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CARACTERIZACIÓN DE COMPUESTOS FENÓLICOS DE SUBPRODUCTOS DE ORIGEN VEGETAL MEDIANTE CROMATOGRFÍA DE LÍQUIDOS DE ALTA RESOLUCIÓN ACOPLADA A ESPECTROMETRÍA DE MASAS Y EVALUACIÓN DE LA ACTIVIDAD BIOLÓGICA

Memoria presentada por

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*"However difficult life may seem, there is always
something you can do and succeed at"*

Stephen Hawking







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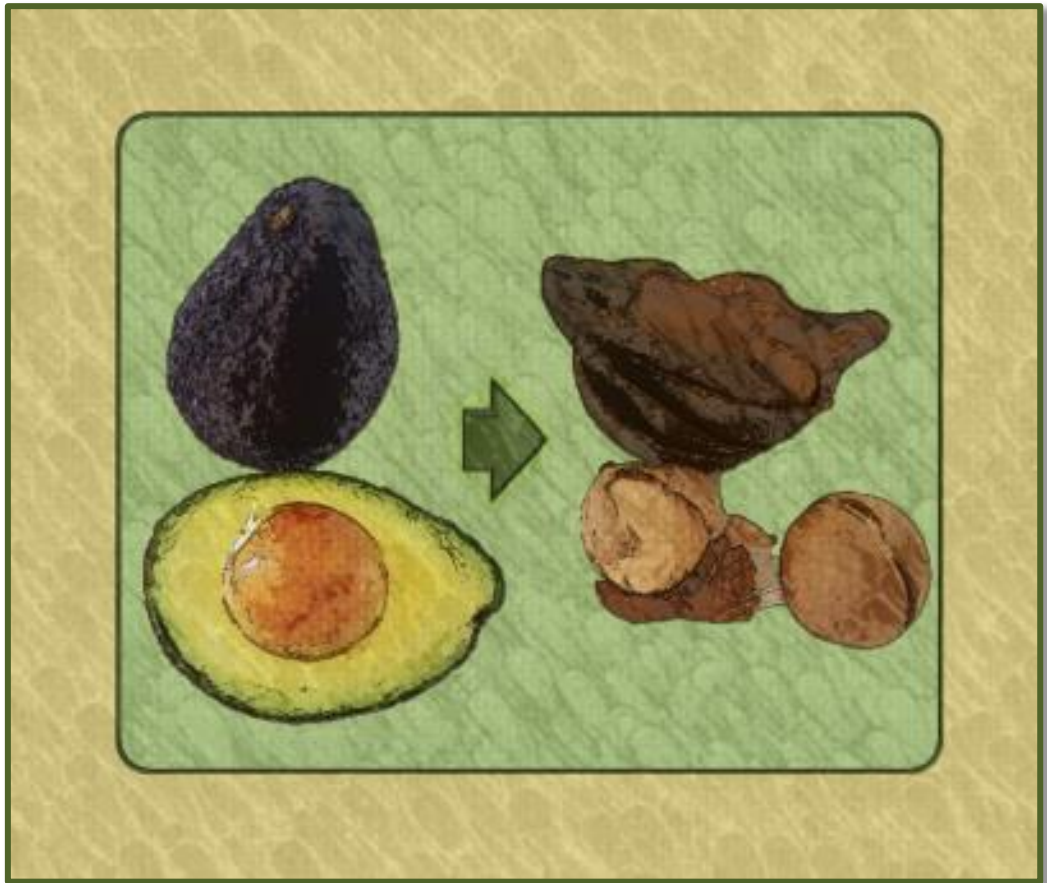
Lista de abreviaturas

Lista de abreviaturas

AAChE	→	Antiacetilcolinesterasa
AAPH	→	Hydrocloruro de 2,2'-azobis(2-amidinopropano)
ABTS	→	2,2'-Azinobis-(3-ethylbenzotiazolín-6-sulfónico)
ACh	→	Acetilcolina
AChE	→	Acetilcolinesterasa
ANOVA	→	Análisis de varianza
AOH	→	Antioxidante
ATCL	→	Yoduro de acetiltiocolina
BHA	→	Butil hidroxianisol
BHT	→	Butil hidroxitolueno
BSA	→	Albúmina de suero bovino
Buffer TRIS	→	Buffer de clorhidrato de tris (hidroximetil) aminometano
CCD	→	Diseño compuesto central
CoQA	→	Isómeros del ácido cumaroilquínico
CQA	→	Ácido cafeoilquínico
DAD	→	Detector de diodos en fila
dm	→	Materia seca
DPPH	→	2,2-difenil-1-picrilhidrazil
DTNB	→	5,5'-ditiobis-(ácido 2-nitrobenzoico)
ESI	→	Ionización por electrospray
ET	→	Transferencia de electrones
FAO	→	Organización de las Naciones Unidas para la Alimentación
FMASE	→	Extracción asistida por microondas focalizado

FQA	→ Ácido feruloilquínico
GAE	→ Equivalentes de ácido gálico
GRAS	→ Generalmente es reconocido como seguro
HAT	→ Tráferencia de átomos
HP	→ Altamente polar
HPLC	→ Cromatografía líquida de alta resolución
HPTLC	→ Cromatografía en capa fina de alta resolución
HRF	→ Ruptura del anillo heterocíclico
IC₅₀	→ Concentración de la muestra necesaria para disminuir en un 50 % la concentración inicial
IT	→ Trampa de iones
LC	→ Cromatografía de líquidos
LD₅₀	→ Dosis letal media
<i>m/z</i>	→ Relación masa/carga
MAE	→ Extracción asistida por microondas
MALDI	→ Ionización por desorción láser asistida por matriz
MP	→ Polaridad intermedia
MS	→ Espectrometría de masas
MS/MS	→ Espectrometría de masas en tándem
P	→ Polar
PLE	→ Extracción con fluidos presurizados
Q	→ Espectrómetro de masas cuadrupolo
QTOF-MS	→ Espectrómetro de masas cuadrupolo-tiempo de vuelo
RDA	→ Reacción inversa de Diels-Alder
RMN	→ Resonancia magnética nuclear

ROS	→ Radicales libres y otras especies reactivas de oxígeno
RSM	→ Metodología de superficie de respuesta
RT	→ Tiempo de retención
SFE	→ Extracción con fluidos supercríticos
SFMAE	→ Extracción asistida por microondas libre de solvente
SPE	→ Extracción en fase sólida
TE	→ Equivalente de trolox
TEAC	→ Ensayo de capacidad antioxidante equivalente de trolox
TLC	→ Cromatografía en capa fina
TOF-MS	→ Espectrómetro de masas de tiempo de vuelo
TPC	→ Contenido total de compuestos fenólicos
Trolox	→ Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
UAE	→ Extracción asistida por ultrasonidos
UHR	→ Ultra alta resolución
UHPLC	→ Cromatografía de líquidos de ultra alta resolución
UHR-MS	→ Espectrometría de masas de ultra alta resolución
UV	→ Región ultravioleta del espectro electromagnético
Vis	→ Región visible del espectro electromagnético
λ	→ Longitud de onda



Resumen

Summary

Resumen

En esta memoria se reúnen los resultados obtenidos durante la realización de la Tesis Doctoral titulada **“Caracterización de compuestos fenólicos de subproductos de origen vegetal mediante cromatografía de líquidos de alta resolución acoplada a espectrometría de masas y evaluación de la actividad biológica”**, donde se valora el potencial de los subproductos de aguacate (piel, hueso y cubierta del hueso) como fuente de compuestos fenólicos con potencial uso para el desarrollo de nutracéuticos o como nuevos ingredientes para la industria alimentaria, cosmética y farmacéutica, así como la bioactividad que éstos presentan. La memoria se ha dividido en dos secciones principales: **Introducción y Parte experimental, resultados y discusión.**

En la **Introducción** se describen, en primer lugar, una visión general de la matriz objeto de estudio, desde su origen, producción mundial, bioactividad hasta su industrialización. En este último punto se profundizó detallando el proceso de elaboración del guacamole como producto estrella derivado del aguacate, su producción en España, la generación de residuos (piel, hueso y cubierta del hueso) y el potencial bioactivo de los mismos. A continuación, se describen las diferentes etapas del procedimiento analítico para la determinación de compuestos fenólicos presentes en los subproductos de aguacate. Como primera etapa se describe el pre-tratamiento de muestra, en este sentido, debido a que las matrices evaluadas en esta Tesis Doctoral poseen una elevada cantidad de agua, se hace referencia al secado de las muestras, incluyendo una breve descripción de los diferentes métodos

existentes y su viabilidad a nivel industrial. Posteriormente, se detallan los métodos de extracción más comunes para compuestos bioactivos de matrices vegetales, entre ellos métodos convencionales y mediante técnicas avanzadas "Green" enmarcadas dentro de la conocida como Química Verde. En las siguientes secciones se profundiza en la separación y detección de compuestos fenólicos, prestando especial atención a las técnicas analíticas usadas en la parte experimental, fundamentalmente como técnica separativa la cromatografía líquida de alta resolución y como sistemas de detección la espectroscopía de absorción UV-Vis y la espectrometría de masas con analizadores de tiempo de vuelo y cuadrupolo-tiempo de vuelo. Por último, se hace un recorrido por la bioactividad de los compuestos fenólicos presentes en los subproductos de las industrias transformadoras de aguacate, prestando especial atención a su papel como antioxidantes y como inhibidores de enfermedades neurodegenerativas.

En la segunda parte se incluye la "**Parte experimental, resultados y discusión**" alcanzados en la presente Tesis Doctoral. Es importante mencionar que los capítulos no se presentan en orden cronológico. El primero y el tercero capítulo tratan de la optimización del proceso de secado y la extracción mediante líquidos presurizados (PLE) de compuestos bioactivos de piel y hueso de aguacate, respectivamente. Los otros dos capítulos restantes describen la caracterización exhaustiva de los extractos obtenidos de piel y hueso de aguacate mediante HPLC-MS.

En el **Capítulo 1**, se evaluó el efecto de la temperatura de secado sobre el contenido total de compuestos fenólicos (TPC) y la concentración individual de los compuestos

identificados en la piel de aguacate. En este sentido, se obtuvieron las cinéticas de secado para diferentes temperaturas ensayadas entre 45 y 85 °C. Además, se validó un método analítico para cuantificar los compuestos fenólicos en piel de aguacate por HPLC-DAD-ESI-TOF-MS evaluando los parámetros de linealidad, sensibilidad y precisión. Además, se utilizó la metodología de superficie de respuesta (RSM) para optimizar la extracción de compuestos fenólicos mediante el uso combinado de PLE y disolventes GRAS y comprobar así que la PLE representa una poderosa herramienta para obtener extractos de piel de aguacate de alta concentración en compuestos bioactivos. En este sentido, se eligió como variables independientes la composición de disolvente de extracción usando mezclas hidroalcohólicas de EtOH:H₂O (0–100%, v/v) y la temperatura (40–200 °C), y como variable respuesta el TPC de los extractos obtenidos. Además, se hizo una comparativa del rendimiento de extracción obtenido mediante PLE con los datos obtenidos a partir del uso de métodos de extracción tradicionales (sólido-líquido) como la maceración dinámica.

A pesar de su gran potencial, el conocimiento sobre el perfil fenólico de los subproductos de aguacate es escaso, especialmente la piel, y es por esto que en el **Capítulo 2** se realiza una caracterización pormenorizada y exhaustiva de los compuestos bioactivos presentes en el extracto de piel de aguacate obtenido mediante las condiciones óptimas de extracción y secado estudiadas en el Capítulo 1. La identificación se realizó mediante una potente plataforma analítica que aúna la excelente separación conseguida mediante HPLC con la sensible detección y capacidad de elucidación estructural del analizados QTOF-MS.

El siguiente capítulo (**Capítulo 3**) describe la optimización de PLE de los compuestos fenólicos del hueso y cubierta del hueso de aguacate. En este estudio, el hueso se secó a cinco temperaturas diferentes. A continuación, para cada muestra de hueso deshidratado, se usó una metodología de RSM basada en un modelo de diseño compuesto central 2^2 para optimizar las condiciones de PLE usando como disolventes agua y etanol. Se utilizó como variables independientes la temperatura de extracción en el rango de funcionamiento del equipo y la composición del disolvente de extracción (0–100 % EtOH), mientras que TPC fue la variable respuesta. Además, los cambios de los perfiles fenólicos de las muestras durante los procesos de secado fueron evaluados por HPLC-DAD-ESI-TOF-MS con una metodología optimizada.

Respecto al hueso de aguacate, a pesar de que algunos estudios sugieren su potencial uso como anticancerígeno, antiinflamatorio, antidiabético, antihipertensivo, hipocolesterolémico, antimicrobiano e insecticida, esta matriz no se ha caracterizado en profundidad en cuanto a su perfil fenólico se refiere. Por lo tanto, en el **Capítulo 4** se realiza un estudio pormenorizado de la composición fenólica del hueso de aguacate mediante el uso combinado de PLE y HPLC-ESI-QTOF-MS. Para ello, el extracto estudiado fue el obtenido usando las condiciones de secado y extracción optimizadas en el Capítulo 3. Además, se evaluó el potencial bioactivo de la semilla de aguacate como antioxidante usando metodologías basadas

tanto en transferencia de electrones (métodos DPPH y TEAC) así como en la transferencia de átomos de hidrógeno (método ORAC).

Finalmente, en el Anexo se recoge la evaluación de la actividad antiacetilcolinesterasa de los extractos obtenidos en los capítulos 1 y 3. Este trabajo se desarrolló con la colaboración del Departamento de Ciencia y Tecnología de los Alimentos de la Universidad de Concepción (Chile).

Summary

This section is a summary of all the results obtained during the PhD Thesis: **“Caracterización de compuestos fenólicos de subproductos de origen vegetal mediante cromatografía de líquidos de alta resolución acoplada a espectrometría de masas y evaluación de la actividad biológica”** (“Characterization of phenolic compounds from vegetable origin by-products by high performance liquid chromatography coupled to mass spectrometry and evaluation of their biological activity”), which assesses the potential of avocado by-products (peel, seed and seed coat) as a source of phenolic compound, which could be used in the pharmaceutical, cosmetic and food industries as bioactive ingredients. The report has been divided into two main sections: Introduction and the Experimental part, results and discussion.

The **Introduction** section presents a global vision of the matrix under study, from the origin, global production and bioactivity to its industrialization. Moreover, guacamole as a star product derived from avocado was described, paying special attention to the Spanish production, industrial processing and by-products generated during its manufacturing. Regarding to this last point, bioactivity of avocado by-products was detailed. Afterwards, the different stages of an analytical procedure for determining phenolic compounds used in the experimental section are described: sample pre-treatment (drying and milling), extraction (conventional and green extraction techniques), separation (HPLC and UHPLC) and several detection systems (DAD, TOF and QTOF). Finally, the bioactivity evaluation of by-

products by *in-vitro* assays to evaluate its potential use was described, focusing on their antioxidant activity and the potential to inhibit the enzyme acetylcholinesterase, related to Alzheimer's disease.

The second part includes the “**Experimental part, results and discussion**” carried out in the current PhD Thesis. It is important to mention that chapters are not presented in chronological order. The first and third chapter deals with the optimization of drying process and pressurized liquid extraction (PLE) for the recovery of bioactive compounds from avocado peel and seed products, respectively. The others recovered the exhaustively characterization by HPLC-MS of the extracts of avocado peel and seed by-products.

In Chapter 1 the effect of air drying temperature on individual phenolic compounds concentration and the total polyphenol content (TPC) of avocado peel were tested. In these sense, drying kinetics for the different temperatures tested (45–85 °C) in the convective oven were studied. As expected, increasing the drying temperature caused an important increase in the drying rate. Besides, an analytical method was validated in order to quantify individual compounds by HPLC-DAD-ESI-TOF-MS. In this sense, linearity, sensitivity, and precision parameters were evaluated. Furthermore, in order to confirm that PLE represents a powerful tool to obtain avocado peel extracts with high concentration in bioactive compounds suitable for its use in the food, cosmetic or pharmaceutical sector a response surface methodology (RSM) was used in order to optimize the extraction of phenolic compounds. In this study, solvent ratio EtOH:H₂O (0–100%, v/v) and temperature

(40–200°C) were chosen for independent variables and TPC was selected as response variable.

Although these by-products could have commercial interest due to their richness in phytochemical substances, they are usually discarded causing environmental problems at disposal sites. Furthermore, these agrowastes are promising inexpensive candidates for the recovery of bioactive compounds, especially phenolic compounds. Despite its great potential, the existing knowledge about the phenolic profile in avocado by-products is scarce, especially in peel. In this sense, **Chapter 2** provides an extend characterization of bioactive compounds present in an avocado peel extract obtained with the optimized drying and extraction process detailed in Chapter 1.

The subsequent chapter (**Chapter 3**) describes the optimization of PLE of phenolic compounds from avocado seed and seed coat. In this study, the seed was dried at five different temperatures. After that, for each dried seed sample, an RSM based on a Central Composite Design 2^2 model was used to optimize the PLE conditions. The independents variables, temperature of extraction and solvent ratio were tested in their fully operational range, whereas TPC was the response variable. Besides, the changes of its phenolic profiles during drying processes were investigated by HPLC-DAD-ESI-TOF-MS.

Regarding avocado seed, despite some studies suggested its potential as anticancer, anti-inflammatory, antidiabetic, antihypertensive, hypocholesterolemic,

antimicrobial and insecticidal, this sample matrix has not been characterized in depth. In this sense, in **Chapter 4** a comprehensive analytical characterization of phenolic and other polar compounds were performed in avocado seed and seed coat using HPLC-ESI-QTOF-MS. Previous optimized drying and PLE conditions in Chapter 3 were used for obtaining the extract. Furthermore, the bioactive potential of avocado seed as antioxidant was evaluated in term of single electron transfer mechanism (DPPH and TEAC methods) and hydrogen atom transfer mechanism (ORAC method).

Lastly, the Annex presents the evaluation the antiacetylcholinesterase activity of extracts obtained in chapter 1 and 3. This work was carried out in collaboration with the “Department of Food Science and Technology” from the University of Concepcion (Chile).



Objetivos

Objectives

Objetivos

En la industrialización del aguacate para producir guacamole, pulpa lista para su consumo, aceite de aguacate, salsas, etc., se aprovecha en el mejor de los casos entre un 60 ó 70 % del peso del fruto completo. En consecuencia, se genera una considerable cantidad de subproductos que actualmente son considerados residuos, pero que tienen un enorme potencial como fuente de compuestos bioactivos naturales. De estudios previos se conoce que tanto la piel como el hueso poseen cantidades considerables de compuestos fenólicos. Sin embargo, estas investigaciones se han llevado a cabo empleando técnicas de extracción convencionales con solventes orgánicos. Además, el perfil fenólico de los subproductos de aguacate no se ha elucidado en profundidad, al emplear en la mayoría de los casos técnicas cromatográficas de baja resolución. Por todo ello, la presente Tesis Doctoral tiene como objetivo global la evaluación de los subproductos de aguacate (piel, cubierta y hueso) como fuente de compuestos fenólicos con potencial uso para el desarrollo de nutraceuticos o como nuevos ingredientes para la industria alimentaria, cosmética y farmacéutica.

Para la consecución de este ambicioso objetivo, se han establecido una serie de objetivos específicos que se describen a continuación:

- Uno de los principales retos que conlleva el aprovechamiento de subproductos hortofrutícolas es su alta cantidad de agua, característica propicia para el desarrollo microbiano y pérdida del potencial funcional de los subproductos.

En este sentido, el primer objetivo específico es optimizar el proceso de deshidratación de los subproductos de aguacate (piel, hueso y cubierta del hueso), conservando su riqueza en compuestos fenólicos. Para ello, mediante el uso de una estufa de convección forzada, se deshidratarán los subproductos a cinco temperaturas diferentes, evaluando el efecto de la temperatura aplicada y el tiempo necesario para el secado sobre la concentración de fenoles totales y compuestos individuales mediante cromatografía de líquidos acoplada a espectrometría de masas de tiempo de vuelo.

- Por otro lado, para que los subproductos de aguacate puedan ser utilizados en diversas industrias, los extractos deben estar libres de disolventes orgánicos que limiten su uso. Es por ello, que se tiene como segundo objetivo específico la optimización de la extracción de compuestos fenólicos con potencial antioxidante mediante técnicas de extracción avanzadas “Green” , como es la extracción con líquidos presurizados (PLE), utilizando para ello disolventes GRAS como agua y etanol. En este proceso se evaluó el contenido de fenoles totales mediante un ensayo espectrofotométrico utilizado como screening o técnica de cribado para seleccionar aquellos extractos más enriquecidos en compuestos bioactivos.
- Una vez obtenidos los extractos de los subproductos de aguacate con una mayor cantidad de compuestos fenólicos es fundamental conocer en profundidad el perfil fenólico que presentan para identificar una potencial

aplicación. Es por ello, que el tercer objetivo consistió en caracterizar pormenorizadamente los compuestos fenólicos presentes en los extractos de subproductos de aguacate (piel, hueso y cubierta del hueso), mediante el uso de la cromatografía de líquidos de ultra alta resolución acoplada a espectrometría de masas cuadrupolo tiempo de vuelo.

- Finalmente, el aprovechamiento de los subproductos de aguacate podrá ser efectivo si los extractos obtenidos tienen actividad biológica destacable. Es por ello que el último objetivo consistió en evaluar la actividad antioxidante y el potencial para inhibir la enzima acetilcolinesterasa, relacionada con la enfermedad de Alzheimer, mediante métodos espectrofotométricos y un bioensayo directo en cromatografía en capa fina de alta resolución respectivamente.

Objectives

Industrially the avocado pulp is exploited principally as guacamole, pulp ready for consumption and avocado oil. Nevertheless, this manufacturing process generates a large amount of by-products, concretely avocado peel and seed, which represented up to 30 % of the fruit weight, with no commercial uses until now. Besides, despite many studies reported high concentration of extractable bioactive compounds, as polyphenols, the majority of these researches have been carried out using conventional extraction techniques with organic solvents. Furthermore, the phenolic profile of avocado by-products has not been elucidated in depth. In fact, those previous studies were carried out using in most cases low resolution chromatographic techniques. Therefore, the main goal of the current Doctoral Thesis is the comprehensive characterization avocado by-products (peel, seed and seed coat) as source of phenolic compounds with potential use for the development of nutraceuticals or as new ingredients for the food, cosmetic and pharmaceutical industries.

In order to achieve this ambitious aim, a series of specific objectives have been established, which are described below:

- One of the main challenges involved in the use of horticultural by-products is their high water activity, a characteristic that produces microbial development and loss of the functional potential of these by-products. In this sense, the first specific aim is to optimize the dehydration process of the avocado by-products

(peel, seed and seed coat), conserving its richness in phenolic compounds. For that purpose, the by-products will be dehydrated at five different temperatures in a convective oven, evaluating the effect of the applied temperature and the time necessary for drying on the concentration of total phenols and individual compounds by liquid chromatography coupled to time of flight mass spectrometry.

- In order to obtain avocado by-products extracts suitable for formulations in the food, pharmaceutical and cosmetic industries, the extracts must be free of organic solvents. Hence, the second specific objective is the optimization of the extraction of phenolic compounds with bioactive potential through advanced "Green" extraction techniques, such as pressurized liquid extraction (PLE), using GRAS solvents like water and ethanol. In this process the total phenolic content was evaluated through spectrophotometric test and it was used as a screening to select those extracts that are richer in phytochemicals.
- Once the extracts of the avocado by-products enriched in phenolic compounds were obtained, the comprehensive characterization of the phenolic profile is essential in order to identify their potential application. Therefore, the third goal was to characterize in detail the phenolic compounds present in avocado by-product extracts (peel, seed and seed coat), by using high resolution liquid chromatography coupled to quadrupole-time of flight mass spectrometry.

- Finally, the use of avocado by-products may be effective if the obtained extracts have remarkable biological activity. Therefore, the last objective was to evaluate the antioxidant activity and the potential to inhibit the enzyme acetylcholinesterase, related to Alzheimer's disease, through spectrophotometric methods and a direct bioassay with high-resolution thin-layer chromatography, respectively.



Introducción

1. Aguacate

El aguacate (*Persea americana*) es una planta perteneciente a la familia *Lauraceae* con origen en los trópicos del hemisferio occidental, desde las tierras altas orientales y centrales de México a través de Guatemala hasta la costa del Pacífico de América Central (Chen, *et al.*, 2008). Restos de semillas encontrados en antiguos asentamientos humanos en el Valle de Tehuacán sugieren que el aguacate podría haberse utilizado entre el 8.000 y el 7.000 A.C. y posiblemente fuera cultivado al menos desde el año 5.000 A.C. por grupos mesoamericanos (Galindo-Tovar, *et al.*, 2007).

Tradicionalmente en la culturas azteca, olmeca y maya se la conocía como ‘regalo de Dios’, y era usada principalmente por los aztecas como un estimulante sexual (Silva & Ledesma, 2014). En otros lugares de América del Sur es conocido por el nombre de palta, ya que los incas trajeron el aguacate de un lugar con este mismo nombre (Popenoe & Zentmyer, 1963). Actualmente, esta fruta se ha extendido desde su hábitat natural a casi todas las regiones tropicales y subtropicales del mundo (**Figura 1**).

Respecto a la evolución del cultivo, el ancestro silvestre *P. americana* fue remplazado por tres ecotipos de aguacate bien delimitados conocidos por sus sitios de origen: el guatemalteco (*Persea americana* var. *guatemalensis* Williams), mexicanas (*Persea americana* var. *drymifolia* Blake) y antillanas (*Persea americana* var. *americana* Mill) (Chen, *et al.*, 2008). No existen barreras de esterilidad entre las tres razas o entre cualquier categoría taxonómica clasificada bajo *Persea americana*.

Por lo tanto, las principales variedades comerciales son híbridos interraciales, desarrollados a partir de plántulas fortuitas, con diferentes grados de hibridación (Hurtado-Fernández, *et al.*, 2018). Entre estas variedades comerciales destacan: Bacon, Fuerte, Gwen, Hass, Lamb Hass, Pinkerton, Reed y Zutano.

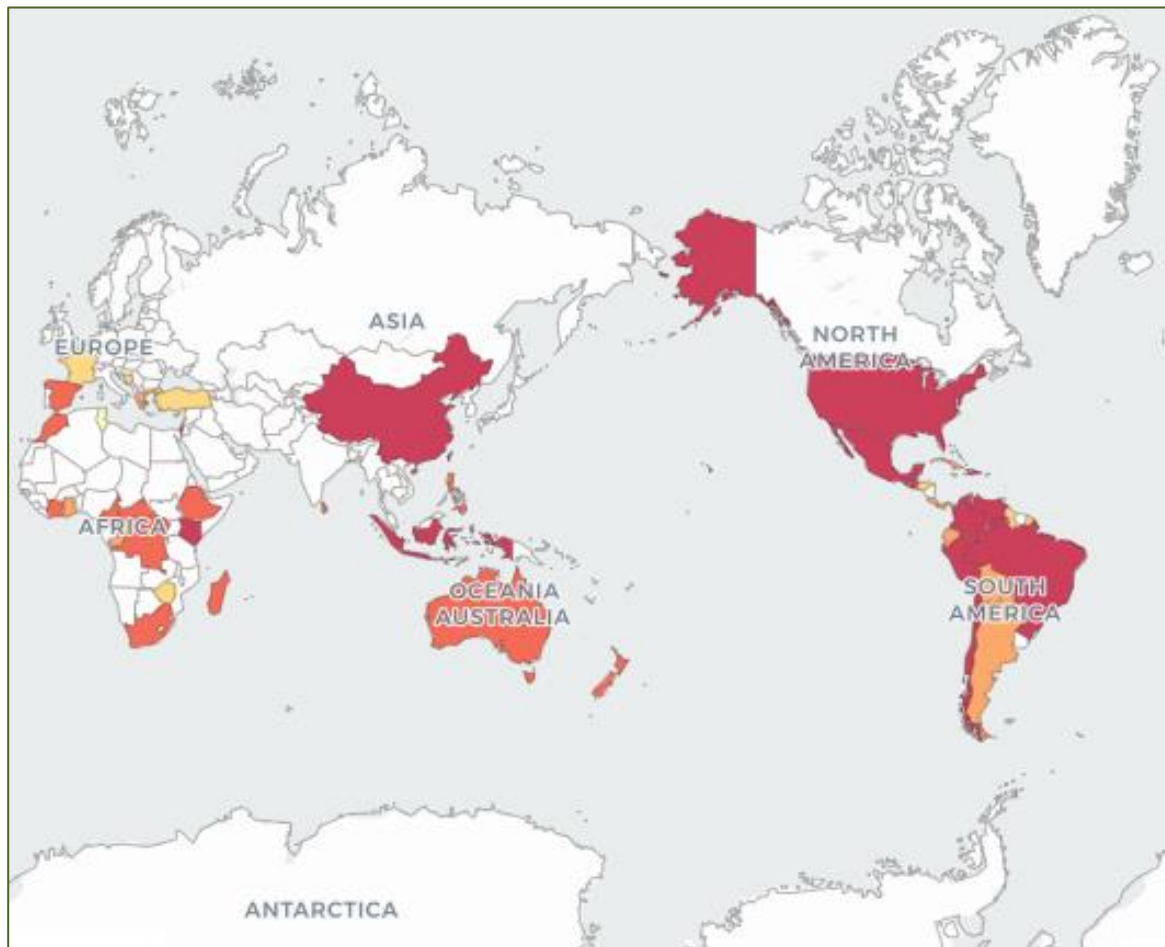


Figura 1.- Países productores de aguacate.

Fuente: FAO (2018)

En cuanto al aspecto del fruto, el aguacate puede mostrar diferentes formas (elíptica, ovalada, esférica, oblata, romboidal, globular, etc.) y muchos colores diferentes (verde pálido, verde oscuro, verde amarillento, amarillo, amarillo rojizo,

morado claro, morado oscuro y negro) en función de la variedad (Domínguez, *et al.*, 2014). Las variedades *Hass*, *Gwen* y *Pinkerton* presentan las mejores características comerciales respecto a almacenamiento, vida útil, respuesta al etileno y características organolépticas.

Sin embargo, más del 85 % de aguacate consumido a nivel mundial pertenece a la variedad *Hass* (Dreher & Davenport, 2013; Silva & Ledesma, 2014), debido principalmente a que presenta una mayor vida útil. Esta variedad es predominantemente guatemalteco (85–90 %) con algunos genes mexicanos (10–15 %) (Domínguez, *et al.*, 2014). Los frutos de esta variedad pesan entre 140 y 340 g, con una relación semilla / piel / pulpa equivalente a 16:12:72 % (Silva & Ledesma, 2014). Poseen una forma de pera a ovoide, con una piel dura y correosa, son de color verde antes de su cosecha y cambian a una tonalidad marrón oscuro o negro cuando alcanzan la madurez sensorial (fruta climatérica). Estos frutos poseen un alto contenido en aceite de entre 15 y 21 %. Además contienen una semilla más pequeña que la encontrada en la mayoría de las otras variedades (Dabas, *et al.*, 2013). Por todas estas características y por ser principalmente la más comercial, la variedad *Hass* fue la escogida para su estudio en la presente Tesis Doctoral.

La producción de aguacate en los últimos años se ha incrementado considerablemente. Especialmente, desde el año 2010 se evidencia un incremento anual del 6.2 % a nivel mundial (**Figura 2**). En el año 2016 la producción mundial alcanzó los 5.6 millones de toneladas. En ese año México fue el país con la mayor producción, en concreto 1.9 millones de toneladas, lo que representa el 34 % de la producción anual.

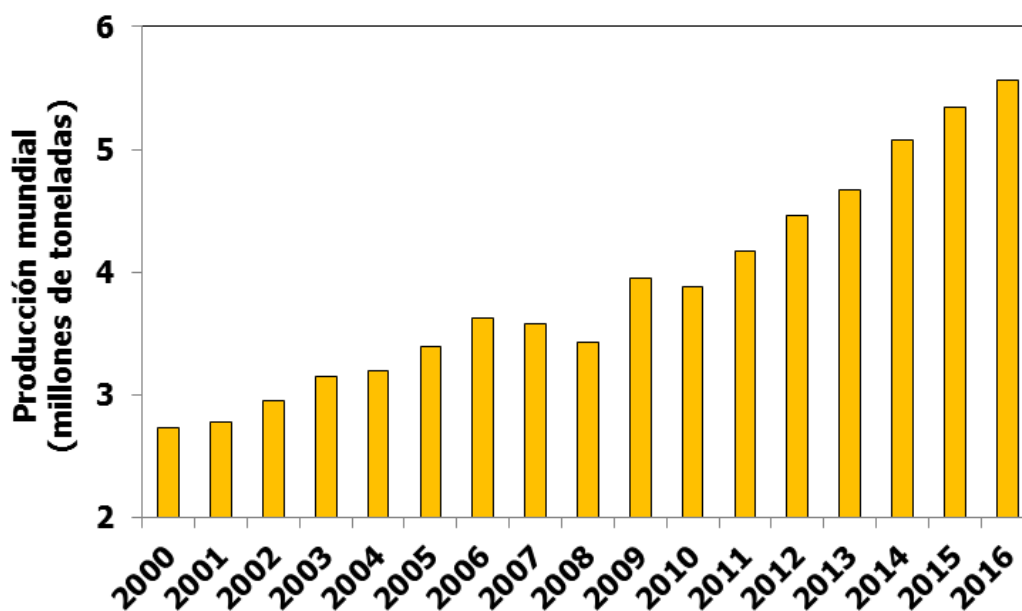


Figura 2.- Producción mundial de aguacate.

Fuente: FAO (2018)

España representa aproximadamente el 2 % de la producción mundial, con 91.000 toneladas (FAO, 2018). Sin embargo, a nivel europeo, la producción española representa el 93 % de la producción del continente, siendo Málaga y Granada las provincias más destacadas (**Tabla 1**) (Ministerio de Agricultura y Pesca, 2016).

La pulpa de aguacate posee además de unas características sensoriales únicas un potencial nutricional excelente. El tamaño de porción es habitualmente medio aguacate (68 g), lo que aporta los siguientes nutrientes: ácidos grasos monoinsaturados (6.7 g), magnesio (19,5 mg), potasio (345 mg), sodio (5,5 mg), ácido pantoténico (1.0 mg), colina (10 mg), fitoesteroles (57 mg), folato (60 mg), luteína/zeaxantina (185 µg), niacina (1.3 mg), riboflavina (0.1 mg), vitamina A (43 g), vitamina B6 (0.2 mg), vitamina C (6.0 mg), vitamina E (1.3 mg), vitamina K1 (14 µg) y fibra dietética (4.6 g) (Dreher & Davenport, 2013). Los principales ácidos grasos

presentes en el mesocarpio incluyen ácido oleico monoinsaturado (50–60 % del contenido total de ácidos grasos), ácido palmítico saturado (15–20 %), ácido palmitoleico insaturado (6–10 %), ácido linoleico insaturado (11–15 %) y ácido linolénico (cerca del 1 %) (Ferreyra, *et al.*, 2016). Por estos contenidos se considera una fuente importante de ácidos grasos, minerales, vitaminas y fibra.

Tabla 1.- Producción de aguacate en España.

Comunidades Autónomas y Provincias	Producción (toneladas)
Andalucía	80.484
Almería	19
Cádiz	4.326
Granada	30.352
Huelva	375
Málaga	45.412
Baleares	117
Canarias	9.427
Las Palmas	1.715
S.C. de Tenerife	7.712
C. Valenciana	1.461
Alicante	1.116
Castellón	60
Valencia	285
Cataluña	20
Tarragona	20
TOTAL	91.509

Fuente: Ministerio de Agricultura y Pesca (2016)

Además, estudios previos han reportado la presencia de persenona A, un compuesto al que se le atribuyen propiedades anticancerígenas, debido al potencial que presenta para inhibir las enzimas óxido nítrico sintasa y ciclooxigenasa (Dabas, *et al.*, 2013; Kim, *et al.*, 2000).

El aceite obtenido a partir de la pulpa de aguacate es conocido por su alto contenido en ácidos grasos monoinsaturados (~30 %), por lo que es utilizado en cosméticos y productos para el cuidado de la piel (Dabas, *et al.*, 2013).

En este sentido, tanto el aguacate como su aceite pueden proporcionar beneficios potenciales para la salud. Estudios previos han encontrado que el consumo de esta fruta puede ayudar a disminuir el riesgo de padecer enfermedades coronarias, cataratas, diabetes, cáncer o enfermedades cardiovasculares. Esta bioactividad demostrada se ha asociado a la presencia de ciertos compuestos en su composición, principalmente fitoesteroles como β -sitosterol, campesterol y estigmasterol, y pigmentos con potencial antioxidante como luteína, zeaxantina, β -criptoxantina, α -caroteno y β -caroteno, además de compuestos fenólicos (Ashton, *et al.*, 2006; Awad & Fink, 2000).

2. Guacamole como producto estrella derivado del aguacate

El estilo de vida de la sociedad actual, caracterizado principalmente por la falta de tiempo fundamentalmente para cocinar, ha generado un incremento de la comercialización y del consumo de alimentos preparados. En este sentido, en los últimos años ha existido una creciente producción de alimentos ricos en calorías,

apetitosos y de bajo coste, denominados ultraprocesados, los cuales han sido incorporados por los consumidores como un alimento usual en su dieta, principalmente por su fácil disponibilidad y bajo coste. Sin embargo, este tipo de alimentos son carentes de nutrientes y ricos en calorías, azúcares y grasa, que contribuyen a un incremento de la incidencia de sufrir sobrepeso de la población.

Por este motivo, la Organización Mundial de la Salud viene impulsando políticas destinadas a alentar a las personas a elegir alimentos con menor cantidad de sal, azúcar y grasas saturadas (ultraprocesados), con la finalidad de reducir la creciente tasa de enfermedades no transmisibles en todo el mundo, principalmente la obesidad, conocida como la pandemia del siglo XXI en los países industrializados. Además, una de sus metas es aumentar la disponibilidad de alimentos funcionales que puedan contribuir de manera beneficiosa a la salud del consumidor (World Health Organization, 2013). El resultado de estas políticas se puede evidenciar en que cada día es mayor la atención que prestan los consumidores hacia las características nutricionales de los alimentos en el momento de la elección para su compra (Spronk, *et al.*, 2014). Bialkova, *et al.* (2014) evaluaron el efecto de las características saludables de un producto alimenticio sobre la preferencia del consumidor, encontrando que aquellos productos más saludables tienen mayor probabilidad de ser elegidos y por tanto mayor aceptación por parte del consumidor. En este sentido, la demanda de frutas y verduras de alta calidad y mínimamente procesadas ha experimentado un aumento en los últimos años, debido por un lado a la conveniencia de este tipo de alimento (preparado y listo para consumir) y por otro al cambio en la actitud del consumidor hacia productos

más saludables (Adiani, *et al.*, 2018). Como consecuencia, una creciente demanda de alimentos funcionales y de frutas y hortalizas ya preparadas para su consumo se espera en los siguientes años.

Derivado del aguacate, el guacamole es un alimento mínimamente procesado tradicional de México obtenido a partir de aguacate. Al ser su principal ingrediente la pulpa de aguacate, posee excelentes características nutricionales que le confieren propiedades funcionales, debido principalmente a su alto contenido en ácidos grasos insaturados, carotenoides como la luteína y vitaminas como el α -tocoferol (vitamina E) o el ácido ascórbico (vitamina C). Por tanto, estas propiedades han contribuido a que el consumo de guacamole se extienda alrededor del mundo (González-Fernández, *et al.*, 2015).

En consonancia con esta tendencia actual, el mercado de salsas en España registra un significativo crecimiento tanto en volumen como en valor comercial. Como se ha comentado previamente, Andalucía es la comunidad con mayor producción de aguacate (**Tabla 1**), y específicamente Málaga es la mayor productora de guacamole con más de 6.000 toneladas al año, lo que la convierte también en la primera de Europa. En la **Figura 3** se presenta el nivel de producción de guacamole en España en los últimos años. Avomix y Montosa, son las empresas con mayor producción, con cerca de 6.000 toneladas por año y con un crecimiento de un 20 % anual gracias al aumento de la demanda. Además estas empresas se caracterizan por usar alta tecnología para lograr un mayor tiempo de vida útil del producto sin deterioro de sus características sensoriales. Las empresas Frumaco, Avos Nerja y Natural Tropic también se han incursionado en el mercado del guacamole con una producción

inferior a 1.000 toneladas por año. La mayoría de la producción se destina para el mercado local, pero cerca del 30 % se exporta, principalmente a Francia y Alemania (Peláez, 2017).

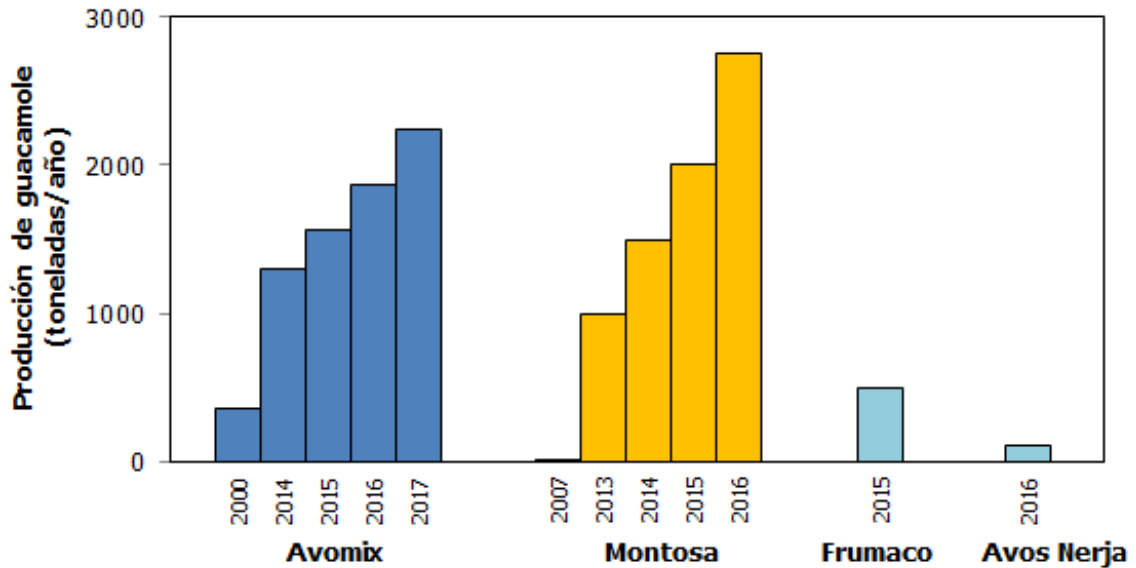


Figura 3.- Producción de guacamole en España.

Fuente: Peláez (2017)

2.1. Proceso de elaboración

Para la elaboración del guacamole, los ingredientes principales son pulpa de aguacate, cebolla, tomate, chile, limón y sal (Iturriaga, *et al.*, 2002). A nivel industrial se utilizan los mismos ingredientes pero muchos de ellos deshidratados, y con la finalidad de alargar la vida útil del producto se usan antioxidantes y conservantes. Desafortunadamente, la pulpa de aguacate es altamente inestable y su vida útil está muy determinada por el deterioro microbiano y los procesos oxidativos. Este proceso de degradación da como resultado ranciedad y la consecuente producción de sabores indeseables y pérdidas de calidad (Aguiló-Aguayo, *et al.*, 2014).

