comparaciones (Ballus y col., 2015). En el presente estudio se identificaron tres grupos principales de compuestos fenólicos: flavonoides (apigenina, luteolina), ácidos fenólicos (naringenina, ácido p-cumárico, ácido vanílico) y alcoholes fenólicos (hidroxitirosol). En nuestras condiciones experimentales se identificó un flavonoide, la naringenina, no detectado

hasta la fecha en aceites de Arbequina al que se le han atribuido efectos beneficiosos en el tratamiento de la osteoporosis, cáncer y enfermedad cardiovascular (Patel y col., 2014). Por otra parte, se encontraron valores muy variables de hidroxitirosol (3-1050 µg/kg), uno de los polifenoles más importantes de los AOVE por su reconocida capacidad antioxidante (Servili et al., 2014) y su capacidad para actuar como estimulante de otros antioxidantes endógenos, como la vitamina C (Lopez-Huertas y Fonollá, 2017). Especialmente llamativa fue la diferencia del contenido en hidroxitirosol entre los aceites brasileños, ya que los valores en las muestras de Rio Grande do Sul superaron en más de 10 veces los del aceite procedente de Minas Gerais (artículo 2).

Hay que destacar que los flavonoides fueron el principal grupo detectado en los aceites estudiados (93-36% del total), seguido de los ácidos fenólicos (10-30% del total), con excepción de la muestra 3 (aceite de Málaga), en la que el alcohol fenólico hidroxitirosol contribuyó con 83% del total de compuestos fenólicos.

Las diferencias encontradas en los componentes minoritarios entre los distintos aceites analizados, nos animaron a estudiar la posible influencia de las condiciones geo-climáticas de las zonas de producción en dicha fracción, que ya había sido propuesta por algunos autores (Dabbou y col., 2009; Bakhouche y col., 2013; Ilyasoglu y col., 2016). Se observó, por una parte, una relación positiva de la altitud con los parámetros estudiados: CoQ_{10}, tocoferoles y algunos de los compuestos fenólicos, especialmente el hidroxitirosol. Por otra parte, la cantidad de precipitaciones pareció tener un efecto negativo en la mayoría de los componentes minoritarios estudiados, mientras que la relación con la temperatura media de las zonas fue en general positiva. Estos hallazgos confirmaron que la fracción no saponificable, además de depender en gran medida de la variedad de la aceituna, se ve afectada por otros factores, entre los que están las condiciones climáticas y la altitud de las áreas de cultivo. Este hecho puede ser debido a que el clima tiene un efecto primordial en las reacciones enzimáticas que se desarrollan durante el crecimiento y la maduración del fruto, particularmente en las que son esenciales para la síntesis de componentes minoritarios como los tocoferoles o polifenoles (Ilyasoglu y col., 2016). Se ha sugerido que el incremento de estos compuestos podría ser un mecanismo de defensa de las plantas ante condiciones de estrés (Beltrán y col., 2010; Ilyasoglu y col., 2016). De todas formas, las relaciones entre los distintos factores geo-climáticos son complejas y el efecto de uno de ellos

puede verse compensado por otro; tal es el caso de la altitud, cuya influencia puede no ser definitiva si se ve contrarrestada con el efecto de otros factores..

Con el objeto de promover una aproximación entre los aceites con características similares, de nuevo se aplicaron análisis quimiométricos (artículo2, figura 2). El dendrograma fue capaz de definir tres grupos y se observó que la muestra brasileña 10 (Río Grande do Sul) mostró según los parámetros evaluados la mayor distancia euclidiana con los otros grupos, es decir, menores similitudes. Igualmente, el análisis factorial permitió explicar el 76% de la variación obtenida en el estudio y además, confirmo la separación la muestra 10 y el impacto del contenido de hidroxitirosol en la clasificación de las muestras en el presente estudio.

Como se ha comentado en esta memoria, las propiedades antioxidantes son uno de los aspectos más valorados en los alimentos, ya que existen hoy día multitud de patologías relacionadas con un aumento de radicales libres, situaciones en las que los antioxidantes de la dieta cobran especial importancia, particularmente desde un punto de vista preventivo. Entre los alimentos con un mayor potencial antioxidante está el AOVE, reconocido desde hace años por sus propiedades beneficiosas sobre la salud. Por estos motivos, nos propusimos en esta memoria el estudio del potencial antioxidante de los aceites de oliva de las diferentes zonas estudiadas, aportando por la primera vez datos sobre las propiedades antioxidantes de los aceites producidos en Brasil (artículo 3).