El pardeamiento u oscurecimiento oxidativo de los alimentos puede ser producto de reacciones químicas enzimáticas y no enzimáticas. Este pardeamiento enzimático es el resultado de la acción de enzimas como la polifenoloxidasas que catalizan la oxidación de mono y difenoles a o-quinonas que luego se polimerizan espontáneamente para formar polímeros de alto peso molecular de color oscuro, lo que provoca el oscurecimiento característico o la formación de manchas oscuras (Brito, 1999). La polifenoloxidasas es la responsable del pardeamiento que sufre la pulpa de aguacate (Bermejo-Prada, *et al.*, 2014), por lo tanto la inhibición de su actividad es un reto que se debe superar en la elaboración del guacamole, ya que esta enzima se activa cuando se produce la rotura de los tejidos del fruto. Hasta la fecha, se han utilizado varios métodos para lograr la inhibición de la actividad de esta enzima en frutas, por ejemplo: adición de productos químicos (sulfitos), descenso del pH, atmósferas modificadas, refrigeración y tratamientos térmicos. Específicamente en el aguacate, en los años 60, los fabricantes de guacamoles utilizaron la congelación como tratamiento de conservación, manteniendo el aroma y textura del producto durante más de un año (Gómez & Bates, 1970). Actualmente hay muchas empresas que utilizan este método de conservación combinado con antioxidantes sintéticos, pero presenta una limitación muy importante, una vez que se ha descongelado el producto este debe consumirse inmediatamente.

Por otra parte, la patente US5384147A describe un método alternativo a los anteriores donde la pulpa se expone a un alto vacío y una posterior liberación lenta del mismo, seguido de un segundo vacío antes de sellar el envase. Este proceso

permite evitar la decoloración, prolongando así la vida útil del guacamole (Hilpert, 1995).

Por otro lado, Brito (1999) patentó un procedimiento donde utiliza antioxidantes o conservantes naturales procedentes del tomate combinado con una reducción del pH. El producto final es estable durante un período de entre dieciocho y veinticuatro meses sin sufrir una oxidación o deterioro sustancial. Por su parte, Orozco y Prieto (2012) evaluaron el efecto de diferentes ingredientes (cebolla 2 % o ajo 0.5 %) combinados con el uso de ultrasonidos (135 KHz, 10 min) sobre la actividad de la polifenoloxidasa en guacamole, encontrando una reducción de entre un 4–39 % asociada a estos ingredientes. Sin embargo, el uso del ultrasonidos provocó un aumento en la actividad de la enzima, hecho previamente descrito por otros autores en pulpa de aguacate (Bi, *et al.*, 2015).

Palou, *et al.* (2000) evaluaron el uso de cuatro ciclos de altas presiones (689 MPa) con un tiempo de retención de 5 minutos cada uno, consiguiendo un valor de actividad de polifenoloxidasa residual menor al 15 %. Se ha demostrado que el procesamiento a alta presión reduce los recuentos microbianos y produce una inactivación enzimática importante sin cambios significativos en las características sensoriales del guacamole (Jacob-Velázquez & Hernández-Brenes, 2012). Actualmente, existen varias procesadoras de aguacate en Estados Unidos, México, Perú, Chile, España, Australia y Nueva Zelanda que utilizan esta tecnología (Bermejo-Prada, *et al.*, 2014). En España, empresas como Avomix y Montosa han implementado el uso de altas presiones como medio de conservación del producto,

lo que permite alcanzar una vida útil del producto de hasta 40 días sin utilizar ningún tipo de conservante.

En la **Figura 4** se presenta el proceso de producción de guacamole, que comienza con la recepción, lavado e inspección de los frutos de aguacate, que son almacenados una vez que se ha comprobado que cumplen con los criterios de calidad hasta que alcancen la madurez organoléptica. En este momento son lavados y desinfectados, cortados en mitades y se procede a separar la pulpa de la piel y el hueso. Normalmente este paso se realiza manualmente para evitar un excesivo desperdicio de pulpa, aunque en la actualidad es cada vez mayor la automatización de esta etapa en las industrias de procesado. Una vez obtenida la pulpa es transportada a un mezclador donde se incorporan el resto de ingredientes según la receta de cada industria, normalmente cebolla, ajo deshidratado, jalapeño, cilantro, azúcar, sal, conservador, antioxidantes, ácido cítrico y ácido ascórbico, (aunque la proporción depende de cada fabricante y receta). Posteriormente una vez homogenizada la mezcla, se procede al envasado y la posterior etapa de conservación. Como se ha comentado anteriormente es cada vez más frecuente el uso de altas presiones, donde los envases son sometidos presiones de hasta 600 MPa con agua fría, lo que permite eliminar la flora microbiana contaminante, conservando las características organolépticas al ser un proceso que no utiliza altas temperaturas.

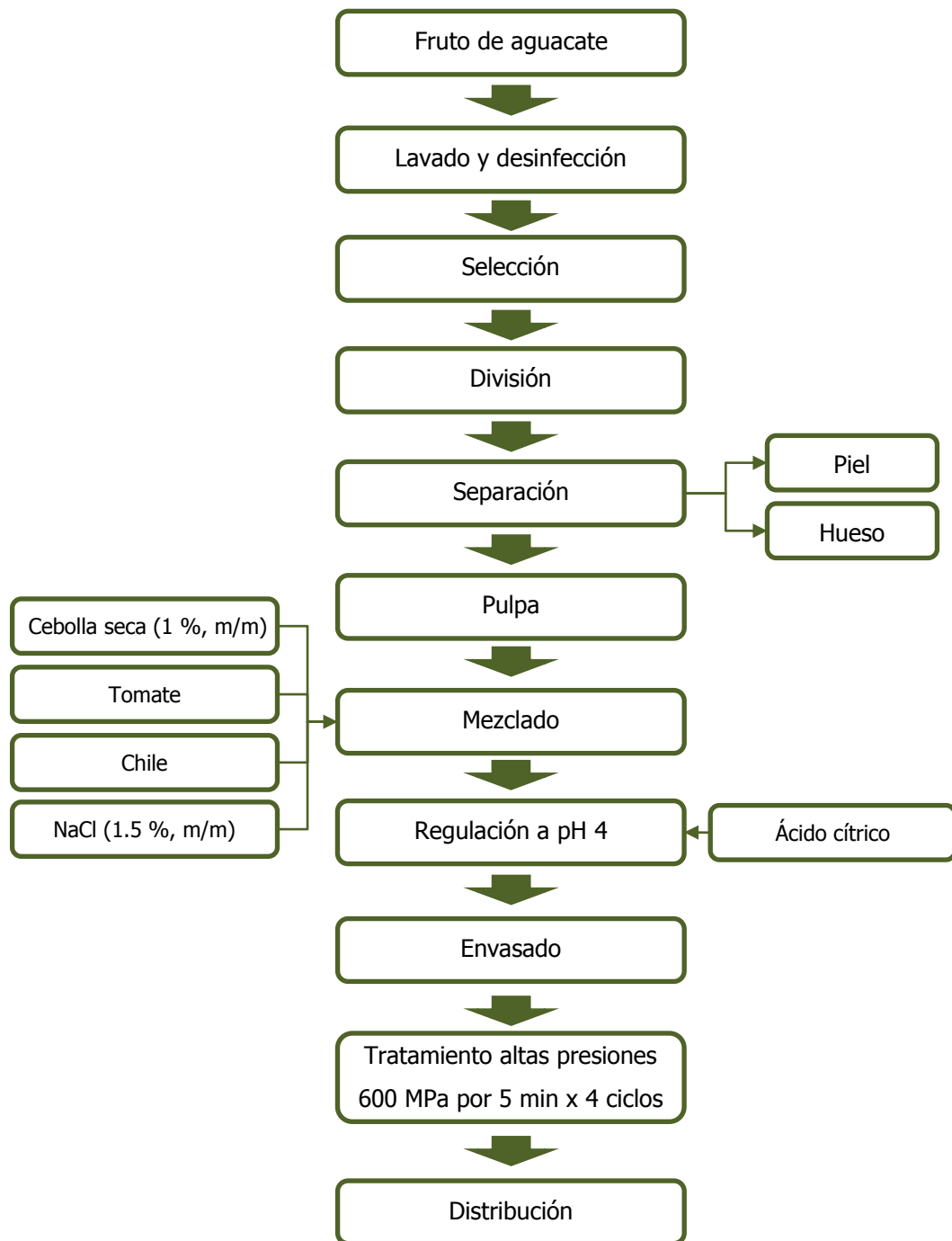


Figura 4.- Esquema del proceso de producción de guacamole.

Como consecuencia de la elaboración del guacamole, teniendo en cuenta la proporción pulpa/piel/semilla descrita anteriormente, se genera una gran cantidad

de subproductos en estas industrias correspondientes aproximadamente al 30 % del peso total de la fruta.

Estos subproductos representan un problema importante a la industria del sector, ya que deben desecharse, lo que se traduce en un gasto importante, y además esto supone un gran impacto medioambiental. Es por esta razón por la que en la presente Tesis Doctoral se utilizarán la piel y el hueso del aguacate como matrices vegetales a estudiar, como potenciales fuentes de compuestos bioactivos que actualmente se encuentran desaprovechadas.

3. Subproductos del aguacate como fuente de compuestos bioactivos

El aguacate está conformado por una cubierta denominada exocarpio o piel (13 % del peso total del fruto), la porción comestible denominada mesocarpio o pulpa (72 %), una fina capa interna que rodea la semilla correspondiente al endocarpio o cubierta del hueso (0.3 %) y la semilla o hueso (14.7 %) (**Figura 5**). La fruta del aguacate se desarrolla en el árbol y madura después de la cosecha en un proceso de maduración que tiene una duración de entre 5 a 7 días a temperatura ambiente (W. Wang, *et al.*, 2010).

La pulpa de esta fruta se consume tradicionalmente de manera directa; sin embargo, el mercado de productos industrializados del aguacate se está expandiendo en los últimos años con la comercialización de productos derivados como guacamole, pulpa lista para consumir, aceite de aguacate, salsas, etc. La producción industrializada de este tipo de alimentos conlleva la generación de una cantidad

considerable de piel y hueso de aguacate como desechos (Kosińska, *et al.*, 2012), que actualmente no son de uso comercial y se gestionan como residuos de la producción en vertederos.

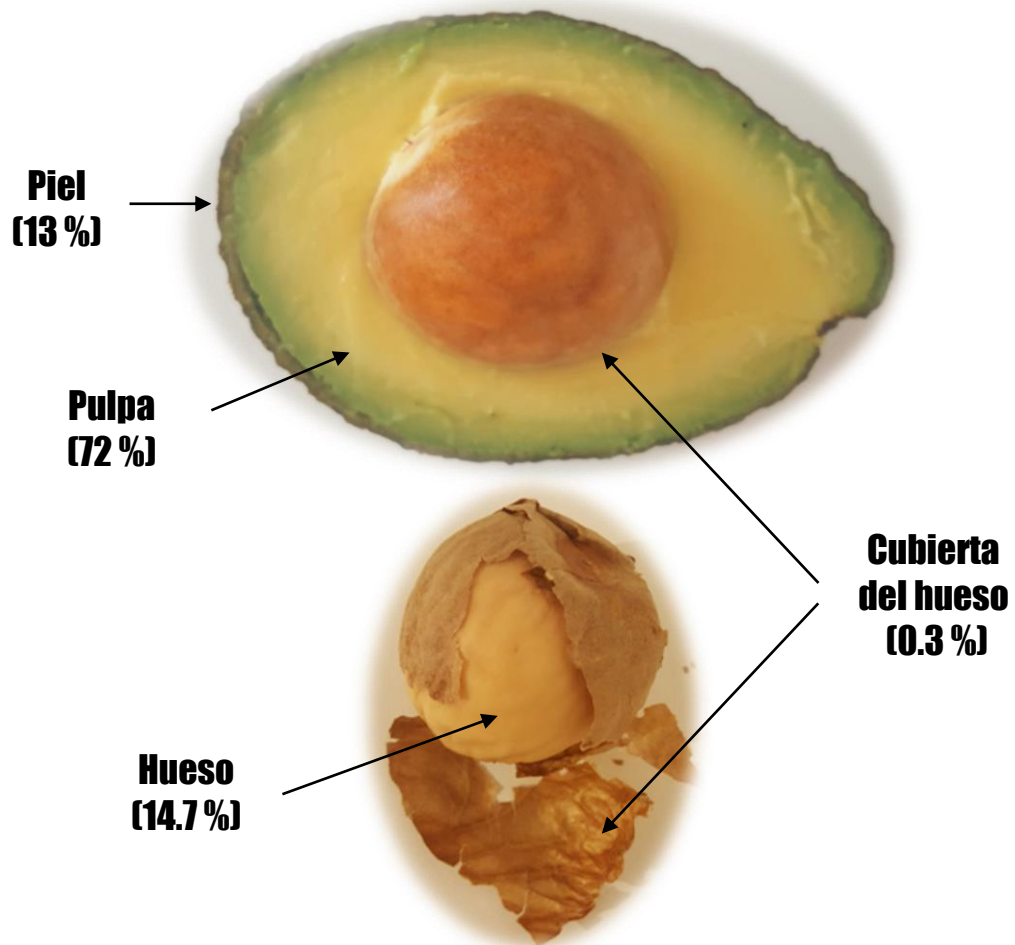


Figura 5.- Partes de la fruta del aguacate.

Como se menciona en el apartado anterior, México es el principal productor de esta fruta con 1.9 millones de toneladas, destinando un 5 % de esta producción a la elaboración de guacamole, generando por tanto un total de 95 mil toneladas de

subproductos. En España, se producen alrededor de 6 mil toneladas de guacamole, desechándose aproximadamente 2 mil toneladas de hueso y piel de aguacate. En este sentido, estos subproductos se han convertido en un problema para las industrias procesadoras debido al gran impacto ecológico que supone su desecho en vertedero. En este sentido, la gestión de estos residuos supone para las industrias del sector una pérdida económica importante debido al alto costo del transporte de estos subproductos a las áreas de eliminación (Leite, *et al.*, 2009). Sin embargo, como puede derivarse de estudios previos, estos subproductos son una fuente muy importante de compuestos bioactivos, entre los que destacan los compuestos fenólicos, carotenoides y clorofilas (W. Wang, *et al.*, 2010). A medida que crece la población mundial y aumenta la presión sobre los recursos finitos, todos tenemos la responsabilidad de adoptar el comportamiento de "reutilizar, reducir y reciclar" cuando se trata de recursos y residuos. En este sentido, los subproductos de aguacate pueden ser aprovechados como fuente de compuestos bioactivos con un valor comercial significativo. En la **Figura 6**, se resumen las principales propiedades de los subproductos de aguacate.

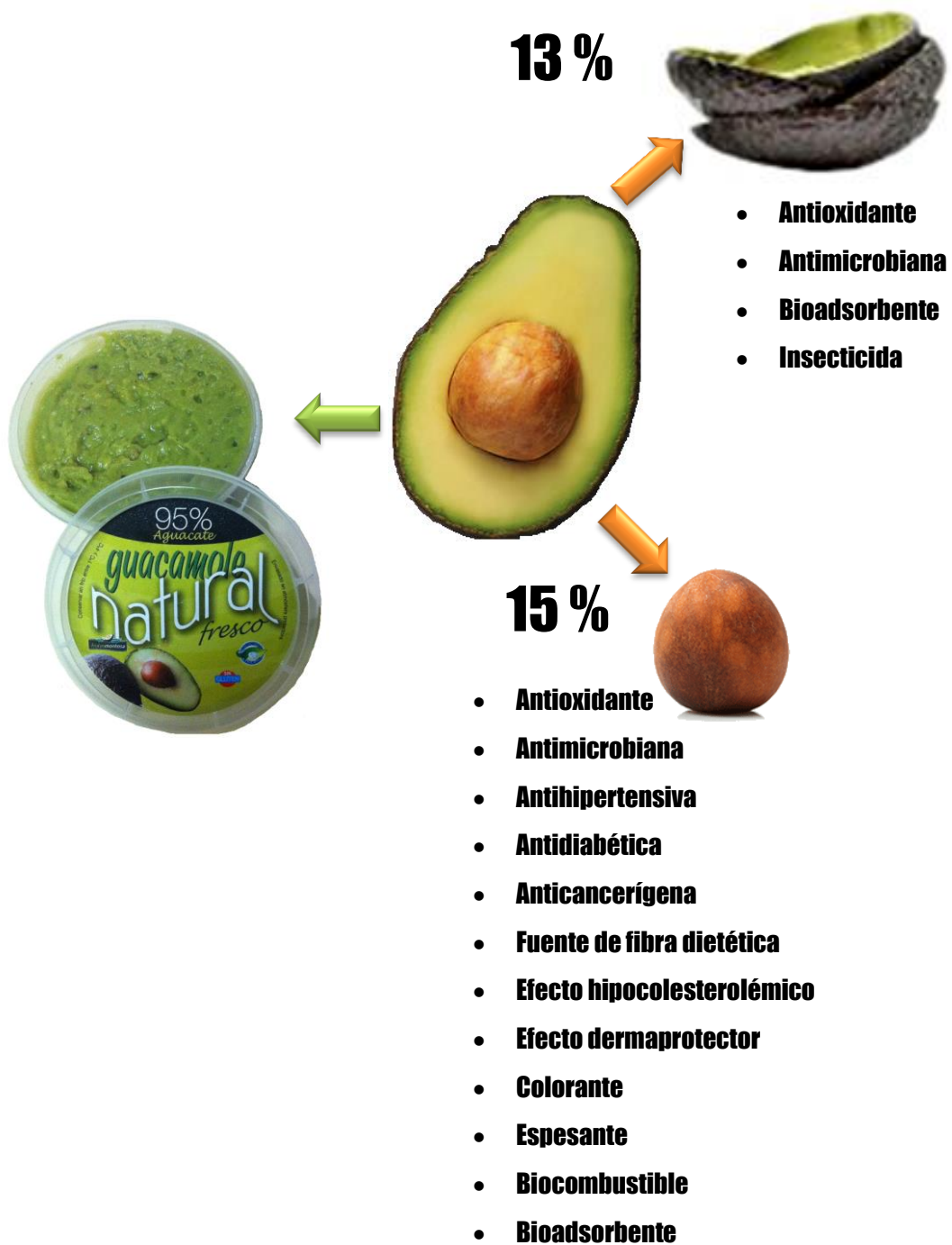


Figura 6.- Propiedades de los subproductos de aguacate.

3.1. Piel

La proporción de piel en el fruto depende de la variedad de aguacate, pero sus valores suelen estar comprendidos entre 3 y 13.5 %, siendo la variedad Choquette la que menos porcentaje de piel presenta y la variedad Booth 8 la que mayor (Domínguez, *et al.*, 2014). Respecto a la variedad Hass, ésta presenta una proporción de piel del 13 %.

De igual manera, la composición química de este subproducto, también depende en parte de la variedad del fruto. En la Tabla 2 se presenta la composición química de la piel de aguacate de las variedades *Hass* y *Fuerte*.

Tabla 2.- Composición química de la piel de aguacate de las variedades Hass y Fuerte.

Parámetro (%)	Hass	Fuerte
Humedad	75.96 ± 1.54	76.76 ± 1.16
Grasa	1.01 ± 0.39	1.91 ± 0.37
Proteína	1.77 ± 0.23	1.33 ± 0.12
Ceniza	0.85 ± 0.38	0.32 ± 0.10

Fuente: Rodríguez-Carpena, *et al.* (2011)

Actualmente, el potencial que presenta la piel de aguacate como fuente de compuestos bioactivos con numerosas actividades biológicas de interés está cada vez más patente. Estudios previos han descrito la riqueza de compuestos fenólicos presentes en esta parte del fruto, principalmente catequinas, epicatequinas y

procianidinas, siendo estos últimos compuestos oligoméricos de alto peso molecular con propiedades antioxidantes, antiinflamatorias, anticarcinogénicas, etc. Estas procianidinas de la piel de aguacate están conformadas por dímeros, trímeros y tetrámeros de catequinas y epicatequinas. Además, la piel posee ácidos fenólicos simples y flavonoles, destacando de cada familia el ácido 5-O-cafeoilquínico y la rutina, respectivamente (Calderón-Oliver, *et al.*, 2016; Kosińska, *et al.*, 2012; Rodríguez-Carpena, *et al.*, 2011; W. Wang, *et al.*, 2010; Widsten, *et al.*, 2014; Wong, *et al.*, 2016). A continuación, se describen las principales actividades biológicas descritas para esta matriz vegetal:

3.1.1. Actividad antioxidante

Actualmente existe una tendencia por la comida libre de aditivos sintéticos perjudiciales para la salud, lo que se traduce en una creciente demanda de ingredientes naturales con propiedades antioxidantes. El potencial de la piel de aguacate para la prevención de la oxidación de alimentos se ha demostrado en varios estudios previos. Se ha comprobado que el extracto de piel obtenido mediante maceración dinámica utilizando como disolvente una mezcla de acetona-agua (70:30 v/v), logra inhibir la formación de compuestos responsables de la oxidación de proteínas durante la cocción y posterior almacenamiento refrigerado de carne de porcino (Rodríguez-Carpena, *et al.*, 2011; Rodríguez-Carpena, *et al.*, 2012; Utrera, *et al.*, 2012). Además, se ha empleado la piel de aguacate para el desarrollo de biofilms en envases activos con potencial uso en la industria

alimentaria gracias principalmente al carácter antioxidante que presenta esta matriz vegetal (Fidelis, Monteiro, Scapim, Monteiro, Morais, Claus, et al., 2015).

Este efecto antioxidante se asocia a la presencia de compuestos fenólicos, principalmente procianidinas, con reconocida capacidad antioxidante que se encuentran en la piel de aguacate (Chávez, et al., 2011; López-Cobo, et al., 2016; Matsusaka & Kawabata, 2010; Morais, et al., 2015; W. Wang, et al., 2010). Estos datos apuntan a que la piel de aguacate puede utilizarse como fuente de compuestos antioxidantes que pueden ser utilizados no sólo en la industria alimentaria, sino en la industria cosmética, donde este tipo de compuestos son cada vez más buscados por su potencial para combatir los radicales libres responsables del envejecimiento de la piel (Soto, et al., 2018).

3.1.2. Actividad antimicrobiana

Al igual que ocurría en el caso de los antioxidantes naturales, existe una creciente demanda en la industria alimentaria por aquellos compuestos de origen vegetal que presentan actividad antimicrobiana. Actualmente los nitritos son los aditivos alimentarios más ampliamente utilizados para controlar el crecimiento de bacterias formadoras de esporas en las carnes procesadas. Sin embargo, estos compuestos pueden producir nitrosaminas, conocidas por ser sustancias carcinógenas. Las alternativas que ha encontrado la industria, principalmente nisina y lauroil arginato de etilo, no presentan la misma funcionalidad que los nitritos aportando además a la carne un sabor y color desagradable, y poseen limitaciones debido a su baja solubilidad y elevado coste (Rodríguez-Sánchez, et al., 2013).

En este caso la piel de aguacate ha demostrado que puede paliar en cierta medida esta creciente demanda de los consumidores e industria por nuevos agentes antimicrobianos a partir de fuentes naturales. Estudios recientes han demostrado que extractos etanólicos obtenidos a partir de la piel de aguacate mostraron actividad antimicrobiana (104.2–416.7 $\mu\text{g}/\text{mL}$) frente a bacterias Gram-positivas y Gram-negativas (excepto *Escherichia coli*); mientras que extractos acuosos sólo mostraron una actividad destacable frente a *Listeria monocytogenes* (93.8-375.0 $\mu\text{g}/\text{mL}$) y *Staphylococcus epidermidis* (354.2 $\mu\text{g}/\text{mL}$) (Raymond Chia & Dykes, 2010).

Otros autores encontraron que un extracto de piel obtenido mediante una maceración con una mezcla acuosa de acetona al 70 % presentaba un moderado efecto frente a *Staphylococcus aureus*, *Escherichia coli*, *Yarrowia lipolytica* y *Pseudomonas spp.* Este hecho se ha asociado al contenido de derivados del ácido *p*-cumárico presente en la piel de aguacate (Rodríguez-Carpena, *et al.*, 2011).

También se ha encontrado relación entre el contenido de procianidinas y el potencial sobre la inhibición de la ureasa, uno de los factores de colonización más importantes de *Helicobacter pylori* (Chávez, *et al.*, 2011). Además, se ha puesto de manifiesto que la riqueza en procianidinas y epicatequina que posee la piel de aguacate combinada con nisina, presenta un efecto sinérgico en la respuesta antimicrobiana frente a *Listeria innocua*, lo que puede permitir reducir la cantidad de nisina empleada a nivel industrial siendo sustituida en parte por sustancias naturales de origen vegetal (Calderón-Oliver, *et al.*, 2016; Calderón-Oliver, *et al.*, 2017).

Sin embargo, el potencial antimicrobiano de la piel de aguacate depende en gran medida de la madurez del fruto, factor a tener en cuenta para la futura aplicación de esta matriz. En este sentido se ha estudiado la evolución de los principales compuestos antimicóticos (1,2,4-trihidroxiheptadec-16-eno, 1,2,4-trihidroxiheptadec-16-ino y 1-acetoxi-2,4-dihidroxiheptadec-16-eno) demostrándose que la piel inmadura posee una mayor concentración de estos activos, cuyos niveles van disminuyendo progresivamente a medida que madura el fruto (Adikaram, *et al.*, 1992; Kobilier, *et al.*, 1993; Dov Prusky, *et al.*, 1994; D Prusky, *et al.*, 1982).

3.1.3. Bioadsorbente

Paralelamente a estas propiedades antioxidante y antimicrobiana se ha estudiado el potencial de la piel de aguacate para su posible uso como bioadsorbente. Mallampati, *et al.* (2015) analizaron este potencial para contaminantes disueltos en agua, encontrando un índice de eliminación de tintes e iones metálicos tóxicos superior al 95 %. De igual manera, el carbón activado obtenido a partir de piel de aguacate puede ser utilizado para la eliminación de colorantes (Palma, *et al.*, 2016) y para la reducción de la demanda química y biológica de oxígeno de aguas residuales (Devi, *et al.*, 2008). En general, la eliminación de contaminantes por adsorción en carbón activo generado a partir de piel de aguacate se presenta como una tecnología prometedora debido a su bajo coste, al proceso de producción directa, las buenas características y capacidad de adsorción comprobada del adsorbente generado, lo que se suma a la fácil disponibilidad del precursor y a la ventaja medioambiental que supondría la reutilización de este subproducto.

3.1.4. Insecticida

Los insecticidas de fuentes naturales se promocionan desde hace tiempo como alternativas atractivas a los insecticidas químicos sintéticos, considerando los peligros asociados al medio ambiente y/o la salud humana que éstos presentan (Isman, 2006). En este sentido, Torres, *et al.* (2014) evaluaron la toxicidad de los extractos de piel de aguacate obtenidos mediante extracciones convencionales con etanol y hexano contra larvas de *Aedes aegypti*, encontrando un LC₅₀ de 10.35 mg/L. Este potencial puede atribuirse a la presencia de varios grupos de fitoquímicos como alcaloides, esteroides, terpenoides, aceites esenciales y compuestos fenólicos presentes en este subproducto. A pesar de que se debe profundizar en el estudio de esta capacidad de los compuestos de piel de aguacate, estos resultados pueden considerarse un indicio del prometedor potencial de la piel de este fruto como una fuente alternativa, no tóxica y ecológica para el control del vector del dengue.

3.2. Hueso

La semilla de aguacate representa entre un 10–26 % del peso total de la fruta. Sin embargo, esta proporción depende al igual que la piel de la variedad de aguacate, siendo de un 10 % para las variedades Nabla, Pinkerton y Lorena; un 15 % para la variedad *Hass* y un 26 % para Zutano, correspondientes a las variedades de menor y mayor tamaño, respectivamente (Domínguez, *et al.*, 2014). La semilla tiene un rico perfil fitoquímico que contiene metabolitos secundarios con un efecto farmacológico y una larga historia de uso etnobotánico. En su composición destacan

la presencia de varias familias de compuestos: alcaloides, saponinas, triterpenoides y compuestos fenólicos como taninos o flavonoides.

Tradicionalmente los huesos de aguacate se han utilizado para tratar micosis, reumatismo, asma, procesos infecciosos, así como la diarrea y la disentería causadas por parásitos intestinales (Jiménez-Arellanes, *et al.*, 2013). Además, se sabe que los efectos anestésicos locales de las preparaciones de semillas de aguacate disminuyen el dolor muscular (Ramos, *et al.*, 2004). A pesar de estos usos, actualmente la semilla de aguacate se considera en gran medida un producto de desecho y es un recurso subutilizado, y al igual que la piel de aguacate podría usarse como fuente de compuestos bioactivos con numerosas actividades biológicas y aplicaciones que se describen a continuación.

3.2.1. Actividad antioxidante

Como ya se ha comentado previamente existe un problema importante en una amplia gama de industrias alimentarias debido a la oxidación de diferentes productos alimenticios. Este fenómeno puede evitarse o retrasarse mediante aditivos antioxidantes, ya sean sintéticos o naturales, de hecho el interés en estos últimos está aumentando constantemente (Garau, *et al.*, 2007).

Existen múltiples estudios que declaran que el hueso de aguacate posee una alta capacidad antioxidante, en parte debido a la presencia de compuestos fenólicos., En su composición destacan ácidos fenólicos simples como ácido cafeico, ácido ferúlico, ácido 3-O-p-cumaroilquínico e isómeros del ácido clorogénico (Calderón-Oliver, *et al.*, 2016; Kosińska, *et al.*, 2012; Rodríguez-Carpena, *et al.*, 2011; Saavedra, *et al.*,

2017; Widsten, *et al.*, 2014). Además se conoce la presencia de derivados del alcoholes fenólicos, entre ellos el hidroxitirosol glucósido y tirosol glucósido (López-Cobo, *et al.*, 2016). De igual manera este potencial se puede atribuir a la presencia de monómeros, dímeros, trímeros y tetrámeros de catequinas, compuestos reconocidos por su alto poder antioxidante (Heim, *et al.*, 2002; Perumalla & Hettiarachchy, 2011).

3.2.2. Actividad antimicrobiana

Desde los años 50, ya se conocía el potencial del hueso de aguacate como antimicrobiano. Jensen (1951) patentó un procedimiento donde sometía el hueso de aguacate a una maceración dinámica con acetona por 24 h a temperatura ambiente. Con este método se obtiene un extracto con actividad antibacteriana contra *Staphylococcus aureus*, *Bacillus subtilis*, *Aspergillus glaucus*, *Penicillium notatum* y *Achromobacter perolens*. En este sentido, Neeman, *et al.* (1970) aislaron ocho compuestos procedente del hueso y pulpa de aguacate con propiedades antimicrobianas, y describieron que el compuesto 1,2,4-trihidroxihepadec-16-eno fue el compuesto más activo, inhibiendo ciertas bacterias gram-positivas a una concentración de 4 µg/mL.

En el estudio de esta actividad otros autores han evaluado el efecto del disolvente (agua, metanol y etanol) y la temperatura usada en la extracción sobre la actividad antimicrobiana de extractos preparados utilizando una proporción fija de muestra-disolvente (en este caso 2:1, p/v). En este trabajo se encontró que la actividad antimicrobiana del extracto de metanol fue más efectiva contra *P. aeruginosa* (halo

de inhibición de 30 mm) y *St. aureus* (halo de inhibición de 20 mm); mientras que el extracto obtenido con agua a elevada temperatura exhibió un efecto antimicrobiano efectivo sobre *P. aeruginosa* (halo de inhibición de 20 mm) y *E. coli* (halo de inhibición de 14 mm). Comparados con los controles usados, estos extractos presentaron mayor actividad que la ciprofloxacina y la perflaxina, y similar a la de ofloxacina (Nwaoguikpe, *et al.*, 2011).

Hernández-Brenes, *et al.* (2012) también patentaron un método para elaborar un concentrado a partir de hueso, pulpa y hojas de aguacate con propiedades antimicrobianas contra *Clostridium*, *Bacillus*, *Alicyclobacillus* y *Listeria*. Este método se basó en una maceración dinámica durante 24 h a 35 °C, utilizando acetona como disolvente. El extracto obtenido fue purificado mediante cromatografía en contracorriente para obtener fracciones ricas en persenonas, compuestos reconocidos por su actividad antimicrobiana.

Por otra parte, Rodríguez-Sánchez, *et al.* (2013) aislaron e identificaron seis compuestos responsables del potencial antimicrobiano del hueso. Entre estos compuestos, los isómeros de persenona A, B y C fueron los principales responsables de la inhibición de *C. sporogenes*, con concentraciones mínimas inhibitorias de entre 7.8 a 15.6 µg/mL.

Estos resultados ponen de manifiesto que los extractos de hueso de aguacate exhibieron amplios espectros de actividad antimicrobiana, por lo tanto podrían tener un uso terapéutico en el tratamiento de muchas enfermedades bacterianas.

3.2.3. Actividad antihipertensiva

Los compuestos bioactivos del hueso de aguacate también han demostrado una actividad frente al tratamiento de la hipertensión. En concreto la actividad de un extracto acuoso de hueso de la variedad Fuerte sobre la presión sanguínea, fue evaluada en ratones albinos hipertensos, a los cuales se les suministró en su dieta diferentes concentraciones de extracto (200 a 700 mg/kg), encontrándose una reducción significativa en los valores de presión arterial ($P < 0.05$) con una dosis de 500 mg/kg (Imafidon & Amaechina, 2010). De esta forma, a falta de seguir profundizando en este estudio el hueso de aguacate puede jugar un papel importante en prevención de problemas cardiovasculares como la hipertensión.

3.2.4. Actividad antidiabética

Otra de las patologías que más afectan a la población actual y para la que se está buscando tratamientos alternativos a los existentes es la diabetes. Estudios previos han encontrado que el extracto acuoso de hueso de aguacate ejerció un efecto hipoglucemiante en ratones albinos, a quienes se les indujo diabetes utilizando alloxan monohidrato (Alhassan, *et al.*, 2012; Ezejiofor, *et al.*, 2013). Este estudio es un primer indicio que apunta al potencial uso que tendría el hueso de aguacate frente al tratamiento de esta patología.

3.2.5. Actividad anticancerígena

En el tratamiento del cáncer, la quimioterapia tiene el inconveniente principal de los muchos efectos secundarios incómodos para el paciente, además del alto coste del tratamiento por paciente y el bajo porcentaje de éxito que presenta en muchos

casos. Por lo tanto, en los últimos años se ha incentivado el incremento de investigaciones que evalúen la efectividad de fitoquímicos naturales como una terapia alternativa o suplementaria para combatir esta enfermedad.

Relacionado con la actividad anticancerígena del hueso de aguacate se han realizado algunos estudios, destacando el llevado a cabo por Abubakar, *et al.* (2017), quienes evaluaron el potencial anticancerígeno de los compuestos presentes en extractos etanólicos de hueso de aguacate. Para ello, tras la obtención del extracto realizaron un fraccionamiento por cromatografía en columna sobre sílica gel utilizando n-hexano:acetato de etilo (3:7, v/v) como fase móvil. Finalmente los compuestos obtenidos en las distintas fracciones fueron aislados mediante Cromatografía en Capa Fina (TLC). El efecto citotóxico de las distintas muestras (extracto crudo, fracciones y compuestos purificados) en varias concentraciones (12.5–200 µg/mL) fue evaluado sobre diferentes líneas celulares, en concreto de cáncer de mama humano MCF-7 (ATCC HTB-22) y cáncer de hígado humano HepG2 (ATCC HB-8065). Se determinó el IC₅₀ del extracto, las fracciones y los compuestos aislados por TLC, y los resultados del estudio revelaron que los triterpenoides presentes en el hueso de aguacate presentan propiedades anticancerígenas frente a estas líneas celulares.

3.2.6. Fuente de fibra dietética

La fibra dietética puede definirse como un conjunto heterogéneo de sustancias que son resistentes a la digestión estomacal y a la absorción en el intestino delgado, y como consecuencia llega inalterado al intestino grueso donde pueden sufrir una fermentación total o parcial. Esta fibra posee numerosos efectos fisiológicos en

todas las funciones del aparato digestivo, desde la masticación hasta la evacuación de heces. Algunas de sus propiedades más destacables son que actúa como agente de carga, normaliza la motilidad intestinal y previene la enfermedad diverticular. Además, algunos tipos de fibra también pueden contribuir en la reducción de la incidencia de cáncer de colon, disminuir los niveles de colesterol sérico y prevenir las hiperglucemias en pacientes diabéticos (Nawirska & Kwaśniewska, 2005).

Por esta razón, en los últimos años se han desarrollado diversos productos que contienen cantidades importantes de fibra (Garau, *et al.*, 2007). El hueso de aguacate posee una proporción relativamente alta de fibra dietética soluble, fibra detergente neutra y hemicelulosa, lo que le permite retener hasta cuatro veces su peso en agua y seis veces su peso en aceite (Barbosa-Martín, *et al.*, 2016). Por lo tanto, es un prometedor ingrediente alimentario debido al equilibrio del contenido de fibra soluble e insoluble, que puede usarse para enriquecer o suplementar alimentos que se consumen habitualmente o para la producción de alimentos ricos en fibra, como cereales, yogurt o tabletas de fibra dietética (Fernández-Ginés, *et al.*, 2003).

3.2.7. Efecto hipocolesterolémico

Uno de los factores de riesgo para desarrollar enfermedades cardiovasculares es el nivel de colesterol en sangre, siendo especialmente peligroso los niveles altos de colesterol unido a lipoproteínas de baja densidad (c-LDL). Es de especial interés la búsqueda de nuevos compuestos naturales que pueden incorporarse a la dieta del

paciente en forma de alimentos funcionales que reduzcan o prevengan estos niveles elevados en sangre.

Varios estudios han identificado que una dieta enriquecida en hueso de aguacate puede reducir significativamente los niveles de colesterol total y de colesterol LDL (c-LDL) en ratones hipercolesterolémicos (Pahua-Ramos, *et al.*, 2012; Shehata & Soltan, 2013). En este último trabajo se encontró que la inclusión de un extracto acuoso de hueso de aguacate en la dieta de ratones albinos durante 4 semanas logró disminuir significativamente los niveles de colesterol total, c-LDL y triacilglicerol. Este efecto podría atribuirse a varios factores, en concreto al contenido en compuestos fenólicos con actividad antioxidante y/o el contenido de fibra dietética de la semilla de aguacate.

3.2.8. Efecto dermoprotector

La radiación UV a longitudes de onda entre 290 y 320 nm afecta predominantemente a las células epidérmicas (queratinocitos), lo que contribuye al envejecimiento y desarrollo de cáncer de piel. Estudios previos han demostrado el potencial del hueso de aguacate para el cuidado de la piel. Werman, *et al.* (1991) demostró que ratones alimentados con componentes lipídicos procedentes de hueso de aguacate exhibieron una reducción en la actividad de la lisil oxidasa de la piel, enzima relacionada con la producción de arrugas debido a su actuación frente al colágeno (Dabas, *et al.*, 2013).

Rosenblat, *et al.* (2011) también describieron que los alcoholes alifáticos polihidroxilados extraídos del hueso de aguacate mejoran la viabilidad celular de la

piel humana después de la irradiación UV. Este potencial ha sido relacionado con la riqueza en ácido quínico, ácido clorogénico, salidroside, derivados del ácido abscísico y proantocianidinas presentes en la semilla de aguacate (Ramos-Jerz, *et al.*, 2013).

Estos efectos demostrados del hueso de aguacate tuvieron como consecuencia que Moy (1999) patentara una formulación dermatológica rica en lípidos insaponificables extraídos del hueso de aguacate que permite mejorar o hacer desaparecer la apariencia de estrías, queratosis y otras lesiones cutáneas mediante la aplicación tópica repetida a la piel afectada.

3.2.9. Aditivo alimentario (colorante y espesante)

El hueso de aguacate puede ser considerado como un potencial aditivo alimentario con varias utilidades. Por un lado es una fuente de almidón, el cual se utiliza en la industria farmacéutica como un vehículo y en la industria alimentaria como agente espesante y gelificante o como ingrediente en polímeros biodegradables para el envasado de alimentos (Chel-Guerrero, *et al.*, 2016; Lacerda, *et al.*, 2014).

Por otro lado, existe la necesidad de remplazar colorantes sintéticos por otros procedentes de fuentes naturales. Se ha encontrado que el extracto acuoso del hueso de aguacate presenta una coloración naranja en presencia de aire (con un máximo de absorción a una longitud de onda de 480 nm), alcanzando su máxima intensidad tras 35 minutos de exposición al ambiente. Un aspecto a tener en cuenta es que la formación del color depende del pH del medio. Desafortunadamente este colorante es termolábil, se ha comprobado que un tratamiento térmico a 100 °C

durante 30 min produce la desaparición de color. Sin embargo se ha observado que esta degradación puede evitarse con la adición de polifenoloxidasas exógenas. Además, se ha confirmado que la coloración de estos extractos se mantiene estable en solución a -18 °C durante un período de tiempo de hasta 2 meses (Dabas, *et al.*, 2011). Como consecuencia de estos resultados puede sugerirse el hueso de aguacate como un colorante natural para su uso en la industria alimentaria salvando ciertas limitaciones.

3.2.10. Alimentación animal

Se sabe que los aguacates de destrío que no pueden ser comercializados se han utilizado en las zonas de cultivo para alimentación animal. Estos frutos han conseguido mejorar la composición de la grasa intramuscular y aumentar la estabilidad oxidativa de la carne de cerdo refrigerada cuando ha formado parte de la dieta de estos animales (Hernández-López, *et al.*, 2016).

Por el contrario, el hueso de aguacate, a pesar de su alto contenido de almidón, no pueden aprovecharse de manera directa como alimento para ganado debido en parte a la alta concentración de polifenoles que presenta, los cuales presentan un sabor amargo siendo rechazados por el animal (Domínguez, *et al.*, 2014). Con la finalidad de superar esta limitación, Ibhaze (2017) evaluó el efecto de un tratamiento de escaldado, estudiando diferentes tiempos de ebullición sobre la composición nutricional y concentración de compuestos bioactivos en huesos de aguacate destinados a alimentación animal. En este estudio se corroboró que existe un descenso significativo en el contenido en fibra y compuestos bioactivos de estas

semillas de aguacate con este tratamiento. Sin embargo, a pesar de esta pérdida, las semillas de aguacate pueden ser incorporadas en la formulación de piensos para alimentación del ganado como fuente de energía y minerales, tras ser sometidas a un tratamiento previo.

3.2.11. Biocombustible

Otra de las aplicaciones tradicionales de las semillas de frutos es para el aprovechamiento en forma de energía calorífica mediante combustión. Perea-Moreno, *et al.* (2016) evaluó el potencial de la semilla de aguacate como biocombustible para la calefacción doméstica o industrial, concluyendo que esta biomasa posee un alto potencial, similar a la semilla del olivo o cáscara de la almendra. Además, el estudio realizado por Rachimoellah, *et al.* (2010) encontró que el biodiesel obtenido a partir de la semilla cumple con los estándares de calidad requeridos para ser considerado un combustible diésel. Desde el punto de vista ambiental, la ventaja de la producción de energía a partir de la biomasa respecto al uso de otros combustibles fósiles es que no contribuye a un aumento de los gases de efecto invernadero, ya que se considera que el balance de emisión de CO₂ a la atmósfera es neutral.

3.2.12. Bioadsorbente

En los últimos años, varios estudios han evaluado el potencial de los subproductos agrícolas para obtener carbón activado que pueda usarse como adsorbente de contaminantes presentes en agua residual como sustitutos del carbón activo generado a partir de madera, coco o huesos de animales. Se ha encontrado que el

carbón activado producido a partir de hueso de aguacate presentó mejor capacidad de adsorción de azul de metileno y fenol que el preparado a partir de mazorca de maíz y granos de ciruela (Álvares Rodrigues, *et al.*, 2011; Elizalde-González, *et al.*, 2007). Además, Devi (2010) encontró que este adsorbente permite una elevada reducción de la concentración de demanda química y bioquímica de oxígeno del efluente de una planta de procesamiento de café.