Las propiedades antioxidantes de los aceites se estudiaron en paralelo con el contenido de polifenoles totales, por su estrecha relación con la actividad antioxidante. Para la evaluación de dichos aspectos del AOV Arbequina de diferentes zonas de Brasil y España se aplicaron diferentes métodos.

En primer lugar, se estudió la actividad antioxidante y el contenido en fenoles en los extractos químicos obtenidos mediante disolventes orgánicos de los aceites. Pero además, ya que la primera condición de un componente de la dieta para ejercer un efecto biológico *in vivo* es el mantenimiento de sus propiedades durante el proceso de digestión (Pastoriza y col., 2011), se analizaron dichas propiedades tras someter a las muestras a un proceso de digestión *in vitro* en dos fases, gástrica e intestinal. Tras este proceso, se obtienen dos fracciones, una soluble o bioaccesible, que contiene los componentes disponibles para ser absorbidos por las células intestinales, y otra no soluble o residual. Ambas fracciones fueron analizadas y la fracción bioaccesible se

utilizó, además, para posteriores ensayos de absorción a través de células Caco-2 y para el estudio de marcadores antioxidantes a nivel celular (Figura 1, artículo 3).

El contenido de polifenoles totales en los extractos varió en un rango de 75 – 302 mg de equivalentes de ácido cafeico/ kg de aceite, cifra similar a estudios anteriores en aceites de oliva (Borges y col., 2015; Uluata y col., 2016). Con respecto a la actividad antioxidante determinada en los extractos químicos, se encontraron diferencias significativas entre los aceites evaluados, pero no sé detecto una diferencia global entre los países. Además, se observaron correlaciones entre los parámetros analizados en los extractos químicos de las muestras y los factores climáticos/geográficos, destacando la encontrada entre la temperatura máxima de la zona con el contenido de polifenoles totales (r=0.801) y las propiedades antioxidantes, determinadas mediante los métodos ABTS, DPPH y FRAP.

La digestión *in vitro* promovió un incremento en la actividad antioxidante evaluada y en el contenido de polifenoles totales respecto a los resultados encontrados en los extractos químicos (artículo 3, Tabla 1-2), apoyando estudios anteriores en aceites realizados por nuestro grupo de investigación (Borges y col., 2015; Seiquer y col., 2015). Estas diferencias son debidas a las transformaciones biológicas del aceite durante el proceso de digestión, que al parecer suponen una liberación de los compuestos responsables de la actividad antioxidante respecto de la matriz alimentaria y que, por tanto, son esenciales para evaluar el potencial antioxidante de los aceites. Hay que señalar que, al contrario de lo observado en los extractos químicos, en la fracción bioaccesible obtenida tras el proceso digestivo de los aceites no se detectó relación estadística entre la actividad antioxidante y la cantidad total de polifenoles. Esto podría indicar que durante la digestión se producen otros compuestos, bien liberados de la matriz o bien resultado de transformaciones intestinales, que también son responsables de las propiedades antioxidantes.

Por otro lado, se debe destacar que en el presente estudio se detectaron en la fracción residual tras la digestión niveles significativos de actividad antioxidante, (aproximadamente 19% ABTS, 7% DPPH y 39% FRAP, calculando respecto a la actividad total en la muestra digerida) y de polifenoles totales (un 20%). Por tanto esta fracción, que tradicionalmente se descarta en estudios de biodisponibilidad, merece ser tenida en cuenta en la valoración del potencial antioxidante de los aceites, ya que algunos compuestos residuales pueden ser catabolizados por la microbiota intestinal y

posteriormente ser absorbidos por el epitelio del colon (Williamson y Clifford, 2017). Además, no se descarta una acción beneficiosa a nivel local de la fracción residual, en virtud de sus propiedades antioxidantes (Martin y Bolling, 2015). La actividad antioxidante de los aceites tras la digestión se analizó también a nivel celular, estudiando los efectos sobre la proliferación celular y sobre la generación de ROS. Estas determinaciones se realizaron tras la incubación de las células Caco-2 durante 2h con la fracción bioaccesible de las muestras, y se estudió tanto el efecto en condiciones basales como la capacidad de prevención ante un estrés oxidativo inducido.

Cuando las células fueron sometidas a un estrés moderado (t-BOOH 3 mM), la preincubación con los aceites digeridos logró una recuperación significativa de la viabilidad, al comparar con las células control. Al forzar las condiciones pro-oxidantes (t-BOOH 5 mM) también se observó un efecto protector en las células previamente tratadas con las muestras, aunque en algunos casos (muestras españolas 6 y 7) la recuperación del daño celular no llegó a ser significativa (Figura 3 del artículo 3). Ante estas condiciones de estrés inducido se observó una reducción en la generación de ROS cuando las células habían sido pre-incubadas con los aceites, significativa en todos los casos (excepto con la muestra 6) al comparar con los niveles producidos en células control estresadas (Figura 4). Esto supone una respuesta positiva en la prevención del daño oxidativo, ya que la disminución de los niveles de ROS actúa como una señal biológica en la regulación del metabolismo redox celular. El tratamiento de las células con los aceites procedentes de Brasil supuso una mayor reducción de la generación de radicales libres, lo que estuvo asociado con una mayor actividad antioxidante de la fracción bioaccesible medida por el método DPPH.

A diferencia de otros estudios, realizados con los polifenoles individuales del AOV o con los extractos químicos (Chiesi y col., 2015; Rodríguez-Ramiro y col., 2011; Incani y col, 2016), nuestros ensayos suponen una aproximación más fisiológica en el estudio del papel antioxidante de los aceites a nivel intestinal, ya que se consideran las modificaciones sufridas por los mismos durante el proceso de digestión.

La absorción de compuestos fenólicos totales y las propiedades antioxidantes de los aceites de oliva Arbequina también fue estudiada en el modelo celular Caco-2. El porcentaje de absorción de los polifenoles totales fue de 32-110%, con diferencias significativas entre las diferentes regiones y entre países (97 % Brasil y 67% España). Aunque la incubación con algunas de las muestras, como las llamadas 1,2,3,4,6 dio

lugar a una absorción relativamente bajo de polifenoles totales, hay que tener en cuenta que la absorción de esos compuestos podría continuar en el intestino grueso (Pereira-Caro y col., 2015) y que otros metabolitos, potencialmente absorbibles, pueden ser generados por las interacciones con la microbiota intestinal.

No obstante, la absorción de antioxidantes a través de las monocapas de células Caco-2, evaluada por el método DPPH, mostró porcentajes de 30-52%, sugiriendo una buena biodisponibilidad de las propiedades antioxidantes, que se han mantenido tras la digestión y ha sido absorbidas por las células intestinales, aunque con diferencias estadísticas entre las muestras. Los datos científicos sobre la absorción de compuestos antioxidantes en alimentos tras la digestión es muy escasa y la información sobre cuáles son los compuestos responsables del potencial bioactivo es controvertida. Los resultados obtenidos en el presente estudio sugieren que, además de los polifenoles totales, otros compuestos bioactivos, como los tocoferoles, carotenoides o la CoQ_{10}, podrían estar implicados y posiblemente actúen de manera sinérgica y compleja.

Las características organolépticas de los aceites son determinantes en la valoración de los AOV por el consumidor y, por tanto, también merecen especial consideración de los productores. La evaluación sensorial de las muestras en este estudio supuso, además de la cata oficial por un panel acreditado, que es preceptiva para la clasificación de los aceites en las distintas categorías según la reglamentación europea (CEE, 2013) la aplicación de la lengua electrónica y la determinación de compuestos volátiles.

Las sensaciones aromáticas olfativas son atribuidas a los compuestos volátiles representados principalmente por aldehídos, alcoholes, cetonas, hidrocarbonos y esteres. Aunque su determinación no sea considerada un parámetro reglamentado juegan un papel fundamental en la clasificación comercial de los aceites de oliva en las diferentes categorías, además de representar un factor clave en la calidad y para evaluar el grado de oxidación, pudiendo afectar la percepción sensorial y su aceptación por los consumidores (Luna y Aparicio, 2002; Kalua y col., 2007; Sinesio y col., 2015). Como parte preliminar para establecer un perfil global de esta fracción, se realizó la puesta a punto de la técnica, los volátiles fueron extraídos por la técnica de espacio de cabeza (Headspace Sampling - HS) empleando la microextracción en fase sólida (SPME), y la fracción volátil fue evaluada por cromatografía de gases masa (GC-MS), teniendo en