Por otra parte, Bhaumik, *et al.* (2014) realizó una carbonización química del hueso obteniendo un carbón activo con una capacidad máxima de adsorción de Cr (VI) de 333,33 mg/g a 25 °C, que es más alta que la mayoría de los adsorbentes carbonizados utilizados previamente. Además, se ha comprobado su efectividad para eliminar flúor del agua, obteniendo resultados satisfactorios que potencian su aprovechamiento (Salomón-Negrete, *et al.*, 2018). Es por estos datos tan esperanzadores por los que el hueso de aguacate puede ser considerado como un material económico para producir carbón activo, que sirva para el tratamiento de aguas residuales.

3.2.13. Insecticida

Como se ha comentado previamente, el uso de insecticidas naturales es una tendencia actual cuya repercusión es cada vez mayor. Al igual que en el caso de la piel de aguacate, los extractos obtenidos mediante maceraciones con metanol y hexano del hueso han mostrado actividad larvicida contra *Aedes aegypti* con un LC₅₀ de 8.87 mg/mL y 16.7 mg/mL, respectivamente (Leite, *et al.*, 2009; Torres, *et al.*, 2014). Adicionalmente, Nzelibé y Albaba (2014) encontraron un mayor poder

larvicida para un extracto de n-hexano (LC_{50} de 0.827 ppm), efecto que podría estar relacionado con el contenido de ácido oleico presente en las muestras. Esto posiciona a las semillas de aguacate como un posible candidato para su uso como agentes alternativos de control del dengue.

Además este efecto ha sido demostrado para otras especies, en concreto diferentes extractos metanólicos de hueso presentaron una actividad moderada ($MC_{100} >500$ mg/mL) contra *Trypanosoma cruzi*, agente etiológico de la enfermedad de Chagas, una de las patologías protozoarias más graves en América Latina. Esta actividad podría tener su origen en los derivados de los compuestos 1,2,4-trihidroxiheptadecano y 1,2,4-trihidroxinadecano presentes en dichos extractos (Abe, *et al.*, 2002).

3.3. Cubierta del hueso

La cubierta del hueso o endocarpio del fruto del aguacate es una pequeña película de color café que recubre el endospermo y embrión (**Figura 5**). El sistema vascular ramificado de la fruta termina en esta cubierta; siendo la única ruta de intercambio entre el embrión en desarrollo, el resto de la fruta y el árbol (Blumenfeld & Gazit, 1971).

Esta capa destaca en las primeras etapas del desarrollo del fruto, representando en el primer mes el 50 % del peso de la semilla y el 5 % del fruto. El embrión en esta etapa es de tamaño diminuto, con forma de varilla y está incrustado en el endospermo gelatinoso amarillo. A medida que la fruta crece, la cubierta también crece pero a un ritmo más lento que el embrión. El encogimiento de este

recubrimiento parece estar fuertemente relacionado con los procesos de crecimiento y la maduración de la fruta, el cual comienza al mismo tiempo que el embrión deja de crecer (Blumenfeld & Gazit, 1971). Cuando la fruta alcanza la madurez organoléptica, la cubierta representa aproximadamente el 0.3 % del peso del fruto completo. Además, en la fruta inmadura la cubierta del hueso es de color claro y succulenta, pero se marchita y oscurece cuando la fruta se acerca a la madurez. Por el contrario, en la fruta madura el recubrimiento de la semilla es un tejido muerto de color café oscuro y de un espesor muy fino (Blumenfeld & Gazit, 1971). Por otro parte, la concentración de citoquinas en esta cubierta; proteínas que regulan la función de las células y cuyos niveles son dependientes del estado fisiológico de la fruta, disminuye progresivamente a medida que la fruta madura. Esta característica es independiente de la variedad de aguacate (Blumenfeld & Gazit, 1970), motivo por el cual es utilizada como índice de madurez.

No existe información relativa a composición de compuestos bioactivos o actividades biológicas de esta parte del fruto de aguacate, sin embargo la información derivada de los estudios previos en hueso de aguacate completo que incluyen este recubrimiento hacen muy posible que sea una parte de los subproductos rica en compuestos bioactivos con diversas bioactividades.

3.4. Evaluación toxicológica de los subproductos del aguacate

Los subproductos de aguacate poseen un gran número de bioactividades a tener en cuenta gracias a la composición en compuestos bioactivos que presentan, por lo que pueden considerarse una buena fuente de obtención de ingredientes para su

aprovechamiento en diversas industrias. Sin embargo, un factor a tener en cuenta antes de evaluar estas aplicaciones es su toxicidad. En este sentido se han realizado evaluaciones toxicológicas y genotóxicas para establecer y verificar la seguridad antes de ser incorporados a cosméticos, fármacos o alimentos, encontrándose en estudios de toxicidad aguda dosis letales medias (LD_{50}) dependientes del disolvente de extracción usado.

Por ejemplo, para extractos etéreos, etanólicos y acuosos de hueso de aguacate en ratas se encontraron valores de LD_{50} de 0.75, 1.2 y 10 g/kg de peso corporal, respectivamente (Ozolua, *et al.*, 2009; Padilla-Camberos, *et al.*, 2013; Taha, *et al.*, 2008). Esta notoria diferencia ratifica la importancia de utilizar disolventes GRAS en la obtención de los extractos. Por otra parte, Taha, *et al.* (2008) evaluaron la toxicidad subaguda de extractos etéreos durante 14 días con dosis de 75 y 150 mg/kg, y concluyeron que este tratamiento reduce significativamente el consumo de alimentos, el peso corporal, la glucosa en sangre, la hemoglobina y los niveles de colesterol hepático de los animales de experimentación. Por el contrario, la creatinina sérica, el ácido úrico, la proteína total y la bilirrubina total no se alteraron significativamente. Además, exámenes histopatológicos del hígado y riñón al final de este estudio mostraron una arquitectura normal en estos órganos, lo que sugiere que no hay alteraciones morfológicas inducidas por el extracto de hueso. Otro estudio más reciente, analizó el potencial genotóxico de un extracto etanólico de hueso a concentraciones de 250 mg/Kg. La frecuencia de micronúcleos en los grupos de animales tratados no mostró diferencias significativas en comparación con el control negativo. Por tanto, la falta de actividad genotóxica *in vivo* de los extractos

permite esperar que el hueso de aguacate pueda usarse como un posible alimento, cosmético o material farmacéutico (Padilla-Camberos, *et al.*, 2013).

4. Determinación de compuestos fenólicos en matrices vegetales

Para el uso de los subproductos de aguacate como fuente de compuestos bioactivos que puedan utilizarse como ingrediente en cualquier aplicación alimentaria, cosmética o farmacéutica, hay una serie de pasos o etapas a los que deben someterse, siendo las principales la etapa de secado, molturación, extracción o purificación y análisis. En la **Figura 7** se ilustra la secuencia de pasos y estrategias junto con las técnicas más empleadas para compuestos fenólicos. A lo largo del manuscrito se describirán todas estas etapas centrándonos en los compuestos fenólicos, que son una de las principales familias de compuestos bioactivos que se encuentran en los subproductos de aguacate y que presentan un abanico más amplio de propiedades farmacológicas beneficiosas para la salud.

4.1. Pre-tratamiento de muestra

Previo a la etapa de extracción las muestras vegetales, especialmente las derivadas de frutos, tienen que someterse a unas etapas de pretratamiento con el objetivo de que estas muestras se encuentren en las condiciones óptimas antes de proceder a la etapa de extracción de compuestos bioactivos. En esta sección se describen dos etapas de pretratamiento de muestra que han sido abordadas en la presente Tesis Doctoral para los subproductos de aguacate, como son la deshidratación y reducción de tamaño partícula de la muestra.

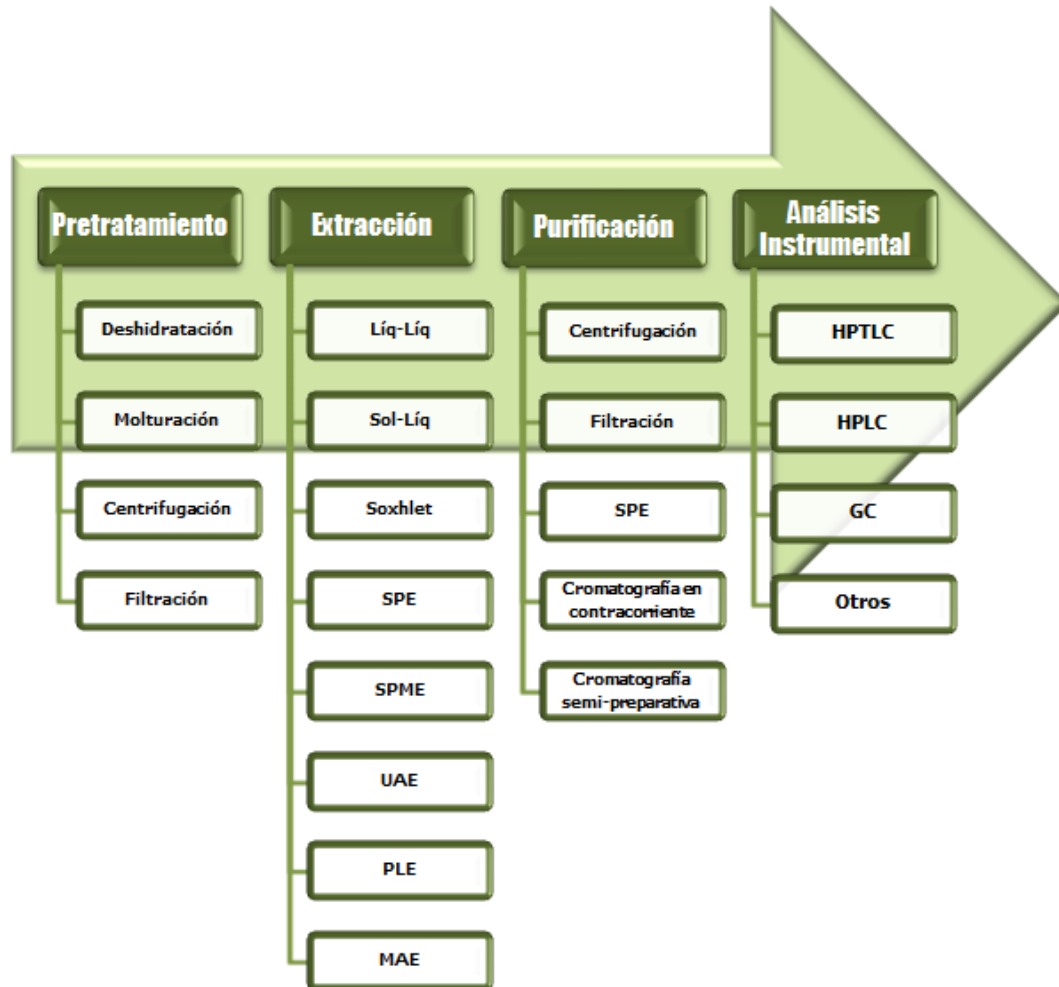


Figura 7.- Esquema de estrategias para la determinación de compuestos fenólicos en matrices vegetales.

Fuente: Tomado y adaptado desde Stalikas (2007)

4.1.1. Deshidratación

Los subproductos del procesamiento industrial del aguacate, principalmente los resultados de la elaboración de guacamole o pasta, son depositados y almacenados en contenedores hasta su posterior transporte hacia el lugar de eliminación o aprovechamiento posterior. Sin embargo, debido al alto contenido en humedad que presentan (mayor al 60 %), estos subproductos son susceptibles de contaminación

microbiana (**Figura 8**). Por esta razón, el primer paso tras la obtención de los subproductos es reducir la cantidad de agua que presentan para permitir alargar su vida útil sin afectar su potencialidad como fuente de compuestos bioactivos. Es una etapa vital ya que muchos de estos compuestos pueden ser degradados por procesos agresivos de secado, principalmente por las altas temperaturas usadas en algunos de ellos.



Figura 8.- Desarrollo microbiano en subproductos de aguacate.

El término secado se refiere generalmente a la eliminación de la humedad de una sustancia. Es el proceso de conservación más común pero también el que consume más energía, existiendo literalmente cientos de variantes utilizadas en el secado de sólidos particulados, pastas, láminas continuas, lechadas o soluciones, siendo la operación más versátil de ingeniería de alimentos (Ratti, 2001).

A nivel de laboratorio, el método menos agresivo para eliminar agua de matrices vegetales es la liofilización, proceso que permite conseguir esta operación minimizando la pérdida de los compuestos bioactivos presentes en la muestra, muchos de ellos termolábiles. Sin embargo los elevados costes del proceso hacen que a nivel industrial no sea rentable, llevándose a cabo otros métodos de secado, entre los que destacan el secado al sol, secado con aire caliente, secado a vacío, mediante bomba de calor, secado con microondas o mediante infrarrojos, entre otros. A continuación, se describen brevemente los métodos usados para la deshidratación de frutas y subproductos más comunes actualmente:

Deshidratación solar.- En este secado se utiliza la energía solar como una fuente de energía alternativa importante para el secado de productos. Esta energía, comparada con otras fuentes es abundante, inagotable y no contaminante, siendo además renovable, económica, popular y medioambientalmente sostenible (Doymaz, 2005). Sin embargo, su aplicación está restringida a ciertos materiales vegetales por el largo tiempo de secado requerido para la mayoría de matrices con niveles de humedad

elevados, la necesidad de un clima favorable y la posible disminución de la calidad o contaminación del producto.

Deshidratación por convección forzada.- En este método, el sólido a secar se expone a una corriente de aire caliente que fluye continuamente permitiendo la evaporación del agua presente en la muestra (Ratti, 2001). El flujo de aire por convección forzada facilita la transferencia de masa, acelerando el proceso de secado y mejorando la calidad del producto final. Las plantas de secado comerciales más ampliamente utilizadas para numerosos subproductos agroalimentarios industriales son los secadores de tipo convectivo, existiendo diferentes tipos, como los secadores de tambor, secadores de banda y secadores de lecho fluido (Celma, *et al.*, 2009).

Deshidratación a vacío.- Este tipo de proceso se utiliza para secar muestras termosensibles que pueden decolorarse o descomponerse a altas temperaturas. Se fundamenta en el uso de vacío para permitir una evaporación a menor temperatura y así disminuir la temperatura del proceso.

Secado por infrarrojos.- En esta metodología la energía de radiación infrarroja se transfiere desde el elemento de calentamiento a la superficie del producto sin calentar el aire circundante. Este tipo de calentamiento es especialmente adecuado para secar capas delgadas de material con una gran superficie expuesta a la radiación (Nowak & Lewicki, 2004). Este procedimiento se ha vuelto más popular en los últimos años debido a sus

grandes ventajas, que consisten en la rapidez del proceso de secado, la calidad del producto seco obtenido y la mayor capacidad de ahorro energético, lo que se traduce en un abaratamiento de costes, además de su menor precio en comparación con los métodos de secado por microondas o a vacío (Riadh, *et al.*, 2015).

Liofilización.- La liofilización de materiales biológicos es uno de los mejores métodos de eliminación de agua. Se basa en bajar la temperatura del producto por debajo de su temperatura de congelación y posteriormente aplicar un vacío de alta presión para extraer el agua en forma de vapor, que se acumula en un condensador en forma de hielo. Por último, un aumento de temperatura gradual extrae toda la humedad restante de la muestra. Este proceso mantiene la estructura física del producto y la conserva para una posible re-hidratación posterior, además de proteger compuestos termolábiles como los compuestos fenólicos, los cuales podrían degradarse a temperaturas superiores como las alcanzadas en otros procesos de secado. El principal inconveniente que presenta es su elevado coste, lo que hace inviable su implantación en procesos industrializados.

Deshidratación asistida por microondas.- La tecnología de microondas utiliza ondas electromagnéticas en el rango de frecuencia de 300 MHz a 300 GHz, que pasan a través del material y hacen que sus moléculas oscilen generando calor. Esta tecnología se puede combinar con unidades de calefacción y convección forzada, alcanzando mejores resultados que con el secado únicamente por aire caliente (Sagar & Kumar, 2010).

El secado al aire proporciona productos que pueden tener una vida útil prolongada de hasta un año, siendo por tanto el método convencional más usado en la industria, principalmente debido a su menor coste de operación. En comparación con la liofilización el coste de la deshidratación con aire forzado es de 4-8 veces menor (Ratti, 2001). Además, la comparativa de tecnologías de secado realizada por Khin, *et al.* (2005) demostró que la liofilización, el secado a vacío y la deshidratación osmótica se consideran demasiado costosos para la producción a gran escala de productos básicos.

Es esta Tesis Doctoral, se escogió el secado por aire como método de secado para la preparación de las muestras de subproductos de aguacate gracias a que es un método económicamente compatible con la industria, evitando encarecer el producto final. Sin embargo, el uso de altas temperaturas puede causar modificaciones irreversibles en los polisacáridos de la pared celular, afectando su estructura original además de reducir la concentración de compuestos bioactivos, muchos de ellos de naturaleza termolábil. Esto puede promover cambios importantes en las propiedades fisiológicas y farmacológicas debido a posibles pérdidas de los compuestos de interés. Por lo tanto, es necesario optimizar el proceso de secado, fundamentalmente la temperatura y tiempo del proceso, con la finalidad de mejorar la calidad de los productos deshidratados.

Estudios previos han evaluado la eficacia de secado de diversos métodos de deshidratación en diferentes tipos de subproductos. Principalmente se han llevado a cabo investigaciones en subproductos de la industria oleícola, residuos industriales

de semillas y piel de tomate y subproductos de cítricos. Los resultados encontrados son bastante diversos, siendo prometedores para el secado de orujo de oliva y almazara mediante un secadero solar (Montero, *et al.*, 2015). La eficacia del secado por infrarrojos ha sido estudiada en residuos industriales de semillas y piel de tomate (Celma, *et al.*, 2009). En el caso de los cítricos se comprobó que para los subproductos de naranja se producían modificaciones importantes en el contenido de fibra dietética y capacidad antioxidante cuando se aplicaron períodos de secado prolongados a temperaturas más bajas en un secador de convección forzada (Garau, *et al.* (2007). También se han llevado a cabo evaluaciones en subproductos de limón utilizando diferentes tipos de secado: mediante microondas, convectivo, infrarrojo y secado de aire combinado microondas. Todos los procesos indujeron la disminución del contenido en fenoles totales, flavonoides y actividad antioxidante, siendo el mejor de ellos el que combinaba energía microondas y aire caliente (M'hiri, *et al.*, 2018).

En los subproductos de aguacate, sólo se han llevado a cabo dos estudios previos donde se ha evaluado el proceso de secado en estas matrices. Avhad y Marchetti (2015) estudiaron el efecto de la temperatura del aire de secado sobre la cinética de secado del hueso de aguacate. Además, evaluaron diferentes pretratamientos basados en la molturación de los huesos sobre la velocidad global de evaporación de la humedad durante el proceso de secado. Concluyeron que con temperaturas mayores a 40 °C se producía una carbonización de la superficie, dañando la apariencia física y calidad del hueso de aguacate. Sin embargo, en este estudio los resultados se centraron únicamente en las características físicas del producto, sin

evaluar el efecto sobre la composición química o concentración de compuestos bioactivos. Un estudio más reciente optimizó el proceso de deshidratación de la piel y hueso de aguacate mediante un secador de bandejas de laboratorio, estudiando la influencia de la temperatura, flujo de aire y densidad de carga. Se encontró que las condiciones optimizadas para un mayor contenido en compuestos fenólicos y capacidad antioxidante para la piel fueron 72.71 °C, 0.8 m/s y 1.77 kg/m², respectivamente; mientras que para el hueso consistieron en 65.52 °C, 1.39 m/s y 2.89 kg/m². Además, se demostró que únicamente la temperatura y flujo de aire tienen efecto en el proceso de secado (Saavedra, *et al.*, 2017). Sin embargo, si se compara los valores de densidad de carga utilizados en este estudio (1 a 3 kg/m²), con otros encontrados en bibliografía, se puede evidenciar que los niveles usados son muy bajos y posiblemente no compatibles a nivel industrial. Por ejemplo, en secaderos de convección forzada se han utilizado cargas de 5.0 ± 0.8 kg/m² en la deshidratación de pimentón (Ramesh, *et al.*, 2001), 7 kg/m² para pimiento rojo (Vega, *et al.*, 2007), 10 kg/m² para cáscaras de orujo de uva roja (Garau, *et al.*, 2007) o 16 kg/m² para zanahoria y patata (Hebbar, *et al.*, 2004),

Es bien conocido que los procesos de secado, y en particular las altas temperaturas y los tiempos prolongados, pueden destruir algunos compuestos bioactivos, entre los que se encuentran los compuestos fenólicos. Respecto al secado por convección de aire hay varios factores pueden influir en la deshidratación, por ejemplo: velocidad y temperatura del aire, difusión del agua a través del material, densidad de carga, espesor y forma del producto a secar. Pocos estudios han evaluado el efecto de la temperatura de secado sobre los compuestos fenólicos, y menos aún en

subproductos de aguacate. En estas matrices sólo Saavedra, *et al.* (2017) han evaluado el efecto de la deshidratación sobre cinco compuestos fenólicos presentes en los subproductos de aguacate. Sin embargo, como se señala en las secciones 3.1.1 y 3.2.1, estos subproductos son ricos en una gran variedad de compuestos fenólicos, por lo cual se hace necesario un estudio que evalúe en profundidad el efecto de estos procesos de secado sobre la fracción fenólica de los subproductos.

4.1.2. Molienda

Otra etapa de pretratamiento de muestra muy importante antes de realizar el proceso de extracción consiste en la adecuación de las características de tamaño y forma para llevar a cabo el proceso de extracción. Esta etapa es vital ya que la estructura de la matriz sólida y el tamaño de partícula de la muestra son algunas variables críticas que afectan la eficiencia de un proceso de extracción. Respecto al tamaño de partícula, menores tamaños incrementan la eficiencia del proceso de extracción gracias a que se facilita la difusión del disolvente a través de la matriz y se incrementa el área de contacto entre ambos, contribuyendo a aumentar la transferencia de masa de los solutos de acuerdo con los principios generales de la Ley de Fick (Franco, *et al.*, 2007; Gião, *et al.*, 2009).

Varios estudios han concluido que con tamaños de partículas inferiores a 500 μm se ha logrado incrementar el rendimiento de extracción de compuestos antioxidantes (Chupin, *et al.*, 2015; Figueroa, *et al.*, 2012; Franco, *et al.*, 2007). Además, Bouras, *et al.* (2015) evaluaron el efecto del tamaño de partícula en la extracción asistida por microondas en corteza de Quercus (*Q. robur* L.), concluyendo que con un tamaño de

partícula de 500 μm se logran mayores rendimientos de extracción comparados con tamaños de 2.5 y 5 cm. Sin embargo, la reducción del tamaño de partícula tiene un límite, ya que partículas muy pequeñas pueden causar problemas durante la separación de la matriz del disolvente u obturaciones y atascos en los equipos de extracción.

4.2. Extracción de compuestos fenólicos

Una de las etapas más importantes en el procedimiento a seguir para el uso de los compuestos fenólicos presentes en los subproductos de aguacate es la etapa de extracción. Sin embargo, la estructura química de estos compuestos es muy variada, pudiendo existir desde compuestos con una estructura fenólica simple hasta taninos complejos con estructura polimérica. Además de esta complejidad estructural, estos compuestos pueden poseer múltiples grupos funcionales hidroxilo que pueden conjugarse con azúcares, ácidos o grupos alquilo (Luthria, 2008) formando toda una variedad de estructuras compuestas. Este amplio espectro estructural de los compuestos fenólicos hace que posean un rango de polaridad bastante amplio, algo que dificulta su extracción y hace necesario un proceso de optimización arduo de los diferentes parámetros o variables que afectan la extracción de estos analitos de interés.

Actualmente han sido descritos en bibliografía numerosos procedimientos para la extracción de compuestos fenólicos en matrices vegetales, en su mayoría utilizando métodos convencionales de extracción líquido-líquido, extracción sólido-líquido o extracción en fase sólida (Harnly, *et al.*, 2007). Sin embargo, debido a los numerosos

inconvenientes que presentan estas técnicas convencionales, en los últimos años se han ido sustituyendo por métodos de extracción más novedosos, que por lo general utilizan alguna fuente de energía para aumentar la transferencia de los analitos al disolvente. Además también se buscan técnicas que sean más respetuosas con el medio ambiente y no utilicen disolventes orgánicos. Es por ello que la extracción con fluidos presurizados (PLE), la extracción con fluidos supercríticos (SFE) y la extracción asistida por microondas (MAE), se han empezado a utilizar para extraer compuestos fenólicos de matrices vegetales (Ignat, *et al.*, 2011). Se puede concluir que debido a la amplia variedad de estructuras y propiedades de los compuestos fenólicos, su distribución desigual en la planta y el hecho de que puedan encontrarse unidos a otros compuestos en las matrices vegetales, no es posible hablar de procedimientos de extracción generales, sino que la elección de un procedimiento concreto dependerá de la matriz de la que se trate, de los compuestos que se quieran determinar y del tipo de información que se desee obtener (cualitativa o cuantitativa) (Luthria, 2006). En las siguientes secciones se describe en mayor profundidad estas técnicas de extracción, haciendo especial hincapié en aquellas utilizadas en la presente Tesis Doctoral.

4.2.1. Técnicas de extracción convencionales

El proceso de extracción en una técnica convencional sigue tres pasos básicos: desorción de los analitos desde una partícula sólida, difusión a través del disolvente que ha penetrado dentro de la partícula y finalmente, la transferencia de los analitos al disolvente circundante. El rendimiento de extracción depende de la naturaleza química del analito de interés, del método de extracción usado, del tamaño de partícula de la muestra, del tiempo, la temperatura y disolvente de extracción, así

como de la presencia de sustancias que pueden interferir en el proceso (Stalikas, 2007). Además, los métodos de extracción deben evitar los procesos de degradación, que pueden ser originados por varios factores como la luz, el aire, el tiempo, la temperatura y la presencia de radicales libres o enzimas que se encuentran en las muestras (principalmente enzimas oxidativas) que se liberan durante la extracción y que pueden causar reacciones de degradación (Biesaga, 2011).

Los métodos convencionales de extracción líquido-líquido y sólido-líquido combinados con calentamiento, ebullición o reflujo, han sido utilizados ampliamente para extraer compuestos bioactivos, principalmente por su facilidad de uso, eficiencia y amplia aplicabilidad (Stalikas, 2007).

La **extracción líquido-líquido** es un proceso de separación para muestras líquidas en el que la distribución del analito se realiza entre dos fases inmiscibles. Los disolventes de extracción comúnmente utilizados son metanol, etanol, acetona, dietil éter y acetato de etilo. Sin embargo, los ácidos fenólicos muy polares (como los ácidos benzoico y cinámico) no se pueden extraer completamente con disolventes orgánicos puros, y se recomienda el uso mezclas de alcohol, agua o acetona (Stalikas, 2007).

Las técnicas de **extracción sólido-líquido** tradicionales se pueden clasificar en extracción Soxhlet, maceración e hidrodestilación. El extractor Soxhlet fue propuesto por primera vez por el químico alemán Franz Ritter Von Soxhlet, y fue diseñado principalmente para la extracción de lípidos. Sin embargo, actualmente sus

aplicaciones son muy variadas, llegando a utilizarse para aislar flavonoides de muestras sólidas (Azmir, *et al.*, 2013). En este método una pequeña cantidad de muestra deshidratada se coloca en un cartucho de material poroso que se sitúa en la cámara del extractor Soxhlet, a continuación se calienta el disolvente situado en el matraz de destilación, condensándose sus vapores que caen, gota a gota, sobre el cartucho que contiene la muestra. Los analitos migran al disolvente y cuando éste alcanza un determinado nivel, es aspirado por un sifón que descarga la solución nuevamente dentro del matraz de destilación. Los analitos se mantienen en este matraz y el disolvente vuelve al ponerse en contacto con la muestra. El proceso se repite varios ciclos hasta que se completa la extracción cuando se alcanza una concentración deseada. En la mayoría de los casos, se emplean metanol acuoso o acetonitrilo como disolvente para la extracción de compuestos fenólicos. Entre las principales desventajas de este método se encuentran los largos tiempos de extracción (de entre 12 a 20 h) y las altas temperaturas empleadas (Hao, *et al.*, 2002). La combinación de estos dos factores puede provocar la degradación de compuestos termolábiles, como los compuestos fenólicos. Además, para algunos de estos compuestos las recuperaciones de extracción son bajas porque el contenido del modificador orgánico no es suficiente para su completo aislamiento, especialmente en el caso de ácidos fenólicos muy polares (Stalikas, 2007). Sin embargo, el método Soxhlet se sigue utilizando como modelo para la comparación de nuevos sistemas de extracción de polifenoles (Azmir, *et al.*, 2013; García-Salas, *et al.*, 2010).

Por otro lado, la maceración consiste en poner en contacto la muestra sólida con el disolvente de extracción en un recipiente cerrado por un determinado tiempo con posibilidad de agitación y calentamiento. El disolvente con los solutos disueltos una vez terminada la extracción se separa de la muestra sólida mediante filtrado o centrifugación. Varios estudios han concluido que la agitación facilita la extracción mediante dos mecanismos: por un lado aumenta la difusión del disolvente entre la muestra y por otro elimina la existencia de una fina capa de disolvente en la superficie de la muestra que se encuentra concentrada en los compuestos de interés, permitiendo que nuevas moléculas de este disolvente pueden entrar en contacto con la muestra y puedan seguir enriqueciéndose en estos compuestos, obteniendo así un mayor rendimiento de extracción. Vongsak, *et al.* (2013) comparó diferentes métodos clásicos de extracción de compuestos fenólicos a partir de hojas de *Moringa oleífera*, concluyendo que la maceración con etanol al 70 % presentó un mayor rendimiento y concentración de fenoles totales comparado con otros métodos como la percolación y el Soxhlet. Además, encontraron un efecto en la eficiencia de extracción por maceración relacionado con la composición del disolvente de extracción y la humedad de la muestra.

Por otro lado, la hidrodestilación es un método tradicional para la extracción de compuestos bioactivos y aceites esenciales de plantas. Las principales ventajas de este método es que no suelen usarse disolventes orgánicos y la extracción puede realizarse antes de la deshidratación de los materiales vegetales. Hay tres tipos de hidrodestilación: destilación de agua, destilación de agua y vapor y destilación directa de vapor (Azmir, *et al.*, 2013). Phutdhawong, *et al.* (2007) realizaron la

extracción de compuestos antioxidantes de hojas de *Cinnamomum iners Reinw.* empleando hidrodestilación y extracción asistida por microondas, concluyendo que no existe diferencia significativa de la actividad antioxidante en función del método de extracción usado.

Estos métodos de extracción convencionales descritos en esta sección se han utilizado durante décadas. Sin embargo, en el caso de compuestos fenólicos y especialmente de flavonoides puede existir una degradación debido a la ionización, hidrólisis y oxidación de los mismos durante el proceso, en parte por los tiempos de extracción tan largos que requieren estas metodologías (Y. L. Wang, *et al.*, 2010). Además, otros inconvenientes son su gran laboriosidad y elevado consumo de disolventes, lo que también es un problema en términos de sostenibilidad y protección del medio ambiente. Por estos motivos, en los últimos años ha habido una demanda creciente de nuevas técnicas de extracción, susceptibles de automatización, con tiempos de extracción más cortos y un menor consumo de disolventes orgánicos, lo que evita la contaminación del medio ambiente y reduce la gestión de residuos en los laboratorios analíticos, disminuyendo además el coste de preparación de muestras (Eskilsson & Björklund, 2000). En la siguiente sección se describen dos métodos avanzados de extracción, como son la extracción con fluidos presurizados y la extracción asistida por microondas. La similitud entre estas técnicas es la posibilidad de trabajar con disolventes a temperaturas y presiones elevadas, lo que mejora drásticamente la velocidad del proceso de extracción y aumenta la eficiencia del mismo. Además, son afines con la tendencia actual de evolucionar hacia la Química Analítica Verde, que busca diseñar nuevos procesos de

extracción, rápidos, más reproducibles y respetuosos con el medio ambiente (Herrero, *et al.*, 2013).

4.2.2. Extracción con líquidos presurizados (PLE)

La extracción con líquidos presurizados (PLE) se basa en el uso de disolventes combinando alta presión y temperatura, de manera que la extracción puede realizarse a una temperatura superior al punto de ebullición del disolvente utilizado manteniendo el estado líquido del mismo. El uso de alta presión y temperatura mejora el rendimiento de extracción debido a numerosos factores, el aumento de la solubilidad del analito de interés se produce gracias a un incremento de la transferencia de masa de la matriz al disolvente y a una modificación de la tensión superficial. En la **Tabla 3** se detallan los principales beneficios del uso de temperatura y presión elevadas en la extracción de compuestos bioactivos.

Además, otras de las ventajas a destacar de la PLE es que permite el uso de disolventes verdes denominados también “Green” que son respetuosos con el medio ambiente, como por ejemplo etanol y agua, ambos considerados disolventes “GRAS” (Generally Recognized As Safe).

Por tanto, se concluyó que esta técnica mejora la eficiencia de extracción comparada con el uso de métodos de extracción convencionales, los cuales emplean disolventes orgánicos que suponen un problema medioambiental y cuyo uso es incompatible con las industrias farmacéutica, cosmética y alimentaria.

Tabla 3.- Efecto del uso de alta presión y temperatura sobre procesos de extracción de compuestos bioactivos.

Temperatura	Presión
<ul style="list-style-type: none"> • Aumenta la capacidad de los disolventes para solubilizar los analitos. • Incrementa la velocidad de difusión. • El aumento de las temperaturas puede alterar las fuertes interacciones existentes entre la matriz y soluto causadas por las fuerzas de Van der Waals y las atracciones dipolo-dipolo de las moléculas de soluto y los sitios activos de la matriz. • La energía térmica puede vencer las interacciones cohesivas y adhesivas, al disminuir la energía de activación requerida para el proceso de desorción. • Los puentes de hidrógeno entre las moléculas de la muestra se debilitan con un aumento de temperatura, con lo cual se facilita el contacto con el disolvente. • Reducción de la viscosidad del disolvente, con lo cual se favorece su penetración en la muestra. • Disminuye la tensión superficial del disolvente, solutos y la matriz, permitiendo que el disolvente interaccione mejor con la muestra. 	<ul style="list-style-type: none"> • El uso de una elevada presión permite usar temperaturas por encima del punto de ebullición del disolvente sin que éste pase a estado gaseoso. • La presión fuerza al disolvente a sitios de la matriz que normalmente no serían alcanzados por disolventes en condiciones de presión atmosférica, favoreciendo por tanto el contacto con la muestra y mejorando la recuperación de los analitos de interés de la matriz en la que se encuentran.

Fuente: Richter, *et al.* (1996).

Las principales variables, además de la presión y temperatura, que se pueden considerar en un proceso de PLE se detallan a continuación:

- **Temperatura:** La temperatura de extracción tiene efecto sobre la constante dieléctrica del disolvente, lo que permite la posibilidad de extraer distintos tipos de compuestos en función de la temperatura usada (Moraes, *et al.*, 2013). El incremento de temperatura produce una modificación de la constante dieléctrica del disolvente de extracción, propiedad de la que depende en gran medida la eficiencia de extracción. En el caso del agua, la constante dieléctrica puede oscilar entre valores de 80 a 25 por efecto de un incremento de temperatura de 25 a 250 °C, debido al debilitamiento de los enlaces de hidrógeno. De esta forma se consiguen valores de constante dieléctrica próximos a la de disolventes menos polares a temperatura ambiente (Herrero, *et al.*, 2013). Por tanto una modificación de la temperatura permite la extracción de compuestos de distinta polaridad.

Las principales ventajas e inconvenientes de las altas temperaturas usadas en esta técnica son:

Ventajas:

- Afecta las propiedades disolventes del fluido.
- Aumenta las tasas de difusión y la capacidad de solubilizar analitos.
- Disminución en la viscosidad y la tensión superficial.

Inconvenientes:

- Disminuye la selectividad de extracción, debido a que componentes de la matriz pueden ser co-extraídos junto con los analitos de interés.
 - Compuestos termolábiles se pueden degradar.
-
- **Presión:** En PLE generalmente se usan altas presiones, lo que permite que el disolvente se mantenga en estado líquido cuando se usan temperaturas por encima del punto de ebullición. También incrementa la eficiencia de extracción al mejorar el contacto de la muestra con el disolvente, llegando a zonas de la matriz que no se alcanzarían usando condiciones atmosféricas (Sun, *et al.*, 2012). Sin embargo, también presenta algunos inconvenientes, por ejemplo la co-extracción de compuestos interferentes presentes en la matriz. Por otro lado existe cierta controversia en el uso de altas presiones, de hecho algunos estudios no han observado ninguna relación entre la presión y la recuperación de analitos de interés, destacando una influencia nula de la presión de extracción, más allá del punto en que el solvente se mantenga en estado líquido (Herrero, *et al.*, 2013; Luthria, 2008).
 - **Disolvente:** En general, las propiedades fisicoquímicas del disolvente, como el punto de ebullición, la polaridad, la densidad específica (influye en la penetración en la matriz de la muestra) y la toxicidad (constituye un riesgo en el lugar de trabajo y en el uso de los extractos obtenidos), son factores a considerar al elegir el disolvente de extracción. En cuanto a los utilizados más comúnmente en PLE, se prefieren aquellos considerados como GRAS (por

ejemplo, etanol, agua o sus mezclas) (Herrero, *et al.*, 2013). Se pueden utilizar una amplia gama de disolventes en PLE, excepto bases y ácidos fuertes, así como aquellos con una temperatura de autoignición de 40–200 °C (por ejemplo, disulfuro de carbono, éter dietílico y 1,4-dioxano).

- **Matriz:** La naturaleza de la muestra y su composición pueden afectar la extracción mediante PLE. Algunos componentes de la matriz pueden ser co-extraídos con los analitos de interés y deben eliminarse generalmente para diversas aplicaciones de los extractos. Además la presencia de otros compuestos como carbohidratos puede modificar las propiedades de la muestra durante la extracción pudiendo afectar la eficiencia de la misma.
- **Relación disolvente/muestra:** Varios estudios han encontrado un efecto significativo de la relación disolvente/muestra sobre la eficiencia de extracción, siendo necesario la optimización de esta relación, ya que los estudios evidencian que el rendimiento no aumenta proporcionalmente con el aumento de la cantidad de muestra (Luthria, 2008).
- **Tamaño de partícula de muestra:** El tamaño de partícula de la muestra es otro factor importante a tener en cuenta. Un menor tamaño de partícula posibilita un área superficial mayor por unidad de masa que está en contacto con el disolvente, dando como resultado una mayor accesibilidad del analito al disolvente de extracción. Luthria (2008) evaluó el efecto del tamaño de partícula sobre la extracción de compuestos fenólicos, encontrando mayores rendimientos de extracción con tamaños de partícula < 0.425 mm.

- **Modo de extracción:** La extracción mediante PLE puede realizarse en diferentes modalidades:
 - **Estático:** En el modo de extracción estático, la muestra y el disolvente se mantienen durante un tiempo programado por el usuario a una presión y temperatura constantes. En este modo, la exposición prolongada a los disolventes permite que la matriz se hinche y mejore la penetración del disolvente en los intersticios de la muestra y el contacto con los analitos. Por lo tanto, en condiciones estáticas, se pueden alcanzar equilibrios entre los componentes de la muestra aún unidos a la matriz y los que ya están solubilizados en el disolvente. Una vez alcanzado este punto, la eficiencia del procedimiento de extracción no aumentará más con el tiempo (Herrero, *et al.*, 2013). Además, deberá optimizarse el tiempo de la extracción en modo estático ya que el rendimiento puede disminuir por una degradación de la muestra al someterse a altas temperaturas durante largo tiempo (Lee & Kim, 2010).
 - **Dinámico:** En este modo de extracción el disolvente fresco se introduce continuamente en la celda, en consecuencia, la eficiencia del procedimiento de extracción aumenta debido al aumento de la transferencia de masa (Herrero, *et al.*, 2013). Sin embargo, este tipo de extracción rara vez se utiliza, principalmente debido al incremento en el consumo de disolvente en comparación con el proceso estático (Sun, *et al.*, 2012).

- **Tiempo de pre-calentamiento:** Es el tiempo en que la celda se mantiene en el horno a la temperatura seleccionada para la extracción antes de producirse el contacto de la muestra con el disolvente. Normalmente 5 minutos es suficiente para garantizar que la celda se encuentre a la temperatura fijada (Sun, *et al.*, 2012)
- **Tiempo de extracción:** La influencia del tiempo de extracción dependerá directamente del modo de PLE aplicado, estático o dinámico. El tiempo estático se define como el tiempo que la muestra interactúa con el disolvente en la celda de extracción por ciclo bajo condiciones definidas. Se ha demostrado que no en todos los casos un aumento del tiempo de extracción produce un aumento de la eficiencia de extracción, ya que puede producirse la degradación de los compuestos de interés. Por ejemplo, Rostagno, *et al.* (2004) reportaron un aumento en la extracción de isoflavonas de soja cuando se aumentó la duración del ciclo de extracción estático de 5 a 7 min. En cambio, Luthria (2008) no observó cambios significativos en los rendimientos de extracción usando tres periodos de tiempo estáticos diferentes (5, 10 y 15 min) en la extracción de compuestos fenólicos de perejil (*Petroselinum crispum*).
- **Ciclos de extracción:** Con la intención de aumentar el rendimiento de extracción pueden utilizarse varios ciclos de extracción consecutivos sobre la misma muestra. Sin embargo, este enfoque no siempre proporciona los mismos resultados, ya que en algunas aplicaciones, un ciclo de extracción es suficiente para extraer todos los compuestos de interés.

- **Agente dispersante:** Antes de la extracción, las muestras pretratadas generalmente deben mezclarse con algún material inerte para evitar la agregación de partículas de muestra que puedan alterar la eficacia de la extracción y aumentar la superficie de contacto muestra-disolvente. El uso de este tipo de materiales contribuye a aumentar el volumen que ocupa la muestra, lo que permite disminuir la cantidad de disolvente necesario para llenar la celda de extracción. Se han utilizado diferentes materiales dispersantes como: tierra de diatomeas (Fredericks, *et al.*, 2013), arena inerte (Luthria, 2012; Taamalli, *et al.*, 2012) o perlas de vidrio (Šťavíková, *et al.*, 2011).
- **Volumen de descarga:** Es la cantidad de volumen de disolvente fresco introducido en la celda después del tiempo de extracción para arrastrar los analitos hacia el vial de recolección. Este volumen asegura que todos los compuestos extraídos se recolecten (Sun, *et al.*, 2012). Diferentes autores no han encontrado gran diferencia en la eficiencia de extracción cuando el volumen de descarga varía del 10 al 100 % del volumen de la celda Luthria (2008). Por lo tanto, un 10 % puede utilizarse para reducir el uso de disolventes de extracción, lo que a su vez disminuye el coste de eliminación de residuos de disolventes generados después de la extracción.

La PLE ha recibido diferentes nombres, tales como: extracción acelerada con disolventes (ASE), extracción con disolvente a presión (PSE), extracción con disolvente a elevada presión (HPSE), extracción con disolventes presurizados a elevadas temperaturas (PHSE), extracción con disolventes a elevada presión y temperatura (HPHTSE), extracción con disolvente sobrecalentado (SHSE), extracción

con agua caliente presurizada (PHWE) y extracción subcrítica con disolventes (SSE). Las dos últimas acepciones son utilizadas para destacar que la extracción se ha realizado únicamente con agua, disolvente no agresivo con el medio ambiente (Moraes, *et al.*, 2013; Sun, *et al.*, 2012). En esta Tesis, se utilizará la designación de PLE, que es la más ampliamente aceptada.