cuenta las características organolépticas específicas del AOVE monovarietal Arbequina (artículo 4). Se utilizó para ello una muestra obtenida directamente del productor (Valladolid) y clasificada como AOVE de acuerdo con los parámetros establecidospor la legislación europea (CEE/2568/91). Se estudiaron las condiciones de análisis de extracción de compuestos volátiles (cantidad de aceite, temperatura y tiempo), aplicando con este objeto la metodología estadística de superficie de respuesta (RSM) y se optimizaron las condiciones mencionadas para el área total de los picos y para algunos de los compuestos volátiles asociados, según estudios previos, con los atributos positivos (almendras, manzana, astringente, plátano, amargo, fresco, verde-afrutado, hojas verdes, picante, dulce y tomate) (Luna y col., 2002; Angerosa y col., 2004; Luna y col., 2006), como Z-3-hexenal, E-2-hexenal, 1-hexanol, Z-3-hexen-1-ol, Z-3-hexen-1-ol acetate, hexyl Acetate y E-2-hexen-1-ol).

Los valores experimentales obtenidos para el área total y para los compuestos volátiles estudiados indicaron que la alta temperatura (50-60°C) y prolongado tiempo de extracción (30-40 minutos) fueron más efectivos para maximizar el área de los picos.

Los valores experimentales fueron similares a los valores predictivos, indicando un ajuste adecuado de los modelos matemáticos (artículo 4, Tabla 2). Por otra parte, los gráficos de superficie de respuesta confirmaron que el tiempo y especialmente, la temperatura, tienen más impacto en el área total evaluada que la cantidad de muestra, El punto óptimo considerado como la condición más favorable para maximizar el área total y de los compuestos se ha establecido en 4,6 g de AOVE Arbequina, 43 minutos y 59 °C.

Los aceites del presente trabajo fueron sometidos al análisis sensorial mediante cata por el Panel Oficial Acreditado del Laboratorio Agroalimentario de Granada (Consejería de Agricultura, Pesca y Desarrollo Rural de la Junta de Andalucía) y todos ellos fueron clasificados como virgen extra. Para las sensaciones de frutado, amargo y picante se obtuvieron valores de 4.4-6.5, 1.8-3.3 y 2.3-3.7, respectivamente.

Los compuestos volátiles, como se ha comentado, se analizaron mediante HS-SPME/ GC-MS, en las condiciones previamente seleccionadas. Se identificaron 19 compuestos volátiles en las muestras evaluadas, agrupados en 6 clases: alcoholes, aldehídos, hidrocarburos, esteres, terpenos y fenoles (artículo 5). En general, destacaron los contenidos de aldehídos y esteres, principalmente de E-2-hexanal (48-380 µg/kg) and Z-

3-hexenyl acetato (9-250 µg/kg). Estos compuestos son obtenidos por la ruta de la lipoxigenasa (LOX) y se asocian a atributos postivos como verde y frutado (Angerosa y col., 2004; García-González y col., 2010).

Tanto los compuestos volátiles como los compuestos fenólicos, también analizados en la presente Memoria, están estrechamente relacionados con las características organolépticas de los aceites de oliva. Ahora bien, el análisis de ambos requiere una infraestructura especializada y las determinaciones correspondientes suponen una gran inversión de tiempo y dinero. Como contrapunto, existen hoy día instrumentos que consisten en un conjunto de sensores electroquímicos que, con un sistema adecuado de reconocimiento de patrones, son capaces de reconocer olores y sabores; son las llamadas nariz y lengua electrónicas que, de una forma más sencilla y rápida, podrían contribuir a la evaluación sensorial de los alimentos. En los últimos años, el análisis potenciométrico de lengua electrónica ha sido propuesto como una herramienta novedosa para evaluación de la calidad y autenticidad de los alimentos (Di Rosa y col., 2017). El empleo de esta técnica tiene muchas ventajas y se ha mostrado útil en la clasificación de los aceites según su variedad, origen geográfico, características físico-químicas y/o atributos sensoriales (Oliveriy col.., 2009; Apetrei y col., 2010; Dias y col., 2014; Slim y col., 2017, Souayah y col., 2017; Veloso y col., 2018). Sin embargo, la lengua electrónica ha sido poco empleada en la evaluación indirecta de compuestos volátiles o fenólicos con el objeto de obtener un perfil organoléptico global de los aceites de oliva vírgenes.

Para la aplicación de la lengua electrónica en el presente trabajo, se utilizaron matrices potenciométricas que contenían 40 sensores, obtenidos a partir de diferentes combinaciones de aditivos grasos, plastificantes y PVC. Los sensores fueron seleccionados, además de por la estabilidad y repetitividad de sus señales, en base a su capacidad para proporcionar respuestas cualitativas y cuantitativas hacia los atributos básicos del sabor (dulce, ácido, amargo, salado y umami) y las sensaciones sensoriales positivas y negativas percibidas normalmente en los aceites de oliva (Dias et al., 2009; Slim et al., 2017; Veloso et al., 2018). Sobre las señales potenciométricas obtenidas de las diferentes muestras, se aplicaron modelo de regresión lineal múltiple, con la finalidad de estimar y/o predecir el contenido en polifenoles y volátiles, previamente determinado. Además, se seleccionaron en cada caso aquellas señales más representativas (entre 8 y 15) de entre las generadas por los 40 sensores aplicando

algoritmos metaheurísticos, y utilizando como criterio de calidad la maximización del coeficiente de determinación (R^2) y la minimización del error de raíz cuadrática media (RMSE) (Cortez, 2014). El valor predictivo del método se estableció al 95% de intervalo de confianza (IC) para los valores de pendiente e intersección de la regresión lineal simple (LR) obtenida entre los contenidos químicos predichos por la lengua electrónica versus los respectivos datos experimentales de concentración de volátiles y polifenoles.

La combinación de las determinaciones analíticas y el tratamiento estadístico mostró que la aplicación de la lengua electrónica supone una aproximación adecuada en la predicción del contenido total de polifenoles y de los niveles de polifenoles individuales, según los valores obtenidos de R^2, RMSE y la validación de interacciones cruzadas (k-fold) ($0.967 \leq R^2 \leq 0.995$; $6.2 \leq \text{RMSE} \leq 109.0$ μg/kg de aceite de oliva; $0.930 \pm 0.031 \leq R^2 \leq 0.983 \pm 0.017$ y $7.2 \pm 2.4 \leq \text{RMSE} \leq 126.0 \pm 26.6$ μg/kg de aceite de oliva). Además, el ajuste del modelo fue comprobado por los valores teóricos esperados de pendiente e intersección obtenidos en la regresión lineal.

Igualmente, se observó un gran potencial en la capacidad predictiva de la lengua electrónica para los compuestos volátiles, con valores de R^2 y RMSE adecuados. Sin embargo, hay que señalar que el poder de esta herramienta para predecir los volátiles fue menor que el observado para los polifenoles, ya que para la mayoría de los compuestos volátiles evaluados; la pendiente y los valores de intersección no fueron estadísticamente iguales a los valores teóricos esperados (artículo 5, Tabla 3).

Aun así, la posibilidad de obtener una estimación preliminar de los perfiles fenólicos y volátiles del aceite de oliva con un único ensayo potenciométrico es de un gran interés práctico. Teniendo en cuenta, además, el bajo costo y la relativa simplicidad del procedimiento electroquímico-quimiométrico, la metodología propuesta puede ser considerada como una herramienta útil en el análisis del aceite de oliva.

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CAPÍTULO 6

Conclusiones y Resumen

CAPÍTULO 6. Conclusiones y Resumen

A partir de resultados de la presente memoria de Tesis Doctoral se pueden obtener las siguientes conclusiones:

1. Los aceites de la variedad Arbequina recientemente implantados en Brasil, así como los aceites españoles evaluados en el presente trabajo, cumplen la normativa para ser clasificados como virgen extra en cuanto a los parámetros de calidad, composición en ácidos grasos y evaluación sensorial, independientemente de la zona de procedencia.
2. Entre los compuestos minoritarios determinados destacan los altos niveles de Coenzima Q_{10}, lo que sugiere que los aceites analizados son una buena fuente dietética de este compuesto. Los aceites brasileños presentan en general niveles intermedios de los componentes menores respecto a los españoles, aunque con diferencias muy marcadas entre las dos zonas productoras analizadas.
3. El proceso de digestión de los aceites promueve el aumento de la actividad antioxidante y del contenido en polifenoles totales en todos los casos, mostrando que las modificaciones a nivel digestivo son decisivas para establecer el potencial antioxidante de los mismos.
4. Los ensayos en cultivos celulares muestran que la fracción bioaccesible obtenida tras la digestión de los aceites, es capaz de ejercer un efecto preventivo ante un estrés oxidativo inducido, reduciendo la producción de ROS y protegiendo la integridad celular, en diferente medida según el origen geográfico de las muestras.
5. Los factores climáticos y geográficos de las regiones productoras pueden afectar a los parámetros estudiados, particularmente las coordenadas de color, la fracción minoritaria y la actividad antioxidante de los aceites, por lo que sus efectos merecen ser considerados en las nuevas zonas de expansión del cultivo del olivo.
6. El uso de nuevos métodos instrumentales, como la lengua electrónica, puede contribuir a la evaluación organoléptica de los aceites, ya que mimetiza los atributos sensoriales normalmente percibidos y es capaz, además, de relacionarlos con posibles compuestos responsables, como los compuestos volátiles o fenólicos.