La evolución de la instrumentación usada para PLE comenzó en 1995, cuando la empresa DIONEX presentó la solicitud de patente de un sistema acelerado de extracción con disolvente. Básicamente consistía en una celda de extracción termostaticada montada entre una bomba de disolvente y un vial de recogida de muestras, que permitía extraer analitos a temperaturas y presiones elevadas por debajo de condiciones supercríticas en tiempos cortos y con bajas cantidades de disolvente (Figura 9). Posteriormente, Richter, *et al.* (1996), desarrollaron un sistema de extracción con líquidos presurizados y evaluaron su eficiencia en la extracción de hidrocarburos totales de petróleo, hidrocarburos aromáticos policíclicos y bifenilos policlorados en muestras certificadas, considerando el efecto de variables como temperatura, presión, volumen de disolvente, material dispersante y precalentamiento sobre el rendimiento de extracción y degradación térmica de los compuestos de interés. Concluyeron que la PLE brinda recuperaciones comparables a las obtenidas con Soxhlet y sonicación, pero con las ventajas de requerir menor tiempo de extracción y baja cantidad de disolvente. Además, no observaron degradación térmica de los analitos de interés durante la extracción. Actualmente la mayoría de los instrumentos comerciales sólo permiten realizar extracciones estáticas, están equipados con carruseles con capacidad para doce o más muestras

que permiten la extracción automática secuencial de un lote de muestras, y para evitar la degradación térmica de los extractos obtenidos estos equipos pueden contar con sistema de refrigeración opcional.



Figura 9.- Sistema de extracción con líquidos presurizados (PLE).

Fuente: Tomado y adaptado desde Dionex (2011).

El procedimiento de trabajo en PLE comienza con la mezcla de la muestra deshidratada con un material dispersante que se empaqueta en una celda de extracción de acero inoxidable. La muestra se extrae con un disolvente adecuado a alta temperatura (40 a 200 °C) y presión (500 a 3000 psi) durante periodos cortos de tiempo (5 a 20 min). Tras este proceso, la muestra se lava con nuevas porciones de

disolvente y el extracto se purga en un vial de recolección con la ayuda de un gas comprimido, usualmente nitrógeno (García-Salas, *et al.*, 2010; Richter, *et al.*, 1996). Estos últimos pasos se realizan para conseguir la eliminación de todos los residuos de la muestra que puedan quedar en el sistema PLE, y así mejorar la recuperación del analito y preparar el sistema para el siguiente proceso de extracción (Sun, *et al.*, 2012). En la **Tabla 4** se detallan las principales ventajas e inconvenientes de PLE frente a métodos de extracción convencionales.

Tabla 4.- Ventajas y desventajas de la extracción con líquidos presurizados (PLE) comparada con métodos tradicionales.

Ventajas	Desventajas
<ul style="list-style-type: none">• Evita la degradación de los analitos de interés por oxidación.• Se obtiene mayores rendimientos que con los métodos convencionales.• Tiempos de extracción bajos.• Bajo consumo de disolvente.• Permite usar disolventes "Green" y "GRAS"• Proceso automatizable.	<ul style="list-style-type: none">• Mayor inversión económica.• Extracciones en ocasiones poco selectivas.

Fuente: Moraes, *et al.* (2013)

En cuanto a la extracción específica de compuestos fenólicos el principal inconveniente es la presencia de oxígeno y luz durante la extracción, que contribuye a la degradación de estos compuestos susceptibles. Por el contrario, en este tipo de técnica la muestra se mantiene en un ambiente libre de oxígeno y luz, evitando la degradación de los analitos de interés por oxidación (Routray & Orsat, 2012; Stalikas, 2007).

En concreto para esta aplicación la PLE se ha utilizado ampliamente en la extracción de compuestos fenólicos de frutas como manzana (Alonso-Salces, *et al.*, 2001), fresas (Fredericks, *et al.*, 2013; Wu, *et al.*, 2004), aguacate (Wu, *et al.*, 2004), arándanos (Heffels, *et al.*, 2015) y en subproductos como orujo de uva (Ju & Howard, 2003) o piel de patata (Luthria, 2012).

4.2.3. Extracción asistida por microondas (MAE)

La extracción asistida por microondas (MAE) es una técnica que parte de una extracción convencional sólido-líquido a la que se le aplica radiación de microondas para mejorar la eficiencia de extracción. Las microondas son ondas electromagnéticas no ionizantes, ubicadas dentro del espectro electromagnético entre el rango de radio-frecuencia e infrarrojo, es decir entre 300 MHz a 300 GHz. La frecuencia de 915 MHz se considera la más útil para aplicaciones industriales por su mayor profundidad de penetración, mientras que la frecuencia de 2.450 MHz se utiliza generalmente en hornos microondas domésticos y para aplicaciones de extracción diseñadas con fines de química analítica (Routray & Orsat, 2012).

A diferencia de otros procesos de extracción en los que interviene una etapa de calentamiento, donde el calor penetra desde el exterior hacia el interior de la matriz, la energía microondas calienta la muestra de manera simultánea y homogénea. El calentamiento por energía microondas se produce a través de dos mecanismos simultáneos, mediante rotación dipolar y conducción iónica, es decir, mediante inversión de dipolos y desplazamiento de iones cargados presentes en el soluto así como en el disolvente (Routray & Orsat, 2012). La rotación dipolar está relacionada con el alineamiento del campo eléctrico con las moléculas de disolvente o muestra que posee un momento dipolar. El calentamiento por microondas ocurre cuando las moléculas del disolvente polar no pueden alinearse lo suficientemente rápido al campo eléctrico de alta frecuencia aplicado del microondas. Esta discrepancia hace que las moléculas de disolvente disipen la energía absorbida en forma de calor (Y. L. Wang, *et al.*, 2010).

Los primeros usos de hornos domésticos de microondas datan de los años 70 para el análisis de humedad (Hesek & Wilson, 1974) y en el tratamiento de muestras biológicas previo al análisis de metales (Abu-Samra, *et al.*, 1975). En los años 80, Ganzler, *et al.* (1986) utilizaron la irradiación de microondas para acelerar la extracción de diversos tipos de compuestos de suelo, semillas, alimentos y piensos, previo al análisis por HPLC, comparando sus resultados con extracciones convencionales por Soxhlet y maceración dinámica. Concluyeron que el método de extracción por microondas fue más eficiente en la extracción de compuestos polares utilizando agua como disolvente comparado con la extracción Soxhlet. Posteriormente, Pare, *et al.* (1991) patentaron un método para acelerar la

extracción de productos naturales de un material de origen vegetal mediante el contacto simultáneo con el medio extractante y la exposición a la radiación de microondas.

Cada sistema de microondas consta normalmente de tres partes básicas: la fuente de microondas, la guía de ondas y el aplicador. Los hornos de microondas pueden ser monomodo o multimodo. La opción monomodo (utilizada en el caso de un horno de microondas enfocado) puede generar una frecuencia que excita sólo un modo de resonancia. Por el contrario, la cavidad multimodo es grande y la onda incidente puede afectar varios modos de resonancia. Esta superposición de modos permite la homogeneización del campo. Además, se incluyen sistemas como placas giratorias para la homogeneización de irradiación (Routray & Orsat, 2012).

La gran ventaja del calentamiento por microondas es que tanto la muestra como el disolvente se calientan, permitiendo que la solución de extracción (disolvente y muestra) alcance la temperatura deseada más rápidamente y evite el gradiente térmico causado por el calentamiento convencional (Biesaga, 2011; Huie, 2002). Además, la aceleración de la extracción se debe a que durante el proceso de extracción por microondas el agua presente en las células de matrices vegetales absorbe energía, produciéndose su calentamiento y aumentando la presión dentro del biomaterial, lo que conduce a una hinchazón e incremento de la porosidad de la matriz, mejorando así la penetración del disolvente de extracción a través de la misma e incluso pudiendo provocar la rotura eventual de las paredes de las células vegetales y la liberación de los compuestos en el disolvente circundante, facilitando la extracción de los analitos de interés (Hao, *et al.*, 2002; Kratchanova, *et al.*, 2004).

También, se ha propuesto que la mejora de eficiencia de extracción observada puede deberse a los gradientes de transferencia de calor y masa que trabajan en la misma dirección (Routray & Orsat, 2012). En la **Tabla 5** se detallan las ventajas y desventajas de MAE frente a extracciones tradicionales.

Existen dos tipos de sistemas de extracción con energía microondas, por un lado la extracción asistida por microondas (MAE presurizado) (Figura 10) y por otro la extracción Soxhlet asistido por microondas (MAE atmosférica). La diferencia entre ambas se encuentra en la focalización de la radiación, en MAE presurizado toda la celda es irradiada y se utiliza una celda cerrada, mientras que en la segunda modalidad sólo una parte de la celda es irradiada y se utiliza una celda abierta. Además, en el primer sistema al utilizar una celda presurizada se pueden alcanzar temperaturas más elevadas, de dos a tres veces superiores al punto de ebullición del disolvente (Routray & Orsat, 2012), lo que permite acortar los tiempos de extracción (Richter, *et al.*, 1996).

Tabla 5.- Ventajas y desventajas de la extracción asistida por microondas (MAE) comparada con métodos tradicionales.

Ventajas	Desventajas
<ul style="list-style-type: none"> • Menor tiempo de extracción (generalmente entre 15–30 minutos). • Mayor rendimiento de extracción. • Menor uso de disolvente (Entre 10 a 40 mL). Estos volúmenes son aproximadamente 10 veces inferiores a los volúmenes utilizados por las técnicas de extracción convencionales. • Pequeña cantidad de muestra necesaria, además permite extracciones de muestra múltiples. • Se evita degradación de los analitos por efecto del uso de altas temperaturas. • Mayor reproducibilidad. 	<ul style="list-style-type: none"> • No es viable para matrices secas o demasiado húmedas cuando se utiliza con disolventes no polares. • Extracciones poco selectivas. • Mayor inversión económica.

Fuente: Eskilsson y Björklund (2000), Huie (2002), Li, *et al.* (2017) y Stalikas (2007).

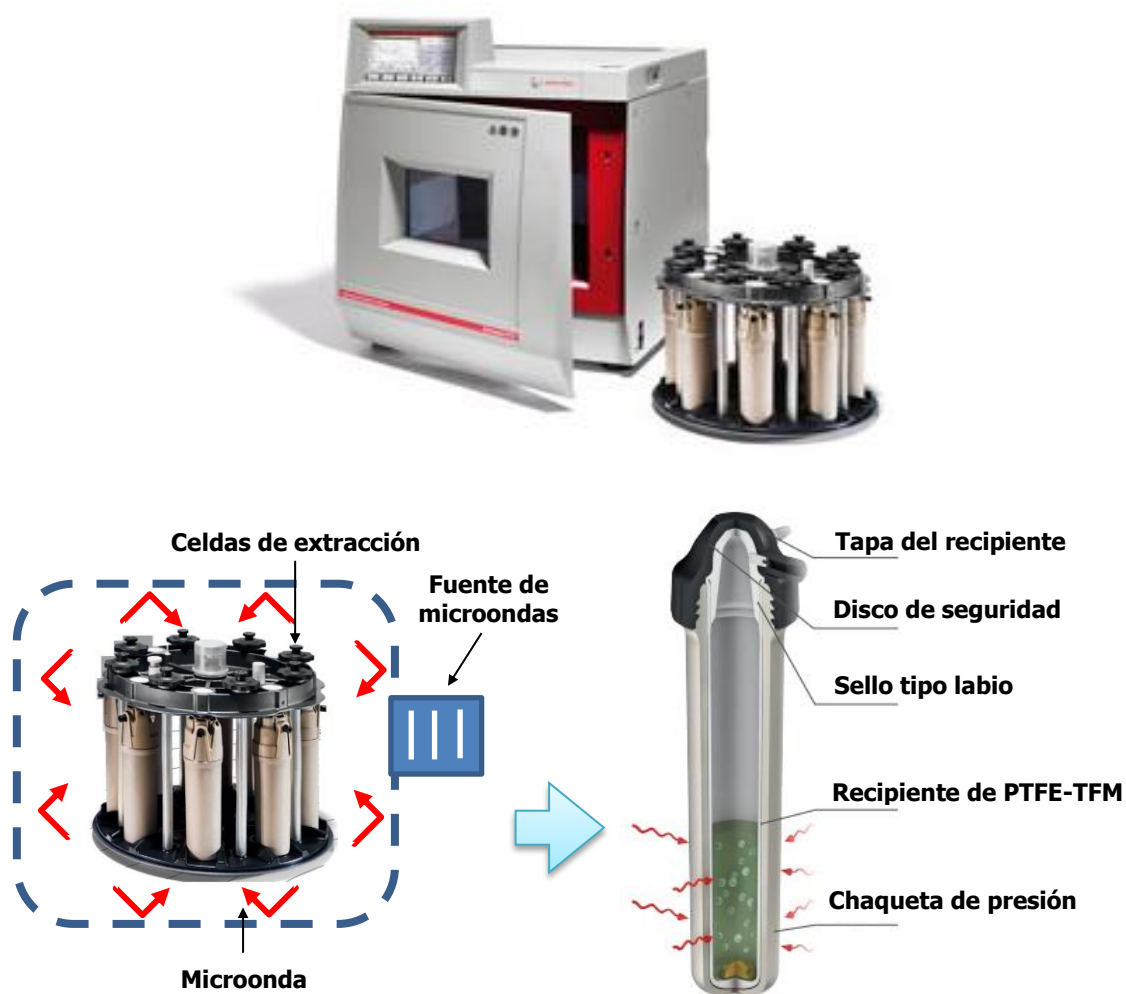
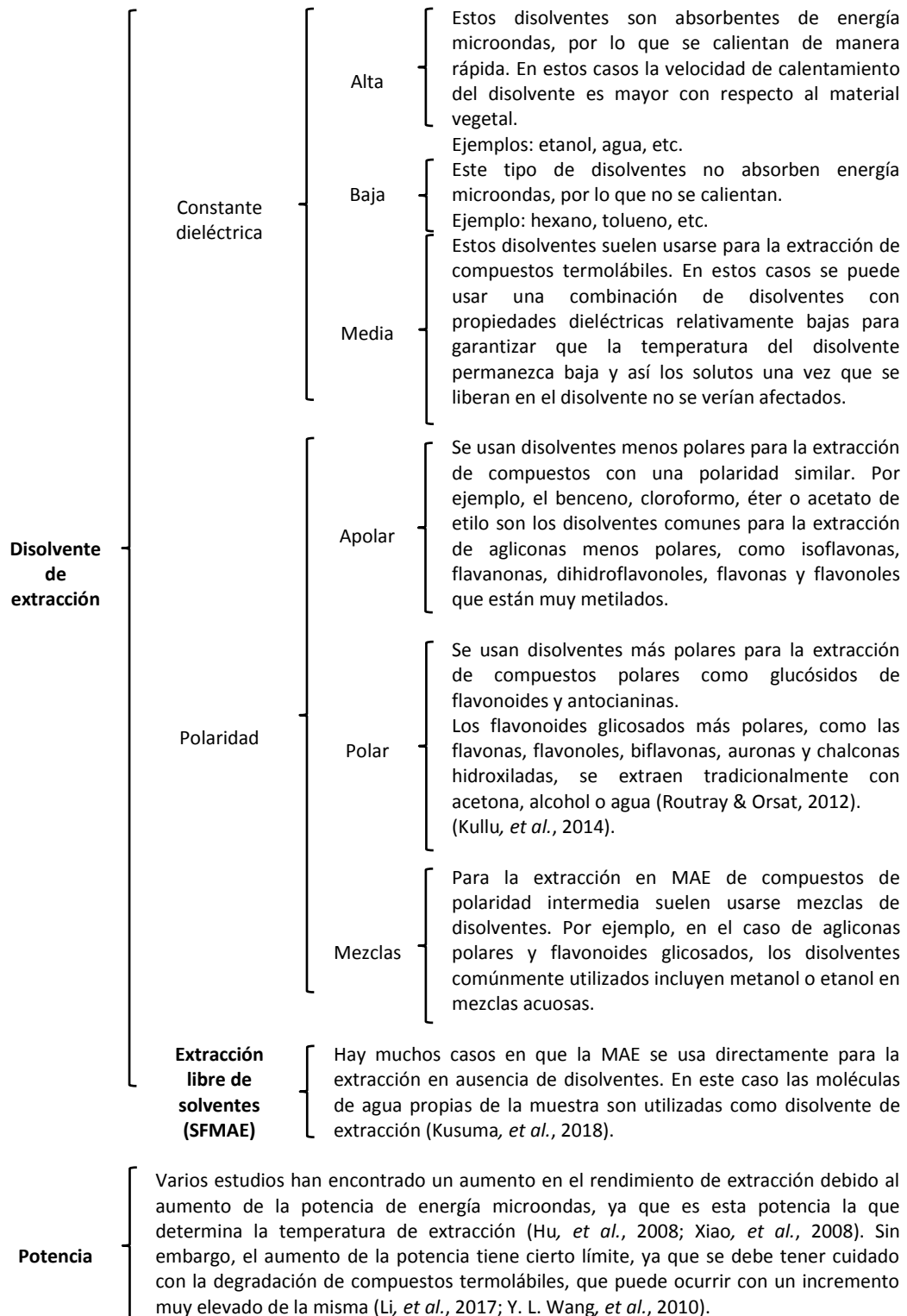


Figura 10.- Sistema de extracción asistida por microondas (MAE presurizado).

Fuente: Tomado y adaptado desde Camel (2001), F. Y. Du, *et al.* (2007) y Paar Anton (2017)

Entre las condiciones comúnmente estudiadas para la optimización del proceso MAE, los efectos de la composición del disolvente, el volumen de disolvente, la temperatura de extracción y las características de la matriz parecen ser los más importantes en la extracción de analitos de matrices vegetales (Huie, 2002). En la **Figura 11** se resumen los factores que afectan a dicha extracción por microondas.



Temperatura	<p>El aumento de temperatura incrementa las interacciones intermoleculares dentro del disolvente, dando lugar a un mayor movimiento molecular que aumenta la solubilidad de los analitos de interés.</p> <p>El aumento de la temperatura también puede causar una acumulación de presión celular que puede provocar la ruptura de la célula vegetal y la apertura de la matriz, dando como resultado una mayor disponibilidad de los componentes para extraerse a la solución (Routray & Orsat, 2012).</p> <p>Por otro lado, la viscosidad del disolvente disminuye con el incremento de la temperatura, lo que aumenta su movilidad y solubilidad, incrementando así la eficacia de la extracción.</p> <p>Este parámetro debe optimizarse dependiendo al tipo de compuesto a extraer, como ya se ha comentado el uso de temperaturas elevadas durante tiempos prolongados puede causar la degradación de compuestos termolábiles. Por ejemplo, los compuestos fenólicos que tienen mayor número de sustituyentes hidroxilo son más susceptibles a la degradación por el uso de temperaturas superiores a 100 °C (Liazid, <i>et al.</i>, 2007).</p>
Tiempo de extracción	<p>Un incremento del tiempo de extracción puede mejorar el rendimiento del proceso, pero se debe tener cuidado cuando se utiliza disolventes con constantes dieléctricas altas ya que la aplicación prolongada de energía microondas puede conducir a la degradación de los analitos de interés por el sobrecalentamiento del sistema soluto-solvente.</p>
Ciclos de extracción	<p>En ocasiones un aumento de los ciclos de extracción puede ayudar a mejorar el rendimiento del proceso. El uso de disolvente fresco en cada ciclo puede minimizar la degradación térmica de los analitos de interés provocada por el sobrecalentamiento del mismo. Además, así se evita la saturación del disolvente con soluto, lo que aumenta la transferencia de materia y la cinética de extracción.</p>
Relación disolvente - muestra	<p>En MAE el disolvente debe estar en cantidad suficiente para que cubra la muestra en la celda de extracción. En el caso de un sistema de extracción de microondas de recipiente cerrado, el calentamiento es más rápido cuando se utiliza un mayor volumen de disolvente. Sin embargo, la alta relación disolvente-muestra no necesariamente mejora el rendimiento, debido a la falta de uniformidad en la distribución y exposición de la energía microondas (Y. L. Wang, <i>et al.</i>, 2010).</p>
Tamaño de partícula	<p>A veces, el proceso de MAE se puede mejorar mediante un módulo de cizallamiento mecánico que utiliza la fuerza física para fracturar las paredes de las células de la matriz, lo que puede acelerar la disolución de los analitos de interés en el disolvente de extracción (Routray & Orsat, 2012). Esto es debido a que cuando se aumenta la superficie de contacto de la matriz, la eficiencia de extracción generalmente aumenta.</p>
Humedad de la muestra	<p>El rápido calentamiento de las moléculas de agua propias de la matriz provoca la ruptura de la membrana celular y los analitos son liberados en el disolvente.</p>

Figura 11.- Variables que afectan la extracción asistida por microondas (MAE).

Como se menciona en la **Figura 11**, la constante dieléctrica es uno de los factores que afectan al proceso de extracción mediante MAE. Esta constante determina la capacidad del disolvente de absorber energía microondas (Hao, *et al.*, 2002). Por tanto, cuanto mayor es la constante dieléctrica mayor es la capacidad de absorción de esta energía y por tanto el calentamiento del disolvente. En la Tabla 6 se detallan las constantes dieléctricas de los principales disolventes utilizados en MAE.

Tabla 6.- Constante dieléctrica de disolventes usados en extracción asistida por microondas.

Disolvente	Constante dieléctrica a 25 °C	Momento dipolar (debye)
Acetonitrilo	37.5	3.37
Acetona	20.7	2.72
Agua	78.5	1.85
Etanol	24.3	1.68
Metanol	32.6	1.66
Cloroformo	4.8	1.15
Éter dietílico	4.3*	1.15
Tolueno	2.4	0.39
Hexano	1.89*	0.1
Benzeno	2.27	0
Ciclohexano	2.25*	0

*constante dieléctrica a 20 °C

Fuente: Brey (2012).

La MAE en comparación con otras técnicas de extracción modernas, como la extracción con fluidos supercríticos o la extracción con líquidos presurizados, es fácil de usar y los sistemas son más baratos. Este tipo de extracción se aplica a

compuestos polares, pero no es viable para matrices secas o demasiado húmedas cuando se utilizan disolventes no polares. Fundamentalmente esta técnica de extracción se ha utilizado para obtener terpenos de la uva, carotenoides del pimiento, aceites esenciales de diversas plantas como romero, salvia, albahaca o menta y en el ámbito de los compuestos fenólicos es una técnica de reciente implantación para la extracción de dichos compuestos de hojas de olivo, romero, orégano, soja, así como de otras plantas medicinales (Careri, *et al.*, 2007; F. Y. Du, *et al.*, 2009; G. Du, *et al.*, 2010; Liu, *et al.*, 2011; Okoh, *et al.*, 2010; Proestos & Komaitis, 2008)

Como se puede deducir de la información recogida antes de proponer un método de extracción para la determinación de compuestos fenólicos, es necesario optimizar las condiciones que afectan al proceso. De hecho, esta optimización es una etapa clave, ya que el uso de técnicas avanzadas no garantiza mayores rendimientos de extracción si no se han utilizado las condiciones de extracción adecuadas. Por ejemplo, Biesaga (2011), evaluó el efecto de diferentes métodos de extracción (calentamiento por reflujo, maceración, extracción asistida con ultrasonidos UAE y MAE) sobre la degradación de flavonoides, encontrando que la menor degradación se obtuvo mediante la extracción por reflujo durante 30 min en baño de agua. Por el contrario, en este caso la UAE, MAE y maceración provocaron una descomposición significativa de miricetina, kaempferol, ramnetina y quercetina. Estos resultados pueden atribuirse a que las condiciones utilizadas en MAE y UAE no fueron las óptimas. A modo de resumen, en la **Tabla 7** se comparan las técnicas de extracción convencionales y más avanzadas.

Tabla 7.- Comparación de técnicas de extracción tradicionales y más avanzadas.

Técnica	MAE	FMASE	PLE	Soxhlet	Sonicación (UAE)
Breve descripción	La muestra se sumerge en un disolvente absorbente de energía microondas en un recipiente cerrado y se irradia con energía de microondas	La muestra se sumerge en un disolvente absorbente de energía microondas en un recipiente abierto y se irradia con energía de microondas.	La muestra y el disolvente se calientan y presurizan en un recipiente de extracción. Cuando finaliza el proceso, el extracto se transfiere automáticamente a un vial de colección.	La muestra se coloca en un cartucho de fibra de vidrio y utilizando un extractor Soxhlet se percola repetidamente con vapores condensados del disolvente.	La muestra se sumerge en disolvente en un recipiente y se coloca en un baño de ultrasonidos.
Tiempo de extracción	Corto 3-30 min	Corto 10-60 min	Corto 5-30 min	Elevado 3-48 h	Corto 10-60 min
Posibilidad de trabajar con temperaturas elevadas	Si	No	Si	No	No
Tamaño de muestra	1-10 g	1-30 g	1-30 g	1-30 g	1-30 g
Consumo de disolvente	Bajo 10-40 ml	Bajo 10-150 ml	Bajo 10-100 ml	Alto 100-500 ml	Alto 30-200 ml

Técnica	MAE	FMASE	PLE	Soxhlet	Sonicación (UAE)
Extracciones múltiples simultáneas	Si	No	No	No	Si
Selectivo a interferencias de la matriz	No	No	No	No	No
Se requiere purificar el extracto	Si	Si	No se requiere filtración	Si	Si
Necesidad de concentración extracto	Si	Si	Si	Si	Si
Posibilidad de automatización	Si	No	Si	No	No
Inversión	Moderada	Moderada	Alta	Baja	Baja

Fuente: Eskilsson y Björklund (2000).

4.3. Técnicas de análisis de compuestos fenólicos

Como ya se ha comentado anteriormente, la amplia variedad de compuestos fenólicos existentes en la naturaleza con polaridad y tamaños tan variados abarcando desde compuestos fenólicos simples (ácidos fenólicos) hasta oligómeros (procianidinas y taninos condensados) sumado a los bajos niveles de concentración en los que pueden encontrarse en las matrices (Motilva, *et al.*, 2013), ha propiciado el desarrollo de poderosas plataformas analíticas capaces de separar, detectar y dilucidar estructuras fenólicas. De todas estas plataformas destaca la cromatografía líquida de alta resolución (HPLC) acoplada a espectrometría de masas (MS), y concretamente a analizadores de tiempo de vuelo (TOF-MS) o los híbridos cuadrupolo-tiempo de vuelo (QTOF-MS), técnica utilizada en esta Tesis Doctoral para la identificación de compuestos fenólicos en piel, hueso y cubierta del hueso de aguacate.

4.3.1. Cromatografía líquida de alta resolución

Tras realizar la extracción, concentración y/o purificación de los extractos vegetales, es necesario realizar la determinación de los componentes presentes en la matriz. Dada la extremada complejidad de estas muestras se hace necesario un paso previo de separación de los analitos antes de su detección. La Química Analítica dispone de un amplio abanico de técnicas separativas que se fundamentan en las distintas propiedades físico-químicas que poseen los componentes de una muestra para lograr su separación y determinación. Dentro de estas técnicas se encuentra la cromatografía de líquidos, una técnica analítica basada en la separación de

compuestos individuales de una mezcla de compleja gracias a la interacción de los analitos con la fase estacionaria y fase móvil. El fenómeno de separación se basa en las distintas afinidades que presentan los constituyentes de la muestra hacia cada una de estas fases. La fase estacionaria puede ser un sólido poroso, generalmente particulado, o bien una sustancia líquida ligada a un soporte sólido formando una capa fina en su superficie. Estas fases estacionarias suelen estar soportadas en placas de vidrio u otro material o contenidas en el interior de un tubo habitualmente metálico, que es lo que se conoce con el nombre de columna cromatográfica (Weston & Brown, 1997a). Por otro lado, la fase móvil es un disolvente o mezcla de ellos a los que en algunas ocasiones se les modifica el pH mediante adición de ácidos, bases o disoluciones reguladoras. En la Tabla 8 se presentan los diferentes tipos de cromatografía y el fundamento de separación.

La variedad de fases estacionarias y móviles que se pueden seleccionar para HPLC permite la separación de una amplia variedad de compuestos que abarcan desde moléculas pequeñas y se extienden a polímeros tales como proteínas (Moldoveanu & David, 2017b).

El tipo de cromatografía líquida más usada es la de reparto, donde dependiendo de la polaridad de la fase móvil y estacionaria se clasifica en fase normal (fase estacionaria polar y fase móvil apolar) y en fase reversa (fase estacionaria apolar y fase móvil polar) (Weston & Brown, 1997b). Esta última es la más usada para la separación de compuestos fenólicos, ya que se usa para separar moléculas neutras en solución en función de su hidrofobicidad (Weston & Brown, 1997a).

Tabla 8.- Descripción de los tipos de cromatografía.

Tipo	Fase estacionaria	Fase móvil	Fundamento
Adsorción	Sólido con propiedades superficiales.	Líquido o gas	Los analitos se adsorben en la superficie de la fase estacionaria.
Reparto	Líquido retenido sobre un soporte sólido.	Líquido o gas	Los analitos se disuelven en la fase estacionaria.
Intercambio iónico	Sólido con propiedades intercambiadoras de iones (generalmente aniones SO_3^- o cationes $\text{N}(\text{CH}_3)_3^+$ covalentemente unidos a la fase estacionaria sólida).	Líquido	Los iones de carga opuesta son atraídos a la fase estacionaria por fuerzas electrostáticas.
Exclusión molecular	Sólido o gel poroso.	Líquido o gas	Separa moléculas por su tamaño, las moléculas grandes pasan más rápidamente que las pequeñas a través de los poros de la fase estacionaria. No hay interacción atractiva entre la fase estacionaria y los analitos.
Afinidad	Sólido con propiedades de retención bioespecíficas.	Líquido	Es el tipo de cromatografía más selectiva. Emplea interacciones selectivas entre los analitos y las moléculas inmovilizadas en la fase estacionaria. El analito retenido se libera posteriormente por modificación del pH o por la fuerza iónica.
Quiral	Reactivo quiral unido a la fase estacionaria	Líquido o gas	Sirve para la separación de enantiómeros, donde uno de ellos se enlaza con más fuerza que el otro a la fase estacionaria, por lo que se mueve más lentamente a través de la columna.

Fuente: Harris (2006).

Generalmente para esos compuestos las fases estacionarias más empleadas son las que tienen rellenos de sílice modificada químicamente con cadenas de hidrocarburos, generalmente de n-octilo (C-8) o n-octadecilo (C-18), lo que le confiere un alto carácter hidrófobo. También se pueden usar como fase estacionaria materiales poliméricos. Respecto a dimensiones de columnas y tamaños de partícula, comercialmente hay disponibles una gran variedad de ellas. En el caso del análisis de polifenoles las columnas de HPLC convencionales son las más usadas, con tamaños de partícula que oscilan entre 3 y 5 μm (Ali, *et al.*, 2015; Dorta, *et al.*, 2014; Vallverdú-Queralt, *et al.*, 2011), aunque recientemente el uso de UHPLC con tamaños de partícula de 1.7 μm se ha extendido ampliamente para el análisis de polifenoles en matrices vegetales (Giambanelli, *et al.*, 2018; Rodríguez-Carrasco, *et al.*, 2018; Viacava, *et al.*, 2017).

Por otro lado, la fase móvil para esta aplicación es típicamente una mezcla de un disolvente orgánico (acetonitrilo, metanol, isopropanol, etc.) y agua, a las que se le pueden agregar pequeñas cantidades de tampones, ácidos o bases (Moldoveanu & David, 2017b). Aunque se han optimizado métodos para separar compuestos fenólicos de una misma familia utilizando gradientes isocráticos (Amakura, *et al.*, 2000; Haghi & Hatami, 2010; Zuo, *et al.*, 2002), la mayoría de aplicaciones emplean elución en gradiente. Los gradientes utilizados para los compuestos fenólicos comienzan con un bajo porcentaje de fase orgánica, entre un 0–10 % (v/v) si se utiliza acetonitrilo ó 5–10 % en el caso del metanol, y terminan con porcentajes elevados, entre 40–100 % de metanol y entre 30–90 % de acetonitrilo.

La HPLC se usó por primera vez para la determinación de flavonoides en 1976 por Fisher y Wheaton (Merken & Beecher, 2000). Desde entonces, esta técnica se ha utilizado para todos los grupos fenólicos (Motilva, *et al.*, 2013).

La HPLC emplea una presión elevada para forzar el paso del disolvente por la fase estacionaria, cuyo tamaño de partícula suele ser muy pequeño, consiguiendo la separación de los analitos mediante la retención selectiva en la fase estacionaria y su liberación secuencial a la fase móvil (Moldoveanu & David, 2017a). En base a este fundamento, la instrumentación de HPLC está conformada por un sistema de suministro de disolvente (contenedores de disolvente y desgasificador), un sistema de bombeo de alta presión, un inyector (frecuentemente incluido en un automuestreador), un soporte de columna termostaticado, una columna cromatográfica, un sistema de detección con uno o más detectores acoplados y una unidad de controladora para adquisición y procesamiento de datos (**Figura 12**) (Merken & Beecher, 2000).

La eficiencia de la separación de los analitos mediante HPLC está determinada por la ecuación de van Deemter, una fórmula empírica que describe la relación entre la velocidad de fase móvil y eficiencia de la columna. En este sentido, el tamaño de partícula de la fase estacionaria es una de las variables que afectan esta relación, es de esperar que a menor tamaño de partícula la eficiencia aumente. Cuando este tamaño de partícula disminuye a valores inferiores a 2.5 μm , se pueden obtener incrementos drásticos en resolución, sensibilidad y velocidad de análisis. Este descubrimiento dio inicio a la cromatografía de líquidos de ultra alta resolución

(UHPLC). En la **Figura 13**, se presenta la relación entre el tamaño de partícula y la eficiencia de separación, descrita por M. E. Swartz (2005).

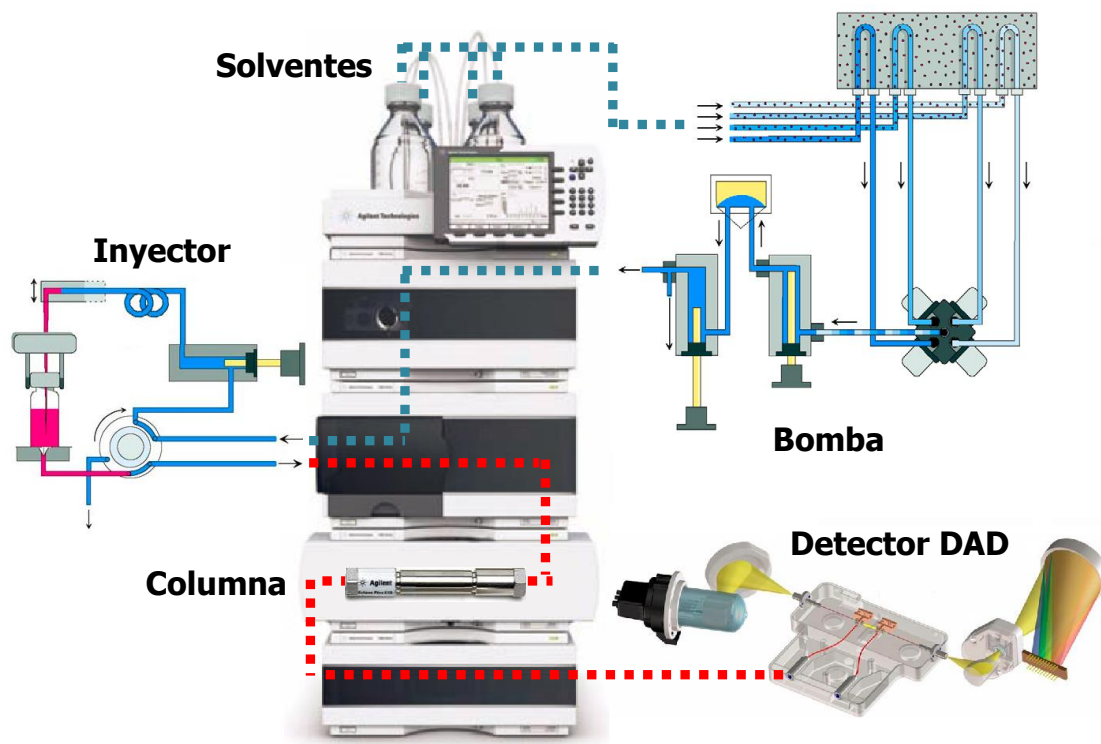


Figura 12.- Esquema de un cromatógrafo de líquidos de alta resolución (HPLC) acoplado a un detector DAD.

Fuente: Tomado y adaptado desde Agilent (2010).

Por otra parte, a menores tamaños de partícula de la fase estacionaria se requiere el uso de altas presiones, lo que puede producir que el calentamiento por fricción de la fase móvil pueda ser bastante significativo y debe considerarse. Por ejemplo, en HPLC utilizando diámetros de columna de 3.0 a 4.6 mm existe una pérdida de rendimiento debido al flujo no uniforme inducido por la temperatura de la fase móvil. En este sentido, para minimizar los efectos del calentamiento por fricción en

UHPLC se usan columnas de diámetro más pequeño (1–2.1 mm) (M. E. Swartz, 2005).

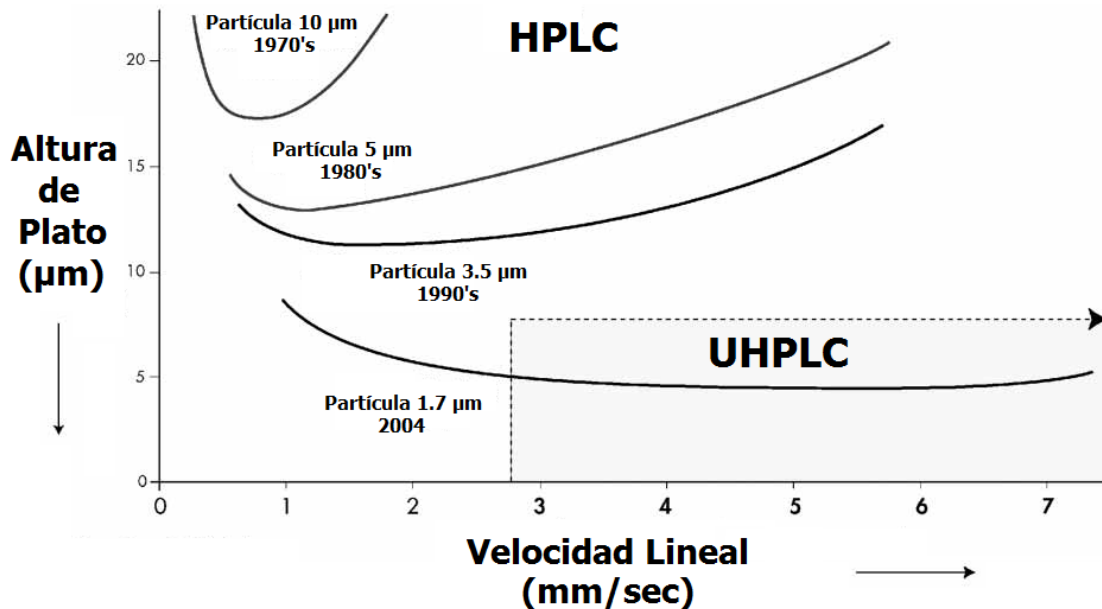


Figura 13.- Relación entre el tamaño de partícula y la velocidad lineal.

Fuente: Tomado y adaptado desde M. E. Swartz (2005).

Sin embargo, la principal desventaja de este sistema es la elevada presión de trabajo, que hace que en ocasiones la tecnología usada en HPLC estándar (bombas, inyector y detectores) no tenga la capacidad necesaria para aprovechar al máximo el uso de fases estacionarias con un tamaño de partícula menor a 2 µm. Por ejemplo, se necesita una presión de 1,035 bar para obtener la máxima eficiencia en una columna de 15 cm de longitud empaquetada con partículas de 1.7 µm. Por lo tanto, se requiere una bomba capaz de suministrar disolvente a esta presión de forma suave y reproducible (M. E. Swartz, 2005). También, el sistema de inyección de la muestra debe operar a esas altas presiones. De igual forma, las válvulas de inyección convencionales, ya sean automáticas o manuales, no están diseñadas para

trabajar a esa presión extrema (M. E. Swartz, 2005). Además, la velocidad de muestreo del detector debe ser lo suficientemente alta como para registrar suficientes puntos de datos a través del pico cromatográfico. La implementación de instrumentación para lograr trabajar con estos niveles de exigencia se consiguió a principios de 2004, cuando se desarrolló el primer sistema de UHPLC (ACQUITY UPLC™ System de Waters), para la separación de pequeñas moléculas, proteínas y péptidos orgánicos relacionados con fármacos (M. E. Swartz, 2005). En la **Tabla 9** se presenta la comparativa entre las principales características de HPLC y UHPLC.

Tabla 9.- Comparación entre HPLC y UHPLC.

Característica	HPLC	UHPLC
Tamaño de partícula	3 a 5 μm	Menor de 1.7 μm
Presión máxima de operación	350–400 bar	1,035 bar
Dimensiones de la columna	150 X 3.2 mm	150 X 2.1 mm
Temperatura de la columna	25–30 $^{\circ}\text{C}$	25–65 $^{\circ}\text{C}$
Volumen de inyección	5–20 μL	5–10 μL

Fuente: Taleuzzaman, *et al.* (2015).

4.3.2. Sistemas de detección

Una vez llevado a cabo la separación de los analitos de interés, es necesario la detección e identificación de los mismos. Los detectores para HPLC/UHPLC (en adelante HPLC) están diseñados para aprovechar algún atributo físico o químico del

analito y convertirlo en una señal medible que corresponda a la concentración o identidad del mismo (M. Swartz, 2010). Un detector ideal, debe poseer las siguientes características:

- Sensible a pequeñas concentraciones de analito.
- Respuesta reproducible y estable.
- Respuesta a todos los analitos o que tenga una especificidad predecible.
- Debe poseer un amplio intervalo de respuesta lineal.
- Respuesta independientemente de la temperatura y composición de fase móvil.
- Que no contribuya al ensanchamiento de la banda extra-columna.
- Seguro y práctico.
- No destructivo.
- Respuesta rápida.
- Bajo nivel de ruido con línea base estable.

A pesar de que hasta el momento ningún detector disponible comercialmente cumple con todas estas características, con el tiempo se han diseñado diferentes tipos de detectores para responder a uno u otro desafío en particular. La elección del detector idóneo para cada aplicación se hace en base a la naturaleza y propiedades de los analitos a determinar así como a la sensibilidad requerida y tipo de información que se desea obtener (estructural, cuantitativa, etc.). Los sistemas de detección más usuales se pueden englobar en tres grandes grupos: ópticos,

electroquímicos y de otro tipo. En la **Tabla 10** se detallan brevemente los diferentes tipos de detectores para cromatografía de líquidos.

Los detectores también pueden clasificarse según sean destructivos (electroquímico, espectrometría de masas, etc.) o no destructivos (absorción UV-Vis, fluorescencia, etc).

Tabla 10.- Tipos de detectores para cromatografía de líquidos.

Tipo	Propiedades	Ejemplo
Propiedades generales	Miden una propiedad física del eluyente.	Índice de refracción
Propiedades específicas	Responden a una característica que es exclusiva de un analito.	Absorción UV-Vis Fluorescencia Conductividad
Cambios en la fase móvil	Se cambia la fase móvil para inducir un cambio en las propiedades del analito.	Detector evaporativo de dispersión de luz

Fuente: Harris (2006).