CONCLUSIONS

The findings of the present Doctoral Thesis allow obtaining the following conclusions:

1. The Arbequina olive oils recently introduced in Brazil, as well as the Spanish olive oils evaluated in the present work, are within the limits established in the regulation for extra virgin olive oils regarding quality parameters, fatty acids composition and sensory analysis, without concerning the area of origin.
2. Among the minor compounds analyzed, the high levels of Coenzyme Q_{10} are remarkable, suggesting that oils evaluated in the present study are a good dietary source of this compound. In general, Brazilian olive oils show intermediate levels of the minor components in comparison with the Spanish oils, although with strong differences between both geographical areas studied.
3. The digestive process promote the increase of the antioxidant activity and the polyphenols content in all the cases, showing that the transformations at the intestinal level are decisive to establish the antioxidant potential of olive oils.
4. Assays in cell cultures showed that the bioaccessible fraction obtained after the in vitro digestion of oils are able to protect against an induced oxidative stress, reducing the generation of ROS and protecting the cells integrity, with significant differences according to the geographical origin of the samples.
5. The climate and geographic conditions of the producing regions may affect the parameters studied, especially colour, minor compounds and antioxidant activity. In this sense, these effects must be taken into account in newly introduced cultivation areas.
6. The use of new instrumental methods, since the Electronic tongue can contribute in the organoleptic evaluation of olives oils, since it mimics the sensory attributes normally perceived and it allows the prediction of related compounds, such as volatile and phenolic fraction.

Resumen

La producción de aceite de oliva es tradicional de la cuenca del mediterráneo, destacando España como el primer productor mundial. Por otro lado, la creciente demanda en el mercado internacional, el conocimiento de las propiedades saludables, las particulares características organolépticas de los aceites de oliva y el avance de nuevas prácticas de cultivo y producción han permitido, en los últimos años, la expansión del olivar y la producción de aceite de oliva en regiones no tradicionales, como Brasil. Actualmente, la producción de aceite en Brasil es muy incipiente comparada con las necesidades del mercado y hay escasos datos sobre su calidad y composición. Además, hay que destacar que hasta la presente fecha, hay un conocimiento científico limitado sobre las características y propiedades de los aceites monovarietales de diferentes áreas geográficas, especialmente los obtenidos en las nuevas zonas de producción. De esta forma, el presente trabajo doctoral tiene por objeto realizar un estudio amplio de los parámetros de calidad, composición y características sensoriales de aceites de la variedad Arbequina producidos en Brasil, en comparación con los procedentes de diferentes regiones españolas. Con ese propósito, se seleccionaron dos regiones productoras de Brasil y nueve regiones de España. En primer lugar, se evaluaron los parámetros de calidad (acidez, índice de peróxidos y coeficiente de extinción específica), parámetros físico-químicos (estabilidad oxidativa y color), el contenido en pigmentos (clorofilas y carotenoides) y el perfil de ácidos grasos. A continuación, se determinó la composición de la fracción minoritaria insaponificable de los aceites (tocoferoles, compuestos fenólicos y Coenzima Q_{10}). Posteriormente, se llevó a cabo un estudio de las propiedades antioxidantes y del contenido de polifenoles totales de los aceites con especial énfasis en su potencial antioxidante tras un proceso de digestión gastrointestinal, estudiando, además, los marcadores y la absorción de antioxidantes a nivel celular.

Con relación a los parámetros organolépticos, los aceites fueron sometidos en primer lugar a la cata mediante panel oficial acreditado. Se realizó también la determinación de compuestos volátiles, para lo que fue necesario optimizar las condiciones analíticas teniendo en cuenta las especificidades del aceite monovarietal Arbequina. Dentro de este apartado, se aplicó el análisis potenciómetico de lengua electrónica para estimar el perfil de compuestos volátiles y el contenido en compuestos fenólicos, empleando técnicas estadísticas multivariantes.

Los resultados obtenidos muestran que todos los aceites evaluados cumplían los límites establecidos en la normativa de la Unión Europea para aceites de oliva virgen extra (AOVE) en los parámetros reglamentados de calidad, perfil de ácidos grasos y características sensoriales. Se encontraron diferencias significativas entre las muestras de distinta procedencia y ciertas diferencias globales al agrupar los aceites por países en algunos de los parámetros evaluados, principalmente en relación al color. Los hallazgos del primer estudio revelan que los factores climáticos y el área geográfica de las zonas productoras juegan un papel importante en las variedades recién introducidas.

Asimismo, se han observado diferencias significativas entre los aceites en relación a los compuestos minoritarios. Es de destacar que todos los aceites analizados mostraron altas cantidades de CoQ_{10} (entre 48 y 85 mg/L) y el α-tocoferol supuso la fracción mayoritaria (algo más del 98%) entre los isómeros de tocoferol cuantificados. Tres grupos principales de compuestos fenólicos fueron identificados: flavonoides (apigenina, luteolina), ácidos fenólicos (naringenina, ácido p-cumárico, ácido vanílico) y alcoholes fenólicos (hidroxitirosol). Además, al igual que en el primer estudio, se observaron relaciones estadísticas entre los compuestos minoritarios y las condiciones climáticas y geográficas de las zonas productoras de las muestras, siendo de especial importancia la altitud y la cantidad de lluvias. Igualmente, los análisis quimiométricos mostraron que, considerando los componentes minoritarios evaluados, las muestras de diferentes procedencias podían ser clasificadas de acuerdo con su origen, siendo el contenido de hidroxitirosol el compuesto que tuvo mayor impacto en esta clasificación.

El estudio de las propiedades antioxidantes mostró que la digestión *in vitro* promueve un incremento en las propiedades antioxidantes de los aceites, así como en el contenido de polifenoles totales. Los ensayos realizados en cultivos celulares revelaron que la preincubación con los aceites digeridos fue capaz de prevenir el efecto citotóxico promovido por las condiciones pro-oxidantes (t-BOOH), preservando la viabilidad celular y reduciendo la generación de radicales libres. Aunque se observaron diferencias significativas en la actividad antioxidante de las distintas muestras, nuestros ensayos ponen de manifiesto que las transformaciones del proceso digestivo son fundamentales para estudiar el potencial antioxidante de los aceites.

La determinación de compuestos volátiles implicó la previa puesta a punto de la técnica de HS-SPME/ GC-MS, mediante la selección de las condiciones experimentales idóneas y aplicando la metodología estadística de superficie de respuesta (RSM). Se

establecieron los valores de cantidad de muestra, tiempo y temperatura de extracción con los que se obtenía la máxima respuesta.

Con objeto de evaluar el perfil organoléptico global de los aceites, se estudió la posibilidad de aplicar el análisis potenciométrico de lengua electrónica para la estimación del perfil de volátiles y de los compuestos fenólicos. Se utilizaron matrices potenciométricas que contenían 40 sensores, seleccionados en base a su capacidad para reproducir las sensaciones sensoriales normalmente percibidas en los aceites de oliva. Aplicando modelos matemáticos se demostró que la aplicación de la lengua electrónica supuso una aproximación adecuada en la predicción del contenido total de polifenoles y de los niveles de polifenoles individuales, y en menor medida, de los compuestos volátiles. Los resultados sugieren que la lengua electrónica es una herramienta analítica útil, ya que permite evaluar en un único ensayo potenciométrico los compuestos relacionados con el perfil organoléptico de los aceites.

El presente estudio ha supuesto una oportunidad única para la evaluación de los AOVE de la variedad Arbequina producidos en Brasil y para su comparación con las variedades españolas. El empleo de multitud de técnicas analíticas y la cooperación de diversos centros de investigación ha permitido la obtención de resultados valiosos que contribuyen al conocimiento de las características de los aceites de oliva. Esperamos que, además, este trabajo consiga favorecer su consumo y suponga un estímulo para investigaciones futuras.