En el caso de la determinación de compuestos fenólicos los más utilizados son la detección espectrofotométrica UV-Vis y la espectrometría de masas (MS). A continuación, se describen los detectores de absorción UV-Vis y analizadores de masas de tiempo de vuelo y cuadrupolo-tiempo de vuelo usados para el desarrollo de la parte experimental de la presente Tesis Doctoral.

4.3.2.1. *Espectroscopía de absorción UV-Vis*

La detección mediante espectroscopía de absorción UV-Vis es el ejemplo más común de detector que utiliza una propiedad específica del analito (cromóforo), en este caso la capacidad de absorber radiación. Son muchas las moléculas que pueden absorber radiación de esta zona del espectro electromagnético, por lo cual, este detector se comporta como un detector universal (M. Swartz, 2010). Los detectores de absorción UV-Vis pueden ser de longitud de onda fija, donde sólo se mide la radiación absorbida de una longitud de onda fijada; o de longitud de onda variable, también conocidos como detectores de diodos en fila o “diodo array” (DAD), los cuales adquieren los espectros completos de absorción de los analitos que pasan a través de la celda de detección. Estos últimos son los más usados para el acoplamiento a técnicas separativas, en especial a HPLC.

La detección UV-Vis es muy robusta, lo que lo convierte en uno de los detectores más usados en análisis cuantitativos. Para análisis cualitativos, dado que no proporciona información estructural, no permite identificar de manera inequívoca compuestos si no se dispone de patrones comerciales, lo cual es un inconveniente. Sin embargo, su sencillez, rapidez y relativo bajo coste hacen que su uso esté ampliamente extendido.

Este tipo de detector es muy usado en el análisis de compuestos fenólicos, ya que debido a la existencia intrínseca de enlaces dobles y aromáticos conjugados en su estructura estos compuestos son cromóforos, lo que le confiere capacidad de absorción de radiación en la región UV–Vis. En general los compuestos fenólicos

presentan un espectro con dos bandas principales de absorción, comúnmente denominadas banda I (300–380 nm) y banda II (240–280 nm) (Merken & Beecher, 2000). A pesar de estas bandas de absorción comunes, cada familia de compuestos fenólicos posee unas bandas de absorción características. En la **Tabla 11**, se presentan la longitud de onda (λ) a la cual los compuestos fenólicos presentan su máxima absorción.

Tabla 11.- Bandas de absorción características de los compuestos fenólicos.

Clasificación	Tipo	λ máxima absorción (nm)	
Ácidos fenólicos	Ácidos hidroxibenzoicos	200–290	
	Ácidos hidroxicinámicos	270–360	
Flavonoides	Auronas	240–270	340–370
	Chalconas	220–27	340–390
	Dihidrochalconas	~220	~280
	Flavonas	250–270	330–350
	Flavonoles	250–270	350–380
	Flavanonas	270–295	
	Isoflavonas	245–270	300–340
	Flavan-3-oles	270–280	
Antocianinas	Antocianidinas y antocianinas	265–275	465–560

Fuente: Merken y Beecher (2000)

Una de las limitaciones del detector DAD es la imposibilidad de reconocer la presencia de azúcares o grupos acilo enlazados a compuestos fenólicos, debido a que estos grupos de compuestos no son cromóforos fuertes. Además, como se ha comentado previamente dado que no proporciona información estructural, es

necesario el uso de patrones comerciales para identificar de manera inequívoca un compuesto. Sin embargo, su sencillez, rapidez y relativo bajo coste hacen que su uso esté ampliamente extendido, acoplado en ocasiones a otros detectores como espectrometría de masas (MS) y resonancia magnética nuclear (RMN) para una identificación inequívoca de compuestos fenólicos en matrices vegetales (Stalikas, 2007).

4.3.2.2. Espectrometría de masas

Los detectores de espectrometría de masas (MS) son sistemas de detección utilizados para identificar y cuantificar compuestos de interés en diferentes productos vegetales, debido a su gran potencial y alta sensibilidad (Dorta, *et al.*, 2014). Este sistema se basa en la separación a vacío de iones en fase gaseosa de acuerdo a su relación masa/carga (m/z). En los últimos años este sistema de detección ha experimentado un auge espectacular, lo cual se debe a las ventajas que posee, principalmente a su selectividad y al hecho de que es uno de los pocos sistemas de detección que proporciona información estructural (Aaby, *et al.*, 2007). A esto se le suma el hecho de que cuando se acopla a una técnica separativa, como puede ser la HPLC, el uso de este detector proporciona una segunda dimensión de separación, ya que tras la separación de los analitos en la técnica separativa se produce una segunda separación de los mismos en el espectrómetro de masas en función de su relación m/z .

Existen diversos tipos de analizadores de masas: analizador de cuadrupolo o filtro de masa cuadrupolar (transmiten los iones de un pequeño rango de m/z a través de un

campo electrodinámico), trampa de iones (los iones pueden ser formados y confinados durante cierto tiempo por la acción de campos eléctricos y/o magnéticos), analizadores de tiempo de vuelo (se fundamentan en la separación en el tiempo en base a la velocidad del ión en un tubo de vuelo), analizadores de sector magnético (utilizan un imán permanente o un electroimán para hacer que el haz de iones de la fuente se mueva en una trayectoria circular de 180, 90 ó 60 grados) y analizadores de resonancia ciclotrónica de iones con transformada de Fourier (la parte esencial de un instrumento de transformada de Fourier es una trampa de iones en la cual los iones circulan en órbitas bien definidas durante largos períodos de tiempo) (Glish & Burinsky, 2008).

El espectrómetro de masas está compuesto básicamente por un sistema de introducción de muestra, un sistema de ionización, un acelerador de iones a través de un campo eléctrico, un analizador que dispersa los iones en función de su relación masa/carga (m/z), un detector de iones y finalmente un sistema de adquisición de datos que procesa la señal generada por el detector. La fuente de ionización es el compartimento donde se producen las especies cargadas, la ionización por electrospray (ESI) es la más empleada en el análisis de compuestos fenólicos, a continuación se describe esta fuente de iones con mayor detalle.

4.3.2.2.1. Sistema de ionización: Ionización por electrospray (ESI)

Para llevar a cabo el acoplamiento de una técnica que trabaja en fase líquida, como es la HPLC, con un espectrómetro de masas en el que las sustancias para ser analizadas deben encontrarse en fase gaseosa, es necesaria una interfase adecuada.

En el proceso de formación del electrospray, el cual se lleva a cabo a presión atmosférica, intervienen diversos mecanismos al mismo tiempo. La muestra en estado líquido es conducida a través de un capilar de acero inoxidable de pequeño diámetro. A medida que la muestra se pulveriza constantemente con la ayuda de un gas nebulizador (generalmente nitrógeno), se aplica un alto potencial eléctrico en la aguja (3–4 kV), dando como resultado la formación de gotitas altamente cargadas (proceso conocido como nebulización). Dos teorías se han planteado para describir el principio de esta técnica de ionización en función de la relación m/z :

- **Modelo de evaporación de iones:** Esta teoría es más favorable para iones con valores de m/z relativamente bajos, donde las gotas se descomponen y, mientras se desplazan dentro de la fuente, su tamaño se reduce continuamente. Eventualmente, las fuerzas de repulsión entre los iones en la superficie de las gotas que se contraen se vuelven muy altas. Estas fuerzas finalmente excederán la tensión superficial del disolvente, lo que dará como resultado iones en fase gaseosa.
- **Modelo de residuo de carga:** Este modelo implica la evaporación continua del disolvente acompañada por la fragmentación de las gotitas de modo que se forma un sólo ion al final de este proceso. Este modelo es el predominante para el caso de iones con alta relación m/z .

La **Figura 14** ilustra los diferentes mecanismos propuestos para la formación de iones durante el proceso ESI.

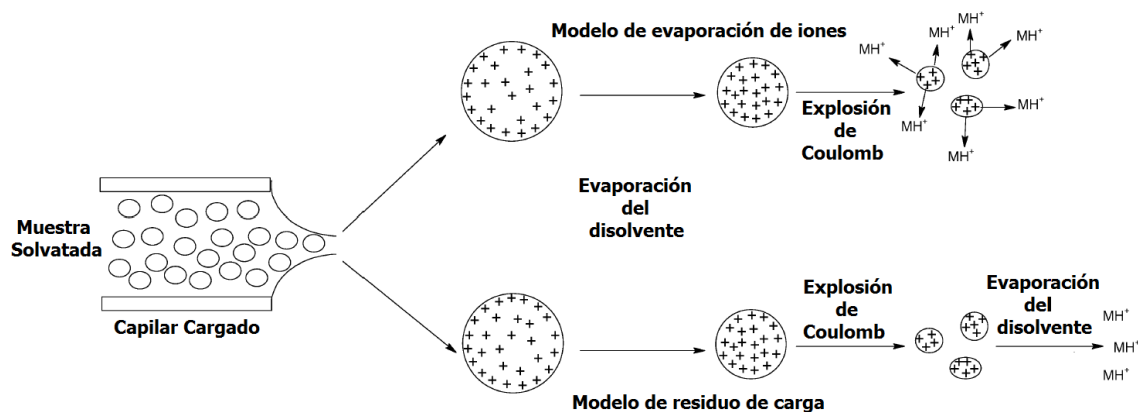


Figura 14.- Formación de iones durante la ionización por electrospray.

Fuente: Tomado y adaptado desde El-Aneed, *et al.* (2009).

Este sistema de ionización se utiliza para estudios cualitativos y cuantitativos de una amplia variedad de productos químicos inorgánicos simples no volátiles y térmicamente lábiles, siendo especialmente útil en la determinación de compuestos fenólicos gracias a que es muy eficaz en el análisis de compuestos polares, lábiles y/o con bajo peso molecular, como lo son estos compuestos.

4.3.2.2.2. *Analizadores de masas*

Una vez que en la interfase se ha llevado a cabo la transformación de los iones procedentes de la técnica separativa desde la fase líquida a la fase gaseosa, los iones son dirigidos hacia el interior del analizador de masas. Estos analizadores de masas permiten la separación, detección y cuantificación de los analitos en estudio con un grado de sensibilidad y selectividad muy elevado, proporcionando información sobre su masa molecular.

Como se ha comentado actualmente existen diferentes tipos de sistemas, en el desarrollo de esta Tesis Doctoral se utilizaron los analizadores de tiempo de vuelo

(TOF) y cuadrupolo-tiempo de vuelo de ultra-alta resolución (UHR-QTOF), que son descritos a continuación:

- **Tiempo de vuelo (TOF)**

El analizador de tiempo de vuelo separa los iones según la distinta velocidad que adquieren en el interior del tubo de vuelo (1–2 m de longitud) en función de su relación m/z . En primer lugar, los iones son extraídos de la cámara de ionización y dirigidos hacia el tubo de vuelo, lugar en el que se aceleran por aplicación de un campo electrostático que les aporta una elevada energía cinética. Si dos iones (A1 y A2) se forman al mismo tiempo con la misma carga pero la masa de $A1 < A2$, A1 alcanzará el detector antes que A2 (Figura 15). El analizador TOF tiene la ventaja de ser capaz de detectar un rango de masas muy amplio. Además, con la finalidad de mejorar la resolución del TOF lineal, fue desarrollado el detector tipo reflector, que incorpora un dispositivo iónico óptico que cambia el camino de los iones (El-Aneed, *et al.*, 2009).

Los iones con mayor energía cinética penetrarán más profundamente en el reflector de iones y, por lo tanto, los iones serán repelidos gradualmente, mejorando la resolución del analizador TOF (Figura 15 b). Un factor muy importante en la resolución de las medidas de masas exacta es la longitud del tubo de vuelo. Debido a que los iones viajan por un camino más largo en el analizador tipo reflector, se puede obtener una mejor resolución y mediciones de masa exacta más precisas debido al aumento en el tiempo de vuelo de los iones. Por tanto, la resolución entre los iones de diferente m/z será mayor cuanto mayor sea la longitud del tubo de vuelo del espectrómetro de masas

(habrá una mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energía cinética de los mismos.

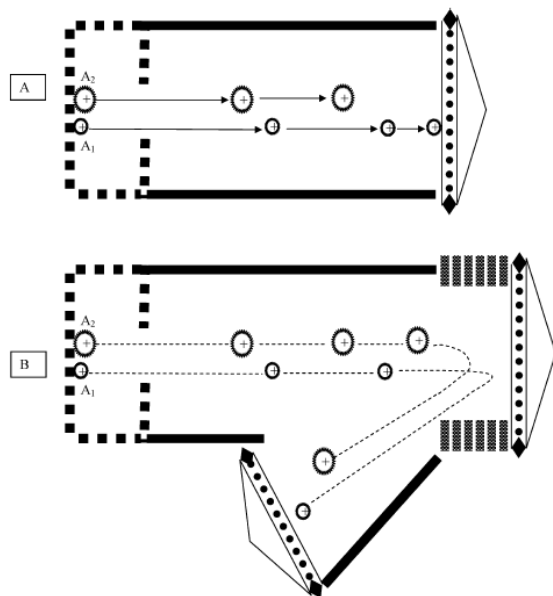


Figura 15.- Representación esquemática de un analizador TOF: a) lineal, b) tipo reflector.

Fuente: Tomado y adaptado desde El-Aneed, *et al.* (2009)

En la **Figura 16** se presenta un esquema de los diferentes componentes presentes en un espectrómetro de masas TOF. Las principales ventajas de este detector es que es un detector rápido, sensible, de gran resolución, que proporciona valores de masa muy exactos. Además la combinación de los valores de masas exactas que proporciona junto con la de la distribución isotópica, permite la determinación de la fórmula molecular del analito, lo cual facilita en gran medida la identificación.

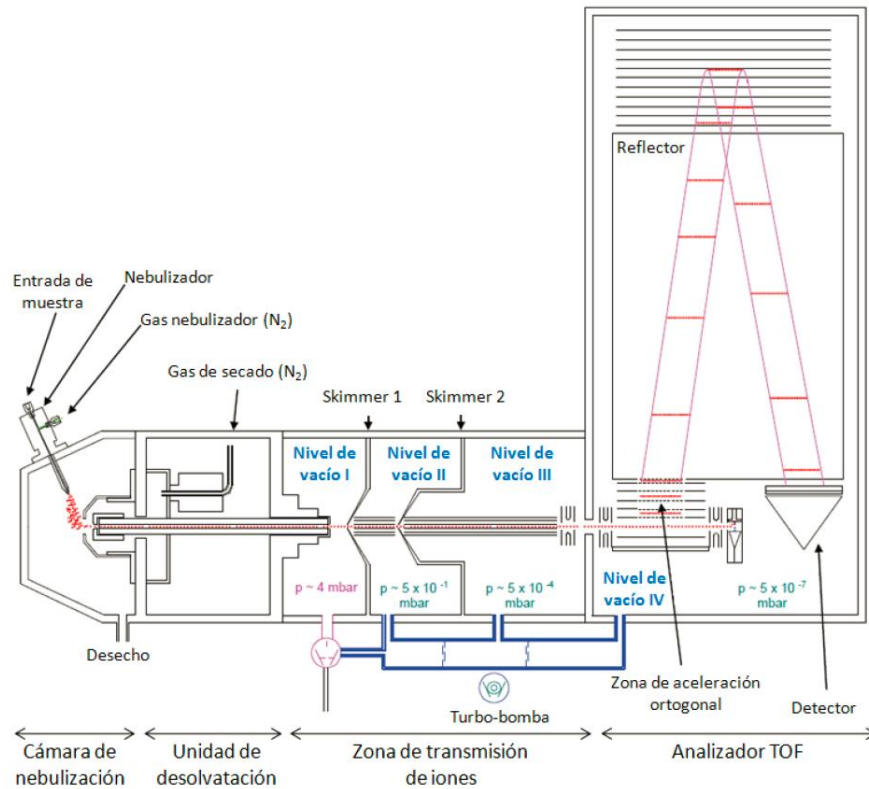


Figura 16.- Esquema de los componentes de un espectrómetro de masas TOF.

- **Cuadrupolo–tiempo de vuelo (Q-TOF)**

El analizador en tándem cuadrupolo–tiempo de vuelo es muy similar al analizador de masas TOF, la principal diferencia es que a los componentes básicos de un TOF se suma un cuadrupolo y una celda de colisión que están geoméricamente alineados en una configuración ortogonal. El cuadrupolo tiene la misión de seleccionar determinados iones para posteriormente fragmentarlos en la celda de colisión con la ayuda de un gas de colisión, que generalmente es nitrógeno. Los iones fragmentados se separan de la misma manera que en un analizador TOF, en función de su relación m/z . Esta modificación permite por tanto obtener espectros de MS y MS/MS en tándem de alta resolución y alta exactitud en la medición de masa (error en un rango

bajo de ppm), siendo hasta 100 veces más sensible que un analizador triple cuadrupolo (El-Aneed, *et al.*, 2009; Glish & Burinsky, 2008).

En la **Figura 17** se presenta un esquema de las diferentes partes de un espectrómetro de masas de cuadrupolo-tiempo de vuelo.

La ventaja principal de un analizador de masas Q-TOF es que añade un tercer nivel de información para la identificación de compuestos con la posibilidad de realizar análisis de MS/MS, lo que se suma a la exactitud de masas y a la distribución isotópica que ya proporcionaba el TOF.

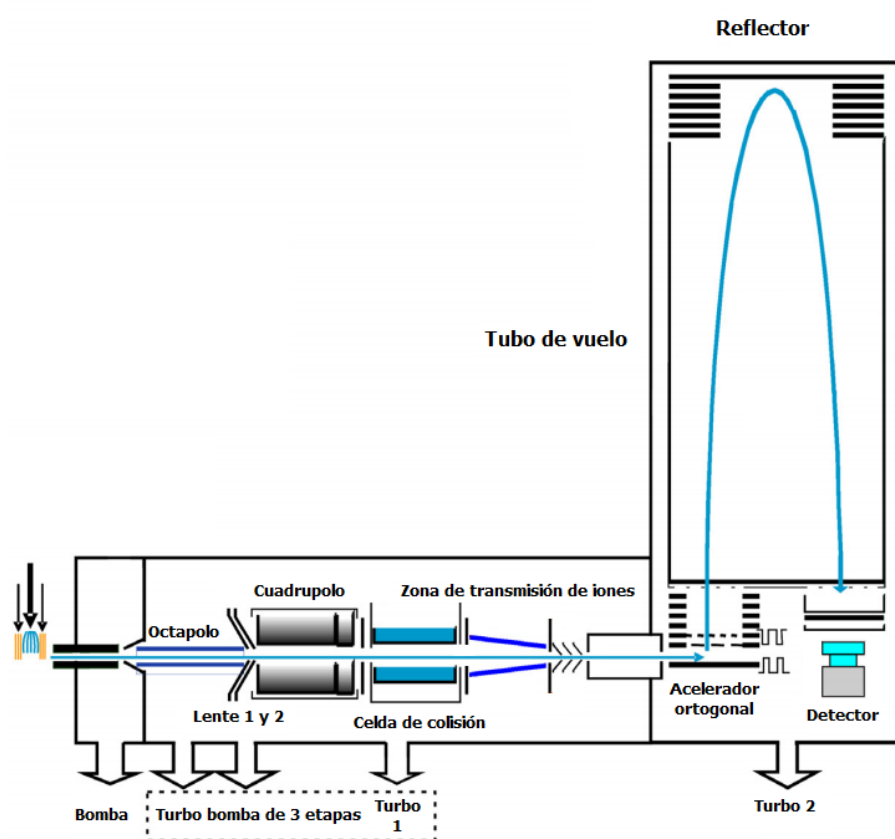


Figura 17.- Esquema de los componentes de un espectrómetro de masas Q-TOF.

Fuente: Tomado y adaptado desde Agilent (2014).

El uso combinado de HPLC con espectrometría de masas de tiempo de vuelo se viene empleando ampliamente en la identificación de compuestos fenólicos en matrices vegetales. Por ejemplo se ha utilizado en muestras como cacao (Ali, *et al.*, 2015), sandía (Abu-Reidah, *et al.*, 2013), tomate (Vallverdú-Queralt, *et al.*, 2011), granada (Gómez-Caravaca, *et al.*, 2013), melocotón (Saidani, *et al.*, 2017), subproductos de mango (Dorta, *et al.*, 2014), subproductos de chirimoya (García-Salas, *et al.*, 2015), hojas de olivo (Quirantes-Piné, *et al.*, 2013), etc. Las condiciones usadas para el análisis de compuestos fenólicos en frutas y subproductos de origen vegetal por HPLC se resumen en la **Tabla 12**.

Tabla 12.- Condiciones usadas para el análisis de compuestos fenólicos en frutas y subproductos de origen vegetal por HPLC.

Matriz	Volumen Inyección	Columna	Temperatura	Fase móvil	Gradiente	Flujo	Detector	Referencia
Avocado	1 µL	C18 (150 x 4.6 mm, 1.8 µm)	50 °C	A: Agua-Ác. fórmico (99.9 : 0.1, v/v) B: Acetonitrilo-Ác. fórmico (99.9 : 0.1, v/v)	0 min, 0% B 20 min, 8% B 40 min, 40% B 45 min 70% B 45.5 min, 100% B	0.60 mL/min	DAD 214, 240, 254 y 280 nm (-) ESI-TOF: 50-1000 m/z	Hurtado- Fernandez, <i>et al.</i> (2011)
Cacao	20 µL	C18 (250 X 4 mm, 5 µm)	25 °C	A: Agua-Ác. trifluoroacético (99.9 : 0.1, v/v) B: Acetonitrilo-Ác. trifluoroacético (99.9 : 0.1, v/v)	0 min, 0 % B 5 min, 10 % B 25 min, 25 % B 5 min, 100 % B	0.8 mL/min	DAD 280-360 nm (-) ESI-IT 100-700 m/z	Ali, <i>et al.</i> (2015)
Tomate	5 µL	C18 (50 x 2 mm, 5 µm)	25 °C	A: Agua-Ác. fórmico (99.9 : 0.1, v/v) B: Acetonitrilo-Ác. fórmico (99.9 : 0.1, v/v)	0 min, 5% B 16 min, 40% B 17 min, 95% B 19.5 min, 5% B	0.4 mL/min	(-) ESI-QTOF: 90-1100 m/z	Vallverdú- Queralt, <i>et al.</i> (2011)
Sandía	5 µL	C18 (150 x 4.6 mm, 1.8 µm)	25 °C	A: Agua-Ác. Acético (99.5 : 0.5, v/v) B: Acetonitrilo	0 min, 1 % B 2 min, 3 % B 15 min, 9 % B 32 min, 35 % B 39 min, 100% B	0.8 mL/min ↓ 0.2 mL/min (masas)	(-) ESI-QTOF- MS: 50-1100 m/z	Abu-Reidah, <i>et al.</i> (2013)
Subproductos de aguacate	3 µL	C18 (100 x 4.6 mm, 2.7 µm)	25 °C	A: Agua-Ác. Acético (99.1, v/v) B: Acetonitrilo	0 min, 5% B 4 min, 15% B 5 min, 16% B 8 min, 18% B 12 min, 100% B 14 min, 100% B 16 min, 5% B 18 min, 5% B	1.2 mL/min ↓ 0.4 mL/min (masas)	DAD 200-600 nm (-) ESI-QTOF: 50-1500 m/z	López-Cobo, <i>et al.</i> (2016)

Matriz	Volumen Inyección	Columna	Temperatura	Fase móvil	Gradiente	Flujo	Detector	Referencia
Subproductos de mango	10 µL	C18 (250 x 4.6 mm, 5 µm)	25 °C	A: Agua-Ác. fórmico (99:1, v/v) B: Acetonitrilo-Ác. fórmico (99:1, v/v)	0 min, 0 % B	1 mL/min	DAD: 280, 320 y 360 nm (-) ESI-QTOF-MS 100-1000 m/z	Dorta, et al. (2014)
					15 min, 25% B			
					30 min, 30 % B			
					60 min, 80 % B			
					65 min, 100 % B			
					70 min, 0 %B			
					0 min, 5% B			
					2 min, 7% B			
					4 min, 9% B			
					7 min, 12% B			
8 min, 15% B								
9 min, 16% B								
12 min, 18% B								
14 min, 20% B								
15 min, 22% B								
16 min, 25% B								
18 min, 28% B								
19 min, 30% B								
20 min 31% B								
21.50 min, 32% B								
25 min, 100% B								
30 min, 100% B								
32 min, 5% B								
0 min, 5 % B								
5 min, 15% B								
25 min, 30% B								
35 min, 95% B								
40 min, 5% B								
45 min, 5% B								
Subproductos de chirimoya	0.5 µL	C18 (100 x 4.6 mm, 2.7 µm)	25 °C	A: Agua-Ác. Acético (99.5:0.5, v/v) B: Acetonitrilo	0.8 mL/min	DAD 200-600 nm 240, 280, 330 y 520 nm (-) ESI-QTOF-MS 50-1200 m/z	García-Salas, et al. (2015)	
					↓			
					0.3 mL/min (masas)			
Hojas de olivo	1 µL	C18 (150 x 2 mm, 3 µm)	25 °C	A: Agua-Ác. Acético (99.5:0.5, v/v) B: Acetonitrilo	0.2 mL/min	(-) ESI-QTOF-MS 50-1100 m/z	Quirantes-Piné, et al. (2013)	

Además, en la mayoría de casos la fase estacionaria, el disolvente y el gradiente cromatográfico deben optimizarse previamente para lograr separar e identificar los diferentes grupos y subclases de compuestos fenólicos presentes. En la **Figura 18**, se resume las diferentes variables que se deben considerar para optimizar el análisis de compuestos fenólicos por cromatografía de líquidos.

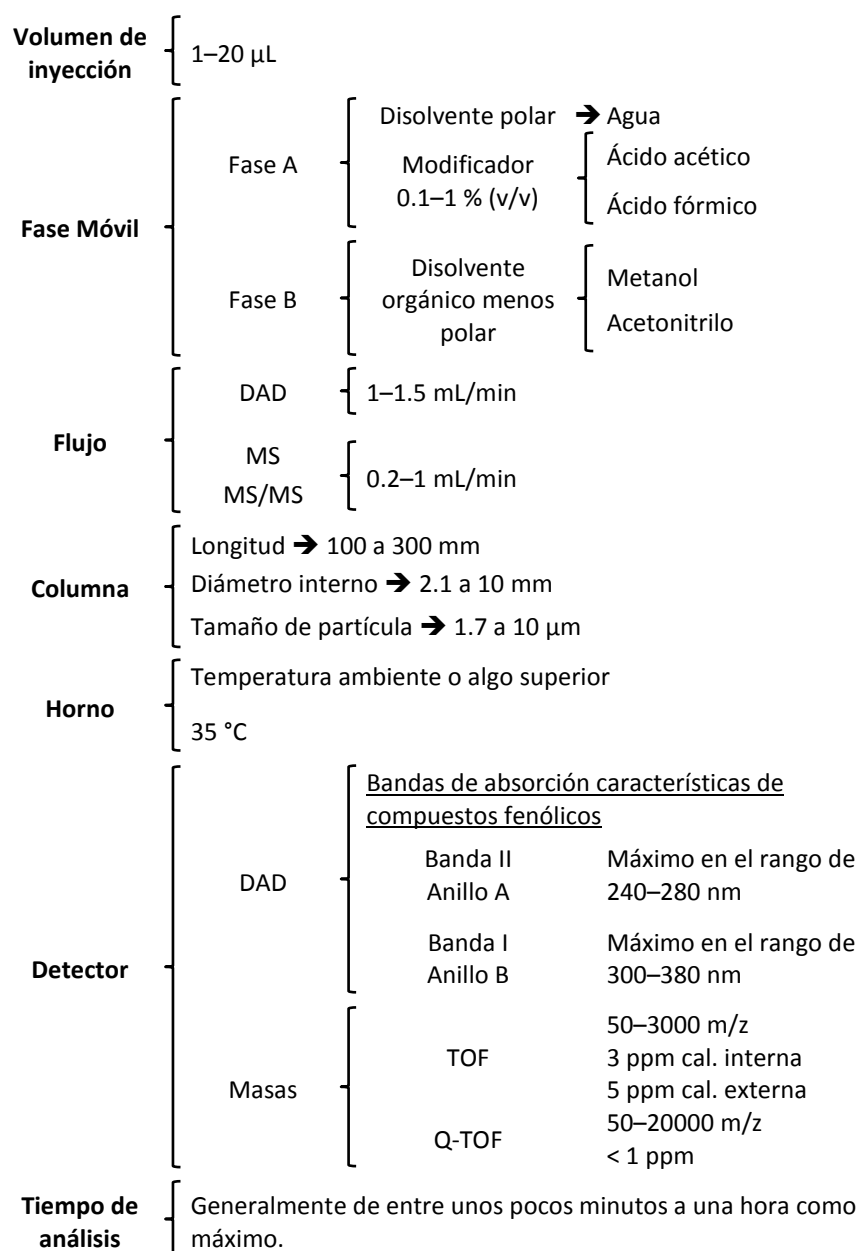


Figura 18.- Variables a considerar para el análisis de compuestos fenólicos por HPLC.

5. Bioactividad *in vitro* de compuestos fenólicos

De acuerdo a estudios previos, los subproductos de aguacate representan una fuente de compuestos bioactivos con propiedades funcionales que pueden aprovecharse a nivel industrial. Sin embargo, esta bioactividad puede disminuir o incrementarse en función de las condiciones de pretratamiento de muestra y extracción utilizadas. Considerando que en esta Tesis Doctoral es la primera vez que se reporta el uso de técnicas de extracción avanzadas combinadas con disolventes "Green", es necesario evaluar la bioactividad de los extractos obtenidos una vez conocida su composición. En este sentido, a continuación se describen los bioensayos utilizados para evaluar la actividad antioxidante y el potencial para inhibir la enzima acetilcolinesterasa, relacionada con la enfermedad de Alzheimer.

5.1. Evaluación *in vitro* de la actividad antioxidante

Los radicales libres y otras especies reactivas de oxígeno (ROS) se forman constantemente en el cuerpo humano. Estos radicales libres se ha demostrado que están implicados en el desarrollo de numerosas patologías, incluyendo cáncer, aterosclerosis, artritis reumatoide y enfermedades neurodegenerativas (Aruoma, 1998), así como en el proceso de envejecimiento.

Además, se conoce que los radicales libres participan en la peroxidación lipídica de los alimentos, proceso causante de malos olores y sabores rancios que disminuyen la calidad nutricional (Scherer & Teixeira Godoy, 2009).

Para combatir la acción de estos radicales libres en la naturaleza existen antioxidantes, que son sustancias que en concentraciones relativamente bajas

previenen, retrasan o reparan los daños oxidativos (Oliveira, *et al.*, 2014). Biológicamente, los antioxidantes desempeñan sus funciones beneficiosas mediante la transferencia de un átomo de hidrógeno (H) o un electrón (e^-) a estas especies reactivas, inactivándolas (Apak, *et al.*, 2016).

La capacidad de inhibir la acción de ROS de cualquier compuesto o extracto puede determinarse por varios modos. De ellos, los métodos *in vitro* se caracterizan por su bajo coste y alto rendimiento, lo que los hace idóneos para el cribado o selección de compuestos o extractos cuando se dispone de una gran batería al proporcionar un valor o índice que permite comparar estas muestras de interés entre sí (López-Alarcón & Denicola, 2013). Existe una variedad de métodos *in vitro* para evaluar la actividad antioxidante en matrices vegetales basados en los diferentes mecanismos de acción que tienen los compuestos antioxidantes para combatir las ROS: transferencia de átomos de hidrógeno (HAT), transferencia de electrones (ET) y modo mixto (HAT/ET) (Apak, *et al.*, 2016; Schaich, *et al.*, 2015). Como la capacidad antioxidante total de una muestra viene determinada por interacciones sinérgicas entre los diferentes compuestos antioxidantes que posee en su composición, así como por el modo de acción concreto de cada uno de ellos, es necesario combinar más de un método de determinación para evaluar de manera correcta la capacidad antioxidante de una muestra (Pérez-Jiménez & Saura-Calixto, 2007). Por lo tanto, la medición de los niveles de actividad antioxidante es útil para determinar el potencial aprovechamiento de los subproductos de aguacate como fuente de compuestos bioactivos en numerosas aplicaciones. En la presente Tesis Doctoral se han utilizado tres métodos *in vitro* diferentes que se describen a continuación.

5.1.1. DPPH (Ensayo de reducción del radical 2,2'-difeníl-1-picrilhidrazilo)

El método 2,2'-difeníl-1-picrilhidrazilo (DPPH[•]) se fundamenta en la medición de la absorbancia ($\lambda_{\text{max}} = 515 \text{ nm}$) de soluciones de un radical estable 2,2'-difeníl-1-picrilhidrazilo (DPPH[•]) antes y después de la adición de diferentes cantidades de un compuesto o extracto antioxidante. Se trata de una reacción de acuerdo a un mecanismo principalmente HAT, pero también hay evidencias que puede ocurrir un mecanismo mixto (Apak, *et al.*, 2016; Schaich, *et al.*, 2015; Zhong & Shahidi, 2015).

El color púrpura original de la solución de DPPH[•] se atenúa a una tonalidad amarilla debido a la reducción de DPPH[•] a DPPH-H por la donación de un átomo de hidrógeno de las sustancias antioxidantes presentes en el medio a los radicales DPPH[•] (Apak, *et al.*, 2016). En la **Figura 19** se presenta la reacción del radical estable DPPH[•] con un compuesto antioxidante (AOH). Una vez transcurrida la reacción, se mide la concentración de DPPH[•] restante tras reaccionar con la muestra empleando una curva de calibrado de DPPH[•], y se representa el % DPPH sin reaccionar frente a la concentración de muestra. Esta actividad se expresa como EC₅₀, que indica la concentración del compuesto antioxidante necesaria para reducir la concentración del DPPH[•] en un 50% (Oliveira, *et al.*, 2014), o como actividad antioxidante equivalente en μM de Trolox.

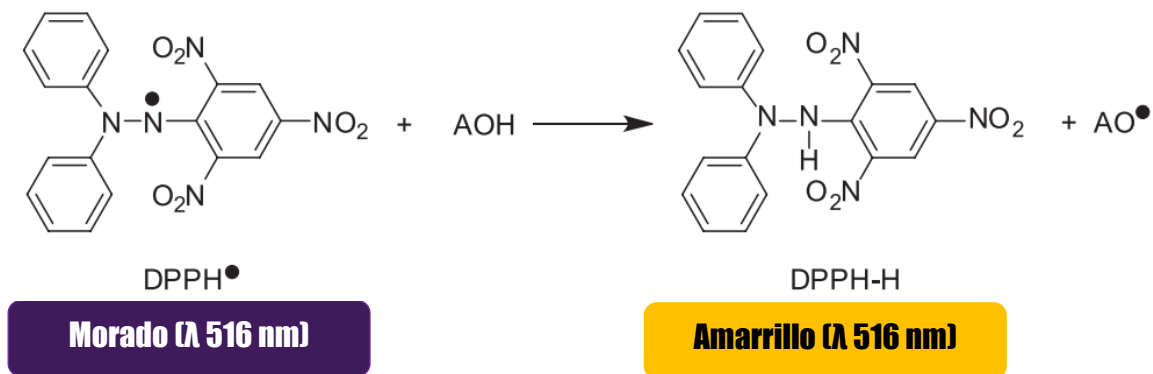


Figura 19.- Reacción del radical estable DPPH[•] con un compuesto antioxidante.

Fuente: Tomado y adaptado desde Oliveira, *et al.* (2014).

Este método tiene las ventajas de que la eficacia antioxidante se mide a temperatura ambiente y, por lo tanto, elimina el riesgo de degradación térmica de las moléculas ensayadas (Bondet, *et al.*, 1997), además de que es un método de bajo coste y alto rendimiento (López-Alarcón & Denicola, 2013). Sin embargo, tiene las limitaciones de que no se consideran datos cinéticos de la reacción por lo que el resultado depende de las condiciones experimentales, además no funciona bien con antioxidantes hidrofílicos y pueden existir efectos por impedimento estérico (Apak, *et al.*, 2016; Bondet, *et al.*, 1997; López-Alarcón & Denicola, 2013; Pérez-Jiménez & Saura-Calixto, 2006).

Este método ha sido ampliamente utilizado en la evaluación de la actividad antioxidante de frutas y sus subproductos, por ejemplo: arándano, ciruela, fresa, limón, mango, manzana, melocotón, naranja, pera, plátano, pomelo, sandía y uva (Floegel, *et al.*, 2011; Kevers, *et al.*, 2007); y en subproductos de aceituna, cacao, mango, guayaba, piña y maracuyá (Ajila, *et al.*, 2010; Herrero, *et al.*, 2011; Martínez, *et al.*, 2012a, 2012b).

5.1.2. TEAC (Trolox Equivalente Antioxidant Capacity)

El ensayo TEAC mide la capacidad antioxidante de una sustancia o extracto para disminuir la concentración del radical catión del ácido 2,2'-azinobis-(3-etilbenzotiazolín-6-sulfónico) (ABTS^{•+}) mediante dos mecanismos: bien por la interrupción de la oxidación inicial y prevención de la producción de ABTS^{•+}, o bien mediante una reacción directa con el radical catión ABTS^{•+} (Schaich, *et al.*, 2015). Esta especie, ABTS^{•+}, es un cromóforo de color azul-verdoso que presenta su máximo de absorción a una longitud de onda de 734 nm. Este radical disminuye su intensidad de absorción en presencia de antioxidantes. La medida en la que disminuye esta coloración, cuantificada mediante el descenso de su absorbancia a 734 nm, depende directamente de la capacidad antioxidante de la sustancia que se encuentre presente en el medio.

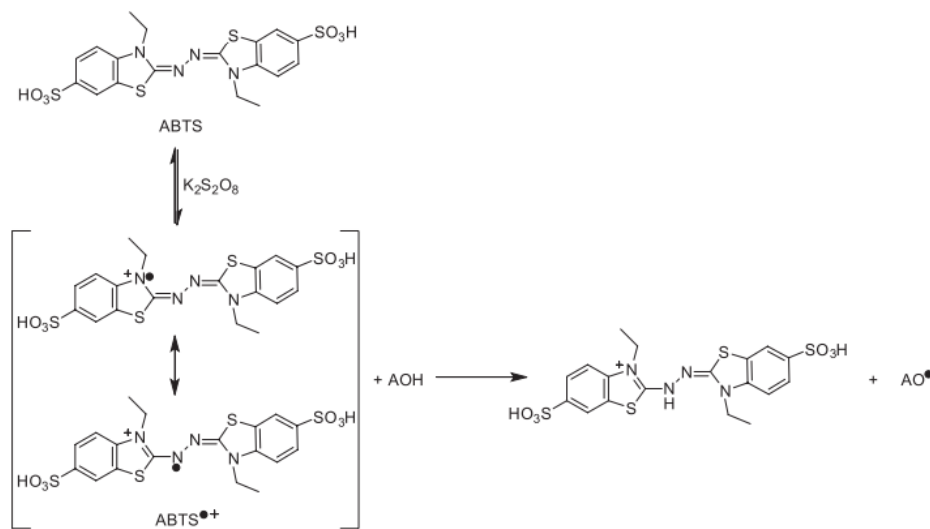


Figura 20.- Oxidación de ABTS por reacción con persulfato de potasio para generar el radical catión ABTS^{•+} y su reacción con un compuesto antioxidante (AOH).

Fuente: Tomado y adaptado desde Oliveira, *et al.* (2014).

A diferencia del radical estable DPPH[•], no se dispone en el mercado del radical ABTS^{•+}, por lo que es necesario generarlo por oxidación del reactivo ABTS usando persulfato de potasio como agente oxidante (Oliveira, *et al.*, 2014), reacción que se presenta en la **Figura 20** junto con la reacción con un compuesto antioxidante.

La principal ventaja de este ensayo, es que es adecuado incluso para muestras muy coloreadas debido a que la absorbancia se mide a una longitud de onda perteneciente a la región del infrarrojo cercano (734 nm), fuera por tanto del rango espectral visible (Oliveira, *et al.*, 2014). Además, el método se puede aplicar para la determinación de sustancias antioxidantes tanto hidrofílicas como lipofílicas (Re, *et al.*, 1999).

5.1.3. ORAC (Capacidad de absorción de radicales de oxígeno)

El ensayo ORAC (Oxygen Radical Absorbance Capacity) se basa en la inhibición de la actividad de las especies reactivas por un antioxidante dando como resultado la disminución de la fluorescencia de una proteína (la fluoresceína) como resultado de la pérdida de su conformación cuando sufre daño oxidativo causado por una fuente de radicales peroxilo (ROO[•]). En concreto mide la capacidad de captación de un radical específico, el peroxilo, generado a partir de la molécula orgánica AAPH o hidrocloreuro de 2,2'-azobis(2-amidinopropano). A diferencia de los ensayos ABTS y DPPH, el método ORAC considera la cinética de la reacción, es decir combina tanto el grado como el tiempo de inhibición en una sola determinación (Galili & Hovav, 2014).

Los radicales se generan calentando AAPH, que por efecto del calor se descompone desprendiendo nitrógeno en estado gaseoso y generando dos radicales de carbono R^\bullet (**Figura 21 a**), que en presencia de oxígeno se convierten casi instantáneamente en radicales peroxilo ROO^\bullet (**Figura 21 b**). Estos pueden atacar moléculas target que presentan fluorescencia como la fluoresceína (**Figura 21 b**) o bien reaccionar con un compuesto antioxidante presente en el medio (**Figura 21 c y d**).

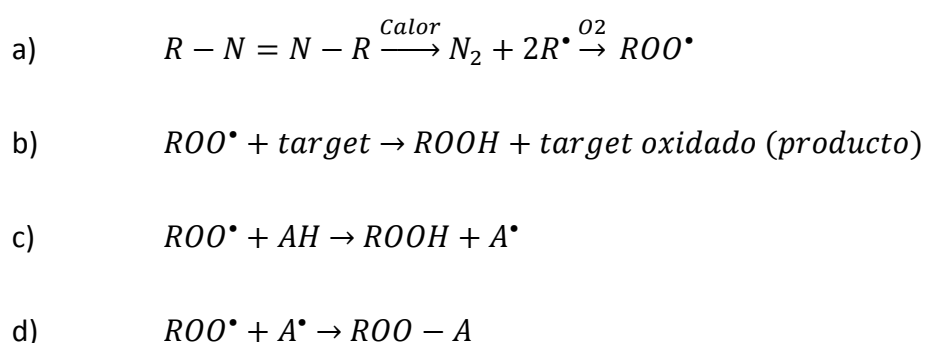


Figura 21.- Reacciones involucradas en el ensayo ORAC.

Fuente: Schaich, *et al.* (2015)

El fundamento del método se basa en la competencia entre el target y los antioxidantes presentes por el radical ROO^\bullet . La fluoresceína, compuesto que presenta una fluorescencia intensa en su forma nativa, cuando es atacada por radicales ROO^\bullet pierde su fluorescencia, de tal forma que si existe un antioxidante en el medio que reaccione con los radicales ROO^\bullet mediante la transferencia de un átomo de hidrógeno (**Figura 21 c**) o la adición de radicales (**Figura 21 d**) se retrasa la pérdida de fluorescencia de la fluoresceína (Prior, *et al.*, 2005; Schaich, *et al.*, 2015). Por tanto, cuando la reacción se realiza en presencia de antioxidantes, estos compuestos captan los radicales peroxilo, lo que hace que el decrecimiento de la

fluorescencia sea menor, por lo que el área bajo la curva de la representación de intensidad de fluorescencia frente al tiempo se incrementa proporcionalmente a la concentración de antioxidantes presentes en el medio.

5.2. Inhibición de la acetilcolinesterasa

La enfermedad de Alzheimer es un trastorno neurodegenerativo crónico que se caracteriza por la pérdida de la capacidad cognitiva, anomalías conductuales graves y, en última instancia, la muerte. Esta enfermedad es una de las formas más comunes de demencia que afecta aproximadamente al 10% de la población mayor de 65 años a nivel mundial (Noridayu, *et al.*, 2011). Una estrategia de tratamiento para mejorar las funciones colinérgicas durante la enfermedad es el uso de inhibidores de la acetilcolinesterasa (AChE) para aumentar la cantidad de acetilcolina presente en las sinapsis entre las neuronas colinérgicas. A este respecto, el uso de un inhibidor selectivo para la AChE ha generado un gran interés para el tratamiento de la demencia provocada por Alzheimer. Se sabe que algunos inhibidores de la AChE, como la fisostigmina o la tacrina, tienen ciertas limitaciones, principalmente una vida media corta o efectos secundarios graves para el paciente como hepatotoxicidad (Jung & Park, 2007). Por lo tanto, la búsqueda de nuevos inhibidores de AChE es de gran interés para combatir esta enfermedad.

Los procedimientos de detección de inhibidores de AChE combinados con cromatografía en capa fina de alta resolución (HPTLC) son los ensayos de enzimas más utilizados hasta el momento (Bräm & Wolfram, 2017). Este bioensayo se basa en el método de Ellman, donde la AChE cataliza la reacción de transformación de la

acetiltiocolina en tiocolina y acetato. El producto de la reacción, concretamente la tiocolina, puede reaccionar con el ácido 5,5'-ditio-bis (2-nitrobenzoico) para formar un anión amarillo (Bräm & Wolfram, 2017; Cieśła, *et al.*, 2015). La acción de las sustancias bioactivas se determina por la desaparición de esta coloración. Las reacciones que se desarrollan durante este bioensayo se detallan en la **Figura 22**.

Brevemente, el método consiste en primer lugar en llevar a cabo la separación de los analitos de interés presentes en el extracto a estudiar mediante HPTLC, empleando una fase estacionaria polar (sílice) y una fase móvil de polaridad intermedia (mezcla de tolueno, acetona y agua). Una vez realizada la separación cromatográfica en placa, mediante calentamiento se elimina el disolvente utilizado como fase móvil. A continuación, se nebuliza sobre la placa de HPTLC una disolución de enzima (3 U/mL) disuelta en buffer de clorhidrato de tris (hidroximetil) aminometano a pH 8 (buffer TRIS), eliminando inmediatamente el disolvente del tampón mediante un flujo de aire. Finalmente, se atomizan disoluciones de sustrato y cromóforo a la placa de HPTLC, yoduro de acetiltiocolina (ATCI) disuelto en agua ultrapura (1 mM) y 5,5'-ditiobis-(ácido 2-nitrobenzoico) (DTNB) disuelta en buffer TRIS, respectivamente. Si en el extracto se encuentran presentes sustancias que contienen propiedades inhibitoras de AChE, aparecerán zonas sin color sobre el fondo amarillo de la placa de HPTLC.

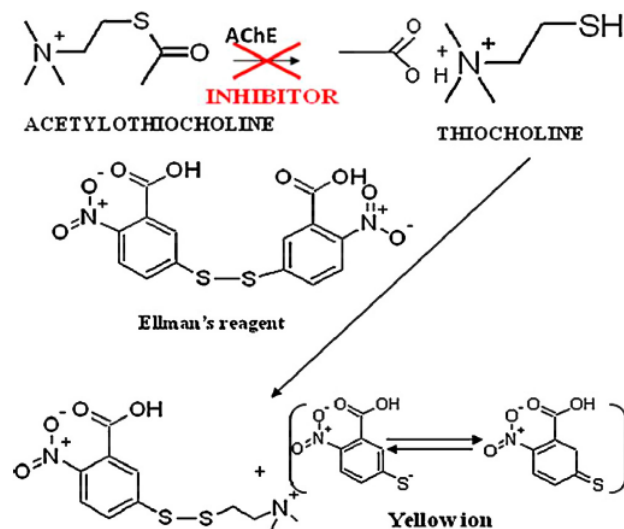


Figura 22.- Método de Ellman para la detección de inhibidores de AChE.

Fuente: Tomado y adaptado desde Cieśla, *et al.* (2015)

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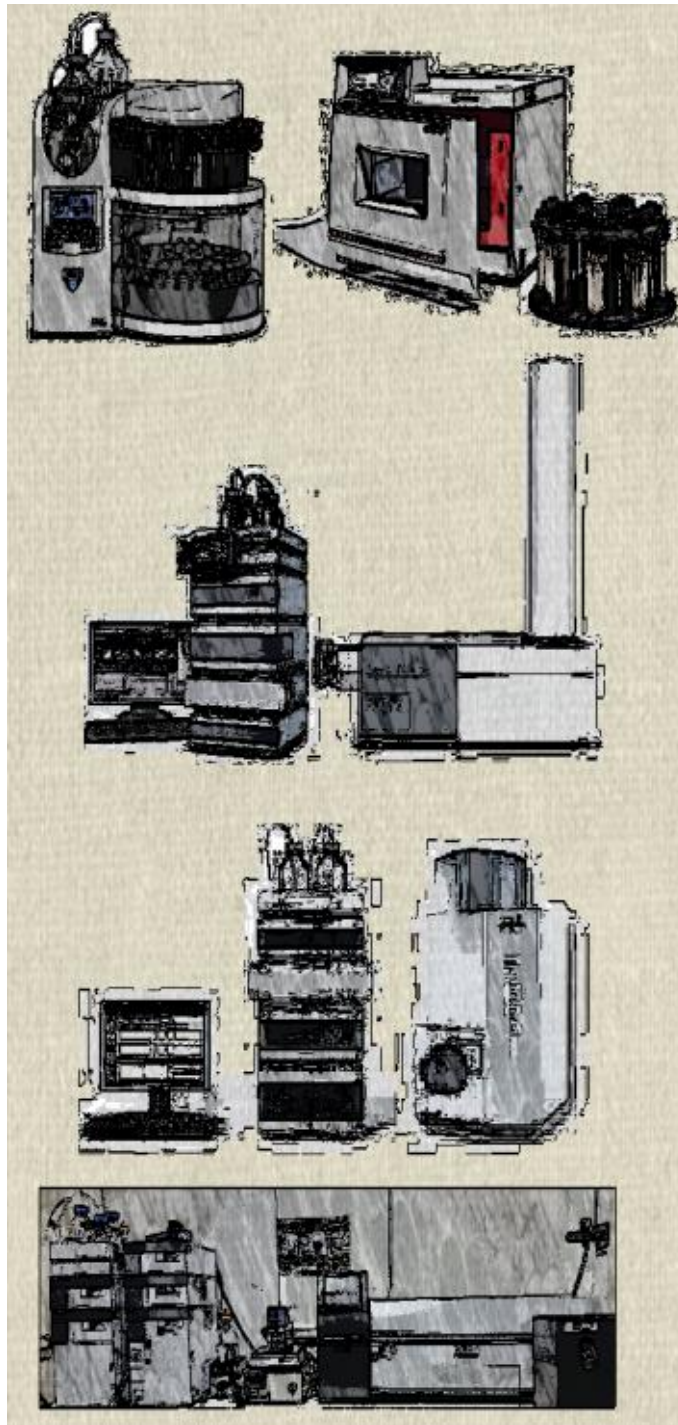
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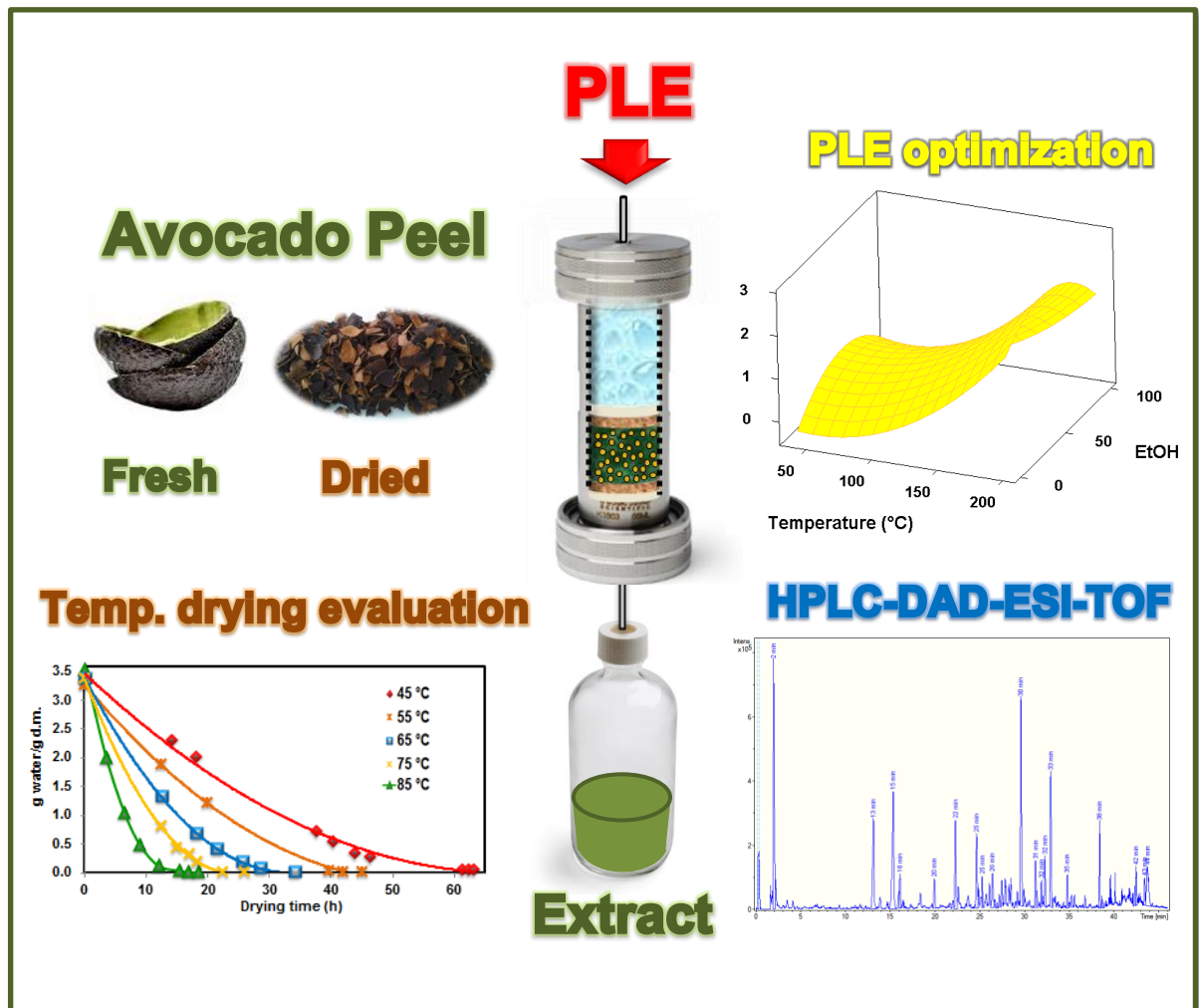
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Parte experimental, resultados y discusión

Capítulo I



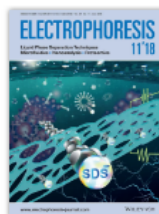
**Optimization of drying process and pressurized liquid extraction
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product**

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Optimization of drying process and pressurized liquid extraction for recovery of bioactive compounds from avocado peel by-product

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Highlights

- PLE was performed with green solvents (ethanol/water).
- A ratio ethanol:water (1:1) and 200 °C were the optimized PLE conditions.
- Drying temperature of 85 °C provided the higher TPC.
- Forty seven phenolic compounds were quantified in avocado peel.
- Phenolic acids were most thermostable compounds.

Abstract

The aim of the present study was to optimize the extraction of phenolic compounds in avocado peel using pressurized liquid extraction (PLE) with GRAS solvents. Response surface methodology (RSM) based on Central Composite Design 22 model was used in order to optimize PLE conditions. Moreover, the effect of air drying temperature on the total polyphenol content (TPC) and individual phenolic compounds concentration were evaluated. The quantification of individual compounds was performed by HPLC-DAD-ESI-TOF-MS. The optimized extraction conditions were 200°C as extraction temperature and 1:1 v/v as ethanol/water ratio. Regarding to the effect of drying, the highest TPC was obtained with a drying temperature of 85°C. Forty seven phenolic compounds were quantified in the obtained extracts, showing that phenolic acids found to be the more stables compounds to drying process, while procyanidins were the more thermolabiles analytes. To our knowledge, this is the first available study in which phenolic compounds extraction was optimized using PLE and such amount of phenolic compounds was quantified in avocado peel. These results confirm that PLE represents a powerful tool to obtain avocado peel extracts with high concentration in bioactive compounds suitable for its use in the food, cosmetic or pharmaceutical sector.

Keywords

Avocado peel; pressurized liquid extraction; drying; phenolic compounds; HPLC-DAD-ESI-TOF-MS

1. Introduction

The avocado (*Persea americana* Mill., Lauraceae) is consumed worldwide as a fresh fruit or processed, existing some avocado value-added products in the market, for instance guacamole, avocado pulp and oil. In this sense, the increase consumption of avocado industrial processed products results in the generation of a considerable amount of solid residue, without any apparent commercial use that is discarded (Kosińska, et al., 2012). The waste of such high quantity of by-products represents an environmental problem with high cost for the food processing industry. In the case of avocado, despite many studies reported high concentration of bioactive compounds in avocado peel, currently it is mainly discarded with no further application or utilized for animal feed (Kosińska, et al., 2012; Morais, et al., 2015; Rodríguez-Carpena, et al., 2011; Saavedra, et al., 2017; Wang, et al., 2010; Widsten, et al., 2014). Therefore, the possible use of this waste as source of bioactive compounds for other applications could be a solution for the processing industries (Rodríguez-Carpena, et al., 2011). Nevertheless, its high moisture content (~75%) and water activity makes it susceptible to decomposition. Therefore, drying is an essential step in the revalorization of this industrial by-product in order to decrease water content to a level at which microbial spoilage and deterioration reactions are minimized.

Furthermore, it should be pointed out that in the majority of experiments described in literature, traditional extractions techniques and incompatible solvents for food or pharmaceutical applications were used. In this sense, many authors have pointed out an excellent antioxidant activity of different avocado peel extracts obtained by

dynamic maceration with solvents not considered as safe (such as methanol or different percentages of acetone-water) (Kosińska, et al., 2012)}(Morais, et al., 2015; Saavedra, et al., 2017; Widsten, et al., 2014). This fact highlights the importance of exploring new green extraction techniques and solvents which are adequate for industrial use and allowed in alimentation or pharmaceutical applications. In this sense, solvents such as ethanol, water or mixtures of them are Generally Recognized as Safe (GRAS) for their use in the food industry. Respect to the extraction techniques, recently Pressurized Liquid Extraction (PLE) combined with GRAS solvents, has demonstrated to be a selective and environmentally friendly extraction technique for the recovery of bioactive compounds from plant matrix. PLE provides several advantages compared with traditional techniques, such as: high extraction efficiencies, less use of solvents and shorter extraction times. Moreover, PLE allows the extraction of compounds with a wide range of polarity, since dielectric constant can be modified by changing the temperature and composition of the extracting fluid, which redefine their selectivity and solvating power (Miguel Herrero, et al., 2013). Furthermore, to the best of our knowledge, this is the first time that an optimization of bioactive compounds extraction, especially phenolic compounds, from avocado peel has been performed.

For these reasons, the aims of this study were, on the one hand, to optimize the effect of extraction conditions, mainly temperature and solvent composition (EtOH:H₂O) for the recovery of phenolic compounds from avocado peel by-product by using PLE with GRAS solvents; and, on the other hand, to assess the influence of

the drying temperature on the TPC and individual phenolic compound concentrations by HPLC-ESI-TOF-MS.

2. Materials and methods

2.1. Chemicals and reagents

For extraction, ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA); methanol, acetone, ethanol and Ottawa sand were supplied from Fisher Scientific (Leicestershire, UK). To measure the TPC, Folin–Ciocalteu reagent, sodium carbonate and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Regarding HPLC analysis, for mobile phase preparation, acetonitrile (HPLC-MS grade) were purchased from Fisher Scientific (Leicestershire, UK) and formic acid was supplied by Sigma-Aldrich (Buchs, Switzerland). For quantification purposes, seven standards were used: procyanidin dimer A2 and trimer B2 were purchased from Extrasynthese (Genay Cedex, France) and (+)-catechin, chlorogenic acid, (-)-epicatechin, quercetin-3- β -glucoside and rutin were supplied from Sigma-Aldrich (Steinheim, Germany). Moreover, protocatechuic acid, quercetin, naringenin and 4-hydroxybenzoic, which were used for identification purposes, were purchased from Extrasynthese (Genay Cedex, France) and Fluka Chemika (Buchs, Switzerland).

2.2. Samples

Fresh avocado fruits (*Persea americana* Mill. cv Hass) were supplied by the commercial group La Caña, Miguel García Sánchez e Hijos, S.A. (Motril, Spain). The fruits were harvested in the subtropical coast of Granada–Spain. Avocados were

allowed to ripen at room temperature. Peels were manually separated, cleaned under continuous flow of tap water and chopped (approximately 4 × 4 cm). As a means of gaining representative results and eliminating factors which could affect the phenolic profile, the peels were separated immediately before the drying process without storage.

2.3. Drying

An oven (Memmert UNE 200, Memmert, Germany) was used in order to evaluate the effect of temperature drying on the total and individual phenolic contents. Five drying temperatures were evaluated (45, 55, 65, 75 and 85°C) in avocado peel. During the process, the samples were turned periodically to ensure uniform dryness. Drying curves were obtained by periodically weighing avocado peels during dehydration. The samples were dried until a final moisture content of approximately 7.0 ± 0.5 g/100 g dm. All drying experiments were performed in triplicate. After that, dried samples were ground using an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting avocado peel powder had an average particle size of 0.5 mm. The obtained material was stored avoiding light exposure and kept at room temperature until its extraction.

2.4. Optimization of pressurized liquid extraction

Response surface methodology (RSM) was used in order to optimize the extraction of phenolic compounds. In this regard, a central composite design (CCD) with two axial points was employed. Solvent ratio EtOH:H₂O (0–100%) and temperature (40–

200°C) were chosen for independent variables and TPC was selected as response variable. The values for those variables are shown in Table 1. For this study the avocado peel dried at 65°C was used. Two extractions were carried out at each experimental design point. Experimental runs were randomised, to minimise the effects of unexpected variability in the observed responses.

Table 1. Central composite design applied for avocado peel phenolic compounds extraction

Experiment	Independent variables		Response
	Temperature (°C)	Solvent ratio (% EtOH)	TPC (mg EAG/g)
1	120	50	9.68
2	120	100	1.96
3	120	0	3.60
4	120	0	4.00
5	120	50	8.48
6	120	50	9.18
7	176	85	7.74
8	40	50	3.80
9	176	15	12.56
10	176	15	13.31
11	63	15	2.17
12	176	85	9.72
13	120	50	9.28
14	120	50	9.03
15	120	50	8.98
16	120	50	9.11
17	63	85	1.37
18	63	15	2.55
19	63	85	1.35
20	40	50	3.48
21	120	50	8.98
22	120	50	9.10
23	200	50	35.09
24	120	50	9.13
25	200	50	33.41
26	120	100	1.91

The extractions were performed using a pressurized liquid extractor (ASE-350, Dionex-Corp., Sunnyvale, CA) equipped with a solvent controller. Briefly, 1 g of dried avocado peel was mixed homogeneously with 5 g of sand and loaded onto 33 mL stainless-steel extraction cells. In order to prevent clogging of the metal frits, disposable cellulose filters were placed at each end of the cell and two portions of sand (5 g) were placed between sample and cellulose filters (Alonso-Salces, et al., 2001). The solvents were previously degassed for 15 min to remove the dissolved oxygen in order to avoid any possible oxidation. The pre-set default conditions were extraction pressure at 11 MPa, with a 20 min static extraction (after a 9 min equilibrium time). Then, the cell was flushed with solvent (60 % of the cell volume) and purged with nitrogen (100 s). The obtained extracts were immediately cooled in ice to attain a temperature of 20–25°C. After that, extracts were centrifuged at 12 000 rpm for 15 min at 4°C in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK). Finally, the supernatants were evaporated under vacuum (13 kPa) at 35°C to dryness in a Savant SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The resulting extracts were stored at –20°C until further analyses.

2.5. Conventional extraction

In order to compare the PLE extracts with conventional extraction methods, two macerations were carried out using the experimental conditions found in bibliography. Briefly, 0.5 g of dried avocado peel dried at 65°C were mixed with 15 ml of extraction solvent (MeOH-water or acetone-water 80:20, v/v) and maintained

in agitation at room temperature during 1 hour. Then the extracts were centrifuged and evaporated until dryness in the same way than the studied PLE extracts. Finally they were stored together with the other PLE extracts at -20°C .

2.6. Determination of total phenolic content (TPC)

The TPC was determined by the Folin–Ciocalteu method with some modifications (Rodríguez-Pérez, et al., 2016). Briefly, the extracts were reconstituted in an appropriated volume of its correspondent extraction solvent mixture. Different concentrations of the extracts were prepared in the range of 1.0–1.5 mg/mL. These dilutions of the studied extracts were tested in order to obtain a value of absorbance comprised in the calibration range obtained with gallic acid as standard. A volume of 10 μL of the diluted extracts was mixed with 600 μL of water and 50 μL of undiluted Folin-Ciocalteu reagent. After 10 min of reaction, 150 μL of Na_2CO_3 (2%, w/v) was added and the volume was made up to 1 mL with water. The mixture was incubated 2 h at room temperature in darkness. After that, 200 μL of the mixture was transferred into a 96 well microplate. A Synergy Mx Monochromator Based Multi-Mode Micro plate reader (BioTek Instruments, Winooski, VT, USA) was used to measure the absorbance at 760 nm. TPC was calculated based on the calibration curve of gallic acid (5–150 $\mu\text{g}/\text{mL}$) prepared in the same manner. The results were corrected with the remain moisture content of peel dried ($7.0 \pm 0.5 \text{ g}/100 \text{ g dm}$) and expressed as mg gallic acid equivalents (GAE)/g dry mass (dm), as mean of three replicates.

2.7. Quantification of phenolic compounds by HPLC-DAD-ESI-TOF-MS

For the HPLC-DAD-ESI-TOF-MS analysis the extracts obtained with the optimized PLE conditions from the avocado peel dried at different temperatures were reconstituted in an appropriated volume of EtOH-H₂O (1:1, v/v) for obtaining solutions at concentrations of 10 mg/mL. Then the extracts were filtered for avoiding possible solid particles using PTFE syringe-filters with a pore size < 0.2 µm. Analyses were performed using an Agilent 1200-RRLC system (Agilent Technologies, Palo Alto, CA, USA) of the Series Rapid Resolution equipped with a vacuum degasser, a binary pump, an autosampler and a UV-vis detector.

The HPLC system was coupled to a microTOF™ mass analyser (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization (ESI) interface operating in negative ionization mode showing the molecular ions [M-H]⁻. The analysis were performed using the instrumental parameters described elsewhere. (Supporting Information). Data were processed using DataAnalysis 4.0 software (Bruker Daltonik) and the molecular formulae were generated using the SmartFormula™ editor.

2.8. Statistical analysis

The data collected for TPC and individual phenolic compound concentration were analysed by one-way analysis of variance (one-way ANOVA) to test the significant effects of five drying temperatures (45, 55, 65, 75 and 85°C). The Tukey's post hoc test was applied for comparisons of means and differences were considered

significant at $p < 0.05$. Statistical analysis and comparisons among means were carried out using the statistical package MINITAB 16.0 software (PA, USA).

3. Results and discussion

3.1. Optimization of pressurized liquid extraction

The present study was conducted to evaluate the optimal conditions for the pressurized liquid extraction of phenolic compounds from avocado peel. A response surface methodology with a CCD was used. Temperature and solvent ratio of EtOH:H₂O mixtures were selected as independent variables and TPC as response variable (Table 1).

The temperature has a high effect on dielectric constant. For instance, water at 25°C has a dielectric constant of ~80 whereas at 250°C the dielectric constant falls to values of 25–27. In these sense, water can be made to resemble different organic solvents (Ollanketo, et al., 2002). Moreover, the water diffusivity increases 10 times when the temperature changes from 25 to 200°C (Miguel Herrero, et al., 2013). On the contrary, high temperatures might have negative effects for the thermo-labile compounds, so in this case different proportions of ethanol and water at several temperatures were used in the experimental design in order to cover a wide range of dielectric constant.

Therefore, to carry out the study of the combined effect of these variables, the following ranges were selected: temperature was set between 40 to 200°C, whereas a fully range of EtOH:H₂O ratio (0–100% EtOH) was evaluated. Furthermore,

extraction pressure were not tested as variable since it was previously reported that the pressure has null effect (Miguel Herrero, et al., 2013; Luthria, 2008). Thus, the pressure was fixed at 11 MPa in order to maintain the solvent in a liquid state. As the extraction was under static conditions, the extraction time was 20 min, a value high enough to reach an equilibrium between those sample components still bound to the matrix and those already solubilized (Luthria, 2008). As mentioned before, TPC was used as response variable.

Nine conditions were evaluated; Figure 1A shows the 3D plot of the response surface for the effects of the temperature and solvent percentage on the TPC. The results showed that the TPC is lower when 100% of pure solvent (ethanol or water) was used compared to solvent mixtures. Moreover, this surface plot shows that the maximum point is outside the experimental region. Nevertheless, the independent variables, temperature of extraction and solvent ratio were tested in their fully operational range. For this reason, it was not possible to move the initial design to achieve this goal. According to the model, the optimal extraction conditions were found at 200°C and using 46% EtOH as extraction solvent. With those values of independent variables, the predicted value of the response variable (TPC) was 27.5 mg GAE/100 g. Nevertheless, as can be seen in Table 2, this value is lower than the experimental one obtained with an axial point at 200°C and 50% EtOH (34 ± 1 mg GAE/g dm). The identical optimal conditions were also observed before for other plant matrix, like during extraction of phenolic compounds from *Stevia rebaudiana* Bertoni leaves (Ciulu, et al., 2017). Moreover, M Herrero, et al. (2010) found the same temperature (200°C) and pure ethanol as the best conditions to extract

phenolic compounds from rosemary, however in that research hydroalcoholic solutions were not evaluated.

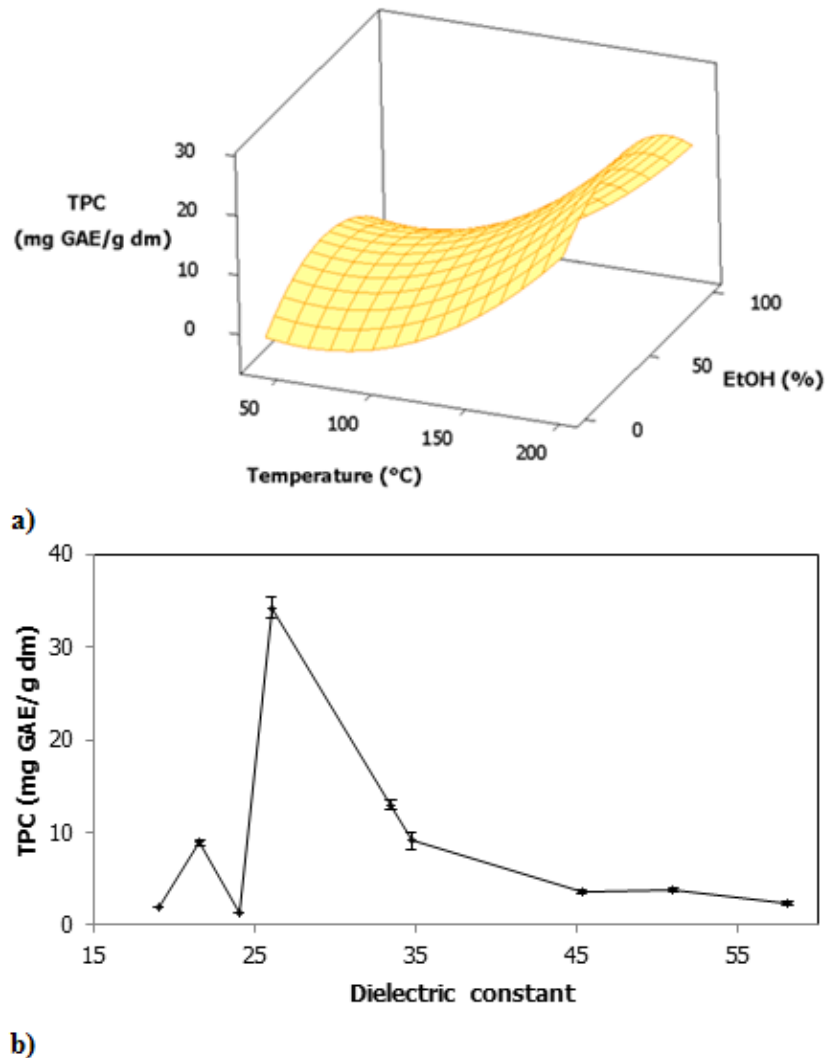


Figure 1. Optimization of phenolic compounds pressurized liquid extraction: (a) Three dimensional plot of response surface (b) effect of dielectric constant on TPC.

The solvent dielectric constants, extraction yields and experimental TPC of the extracts obtained at different conditions are shown in Table 2. The dielectric constant values were obtained from previous studies (Akerlof, 1932; Ciulu, et al., 2017; Lozano-Sánchez, et al., 2014; Uematsu & Frank, 1980). The highest extraction yield was achieved with a solvent dielectric constant of 26.0. In addition, the results

showed that the extraction yield increases when the percentage of water in the solvent mixing ratio is higher. Nevertheless, the TPC in the extracts decrease with high concentrations of water. This could be explained by the fact that too high water content facilitated the concomitant extraction of other compounds, such as sugar and organic acids (Zhao, et al., 2006).

Table 2. Dielectric constant, extraction yield and TPC in the extracts under different experimental conditions.

Temperature (°C)	EtOH (%)	Dielectric constant	Yield (%)	TPC (mg/g extract)
40	50	45.3	11.7 ± 0.7 ^e	31.1 ± 0.1 ^{cd}
63	15	58.1	14.7 ± 0.3 ^{de}	16 ± 2 ^e
63	85	24.0	6.5 ± 0.6 ^f	21 ± 2 ^{de}
120	0	50.9	20.4 ± 0.9 ^c	19 ± 2 ^e
120	50	34.7	16 ± 0.3 ^d	57 ± 6 ^b
120	100	19.0	5.8 ± 0.5 ^f	34 ± 3 ^c
176	15	33.4	33 ± 2 ^b	39.6 ± 0.6 ^c
176	85	21.6	16.5 ± 0.7 ^d	54 ± 4 ^b
200	50	26.0	39 ± 0.2 ^a	88 ± 3 ^a

Mean values with superscript different letters indicate significantly different values ($p < 0.05$).

In the Figure 1 (b) the relation between dielectric constant and TPC is presented. The highest TPC was found with a middle dielectric constant of 26 (200°C and 50% EtOH). It can be seen that highest and lowest dielectric constants provided lowers TPC.

Comparing the PLE extractions with the macerations performed with organic solvents in terms of extraction yield, we found that in all PLE experiments the recovery was higher than the yields obtained in solid-liquid extractions. In fact, the values obtained for the extraction performed with acetone-water and methanol-water was 4.61 and 5.36% respectively. Respect to TPC, the results highlighted a big difference between the values obtained for PLE and the ones obtained for solid-liquid extractions. The first ones ranging between 16 ± 2 and 88 ± 3 mg GAE/g dm whereas the TPC of macerations were 8 ± 2 and 7 ± 2 mg GAE/g dm for acetone and methanol aqueous mixtures, respectively. Those results are reduced by half the lower TPC value for PLE extracts, obtained at 63°C with 15% EtOH. Those results highlight the big potential of PLE for obtained higher yields of extracts enriched in phenolic compounds respect to conventional extraction methods with organic solvents.

Therefore, the effect of drying temperature on phenolic compounds content was evaluated using these last optimum PLE conditions for the phenolic compounds extraction (200°C and 50% EtOH).

3.2. Effect of drying temperature on TPC

Drying kinetics for the different temperatures tested in the convective oven are shown in Fig. 2A. Two drying periods were observed, a first period where the water loss is relatively rapid and the relation between temperature and time could be described by a linear function; then, once the sample reached a moisture ratio of

~0.4 (namely critical moisture content), a second period began until the peels reached a constant weight, this period was described by an exponential function.

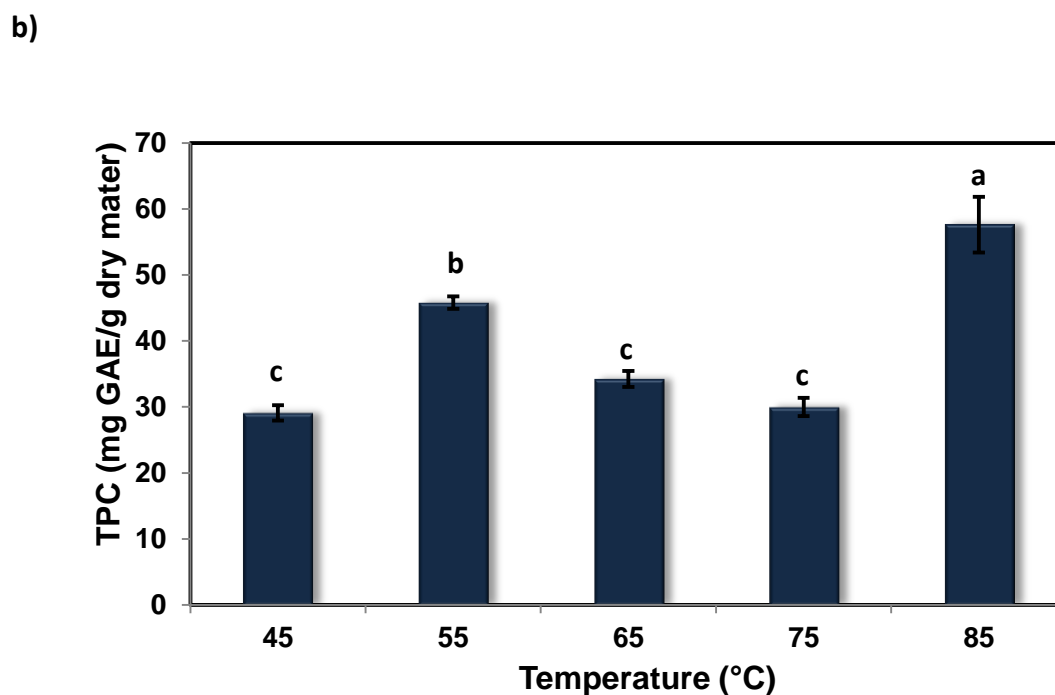
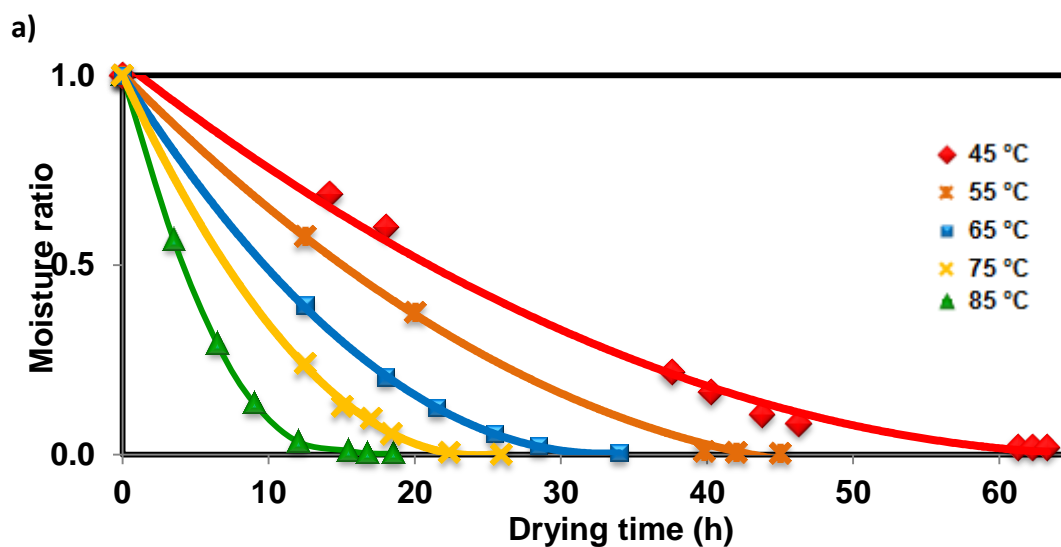


Figure 2. (a) drying kinetics of avocado peel at different temperatures and (b) effect of temperature drying on TPC. Different letters indicate significantly different values ($p < 0.05$).

These results were in agreement with the findings of Saavedra, et al. (2017) who optimized the drying of the avocado peel using an laboratory type tray drier at different temperatures (45–75°C), air-flow (0.8–1.8 m/s) and loading density (1–3 kg/m²). Nevertheless, lower drying times in both phases were found by Saavedra (45–50 and 250–350 min, respectively), these differences could be attributed to the drying method. In this sense, a previous study conducted in willow bark reported shorter drying time when tray drier was used than when the product was dried in oven (Harbourne, et al., 2009). Furthermore, similar behaviour in terms of drying kinetics at different temperatures and the reduction of the moisture content have been previously reported for the drying processes of different food and by-products: such as strawberry fruits (Wojdyło, et al., 2009), pears and orange by-products (Garau, et al., 2007; Mrad, et al., 2012).

As expected, increasing the drying temperature caused an important increase in the drying rate. For instance, when the avocado peel was dried at 85°C, the time necessary for decreasing the moisture content was four-fold less than when the drying temperature was set at 45°C. The initial moisture content of avocado peel was $76.0 \pm 0.8\%$, so the time required to reduce this moisture to a final content of $7.0 \pm 0.5\%$ were 61, 41, 28, 20 and 12 h at 45, 55, 65, 75 and 85°C, respectively. Figure 2B shows the TPC after drying at the five tested temperatures (45–85°C). The results ranged from 29 to 57 mg GAE/g dm, obtaining the highest TPC in the sample dried at 85°C. Different authors have reported the TPC of different vegetables and fruits by-products after drying temperatures. Larrauri, et al. (1997) reported a decrease on TPC of red grape pomace peels after oven drying at 60°. Moreover,

Garau, et al. (2007) indicated a case-hardening effect, when the temperature of air-drying process was evaluated on antioxidant capacity of orange pulp at 90°C, suggesting that beyond this temperature no significant increase in the drying rate would probably be observed. Therefore, temperatures over 85°C were not evaluated in this research. Despite this result, according to several studies, in order to avoid a decrease in TPC during the drying process, this step should be carried out with a temperature around 60°C (Garau, et al., 2007; Larrauri, et al., 1997; Mrad, et al., 2012). This fact was corroborated in the present study, with 55°C as optimum temperature of drying within a range between 45 to 75°C. Nonetheless, it should be pointed out that the highest TPC was found when a higher temperature (85°C) was used. Moreover, when the avocado peel was dried at 45, 65 and 75°C no statistically significance effect was detected on the TPC. Similar results were observed by López, et al. (2010) when the authors evaluated the effect of air temperature on TPC of blueberries. This behaviour could be explained because long drying times contribute to a notable reduction in the TPC due to a possible generation of different degradation products with lower antioxidant activity (Garau, et al., 2007; López, et al., 2010).

In this sense, Saavedra, et al. (2017) optimized the drying process of avocado peel before carrying out a conventional extraction with acetone/water (70:30, v/v). The TPC of the obtained extract was 52 mg GAE/g dm. Similar values were reported by Wang, et al. (2010). In addition, other authors have evaluated the TPC in extracts obtained with solvents such as methanol or acetone/water/acetic acid mixtures, reaching a value of 90 mg GAE/g dm (Rodríguez-Carpena, et al., 2011). Nevertheless,

employment of organic solvents in the extraction process limits the potential food or pharmaceutical applications of avocado by-products. Exploring new green extraction techniques is thus of high importance. In this research, the recovery of phenolic compounds from agro-industrial by-product applying accelerated extraction and using GREEN and GRAS solvents allowed to obtain an extract with a TPC of 58 mg GAE/g dm. In fact, the TPC found in the present research for avocado peel was comparable to that reported by other authors that used conventional extraction techniques with acetone-water or methanol as solvents (Rodríguez-Carpena, et al., 2011) and much higher than the value reported by Morais, et al. (2015) of 13 mg GAE/g dm, who used freeze-dried peels and acetone–water for the extraction of total phenols. However, it is lower than that reported by Rodríguez-Carpena et al., who used freeze-dried peels and employed PLE with acetone-water, predictable results taking into account the type of drying used in this study. Nevertheless, this drying process has as main disadvantage its high energy consume. For this reason, the present technological process that combines an economical drying step (oven-drying) and a powerful extraction technique (PLE) seem to be a potent alternative to extract phytochemicals from avocado by-products balancing cost and efficiency.

3.3. Quantification of bioactive compounds

In order to quantify the individual amount of each compound in the extracts, seven calibration curves with nine points were prepared using catechin, 5-O-caffeoylquinic acid, epicatechin, procyanidin A2, procyanidin B2, quercetin-3- β -glucoside and rutin. Neohesperidin was used as an internal standard and the calibration points were analysed in triplicate. The compound concentrations were determined using the

corrected area of each individual compound (area standard/area internal standard) and by interpolation in the corresponding calibration curve (Supplementary Information).

The validation of the proposed method was performed with linearity, sensitivity, and precision parameters (Supplementary Information).

Forty seven phenolic compounds were identified in dried avocado peel extracts, forty four of them were classified into four phenolic compounds class. Procyanidins was the family with a higher number of compound identified (19 compounds), followed by flavonoids (15 compounds), phenolic acids (seven compounds) and catechins (three compounds) at the end of the list. The other phenolic compounds identified were tyrosol-glucosyl-pentoside, pyrocatechol and vanillin (See Supporting Information Table 2).

Regarding concentration, after the drying at 85°C phenolic acids were the major phenols with 330 ± 10 mg/100 g dm, followed by procyanidins with 269 ± 8 mg/100 g dm, flavonoids with 221 ± 3 mg/100 g dm and finally catechins with 208 ± 4 mg/100 g dm. Flavonols were the main polyphenol subclass presented into the flavonoids family, with a concentration of 212 ± 4 mg/100 g dm.

Rodríguez-Carpena, et al. (2011) used PLE with acetone/water (70:30, v/v) to extract phenolic compounds from freeze dried avocado peel and reported a lower content of phenolic acids (329 mg/100 g dm), flavonols (129 mg/100 g dm) and slightly higher catechins content (228 mg/100 g dm). Regarding to procyanidins (dimers and trimers) content, it seems that a slight degradation occurs compared to other drying

process (Fig. 3). In these sense, the content found at 85°C was lower than the values reported by López-Cobo, et al. (2016) and Wang, et al. (2010) for freeze dried avocado peel. The extractions in these researches were carried out in an ultrasonic bath with methanol/water (80:20, v/v) and acetone/water/acetic acid (70:29.7:0.3, v/v/v), respectively.