Abstract

The olive oil production is traditionally from the Mediterranean area; mainly Spain is recognized as the first producer in worldwide. On the other hand, the increasing demands of the international market, the knowledge of healthy proprieties, the particular organoleptic characteristics of olive oils and the development of new cultivation practices and systems of production allowed, in the last years, the olive cultivation expansion and olive oil production in no traditional regions, such as Brazil. Recently, the Brazilian olive oil production is in an initial stage in comparison with the market necessities and there is a lack of data about the quality and composition. In addition, it is remarkable that to the present date, there is limited scientific knowledge about monovarietal olive oil characteristics and proprieties from different geographic areas, especially from those obtained from new production regions. In this sense, the present doctoral work aims to carry out a comprehensive study of the quality parameters, composition and sensorial characteristics of Arbequina olive oils produced in Brazil, in comparison with those produced in different Spanish regions. For this purpose, two producing regions of Brazil and nine regions of Spain were selected. Firstly, quality parameters (acidity, peroxide index and specific extinction coefficient), physical-chemical parameters (oxidative stability and colour), pigment content (chlorophylls and carotenoids) and fatty acid profile were evaluated. Secondly, the composition of the minor unsaponifiable fraction of oils (tocopherols, phenolic compounds and Coenzyme Q_{10}) was determined. Then, a study of the antioxidant properties and the total polyphenol content of the oils was carried out, emphasizing on their antioxidant potential after a gastrointestinal digestion process, also studying the markers and the absorption of antioxidants at the cellular level.

Regarding the organoleptic parameters, the olive oils were evaluated by an official sensory panel. Moreover, the determination of volatile compounds was also carried out, for which it was necessary to optimize the analytical conditions taking into account the specificities of the monovarietal Arbequina olive oils. Within this section, the analysis of the potentiometric electronic tongue was applied to estimate the profile of volatile compounds and the content of phenolic compounds, using multivariate statistical techniques.

The results showed that all olive oils analyzed complied the limit established by European regulation for extra virgin olive oils (EVOO) concerning quality parameters,

fatty acids profile and sensory characteristics. Statistical differences were found between samples from different origin and also between countries regarding some parameters evaluated, mainly for colour. The findings of this first study revealed that the climate conditions and geographic area play an important role in the newly introduced varieties production.

Moreover, significant differences were found between oils in relation to minor compounds. It is noteworthy that all analyzed oils presented high amounts of CoQ_{10} (48-85 mg/L) and α-tocopherol was the major fraction found (>98%) among the quantified tocopherol isomers.

Three main groups of phenolic compounds were identified: flavonoids (apigenin, luteolin), phenolic acids (naringenin, p-coumaric acid, vanillic acid) and phenolic alcohols (hydroxytyrosol). In addition, in the same line of the first study, statistical relationships were observed between minor compounds and geoclimate conditions of the production areas of samples, mainly in relation to altitude and rainfalls.

Similarly, the chemometric analysis performed, considering the minor compounds evaluated, allowed the classification of samples according to their origin, having the hydroxytyrosol content the highest impact in this classification.

The study of antioxidant properties proved that the in vitro digestion promotes an increase in the antioxidant activity of oils and in the total polyphenol content. The cell culture assays revealed that the preincubation with the digested oils was able to prevent the cytotoxic damage generated by the pro-oxidant conditions (t-BOOH), preserving cell viability and reducing the ROS generation. Although significant differences were observed in the antioxidant activity of samples, the assays performed confirmed that transformations during the digestive process are vital to study the antioxidant potential of oils.

The volatile compounds determination implied the prior development of the HS-SPME/GC-MS technique, by the selection of the optimum experimental conditions of extraction and applying statistical methodologies of response surface model (RSM). The response was obtained for the quantity of sample, time and temperature conditions were established.

In order to evaluate the global organoleptic profile of oils, it was studied the possibility of applying the electronic tongue potentiometric analysis for prediction of the volatile profile and the phenolic compounds. potentiometric signals based on 40 sensors were

used, on the basis of their ability to reproduce the sensory sensations normally perceived in olive oils. Mathematical models were carried out and it was found that the employment of the electronic tongue was an adequate approach in the prediction of the total content of polyphenols and the levels of individual phenols, but less accuracy of volatile compounds was verified. In summary, the results suggest that the electronic tongue is a useful analytical tool since it allows evaluating in a single analysis the compounds related to the organoleptic profile of the oils.

The present study has provided a unique opportunity for evaluation of Arbequina EVOO produced in Brazil and for the comparison with original Spanish variety. The employment of many analytical techniques and the collaboration of several research centres allowed the valuable results that contribute to the knowledge about the olive oil characteristics. We believed that this work could promote its consumption and encourage future research.

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