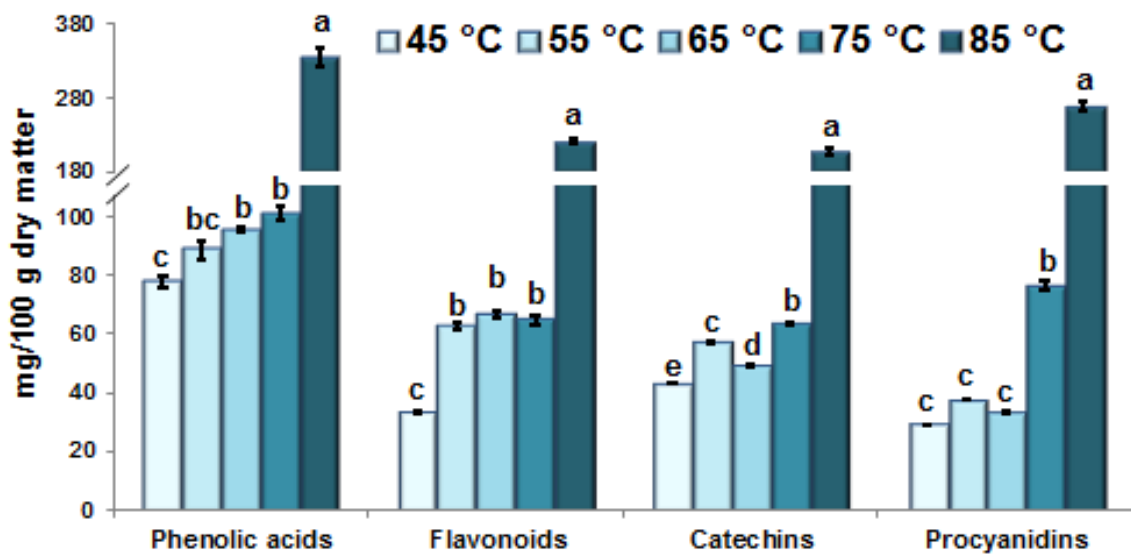


Figure 3. Effect of temperature drying on phenolic compounds content. Different letters indicate significantly different values ($p < 0.05$).

As can be seen in Fig. 3, the drying temperature had a significant effect on phenolic compounds content. For all phenolic classes, the concentrations obtained after an oven drying at 85°C were at least three-fold greater than with others temperatures. In the opposite, 45°C was the treatment that had the most negative effect on the phenolic compounds content. Moreover, these results were in agreement with TPC detailed above. Therefore, long drying times at lower temperatures contributed to a notable reduction in the concentration of phenolic compounds.

On the other hand, the compounds with a more stable behaviour were the phenolic acids, showing a slight increase with temperature. Taking into account that the highest TPC were obtained at higher drying temperature, it could be assumed that the antioxidant activity of avocado peel extracts is related to higher concentration of phenolic acids. The rising of phenolic acids due to drying temperature increase (from 50 to 90°C) was also reported for the rhizome of *Cimicifuga dahurica* (Qin, et al., 2017). Meanwhile, procyanidins seemed to be the phenolic compounds whose content varied the most with temperature, increasing their concentrations 11 and 45 times at 75 and 85°C respectively compared to the values obtained at lower temperatures. Regarding to this behaviour, Ciulu, et al. (2017) found an increase in the amount of phenolic compounds thanks to an increase in drying temperature for *Stevia rebaudiana* Bertoni leaves.

The effect of drying temperature on the concentration of individual phenolic compounds is showed in Table 3. (+)-Catechin was the major compound found in avocado peel after being dried at 85°C, whereas 4-hydroxybenzoic acid was the most important for others treatments (45–75°C).

Respect to phenolic acids, the stability is not the same for all the identified phenolic acids. For instance, the isomers of caffeoylquinic acid were the major phenolic acids found after drying at 85°C. On the other hand, when the avocado peel was dried at lower temperature (< 85°C) these compounds were not detected, which highlight that these compounds could suffer degradation with longer drying process. In addition, 3,4-dihydroxyphenylacetic acid, 4-hydroxybenzoic acid and vanillic acid showed the highest concentration when the avocado peels were dried at higher

temperature (85°C). Meanwhile, other acids seem to suffer a decrease with higher temperatures, such as protocatechuic acid, which showed its major concentration at 65°C.

About 82% of flavonoids identified in the avocado peel extracts were highly affected by longer drying process. Thus, a reduction in its concentration above 70% compared with the value found at 85°C was found when a temperature of 45°C was used. As an example, the quercetin derivative with a $[M-H]^-$ ion at m/z 609.148, tentatively identified as multinoside A, was the major flavonoid identified. This compound suffered a reduction in its content of 89% when the peel was drying at 45°C compared to the concentration obtained in the sample dried at 85°C.

Furthermore, about 74% of the procyanidins were completely degraded after the drying at 45°C. This behaviour could be related to the long drying process necessary to decrease the moisture content at lower temperatures, which may cause a decrease of compounds concentration (Garau, et al., 2007).

Table 3. Concentration of phenolic compounds after drying process at different temperatures

Analyte	RT (min)	m/z	Concentration (mg/100 g dm)				
			45 °C	55 °C	65 °C	75 °C	85 °C
3,4-dihydroxyphenylacetic acid	8.05	167.035	11.7 ± 0.4 ^d	12.8 ± 0.4 ^{cd}	13.9 ± 0.6 ^c	15.1 ± 0.4 ^b	16.6 ± 0.3 ^a
Protocatechuic acid	8.61	153.019	11.4 ± 0.1 ^c	11.2 ± 0.3 ^c	14.8 ± 0.3 ^a	12.7 ± 0.1 ^b	10.4 ± 0.2 ^d
Pyrocatechol	9.64	109.030	8.85 ± 0.05 ^c	10.1 ± 0.1 ^a	10.1 ± 0.1 ^a	9.5 ± 0.1 ^b	9.58 ± 0.09 ^b
3-O-caffeoylquinic acid	10.77	353.089	n.d.	n.d.	n.d.	n.d.	21.3 ± 0.9
Proc. dimer B isomer 1	13.17	577.135	n.d.	n.d.	n.d.	3.66 ± 0.07	8.4 ± 0.3
Tyrosol-glucosyl-pentoside	13.30	431.157	7.4 ± 0.2 ^e	8.9 ± 0.3 ^d	10.8 ± 0.2 ^c	16.1 ± 0.6 ^b	28.2 ± 0.6 ^a
4-hydroxybenzoic acid*	13.37	137.025	47 ± 2 ^c	57 ± 4 ^b	56.6 ± 0.4 ^b	64 ± 2 ^b	83 ± 6 ^a
Proc. dimer B isomer 2	14.24	577.135	n.d.	n.d.	n.d.	n.d.	14.1 ± 0.9
5-O-caffeoylquinic acid	14.53	353.089	n.d.	n.d.	n.d.	n.d.	131 ± 4
Vanillic acid	15.19	167.035	7 ± 0.2 ^e	7.67 ± 0.05 ^d	10.3 ± 0.1 ^b	9.46 ± 0.07 ^c	11.42 ± 0.09 ^a
(+)-Catechin	15.53	289.073	21.7 ± 0.1 ^c	31.1 ± 0.3 ^b	22.6 ± 0.3 ^c	32.4 ± 0.3 ^b	135 ± 2 ^a
4-O-caffeoylquinic acid	15.84	353.089	n.d.	n.d.	n.d.	n.d.	61 ± 2
Proc. dimer B isomer 3	16.12	577.135	n.d.	n.d.	n.d.	n.d.	11.3 ± 0.4
Proc. dimer B isomer 4	17.70	577.135	n.d.	n.d.	n.d.	3.7 ± 0.1 ^b	51 ± 5 ^a
(-)-Epicatechin	19.52	289.073	7.81 ± 0.05 ^d	10.92 ± 0.05 ^{bc}	8.98 ± 0.08 ^{cd}	12.6 ± 0.1 ^b	51 ± 2 ^a
Vanillin	19.72	151.040	30.2 ± 0.9 ^{ab}	26 ± 2 ^{bc}	25.3 ± 0.6 ^c	31 ± 2 ^a	25.9 ± 0.07 ^c
Proc. dimer B isomer 5	20.83	577.135	n.d.	n.d.	n.d.	3.8 ± 0.1 ^b	16.7 ± 0.5 ^a
Proc. trimer B isomer 1	20.95	865.198	n.d.	n.d.	n.d.	n.d.	19.7 ± 0.5
Quercetin-diglucoside isomer 1	21.73	625.140	2.8 ± 0.1 ^c	6.1 ± 0.3 ^b	6.2 ± 0.1 ^b	6.3 ± 0.1 ^b	23.5 ± 0.8 ^a
Quercetin-diglucoside isomer 2	21.91	625.140	1.2 ± 0.01 ^c	1.97 ± 0.07 ^b	2.15 ± 0.04 ^b	2.03 ± 0.08 ^b	6.3 ± 0.2 ^a
Proc. dimer B isomer 6	22.00	577.135	n.d.	n.d.	n.d.	2.4 ± 0.3 ^b	7.9 ± 0.2 ^a
Proc. trimer B isomer 2	22.79	865.205	n.d.	n.d.	n.d.	n.d.	7.7 ± 0.2
Quercetin-O-arabinosyl-glucoside	23.71	595.127	2.7 ± 0.1 ^d	5.9 ± 0.2 ^{bc}	5.3 ± 0.2 ^c	6.3 ± 0.4 ^b	23.3 ± 0.3 ^a
Rutin	24.23	609.148	1.59 ± 0.04 ^d	3.39 ± 0.03 ^c	3.61 ± 0.03 ^c	3.92 ± 0.05 ^b	8.2 ± 0.2 ^a
Proc. dimer B isomer 7	24.58	577.135	n.d.	n.d.	n.d.	4 ± 0.1 ^b	15 ± 1 ^a
Proc. dimer A isomer 1	25.28	575.120	6.37 ± 0.07 ^d	8.42 ± 0.04 ^c	7 ± 0.1 ^d	9.9 ± 0.1 ^b	21 ± 1 ^a
Luteolin pentosyl-hexoside	26.08	579.138	1.35 ± 0.02 ^d	2.6 ± 0.02 ^c	2.74 ± 0.02 ^c	3.02 ± 0.05 ^b	4.8 ± 0.2 ^a
Proc. dimer B isomer 8	26.30	577.135	n.d.	n.d.	n.d.	n.d.	11 ± 1
Quercetin-3-β-glucoside	26.68	463.089	n.d.	0.35 ± 0.03 ^b	0.58 ± 0.08 ^b	0.25 ± 0.06 ^b	10.6 ± 0.4 ^a
Quercetin hexose "B"	26.84	463.089	1.1 ± 0.02 ^c	1.5 ± 0.04 ^b	1.6 ± 0.03 ^b	1.5 ± 0.05 ^b	3.9 ± 0.1 ^a
Quercetin glucuronide	27.08	477.065	1.23 ± 0.02 ^c	2.42 ± 0.05 ^b	2.1 ± 0.1 ^b	2.3 ± 0.1 ^b	5.7 ± 0.3 ^a
Proc. dimer A isomer 2	28.22	575.120	6 ± 0.04 ^d	7.7 ± 0.1 ^b	6.7 ± 0.1 ^c	8.1 ± 0.2 ^b	15 ± 0.4 ^a
Proc. dimer A isomer 3	28.28	575.120	5.39 ± 0.07 ^d	6.42 ± 0.04 ^c	6.7 ± 0.1 ^c	7.6 ± 0.1 ^b	12.9 ± 0.5 ^a
Multinoside A	28.40	609.148	9.7 ± 0.2 ^d	19.7 ± 0.7 ^{bc}	21.6 ± 0.9 ^b	18.2 ± 0.6 ^c	85 ± 1 ^a
Proc. dimer A isomer 4 (A2)	29.42	575.120	5.86 ± 0.02 ^d	7.76 ± 0.08 ^b	6.74 ± 0.09 ^c	8.53 ± 0.09 ^b	13.4 ± 0.6 ^a
Quercetin-xylosyl-rhamnoside	30.22	579.138	2.08 ± 0.08 ^c	4.35 ± 0.05 ^b	4.6 ± 0.2 ^b	4.3 ± 0.2 ^b	11 ± 0.2 ^a
(Epi)gallocatechin	30.28	305.068	13.49 ± 0.09 ^e	15.4 ± 0.1 ^d	17.38 ± 0.06 ^c	18.3 ± 0.3 ^b	21.1 ± 0.1 ^a
Proc. dimer A isomer 5	30.85	575.120	n.d.	n.d.	n.d.	6.5 ± 0.02 ^b	9.5 ± 0.3 ^a
Proc. dimer A isomer 6	31.09	575.120	5.58 ± 0.02 ^d	7.4 ± 0.2 ^b	6.54 ± 0.05 ^c	7.2 ± 0.2 ^b	12.2 ± 0.2 ^a
Kaempferol-O-rhamnosyl-glucoside	31.40	593.151	2.4 ± 0.1 ^d	4.61 ± 0.05 ^c	5.3 ± 0.1 ^b	4.6 ± 0.2 ^c	12.4 ± 0.1 ^a
Proc. dimer A isomer 7	32.57	575.120	n.d.	n.d.	n.d.	6.28 ± 0.03	8.1 ± 0.1
Proc. dimer A isomer 8	35.88	575.120	n.d.	n.d.	n.d.	n.d.	7.35 ± 0.07
Quercetin	37.84	301.036	2.2 ± 0.08 ^d	4.1 ± 0.2 ^c	5 ± 0.2 ^b	4.5 ± 0.2 ^c	15.2 ± 0.2 ^a
Kaempferol	38.97	285.042	1.8 ± 0.06 ^e	2.3 ± 0.05 ^d	3.4 ± 0.07 ^b	3.1 ± 0.1 ^c	5.1 ± 0.08 ^a
(±)-Naringenin	39.08	271.063	1.2 ± 0.03 ^c	1.6 ± 0.04 ^b	1.2 ± 0.02 ^c	1.9 ± 0.08 ^a	1.9 ± 0.02 ^a
Proc. dimer B isomer 9	39.55	577.135	n.d.	n.d.	n.d.	5 ± 1 ^b	6.8 ± 0.2 ^a
Sakuranetin	40.10	285.079	2.1 ± 0.01 ^b	2.2 ± 0.05 ^{bc}	1.7 ± 0.03 ^d	2.8 ± 0.02 ^a	2.2 ± 0.02 ^b

Mean values with superscript different letters indicate significantly different values ($p < 0.05$).

n.d. Compound not detected.

4. Conclusions

It can be concluded that in general shorter drying times at higher temperatures maintained the content of bioactive compounds in a better way. In this research, the optimized drying process at 85°C in a forced air oven seems to be a suitable process for industrial scale in order to dry the avocado peel by-product before its transport or processing.

To our knowledge, this is the first available study that optimized the phenolic compounds extraction from avocado peel using PLE with GRAS solvents. The optimum extraction conditions were established for the PLE process with a mixture of EtOH/H₂O 50:50 (v/v) at a temperature of 200°C. Moreover, the drying temperature effect on TPC and individual phenolic compounds contents was also evaluated. The better results were obtained with the shortest drying process, performed at 85°C for 12 hours, that showed the highest TPC value (58 ± 4 mg GAE/g dm) and in general the highest concentrations of all the compounds identified in the extracts. Respect to individual concentrations, phenolic acids were the more stable compounds during the drying process regardless of the applied temperature, although its levels decrease with longer drying process at lower temperatures. On the opposite, procyanidins suffered an important degradation with longer drying steps, showing a decrease in their content of around 75%.

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Supplementary Information

1. Quantification of phenolic compounds by HPLC-DAD-ESI-TOF-MS

For the analysis, a Zorbax Eclipse Plus C18 column (4.6×150 mm, 1.8 μm particle size) was used as stationary phase. Pre-filters of the same packing were used as pre-column, RRLC in-line filters, 4.6 mm, 0.2 μm (Agilent Technologies, Palo Alto, CA, USA). The temperature of the column was maintained at 25°C and the injection volume was 10 μL . The separation was achieved at a flow rate of 0.8 mL/min of the following mobile phases: water plus 0.1% of formic acid as mobile phase A and acetonitrile as mobile phase B. The chromatographic run was conducted using the following multi-step gradient: 0.0 min [A:B 95/5%], 30.0 min [A:B 75/25%], 35.0 min [A:B 65/35%], 40.0 min [A:B 5/95%], 45.0 min A:B 95/5% and finally at 50 min a conditioning cycle of 5 min with the initial conditions before the next analysis.

The effluent from the HPLC column was reduced using a “T” type splitter before being introduced into the mass spectrometer (split ratio 1:3) to provide a stable spray and, consequently, reproducible results. Detection was carried out considering a mass range of 50-1000 m/z . The ionization parameters were: capillary voltage, 4500 V; drying gas temperature, 190°C; drying gas flow, 9 L/min; nebulizing gas pressure, 2.0 Bar; and end plate offset, -500 V. The values of transfer parameters were: capillary exit, -150 V; skimmer 1, -50 V; hexapole 1, -23 V, RF hexapole, 199 V; skimmer 2, -22.5 V; the trigger time was set to 53 μs (50 μs for transfer time and 3 μs for pre-pulse storage time).

For the necessary mass accuracy to identify compounds, external mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface. A sodium formate cluster solution (5 mM sodium hydroxide and water-2-propanol 1:1 (v/v) with 0.1% (v/v) of formic acid) in quadratic plus high-precision calibration regression mode was used. The mixture was injected at the beginning of each run and all the spectra were calibrated prior to compound identification. Because of the compensation for temperature drifts inside the instrument, this external calibration provided accurate mass values that were better than 5 ppm.

2. Quantification of bioactive compounds

For this purpose, catechin, 5-O-caffeoylquinic acid, epicatechin, procyanidin A2, procyanidin B2, quercetin-3- β -glucoside and rutin were quantified with the calibration curves of their own commercial standards. The remaining compounds were tentatively quantified on the basis of calibration curves from other compounds with structural similarities. It should be taken into account that the response of the standards can differ from that of the analytes found in the extracts, and consequently the quantification of these compounds is only an estimation of their actual concentrations although it can be considered a useful approximation. In this sense, the calibration curve of catechin was used to quantify catechin derivatives, 5-O-caffeoylquinic for phenolic acids and phenolic alcohol derivatives, procyanidin A2 for procyanidins type A, procyanidin B2 for procyanidins type B, and finally rutin for flavonols derivatives. Supplementary Table 2 shows the limits of detection (LODs) and quantification (LOQs), calibration range, calibration equations, and regression

coefficient (r^2) for all standards used. All calibration curves showed good linearity between different concentrations depending on the analytes studied. The LODs and LOQs for individual compounds in standard solutions were also calculated as $S/N = 3$ and $S/N = 10$, respectively, where S/N is the signal-to-noise ratio. The repeatability of the method was measured as the relative standard deviation (RSD %) in terms of concentration. An avocado-peel extract was injected several times ($n = 6$) on the same day (intraday precision) and 3 times on 2 consecutive days (interday precision, $n=12$). The intraday repeatability of the developed method for all analytes ranged from 0.81 to 7.87%, whereas the interday repeatability ranged from 1.03 to 9.60%.

Supplementary Table 1. Analytical parameters of the proposed method.

Analyte	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)	Calibration range ($\mu\text{g/mL}$)	Calibration equations	r^2
Catechin	0.95	3.17	LOQ–120	$y = 0.1231 x - 0.0399$	0.997
Chlorogenic acid	0.19	0.63	LOQ–100	$y = 0.0055 x - 0.0075$	0.981
Epicatechin	0.57	1.89	LOQ–120	$y = 0.2333 x - 0.0413$	0.997
Procyanidin A2	0.07	0.22	LOQ–120	$y = 0.2981 x - 0.0440$	0.994
Procyanidin B2	0.32	1.08	LOQ–120	$y = 0.1095 x - 0.0062$	0.993
Quercetin-3- β -glucoside	0.07	0.22	LOQ–120	$y = 0.0634 x + 0.0167$	0.998
Rutin	0.02	0.06	LOQ–120	$y = 0.1155 x - 0.0273$	0.996

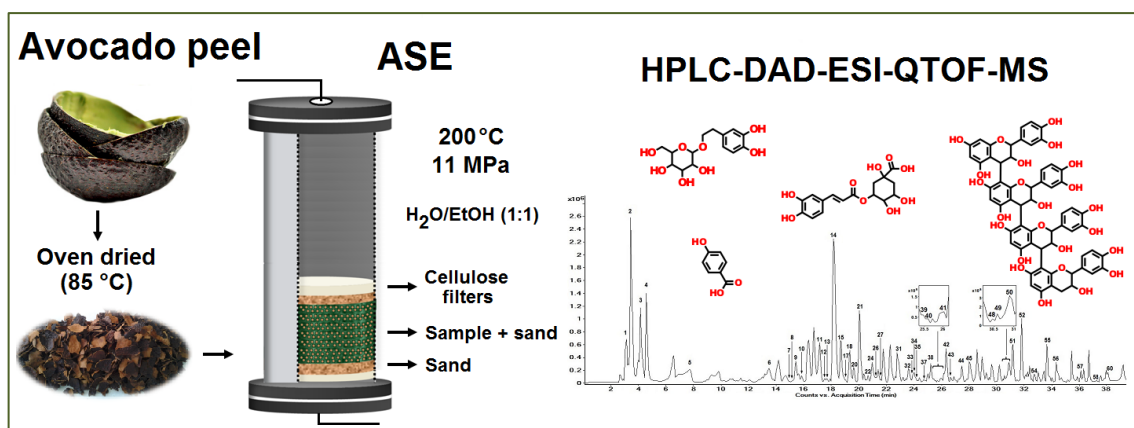
Supplementary Table 2. Identification of phenolic compounds in the PLE peel extracts of avocado by HPLC-DAD-ESI-TOF-MS.

Analyte	RT (min)	Experimental m/z	Calculated m/z	Molecular formula	Error (ppm)	Score	mSigma
3,4-dihydroxyphenylacetic acid	8.046	167.0354	167.0350	C ₈ H ₈ O ₄	-2.4	100	13.6
Protocatechuic acid	8.610	153.0220	153.0193	C ₇ H ₆ O ₄	-17.4	100	5.1
Pyrocatechol	9.639	109.0307	109.0295	C ₆ H ₆ O ₂	-11.3	100	37.5
3-O-caffeoylquinic acid	10.768	353.0879	353.0878	C ₁₆ H ₁₈ O ₉	-0.4	100	19.5
Procyanidin dimer B isomer 1	13.174	577.1351	577.1351	C ₃₀ H ₂₆ O ₁₂	0	100	8.4
Tyrosol-glucosyl-pentoside	13.304	431.1544	431.1559	C ₁₉ H ₂₈ O ₁₁	3.4	100	5.9
4-hydroxybenzoic acid*	13.369	137.0262	137.0244	C ₇ H ₆ O ₃	-12.7	100	3.6
Procyanidin dimer B isomer 2	14.241	577.1305	577.1351	C ₃₀ H ₂₆ O ₁₂	8.1	100	27.5
5-O-caffeoylquinic acid*	14.531	353.0875	353.0878	C ₁₆ H ₁₈ O ₉	0.8	100	1
Vanillic acid	15.191	167.0357	167.0350	C ₈ H ₈ O ₄	-4.1	100	16.5
(+)-Catechin*	15.534	289.0722	289.0718	C ₁₅ H ₁₄ O ₆	-1.6	100	12.4
4-O-caffeoylquinic acid	15.836	353.0879	353.0878	C ₁₆ H ₁₈ O ₉	-0.4	100	3.3
Procyanidin dimer B isomer 3	16.122	577.1372	577.1351	C ₃₀ H ₂₆ O ₁₂	-3.6	100	13.4
Procyanidin dimer B isomer 4	17.699	577.1372	577.1351	C ₃₀ H ₂₆ O ₁₂	-3.6	100	4.4
(-)-Epicatechin*	19.519	289.0688	289.0718	C ₁₅ H ₁₄ O ₆	10.4	100	7.8
Vanillin	19.717	151.0390	151.0401	C ₈ H ₈ O ₃	7	100	18.5
Procyanidin dimer B isomer 5	20.827	577.1371	577.1351	C ₃₀ H ₂₆ O ₁₂	-3.5	100	36.8
Procyanidin trimer B isomer 1	20.949	865.1950	865.1985	C ₄₅ H ₃₈ O ₁₈	4.1	100	27.6
Quercetin-diglucoside isomer 1	21.727	625.1345	625.1410	C ₂₇ H ₃₀ O ₁₇	10.5	100	9
Quercetin-diglucoside isomer 2	21.906	625.1317	625.1410	C ₂₇ H ₃₀ O ₁₇	9.3	100	7.9
Procyanidin dimer B isomer 6	22.004	577.1393	577.1351	C ₃₀ H ₂₆ O ₁₂	-7.2	100	16.1
Procyanidin trimer B isomer 2	22.791	865.2047	865.1985	C ₄₅ H ₃₈ O ₁₈	-7.1	100	15
Quercetin-O-arabinosyl-glucoside	23.713	595.1274	595.1305	C ₂₆ H ₂₈ O ₁₆	5.2	91.99	15.3
Rutin*	24.228	609.1500	609.1461	C ₂₇ H ₃₀ O ₁₆	-6.4	100	18.6
Procyanidin dimer B isomer 7	24.576	577.1190	577.1351	C ₃₀ H ₂₆ O ₁₂	28	100	11.7

Analyte	RT (min)	Experimental m/z	Calculated m/z	Molecular formula	Error (ppm)	Score	mSigma
Procyanidin dimer A isomer 1	25.277	575.1222	575.1195	C ₃₀ H ₂₄ O ₁₂	-4.6	100	27.9
Luteolin pentosyl-hexoside	26.076	579.1366	579.1355	C ₂₆ H ₂₈ O ₁₅	-1.8	100	11.8
Procyanidin dimer B isomer 8	26.295	577.1349	577.1351	C ₃₀ H ₂₆ O ₁₂	0.5	100	48.6
Quercetin-3-β-glucoside*	26.680	463.0891	463.0882	C ₂₁ H ₂₀ O ₁₂	-1.8	100	6.7
Quercetin hexose "B"	26.838	463.0912	463.0882	C ₂₁ H ₂₀ O ₁₂	-6.5	100	14.8
Quercetin glucuronide	27.079	477.0691	477.0691	C ₂₁ H ₁₈ O ₁₃	-3.5	100	8.4
Procyanidin dimer A isomer 2	28.216	575.1193	575.1195	C ₃₀ H ₂₄ O ₁₂	0.3	100	27.9
Procyanidin dimer A isomer 3	28.284	575.1195	575.1195	C ₃₀ H ₂₄ O ₁₂	3.6	100	17.2
Multinoside A	28.399	609.1473	609.1461	C ₂₇ H ₃₀ O ₁₆	-1.9	100	26.2
Procyanidin dimer A isomer 4 (A2)*	29.416	575.1174	575.1195	C ₃₀ H ₂₄ O ₁₂	2	100	15.2
Quercetin-xylosyl-rhamnoside	30.217	579.1353	579.1355	C ₂₆ H ₂₈ O ₁₅	0.5	100	11.5
(Epi)galocatechin	30.282	305.0649	305.0667	C ₁₅ H ₁₄ O ₇	5.8	100	20.7
Procyanidin dimer A isomer 5	30.848	575.1175	575.1195	C ₃₀ H ₂₄ O ₁₂	-0.3	100	22.8
Procyanidin dimer A isomer 6	31.089	575.1197	575.1195	C ₃₀ H ₂₄ O ₁₂	7.5	100	16.5
Kaempferol-O-rhamnosyl-glucoside	31.403	593.1501	593.1512	C ₂₇ H ₃₀ O ₁₅	1.8	100	5.2
Procyanidin dimer A isomer 7	32.569	575.1152	575.1201	C ₃₀ H ₂₄ O ₁₂	-1	100	53.9
Procyanidin dimer A isomer 8	35.875	575.1200	575.1195	C ₃₀ H ₂₄ O ₁₂	-0.9	100	39.3
Quercetin*	37.839	301.0354	301.0354	C ₁₅ H ₁₀ O ₇	-5.4	100	2.4
Kaempferol	38.971	285.0440	285.0405	C ₁₅ H ₁₀ O ₆	-12.5	100	8.7
(±)-Naringenin*	39.084	271.0633	271.0612	C ₁₅ H ₁₂ O ₅	-7.8	100	27.9
Procyanidin dimer B isomer 9	39.552	577.1328	577.1351	C ₃₀ H ₂₆ O ₁₂	4.2	100	39.1
Sakuranetin	40.096	285.0794	285.0768	C ₁₆ H ₁₄ O ₅	-9	100	14.2

* Compounds identified by comparison with commercial standard.

Capítulo II

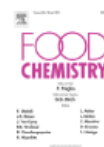


Comprehensive identification of bioactive compounds of avocado peel by liquid chromatography coupled to ultra-high-definition accurate-mass Q-TOF

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Comprehensive identification of bioactive compounds of avocado peel by liquid chromatography coupled to ultra-high-definition accurate-mass Q-TOF

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Highlights

- Avocado seed coat phenolic profile has been studied for the first time.
- Accelerated solvent extraction was performed with green solvents (water/ethanol).
- Eighty four compounds from eight families were identified.
- Forty five phenolic compounds were identified for the first time in avocado seed.
- Avocado seed and seed coat have potential as a source of phenolic compounds.

Abstract

Industrially the avocado pulp is exploited principally as oil and paste, generating a huge quantity of peel and seed as by-products. Avocado peel is a promising inexpensive candidate for recovery phenolic compounds. The aim of this work was to identify the bioactive compounds present in an extract of avocado peel obtained by a green extraction technique. Accelerated solvent extraction was performed using water and ethanol as extraction solvents. Liquid chromatography coupled to ultra-high-definition accurate-mass spectrometry was used in order to identify the bioactive compounds. A total of sixty-one compounds belonging to eleven families were identified. Procyanidins, flavonols, hydroxybenzoic and hydroxycinnamic acids were the most common compounds. A sum of thirty-five compounds has been identified here for the first time in avocado peel. These results confirm the potential of avocado peel as a source of bioactive ingredients for its use in the food, cosmetic or pharmaceutical sector.

Keywords

Avocado peel; Green solvent; PLE; HPLC-DAD-ESI-QTOF-MS; Polyphenols; Procyandins

1. Introduction

Avocado, (*Persea americana* Mill., Lauraceae), is a very nutritious fruit endemic to the humid tropical areas of Mexico, although nowadays, it is widely grown worldwide on large scale in various subtropical countries (Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011). In 2014 the world production was 5 million tons, and Mexico was the largest producer with 1.5 million tons (FAO, 2017). The European Union is the major importer in the world (Rodríguez-Carpena et al., 2011), while the principal cultivars are Mexican, West Indian and Guatemalan. Current commercial varieties are hybrids of these cultivars, such as the 'Hass' variety, one of the most popular type grown and imported (Kosińska et al., 2012), which belongs to the Guatemalan-Mexican hybrid group (Wang, Bostic, & Gu, 2010).

Currently, the food and cosmetic industries are experiencing a constantly growing demand for new ingredients from natural sources as alternatives to synthetic substances. An example is the case of BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene), whose use in the food industry have been restricted because of their suspected carcinogenic effect and the growing consumer demand for natural products (Jayaprakasha, Ohnishi-Kameyama, Ono, Yoshida, & Jaganmohan Rao, 2006). In this sense, food industry by-products have a great potential to be used as a source of these demanded natural ingredients (Wang et al., 2010).

Industrially, only the avocado pulp is exploited as oil and paste generating a large quantity of seed and peel, which are discarded with no further applications. These

by-products represent about 25% of the total fresh weight of the fruit, so its waste could cause environmental problems (Rodríguez-Carpena et al., 2011). Nevertheless, the avocado seed and peel had high amounts of extractable bioactive compounds, as polyphenols (Wang et al., 2010), which could be used in many applications due to their well-known bioactivity (Calderón-Oliver et al., 2016; Rodríguez-Carpena et al., 2011). Wang et al. (2010) reported that the avocado peel and seed showed much higher phenolic content than the pulp. Therefore, these by-products could be promising inexpensive candidates for recovery phenolic compounds, which could be used in the pharmaceutical, cosmetic and food industries as bioactive ingredients. Moreover, the utilization of these by-products add value to the avocado industry, minimize cost and environmental impact (Rodríguez-Carpena et al., 2011; Wang et al., 2010), whereas its extracts do not contain potentially toxic or harmful components (Rodríguez-Carpena et al., 2011).

Despite its great potential, the existing knowledge about phenolic profile in avocado by-products is scarce, especially in peel. Furthermore, the majority of existing literature use conventional extraction techniques based on maceration (Calderón-Oliver et al., 2016; Fidelis et al., 2015; Kosińska et al., 2012; Morais et al., 2015; Saavedra et al., 2017; Widsten, Cruz, Fletcher, Pajak, and McGhie, 2014; Wong et al., 2016) or this extraction procedure assisted by ultrasonic bath (López-Cobo et al., 2016; Wang et al., 2010). In this sense, only Rodríguez-Carpena et al. (2011) have used pressurized liquid extraction, but they do not presented an individual identification of the obtained extract. Generally, the extraction of phenolic compounds in these studies was based on the use of organic solvents, such as

methanol and acetone. On the contrary, Pressurized Liquid Extraction (PLE) is a solid–liquid extraction process performed at high temperatures (50–200 °C) and high pressures (10–15 MPa), being its main advantages over traditional extraction methods the dramatic decreases in the amount of solvent used and the extraction time (H. Sun, Ge, Lv, & Wang, 2012). Moreover, PLE is a “green” technology that allows the use of ethanol, water or their mixtures, (generally recognized as safe or GRAS solvents) for the extraction of different classes of compounds, such as polyphenols. Furthermore, several studies have pointed out that PLE obtains similar or greater yields than conventional or ultrasonic assisted extraction (UAE) (Luthria, Biswas, & Natarajan, 2007; H. Sun et al., 2012). The aim of the present study was to determine the polar fraction of avocado peel using a powerful green extraction technique (PLE) with GRAS solvents in combination with a potent analytical platform (high resolution liquid chromatography coupled to ultra-high-definition accurate-mass spectrometry, HPLC-ESI-QTOF-MS) for the individual identification of bioactive compounds.

2. Material and methods

2.1. Chemicals and reagents

For identification purposes, the standards compounds benzoic acid, (+)-catechin, chlorogenic acid, citric acid, (-)-epicatechin, gentisic acid, protocatechuic acid, quercetin, quercetin-3- β -glucoside, rutin and (\pm)-naringenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Moreover, 4-hydroxybenzoic acid was sourced from Fluka Chemika (Buchs, Switzerland), quinic acid was supplied from Acros

Organics (Geel, Belgium), while dihydrocaffeic acid, procyanidin dimer A2 and B2 standards were purchased from Extrasynthese (Genay Cedex, France). For mobile phase preparation, formic acid was purchased from Fluka, Sigma-Aldrich (Steinheim, 126 Germany), HPLC-MS-grade acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Ethanol and Ottawa sand for extraction were purchased from Fisher Scientific (Leicestershire, UK).

2.2. Samples

'Hass' avocados from the subtropical coast of Granada were provided by the commercial group La Caña, Miguel García Sánchez e Hijos, S.A. (Motril, Spain). All fruits were kept at room temperature until full maturity. Fully ripened fruits were manually separated into pulp, peel and seed. Peel was cleaned under continuous flow of tap water. Then, the obtained by-product was chopped and oven-dried at 85 °C while turned periodically to ensure uniform dryness until a percentage moisture content of less than 10%. After that, the dried peel was milled in an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting avocado peel powder had an average particle size of 0.5 mm. The material was stored at room temperature and protected from light until its extraction and analysis.

2.3. Pressurized liquid extraction (PLE)

For bioactive compounds extraction, a pressurized liquid extractor model ASE 350 (Dionex Corp., Sunnyvale, CA) equipped with a solvent controller was used. A portion of dried peel (1.5 g) was mixed homogeneously with 5 g of sand and

transferred into 34 mL stainless-steel extraction cells. Disposable cellulose filters were placed into the cell's inlet and outlet in order to prevent clogging in the metal frits of the extraction cell. The extractions were performed at 200 °C with a mixture of ethanol/water (1:1, v/v) as solvent. Prior to use, solvents were sonicated for 15 min to remove the dissolved oxygen in order to avoid any possible oxidation. The extraction of avocado peel bioactives was performed with a static extraction cycle of 20 min at 11 MPa. An extraction cell heat-up step of 9 min was carried out prior to the static cycle. After that, the cell was flushed with solvent (60% of the cell volume), purged with nitrogen (100 s) and immediately cooled in ice to attain a temperature of 20–25 °C. The obtained extracts were centrifuged at 12,000 rpm for 15 min at 4 °C in a Sorvall ST 16 R centrifuge (Thermo Scientific, Leicestershire, UK) and the supernatants were evaporated at 35 °C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The extract was stored at –20 °C until further use. All experiments were performed in duplicate.

2.4. Determination of phenolic and other polar compounds by HPLC-DAD-ESI-QTOF-MS

The extracts were reconstituted with the same solvent used in the extraction at a concentration of 10,000 mg/L. Then, the extracts were filtered with regenerated cellulose syringe-filters of 0.2 µm pore size (Millipore, Bedford, MA, USA). The purified extracts were analysed using an Agilent 1260 series Rapid Resolution LC coupled to a diode-array detector (DAD) and an Agilent 6540 Ultra High Definition (UHR) Accurate Mass Q-TOF with a Jet Stream dual ESI interface (Agilent Technologies, Palo Alto, CA, USA). The instrument was equipped with a vacuum

degasser, a binary pump, and a thermostated autosampler and column compartment. Compounds were separated using a Zorbax Eclipse Plus C18 column (4.6 × 150 mm, 1.8 μm; Agilent Technologies, Palo Alto, CA, USA). Acidified water (0.1% formic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively. The elution gradient was conducted at a constant flow rate of 0.5 mL/min as follows: 0 min, 95% A; 25 min, 50% A; 33 min, 0% A; 36 min, initial conditions until 40 min as a re-equilibration step. The sample volume injected was 10 μL. The autosampler and column temperatures were maintained at 4 and 25 °C, respectively. The UV spectra were recorded from 190 to 600 nm, whereas the compounds were monitored at 240, 280 and 340 nm.

MS analyses were carried out using the following operating conditions: drying nitrogen temperature at 325 °C with a flow of 10 L/min; nebulizer pressure, 20 psi; sheath gas temperature at 400 °C with a flow of 12 L/min; capillary, nozzle, fragmentor, skimmer and octopole radiofrequency voltages of 4000, 500, 130, 45 and 750 V, respectively. The spectra were acquired in negative ionization mode over a mass-to-charge (m/z) range 100–1700, and the detection window was set to 100 ppm. Data acquisition (2.5 Hz) in the centroid mode was performed by MassHunter Workstation software (Agilent Technologies, Palo Alto, CA, USA). To maintain mass accuracy during the runtime, a continuous infusion of Agilent TOF mixture containing two reference masses was performed: trifluoroacetic acid ammonium salt (m/z 112.9856) and hexakis (1H, 1H, 3H–tetrafluoropropoxy) phosphazine (m/z 1033.9881) was used. Data analysis was realized on MassHunter

Qualitative Analysis B.06.00 (Agilent Technologies, Palo Alto, CA, USA). Literature search for published spectral information was carried out by using SciFinder®.

3. Results and discussion

Avocado peel extract obtained by PLE were analysed by HPLC-DAD-ESI-QTOF-MS in order to characterize the polar fraction. A total of 61 phenolic and other polar compounds were tentatively identified in avocado peel (Fig. 1). These compounds were identified on basis on their retention times, UV spectra and by interpreting the accurate mass information obtained via their MS and MS/MS spectra, taking into account the registered mass spectra fragmentation patterns in data bases and information previously reported in literature. These detected compounds belonged to various metabolite families that included catechins, flavanones, flavones, flavonols, organic acids, hydroxycinnamic acids, hydroxybenzoic acids, glucosylated acid derivates, proanthocyanidins and phenolic alcohol derivatives. Some of these compounds had been previously reported in avocado, however, the high-resolution of QTOF-MS revealed the presence of twenty-nine polyphenols that have never been reported in this sample matrix.

3.1. Organic acids

Four organic acids were identified; quinic acid (RT = 3.44 min and m/z = 191.0570), citric acid (RT = 4.57 min and m/z = 191.0205), malic acid (RT = 4.14 min and m/z = 133.0146) and succinic acid (RT = 7.69 min and m/z = 117.0195); which is in agreement with previous studies in avocado peel (Calderón-Oliver et al., 2016; Fidelis et al., 2015; López-Cobo et al., 2016; Morais et al., 2015). Quinic acid and

citric acid exhibited similar deprotonated molecular ion at m/z 191, nevertheless, those compounds were distinguished by their fragmentation patterns. Quinic acid presented the main product ion at m/z 127 (Friščić, Bucar, & Hazler Pilepić, 2016), while citric acid gave a fragment at m/z 111 corresponding to $[M-H-CO_2-2H_2O]^-$ (Mata et al., 2016). Furthermore, they matched their retention time and MS data with the commercial standards. Malic acid showed a precursor ion $[M-H]^-$ 133 and a main product ion at m/z 115 corresponding to $[M-H-H_2O]^-$, in agreement with data previously reported by Mata et al. (2016). With regard to succinic acid, this compound did not presented fragmentation ions.

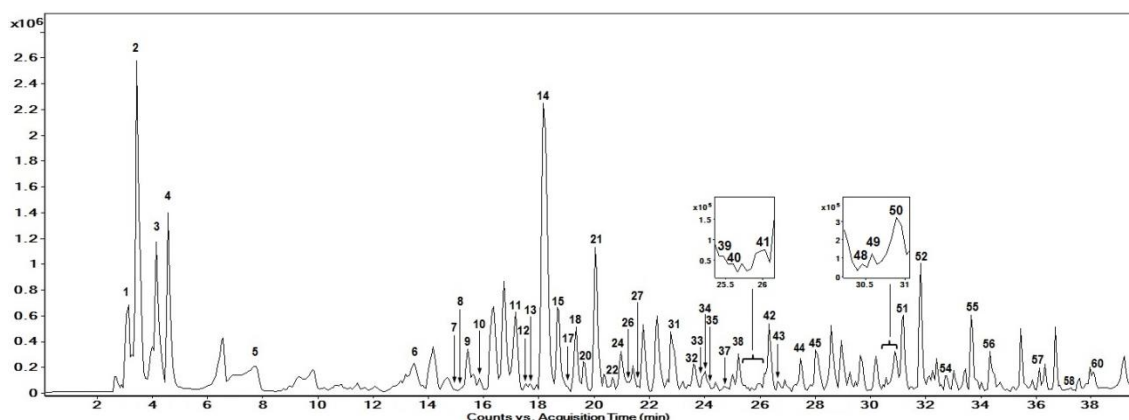


Fig. 1. Base peak chromatogram of avocado peel extract obtained by HPLC-ESI-QTOF-MS.

3.2. Hydroxybenzoic acids

Protocatechuic and gentisic acids displayed the same precursor ions $[M-H]^-$ at m/z 153.02 and MS/MS product ion at m/z 109 corresponding to $[M-H-CO_2]^-$. Therefore, the identification was made based on the elution order according to reference standards, protocatechuic acid with a RT 13.08 min and gentisic acid at RT 15.42 min. Moreover, 4-hydroxybenzoic acid (RT = 18.18 min and m/z = 137.025)

was unambiguously identified since it matched its retention time and MS data with the commercial standard. Peak 28 with a RT 22.27 min and m/z 121.0298 was characterized as benzoic acid by comparison of its retention time, UV and MS data to that of commercial standard. Besides, vanillic acid (RT = 13.19 min and m/z = 167.0357) was also found and confirmed by MS/MS spectra, with a precursor ion at m/z 123 due to a loss of CO₂, a characteristic feature of hydroxybenzoic acid derivatives as well as for other phenolic acids (J. Sun, Liang, Bin, Li, & Duan, 2007).

3.3. Hydroxycinnamic acids

Concerning caffeic acid (RT = 24.65 min and m/z = 179.0353), its MS/MS spectra showed a precursor $[M-H]^-$ ion at m/z 179, with a product ion at m/z 107 corresponding to $[M-H-CO_2-C_2H_4]^-$ (Mata et al., 2016). Peak 29, detected at 22.35 min and m/z 163.0402, was tentatively assigned to p-coumaric acid according to the molecular formula provided for its accurate mass. Its major fragment was found at m/z 119, in accordance with the value reported by Hossain, Rai, Brunton, Martin-Diana, and Barry-Ryan (2010).

In addition, three peaks were assigned to different isomers of caffeoylquinic acids derivatives: 3-O-caffeoylquinic acid (RT = 15.85 min and m/z = 353.0892), 5-O-caffeoylquinic acid (RT = 20.03 min and m/z = 353.09) and 4-O-caffeoylquinic acid (RT = 20.97 min and m/z = 353.0903). The identification was made based on the relative intensities of fragment ions in MS/MS spectra (Clifford, Knight, & Kuhnert, 2005). The 4-O-caffeoylquinic acid, also named as cryptochlorogenic acid, presented a MS/MS base peak of m/z 173 corresponding to $[quinic-H-H_2O]^-$. On the other

hand, 3-O-caffeoylquinic and 5-caffeoylquinic acids present two fragments of m/z 191 and 179, but they could be differentiated on the basis of the relative abundance of the fragment ion m/z 179, which is more intense in the isomer 3-O-caffeoylquinic acid compared to 5-O-caffeoylquinic acid, (the found relative abundances were 53% and less than 3%, respectively). Also the isomer 5-O-caffeoylquinic acid was confirmed by comparing its spectrum and retention time with those of the commercial standard.

3.4. Phenolic alcohol derivatives

The mass spectrum of peak 7 at RT = 14.69 min showed a precursor ion in the negative ionization mode at m/z 315.1093 $[M-H]^-$, and daughter ions peaks at m/z 135 and 153, as a result of an O-glycosidic cleavage and hydroxytyrosol moiety (Fig. 2). It was tentatively assigned as hydroxytyrosol glucoside according to the molecular formula provided for its accurate mass and fragmentation pattern.

Likewise, peak 13 (RT = 17.64 min) had $[M-H]^-$ at m/z 299.1142, which gave daughter ions at m/z 119 and 137. These fragment ions were derived from an O-glycosidic cleavage and a tyrosol moiety, respectively (Fig. 2). The fragmentation pattern was consistent with a previous report (López-Cobo et al., 2016). Thus, this compound was tentatively assigned as tyrosol glucoside. In addition, the ion found at m/z 431.1577 was assigned to tyrosol hexoside-pentoside (RT = 18.97 min), which presented three product ions at m/z 119, 137 and 299. The ions at m/z 119 and 299 corresponded to an O-glycosidic cleavage and the m/z 137 was related to a tyrosol moiety (Fig. 2).

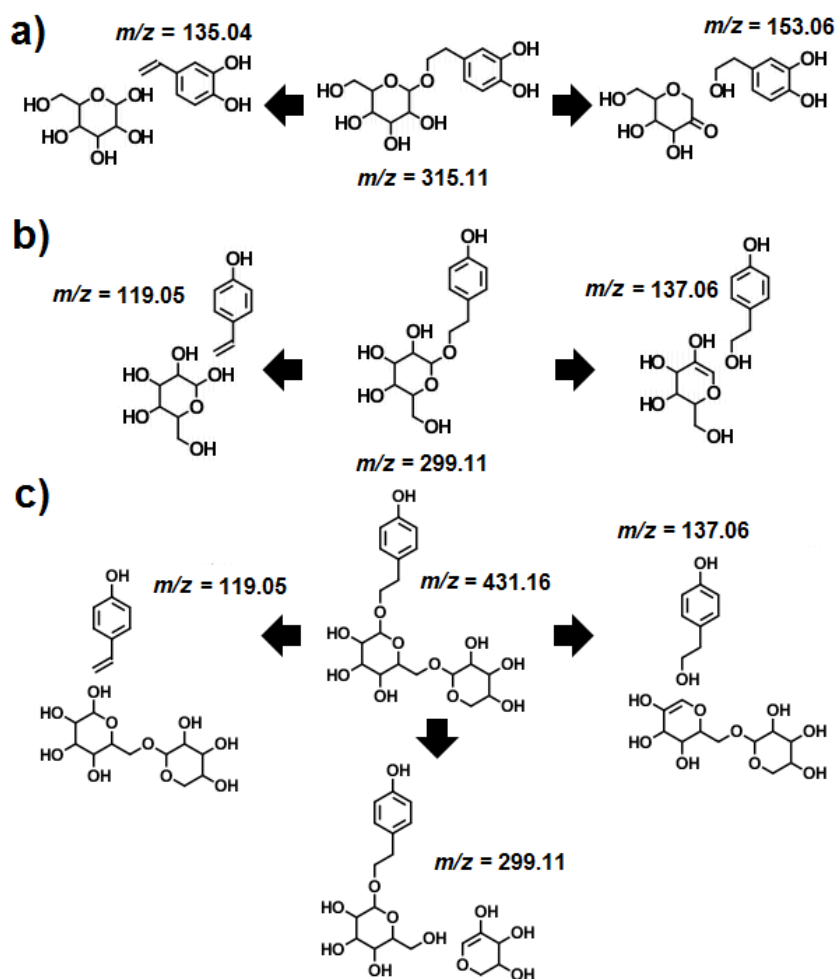


Fig. 2. The fragmentation pathway: (a) hydroxytyrosol glucoside, (b) tyrosol glucoside, c) tyrosol hexoside-pentoside.

3.5. Flavanoids

Naringenin with a $[M-H]^-$ ion at m/z 271.0633 was identified by comparing its retention time and characteristic MS spectral data with that of authentic standard. Peak 48 (RT = 30.45 min and m/z = 579.1384) had a fragmentation pattern in accordance with Mudrić et al. (2017), thus, this compound was tentatively identified as luteolin 7-O-(2''-O-pentosyl) hexoside. Quercetin was found at m/z 301.0369 and

RT 33.66 min, it yielded a major fragment ion at m/z 151 due to a RDA reaction. It is in agreement with the fragmentation pattern reported by Rojas-Garbanzo, Zimmermann, Schulze-Kaysers, and Schieber (2016). Concerning to flavonol derivatives, eight different flavonols glucosides were characterized in the studied extract. In this sense, Kosińska et al. (2012) reported several quercetin derivatives in avocado peel. Thus, in the studied extract, a sum of seven different quercetin derivatives were detected corresponding to peaks 42, 43, 45, 46, 49, 50 and 52. Two quercetin diglucosides (peaks 42 and 43) were found with RT 26.34 and 26.66 min, and experimental m/z 625.1440 and 625.1431, respectively. As occurs in quercetin derivative compounds, a major fragment ion was found at m/z 300 in the fragmentation profile, in accordance with an O-glycosidic cleavage. Peak 45 with a RT 28.01 min had $[M-H]^-$ of m/z 595.1342, which gave a daughter ion at m/z 300, derived from a cross-ring cleavage of its sugar moiety. It was tentatively assigned to quercetin-O-arabinosyl-glucoside. Peak 46 was unambiguously identified as rutin by comparison of its retention time, UV and MS/MS spectra to that of commercial standard. Besides, peak 49 was tentatively identified as quercetin glucuronide (RT = 30.57 min and m/z = 477.0698), yielded the major fragment ion at m/z 300, which corresponds to an O-glycosidic cleavage (Vallverdú-Queralt et al., 2015). Peak 50 (RT = 30.78 min and m/z = 463.0905), was unambiguously identified as quercetin-3-glucoside by comparison of its retention time and spectra data to that of the reference standard. Peak 52 was identified as the predominant flavanol glucoside with a $[M-H]^-$ ion at m/z 609.1487 and a RT 31.81 min. The fragmentation profile showed the odd ion at m/z 301 and the even ion at m/z 300, which corresponded to

an O-glycosidic cleavage (Liu et al., 2016). It was tentatively identified as multinoside A.

Moreover, peak 54, detected at 32.82 min with m/z 593.1541, was tentatively assigned to kaempferol O-glucosyl-rhamnoside according to the molecular formula provided for its accurate mass. Indeed, the major fragment ion at m/z 284 revealed a kaempferol moiety, and losses of one hexose and methyl pentose.

3.6. Catechins

The monomers catechin (RT = 18.66 min and m/z = 289.0736) and epicatechin (RT = 22.78 min and m/z = 289.0737) were unambiguously identified since they matched retention times and MS data with those of reference substances. Furthermore, the MS/MS fragments at m/z 109, 125, 151, 137 and 221 were in agreement with those reported by Rojas-Garbanzo et al. (2016).

3.7. Proanthocyanidins

Proanthocyanidins, also known as condensed tannins, are the second most abundant class of natural phenolic compounds after lignins. Belonging to proanthocyanidins, there is a class of oligomeric compounds formed exclusively from catechin and epicatechin, called procyanidins (Gu et al., 2003). There are different classes of procyanidins, the type A are linked through a C4–C8 or C4–C6 bond, which can coexist with an additional C2–O–C7 or the less abundant C2–O–C5 bond; whereas the procyanidins type B are linked by a single bond (Appeldoorn et al., 2009). The procyanidins type A are identified on MS by their $[M-H]^-$ ions being 2 Da less than those of the procyanidins type B (Gu et al., 2003). In previous works,

only procyanidins had been reported in avocado peel. In this sense, Gu et al. (2003) found only this type of proanthocyanidins in avocado fruit.

3.7.1. Procyanidin dimers

Five procyanidins type A dimers were tentatively identified with m/z 575.121 and RT of 22.47, 24.03, 25.27, 25.52 and 27.52 min. The main MS/MS ions were m/z 285 and 289 corresponding to [(epi)catechin-2H₂-H]⁻ and [(epi)catechin-H]⁻, respectively. This fragmentation pattern generated by the cleavage of the interflavanoid bonds is in agreement with Cádiz-Gurrea et al. (2014).

In addition, seven procyanidins type B dimers were also experimentally identified with m/z 577.13 and RT 15.29, 17.04, 19.35, 20.6, 21.22, 23.9 and 26.02 min. The main MS/MS fragments were m/z 289, 125, 407, 245, 425 and 287. The fragmentation pattern was consistent with previous reports (Rockenbach et al., 2012). The ions m/z 425 and m/z 289 correspond to a [retro-Diels–Alder reaction (RDA)] and a loss of an (epi)-catechin unit, respectively; while the fragment at m/z 407 resulted from a water elimination of the moiety m/z 425.

3.7.2. Procyanidin trimers

Four procyanidins trimers were found in avocado peel, two of each type. The trimers type A yielded an ion at m/z 863.18 [M-H]⁻, with RT of 18.85 and 21.90 min, respectively. The fragment ions with m/z 411, 711, 693 and 573 were observed in the MS/MS spectrum, which was consistent with previous researches (Jayaprakasha et al., 2006). The MS/MS secondary peaks at m/z 711 [M-H + -152 Da]⁻ were originated from RDA reactions (152 Da). Besides, the fragment ion at m/z 573 from a

quinone methide (QM) cleavage indicate that the A-type interflavan linkage of the trimer was between the top and middle units (Wang et al., 2010). Moreover, the observed m/z 411 ion was formed after the cleavage of the middle unit through the heterocyclic ring fission (HRF).

On the other hand, the peaks with RT at 17.48 and 20.91 min, were identified as procyanidins type B trimers, with m/z 865.2001 $[M-H]^-$. The mayor MS/MS product ions were m/z 287, 289, 407, 425, 577, 695 and 713, in agreement with the fragments reported by Rockenbach et al. (2012). The fragment ion at m/z 713 is a result of a RDA fission of the heterocyclic ring system, while the m/z 713 ion loosed a water molecule to form a more stable ion at m/z 695. RDA on the middle unit of the trimer gave rise to m/z 407 (after water elimination). Moreover, the observed fragments m/z 577 and m/z 287 were a result of QM cleavages. Finally, the product ions at m/z 289 indicated that the monomeric unit is (epi)catechin.

3.7.3. Procyanidin tetramer

One procyanidin type B tetramer was found in the avocado peel extract (RT = 21.09 min and m/z = 1153.262). The MS/MS fragmentation pattern showed several fragment ions at m/z 287, 575, 577, 413, 863, 865, 739, 1001 and 285. These fragments were suitable for a fragmentation pathway resulting of a RDA fission of the heterocyclic ring system, QM cleavage and heterocyclic ring fission (HRF), as can be observed in Fig. 3.

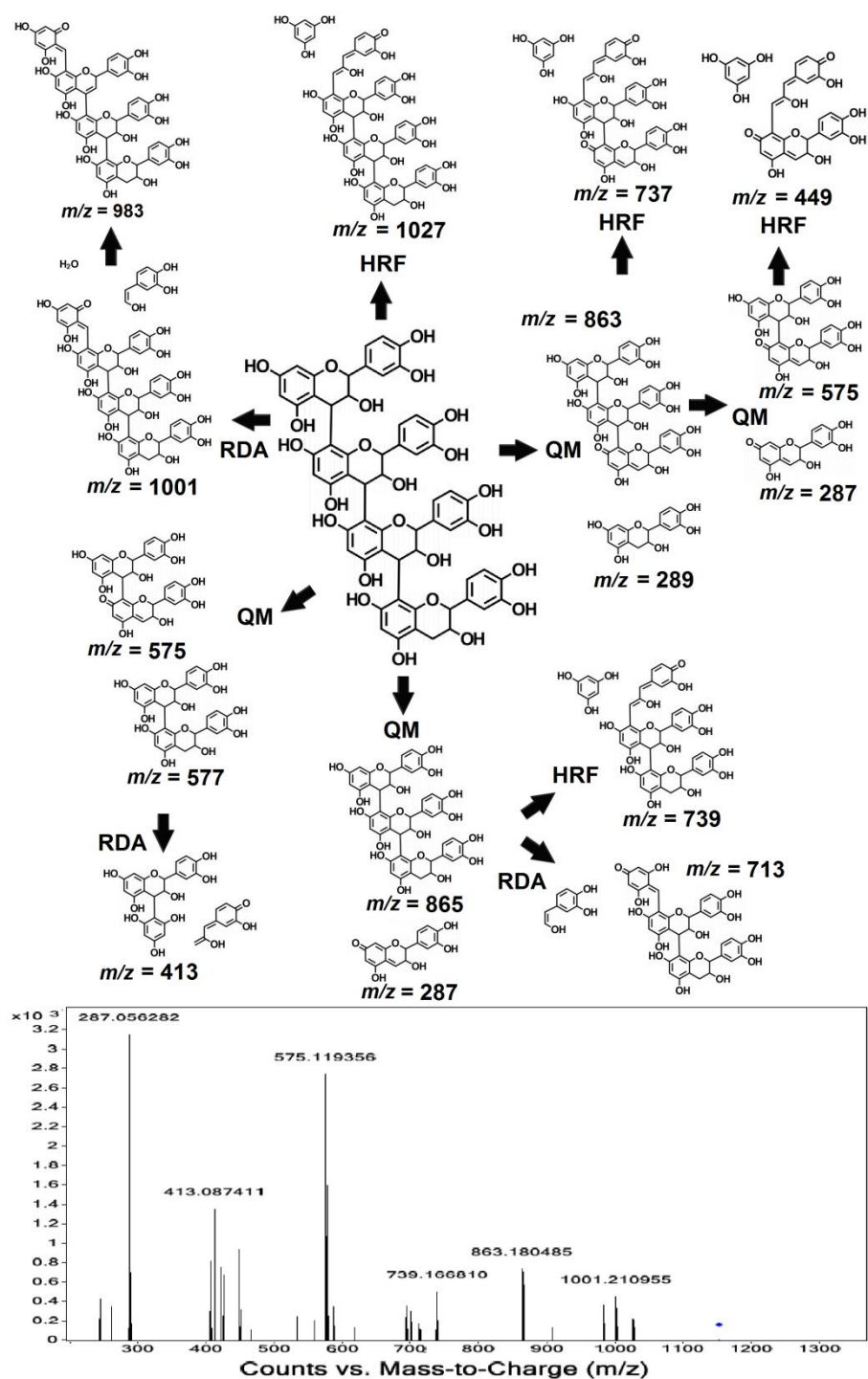


Fig. 3. The fragmentation pathway and MS/MS spectra of procyanidin tetramer B.

3.8. Others polar compounds

Moreover, other five phenolic compounds were identified in the avocado peel extract. Peak 32, with a RT 23.62 min and $m/z = 151.0406$, showed a main fragment at m/z 122, in agreement with the information reported by de la Torre-Carbot et al. (2005). Based on the fragmentation pathway obtained, compound 32 was tentatively identified as vanillin. Peak 34 (RT = 24.01 min and $m/z = 181.0514$) had a fragmentation pattern in accordance with Farrell, Poquet, Dionisi, Barron, and Williamson (2011), thus, this compound was identified as dihydrocaffeic acid. Furthermore, peaks 36 (RT = 24.27 min) and 53 (RT = 31.87 min) with the same precursor ion m/z 451.105 and main product ions (m/z 341, 217 and 189) were tentatively identified as isomers of cinchonain I. The MS/MS fragments were in agreement with the fragments reported by Beltrame, Rodrigues Filho, Barros, Cortez, and Cass (2006). This compound had been previously found in cacao extract with the same fragmentation pattern (Cádiz-Gurrea et al., 2014). Besides, peak 40 (m/z 551.2152, RT 25.58 min) with fragment ions of m/z 419, 551, 153 and 233 was tentatively assigned as nudiposide. The first one, at m/z 419, corresponded to O-glycosidic cleavage. The fragment ions at m/z 153 and 233 were derived from a QM cleavage (Fig. 4).

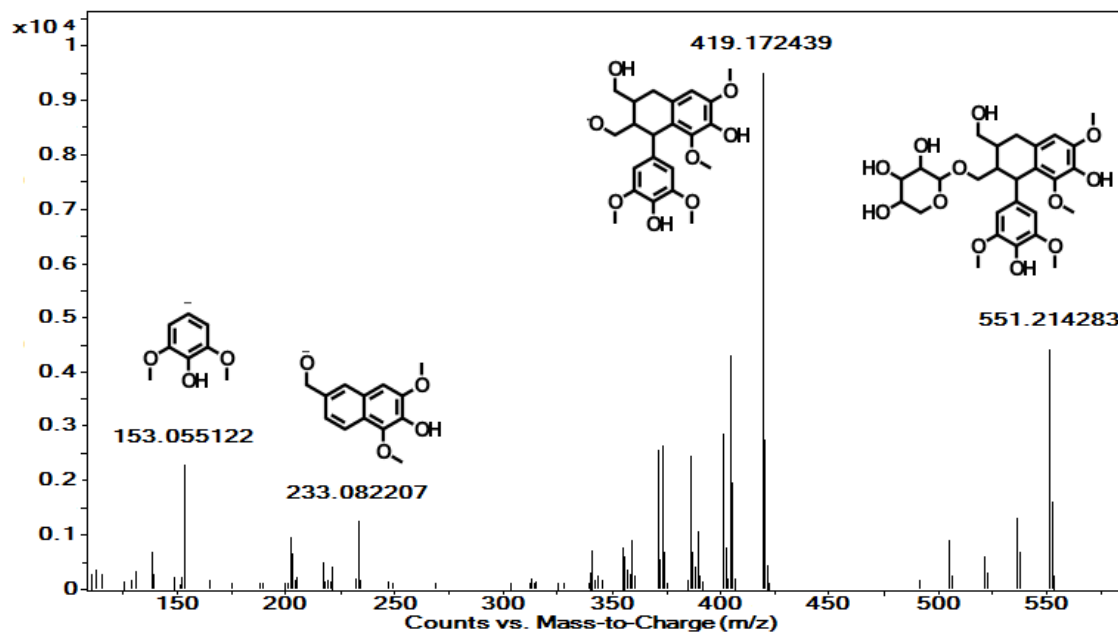


Fig. 4. The fragmentation pathway and mass/mass spectra of nudiposide.

The sugar perseitol with RT 3.07 min was found with $m/z = 211.0831$ and MS/MS fragment ion at $m/z 101$. Compound 18 (RT = 19.35 min), with a molecular mass of $m/z 443.1947$, was tentatively assigned as penstemide according to the molecular formula provided for its accurate mass and corroborated by the MS/MS main fragments at $m/z 101$ and 113 (López-Cobo et al., 2016). Another compound (compound 47) was found with $m/z 441.1796$, and fragments at $m/z 139$, 330 , 161 , 397 . Based on the fragmentation pathway obtained, compound 47 was tentatively identified as (1'S, 6'R)-8'-hydroxyabscisic acid β -d-glucoside. In this case, this compound has been previously found in avocado but in the seeds (López-Cobo et al., 2016).

3.9. Other non-polar compounds

Due to the oily nature of avocado fruit, some non-polar compounds have also been characterized in the less hydrophilic segment of the chromatographic profile. Peak

57 eluted at RT 36.13 min with m/z 329.2349, and it had a fragment pattern formed of m/z 171, 201, 127 and 329 (Nording et al., 2010). It was tentatively assigned to trihydroxyoctadecenoic acid according to the molecular formula provided for its accurate mass and molecular structure correlator software. In the same way, based on the fragmentation pathway obtained and molecular structure correlator software, compound 58 was tentatively identified as dihydroxyoctadecenoic acid. In addition, palmitic acid was also found as well at RT 37.75 min and m/z = 255.2336. Its MS/MS spectrum showed fragments at m/z 255, 237, 183 and 212, which were in agreement with the information reported by Yang, Wu, Rui, Guo, and Feng (2015). Furthermore, peak 60 at RT 38.04 min and m/z 295.2293 was tentatively identified as hydroxyoctadecadienoic acid. The main MS/MS ions were 277, 171, 195 and 251, a fragmentation pattern already reported by Geng et al. (2015). Peak 61, tentatively assigned to hydroxy-oxo-octadecenoic acid, eluted at RT 38.18 min with m/z 311.2239 and MS/MS pattern fragment of m/z 183, 311, 184, 249, 293, which were in agreement with the fragments reported by Geng et al. (2015).

3.10. Comparison with previous studies

Studies based on the phytochemical composition of avocado peel are scarce, and most of them used dynamic maceration as extraction procedure. Additionally, these researches have described compositions of no more than 16 compounds (Calderón-Oliver et al., 2016; Fidelis et al., 2015; Kosińska et al., 2012; Morais et al., 2015; Saavedra et al., 2017; Widsten et al., 2014; Wong et al., 2016). On their behalf, López-Cobo et al. (2016) focused on the composition of different extracts obtained using UAE from different parts of avocado, however, they achieve the identification

of only 7 compounds in the peel extracts. On the other hand, Rodríguez-Carpena et al. (2011) used PLE to extract phenolic compounds, nevertheless, the chemical characterization of the extracts was developed by HPLC using PDA and FLD as detectors. Consequently, the results were reported as a sum of compounds belonging to the same families, not individually as in the present research. On the contrary, thanks to the methodology proposed in the present study, PLE in combination with HPLC-DAD-ESI-QTOF-MS, a total of 61 compounds (Table 1) from several structural classes have been identified in avocado peel extract.

In this research, five hydroxybenzoic acids were identified in the avocado peel extract. In literature, Wong et al. (2016) found three of these hydroxybenzoic acids (peaks 6, 14 and 20) using dynamic maceration solely. On the contrary, to the best of our knowledge, this is the first time that gentisic acid and benzoic acid, described above, have been reported in avocado peel.

A total of five hydroxycinnamic acids have been characterized in the studied extract of avocado peel, corresponding to caffeic acid, p-coumaric acid and three different isomers of caffeoylquinic acid. Respect to the data found in literature, it can be resumed that caffeic acid and p-coumaric acid were reported before in avocado peel by Wong et al. (2016) and Saavedra et al. (2017), respectively. Furthermore, 3O-caffeoylquinic acid was identified in avocado peel extract obtained by PLE (Rodríguez-Carpena et al., 2011). Nevertheless, this compound has not been detected in extracts obtained by using dynamic maceration as extraction technique (Kosińska et al., 2012; Widsten et al., 2014; Wong et al., 2016). Chlorogenic acid, the most common hydroxycinnamic acid, has been reported before in several

researches (Calderón-Oliver et al., 2016; Kosińska et al., 2012; López-Cobo et al., 2016; Rodríguez-Carpena et al., 2011; Saavedra et al., 2017; Widsten et al., 2014; Wong et al., 2016). On the contrary, to the best of our knowledge, the isomer 4-O-caffeoylquinic acid (cryptochlorogenic acid) has never been reported in this sample.

Concerning to phenolic alcohol derivatives, hydroxytyrosol glucoside, tyrosol-glucoside and tyrosol-hexoside-pentoside, it could be pointed out that these compounds have been reported for the first time in avocado peel.

Regarding to flavonoid, the peak 56 (a flavanone) and the peak 48 (a flavone) have never been reported in avocado peel. In the obtained extract, flavonols resulted to be the second main family, with nine compounds identified in the analysis. Some of them (compounds 42, 45, 46, 52 and 55) had been previously reported in avocado peel (Kosińska et al., 2012; López-Cobo et al., 2016; Widsten et al., 2014; Wong et al., 2016), while the compounds 52 and 55 were not identified. Furthermore, to the best of our knowledge, the peaks corresponding to quercetin glucuronide, quercetin 3-glucoside and kaempferol O-glucosyl-rhamnoside have never been characterized in avocado peel variety 'Hass'.

Table 1. Identification of phenolic and other polar compounds in the PLE peel extract of avocado by HPLC-DAD-ESI-QTOF-MS.

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS fragments (m/z, relative abundance)	Proposed compound	Ref.
1	3.07	C ₇ H ₁₆ O ₇	211.0831	99.77	3.25	101 (100)	Perseitol	(López-Cobo, et al., 2016)
2	3.44	C ₇ H ₁₂ O ₆	191.0570	99.87	4.25	127 (100)	Quinic acid*	(Friščić, et al., 2016)
3	4.14	C ₄ H ₆ O ₅	133.0146	99.93	2.37	115 (100)	Malic acid	(Mata, et al., 2016)
4	4.57	C ₆ H ₈ O ₇	191.0205	99.72	3.85	111 (100)	Citric acid*	(Mata, et al., 2016)
5	7.69	C ₄ H ₆ O ₄	117.0197	99.91	3.25	-	Succinic acid	
6	13.08	C ₇ H ₆ O ₄	153.0197	98.44	2.51	109 (100)	Protocatechuic acid*	(Sun, et al., 2007)
7	15.03	C ₁₄ H ₂₀ O ₈	315.1083	99.79	-2.27	135 (100), 153 (12)	Hydroxytyrosol glucoside	(López-Cobo, et al., 2016)
8	15.29	C ₃₀ H ₂₆ O ₁₂	577.1378	93.76	4.28	289 (100), 125 (86), 407 (71), 245 (18), 425 (6), 287 (5)	Procyanidin dimer B (isomer 1)	(Rockenbach, et al., 2012)
9	15.42	C ₇ H ₆ O ₄	153.0194	99.42	-0.27	109 (100)	Gentisic acid*	(Sun, et al., 2007)
10	15.85	C ₁₆ H ₁₈ O ₉	353.0892	99.36	3.87	191 (100), 135 (71), 179 (53)	3-O-caffeoylquinic acid	(Clifford, et al., 2005)
11	17.04	C ₃₀ H ₂₆ O ₁₂	577.1368	97.01	2.84	289 (100), 125 (99), 407 (76), 245 (25), 425 (5), 287 (3)	Procyanidin dimer B (isomer 2)	(Rockenbach, et al., 2012)
12	17.48	C ₄₅ H ₃₈ O ₁₈	865.2001	99.72	1.67	287 (100), 289 (56), 577 (44), 449 (25), 299 (16), 739 (16)	Procyanidin trimer B (isomer 1)	(Rockenbach, et al., 2012)
13	17.64	C ₁₄ H ₂₀ O ₇	299.1142	99.93	1.32	119.050 (100), 113.024 (37), 101.025 (36), 179.034 (1), 131.035 (1)	Tyrosol glucoside	(López-Cobo, et al., 2016)
14	18.18	C ₇ H ₆ O ₃	137.0250	99.78	4.3	108 (100), 119 (7)	4-hydroxybenzoic acid*	
15	18.66	C ₁₅ H ₁₄ O ₆	289.0736	98.50	6.13	109 (100), 125 (58), 151 (38), 137 (29), 221 (19)	(+)-Catechin*	(Rojas-Garbanzo, et al., 2016)
16	18.85	C ₄₅ H ₃₆ O ₁₈	863.1840	99.01	3.07	411 (100), 451 (30), 711 (25), 573 (23), 531 (4)	Procyanidin trimer A (isomer 1)	(Jayaprakasha, et al., 2006)
17	18.97	C ₁₉ H ₂₈ O ₁₁	431.1577	99.10	4.11	119 (100), 431 (31), 137 (13), 299 (12)	Tyrosol-hexoside-pentoside	(López-Cobo, et al., 2016)
18	19.35	C ₂₁ H ₃₂ O ₁₀	443.1957	96.98	7.47	101 (100), 113 (49)	Penstemide	(López-Cobo, et al., 2016)
19	19.35	C ₃₀ H ₂₆ O ₁₂	577.1399	94.65	6.04	289 (100), 125 (89), 407 (70), 245 (21), 425 (5), 287 (3)	Procyanidin B2*	(Rojas-Garbanzo, et al., 2016)
20	19.47	C ₈ H ₈ O ₄	167.0354	99.87	2.83	123 (100)	Vanillic acid	(Sun, et al., 2007)
21	20.03	C ₁₆ H ₁₈ O ₉	353.0900	98.57	5.8	191 (100), 135 (1), 179 (1)	5-O-caffeoylquinic acid*	(Clifford, et al., 2005)
22	20.60	C ₃₀ H ₂₆ O ₁₂	577.1368	89.69	1.74	125 (100), 289 (91), 245 (70), 207 (36), 287 (19), 407 (15), 425 (5)	Procyanidin dimer B (isomer 3)	(Rockenbach, et al., 2012)

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS fragments (m/z, relative abundance)	Proposed compound	Ref.
23	20.91	C ₄₅ H ₃₈ O ₁₈	865.2004	99.66	1.83	287 (100), 289 (67), 407 (61), 425 (49), 577 (46), 695 (28), 713 (17)	Procyanidin trimer B (isomer 2)	(Rockenbach, et al., 2012)
24	20.97	C ₁₆ H ₁₈ O ₉	353.0899	98.84	-5.22	173 (100), 191 (95), 179 (72)	4-O-caffeoylquinic acid	(Clifford, et al., 2005)
25	21.09	C ₆₀ H ₅₀ O ₂₄	1153.2620	100	-0.14	287 (100), 575 (87), 577 (50), 413 (42), 863 (23), 865 (22), 739 (15), 1001 (14), 285 (3)	Procyanidin tetramer B	
26	21.22	C ₃₀ H ₂₆ O ₁₂	577.1371	96.16	3.02	245 (100), 289 (81), 125 (55), 207 (38), 407 (6), 287 (4), 425 (3)	Procyanidin dimer B (isomer 4)	(Rockenbach, et al., 2012)
27	21.9	C ₄₅ H ₃₆ O ₁₈	863.1852	96.51	5.82	411 (100), 451 (34), 573 (28), 711 (27), 531 (5)	Procyanidin trimer A (isomer 2)	(Jayaprakasha, et al., 2006)
28	22.27	C ₇ H ₆ O ₂	121.0298	99.86	3.78	121 (100)	Benzoic acid*	
29	22.35	C ₉ H ₈ O ₃	163.0402	99.74	0.78	163 (100), 135 (30), 119 (6)	p-Coumaric acid	(Hossain, et al., 2010)
30	22.47	C ₃₀ H ₂₄ O ₁₂	575.1211	98.47	4.59	285 (100), 289 (59)	Procyanidin dimer A (isomer 1)	(Cádiz-Gurrea, et al., 2014)
31	22.78	C ₁₅ H ₁₄ O ₆	289.0737	98.62	6.43	109 (100), 125 (54), 151 (38), 137 (30), 221 (18)	(-)-Epicatechin*	(Rojas-Garbanzo, et al., 2016)
32	23.62	C ₈ H ₈ O ₃	151.0406	99.85	3.32	122 (100)	Vanillin	(de la Torre-Carbot, et al., 2005)
33	23.9	C ₃₀ H ₂₆ O ₁₂	577.1367	94.17	2.88	289 (100), 245 (86), 125 (73), 207 (34), 407 (21), 287 (5), 425 (3)	Procyanidin dimer B (isomer 5)	(Rockenbach, et al., 2012)
34	24.01	C ₉ H ₁₀ O ₄	181.0514	99.68	4.22	137 (100)	Dihydrocaffeic acid*	(Farrell, et al., 2011)
35	24.03	C ₃₀ H ₂₄ O ₁₂	575.1227	91.75	10.85	285 (100), 289 (63)	Procyanidin dimer A (isomer 2)	(Cádiz-Gurrea, et al., 2014)
36	24.27	C ₂₄ H ₂₀ O ₉	451.1059	96.27	8.17	341 (100), 217 (42), 189 (26), 231 (26), 451 (4)	Cinchonain (isomer 1)	(Beltrame, et al., 2006)
37	24.65	C ₉ H ₈ O ₄	179.0354	99.68	2.09	108 (100)	Caffeic acid	(Mata, et al., 2016)
38	25.27	C ₃₀ H ₂₄ O ₁₂	575.1217	98.29	-4.89	285 (100), 289 (54)	Procyanidin dimer A (isomer 3)	(Cádiz-Gurrea, et al., 2014)
39	25.52	C ₃₀ H ₂₄ O ₁₂	575.1215	98.13	-5.10	285 (100), 289 (52)	Procyanidin dimer A (isomer 4)	(Cádiz-Gurrea, et al., 2014)
40	25.58	C ₂₇ H ₃₆ O ₁₂	551.2152	99.33	3.13	419 (100), 551 (47), 153 (24), 233 (13)	Nudiposide	
41	26.02	C ₃₀ H ₂₆ O ₁₂	577.1370	75.34	4.63	125.024 (100), 289.072 (77), 407.079 (42)	Procyanidin dimer B (isomer 6)	(Rockenbach, et al., 2012)
42	26.34	C ₂₇ H ₃₀ O ₁₇	625.1440	98.48	-4.46	300 (100), 625 (35)	Quercetin-diglucoside	(López-Cobo, et al., 2016)
43	26.66	C ₂₇ H ₃₀ O ₁₇	625.1431	99.23	-3.16	300 (100), 625 (33)	Quercetin-diglucoside	(López-Cobo, et al., 2016)
44	27.52	C ₃₀ H ₂₄ O ₁₂	575.1213	97.85	5.44	285 (100), 289 (66)	Procyanidin dimer A (isomer 5)	(Cádiz-Gurrea, et al., 2014)
45	28.01	C ₂₆ H ₂₈ O ₁₆	595.1343	97.23	6.18	300 (100), 301 (22), 595 (18)	Quercetin-O-arabinosyl-glucoside	(López-Cobo, et al., 2016)
46	28.45	C ₂₇ H ₃₀ O ₁₆	609.1482	99.53	3.21		Rutin*	

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS fragments (m/z, relative abundance)	Proposed compound	Ref.
47	28.57	C ₂₁ H ₃₀ O ₁₀	441.1796	97.89	6.24	139 (100), 330 (91), 161 (22), 397 (22)	(1'S, 6'R)-8'-hydroxyabscisic acid β-D-glucoside	(López-Cobo, et al., 2016)
48	30.45	C ₂₆ H ₂₈ O ₁₅	579.1384	98.25	4.96	284 (100), 285 (39), 579 (21)	Luteolin 7-O-(2''-O-pentosyl)hexoside	(Mudrić, et al., 2017)
49	30.57	C ₂₁ H ₁₈ O ₁₃	477.0698	98.60	4.87	301 (100)	Quercetin glucuronide	(Vallverdú-Queralt, et al., 2015)
50	30.78	C ₂₁ H ₂₀ O ₁₂	463.0905	97.86	6.10	300 (100), 301 (44), 271 (10), 255 (6)	Quercetin-3-glucoside*	(Rojas-Garbanzo, et al., 2016)
51	31.17	C ₉ H ₁₀ O ₄	181.0516	99.51	5.27	108 (100), 109 (33), 110 (3), 153 (2), 107 (2), 152 (1), 181 (1)	Ethyl Protocatechuate	
52	31.81	C ₂₇ H ₃₀ O ₁₆	609.1487	98.86	3.91	300 (100), 609 (56), 301 (26), 610 (18), 178 (4)	Multinoside A	(Liu, et al., 2016)
53	31.87	C ₂₄ H ₂₀ O ₉	451.1046	99.34	2.68	341 (100), 217 (50)	Cinchonain (isomer 2)	(Beltrame, et al., 2006)
54	32.82	C ₂₇ H ₃₀ O ₁₅	593.1539	98.52	4.51	593 (100), 284 (1)	Kaempferol O-glucosyl-rhamnoside	
55	33.66	C ₁₅ H ₁₀ O ₇	301.0375	98.44	6.68	151 (100), 121 (43), 107 (40), 179 (14), 273 (4)	Quercetin*	(Rojas-Garbanzo, et al., 2016)
56	34.33	C ₁₅ H ₁₂ O ₅	271.0633	98.27	7.51	199 (100), 200 (71), 228 (45), 271 (21)	(±)-Naringenin*	(Sun, et al., 2007)
57	36.13	C ₁₈ H ₃₄ O ₅	329.2351	98.95	5.19	171 (100)	Trihydroxyoctadecenoic acid	(Nording, et al., 2010)
58	37.31	C ₁₈ H ₃₄ O ₄	313.2403	98.54	5.74	201 (100), 267 (34), 127 (21), 171 (19), 313 (18), 125 (16), 317 (12)	Dihydroxy-octadecenoic acid	
59	37.75	C ₁₆ H ₃₂ O ₂	255.2332	99.72	1.07	255 (100), 237 (3), 183 (2), 212 (2)	Palmitic acid	(Yang, et al., 2015)
60	38.04	C ₁₈ H ₃₂ O ₃	295.2296	99.53	5.81	277 (100), 171 (54), 195 (15), 251 (4)	Hydroxy-octadecadienoic acid	(Geng, et al., 2015)
61	38.18	C ₁₈ H ₃₂ O ₄	311.2239	99.3	3.50	183 (100), 311 (71), 184 (13), 249 (2), 293 (1)	Hydroxy-oxo-octadecenoic acid	(Geng, et al., 2015)

*Compounds identified by the comparison with an authentic standard.

Respect to flavanols, catechin (peak 15) and epicatechin (peak 31) have been previously reported in avocado peel (Calderón-Oliver et al., 2016; Fidelis et al., 2015; Kosińska et al., 2012; Morais et al., 2015; Rodríguez-Carpena et al., 2011; Widsten et al., 2014; Wong et al., 2016). Previous studies have described the presence of procyanidins in avocado peel using dynamic macerations as extracting procedure. Rodríguez-Carpena et al. (2011) found procyanidins in avocado peel, however, its

results were reported as a sum of dimers, oligomers and polymers, not individually as mentioned above. Moreover, other authors also detected a low number of procyanidins in avocado, for example Calderón-Oliver et al. (2016) found only one procyanidin dimer type B. Furthermore, Kosińska et al. (2012) detected solely two procyanidin dimers, one type A and another type B. On their behalf, Widsten et al. (2014) reported two procyanidin dimers, two trimers and two tetramers, all type B, not being detected any type A procyanidins in this research. Although some work have showed procyanidins of different polymerization degree in avocado peel using normal phase column (López-Cobo et al., 2016; Wang et al., 2010), in this methodology only one peak is emerging from this type of column for each polymerization degree. On the contrary, the reversed phase column used in this research allowed the separation of different oligomers that had the same molecular weight (Callemien & Collin, 2008). In this sense, in the avocado peel extract studied in this research seventeen procyanidins corresponding to five dimers type A, seven dimers type B, two trimers type A and two type B, and one type B tetramer have been characterized. Around 37 and 25% of total dimer and trimer were A-type, respectively. Those values were higher than the ones reported in avocado pulp by Gu et al. (2003) and Wang et al. (2010) in avocado peel, which showed a content of this type of procyanidins of less than 12%.

The polar compounds perseitol and penstemide have been described before in avocado peel by López-Cobo et al. (2016). Nevertheless, as far as we are concerned, the others polar compounds found (peaks 32, 34, 36, 40, 47 and 53) have detected for the first time in avocado peel in the present work.

Respect to the non-polar compounds tentatively identified (peaks 57, 58, 59, 60 and 61), despite there was a report in avocado pulp by Hurtado-Fernández, Carrasco-Pancorbo, and Fernández-Gutiérrez (2011), to the best of our knowledge, this is the first time that they have been identified in avocado peel.

Summarizing, a number of thirty-five compounds were identified for first time in avocado peel, which highlight the potential of avocado peel as a source of bioactive compounds and lay bare the potent combination of the extraction and identification techniques used in the present work.

3.11. Potential use of avocado peel extract as functional ingredient

The identification carried out showed that hydroxybenzoic and hydroxycinnamic acids, flavonols and procyanidins were the most representative families of compounds found in the avocado peel extract. Those compounds possess well-known bioactive properties, which turned into the potential use of avocado peel by-product as functional ingredient. In this sense, cinnamic acids have been reported to offer many health properties, such as antioxidant, antiinflammatory, antibacterial, antidiabetic, anticarcinogenic, antiaging, and neuro-protective effects (Mata et al., 2016; Shahidi & Ambigaipalan, 2015). Among these, chlorogenic acid was reported to exhibit hypoglycemic, hypolipidemic and antioxidant properties (Ibrahim et al., 2015); p-coumaric acid exhibit antimicrobial activity (Rodríguez-Carpena et al., 2011), while caffeic acid might possess antitumour activity against colon carcinogenesis (Shahidi & Ambigaipalan, 2015). Moreover, in the field of food

manufacturing, hydroxybenzoic acids, as vanillic acid, are used as flavouring enhancer (Mudrić et al. 2017).

The presence of flavonols had been related to antioxidant, anti-inflammatory and cardioprotective effects (Mata et al., 2016). Quercetin was found to improve insulin-stimulated glucose uptake in mature adipocytes, whereas rutin was reported to possess potent hypoglycemic and hypolipidemic activities (Ibrahim et al., 2015).

Catechin has been described as potential agonist characteristic to insulin receptor (Ibrahim et al., 2015). Procyanidins have been reported to exhibit a wide range of health benefits, such as antioxidant, anti-carcinogenic and anti-inflammatory (Gu et al., 2003; Wang et al., 2010). Cádiz-Gurrea et al. (2014) found that the antioxidant activity increase with the polymerization degree of procyanidins. In this sense, dimers, trimers and tetramers are more effective than monomeric flavonoids against superoxide anion. Furthermore, procyanidins have presented higher antioxidant activities compared to others flavonoids, such as quercetin. Moreover, some studies suggests that the presence of procyanidin type A could possess several health benefits, as aid in maintaining urinary tract health, anti-allergic and anti-asthmatic effects (Aswar, Kandhare, Mohan, & Thakurdesai, 2015).

Overall, these results exemplify the usefulness of avocado peel as a source of this kind of compounds for its use as ingredient in food, cosmetic and pharmaceutical industries.

4. Conclusions

To our knowledge, this is the first available study that provides an exhaustive identification of a total of sixty-one phenolic and other polar compounds in avocado peel, which were classified into eleven compound families. Procyanidins, flavonols, hydroxybenzoic and hydroxycinnamic acids were the most representative groups. On this sense, seventeen procyanidins with different polymerization degree, as well as thirty-five compounds have been identified for the first time in avocado peel. These results highlight the potential combination of PLE and HPLC-DAD-ESI-QTOF-MS to extract and identify bioactive compounds in avocado peel by-product. Overall, these results exemplify the huge possibility of avocado peel as an ingredient in the food industry or cosmetic sector.

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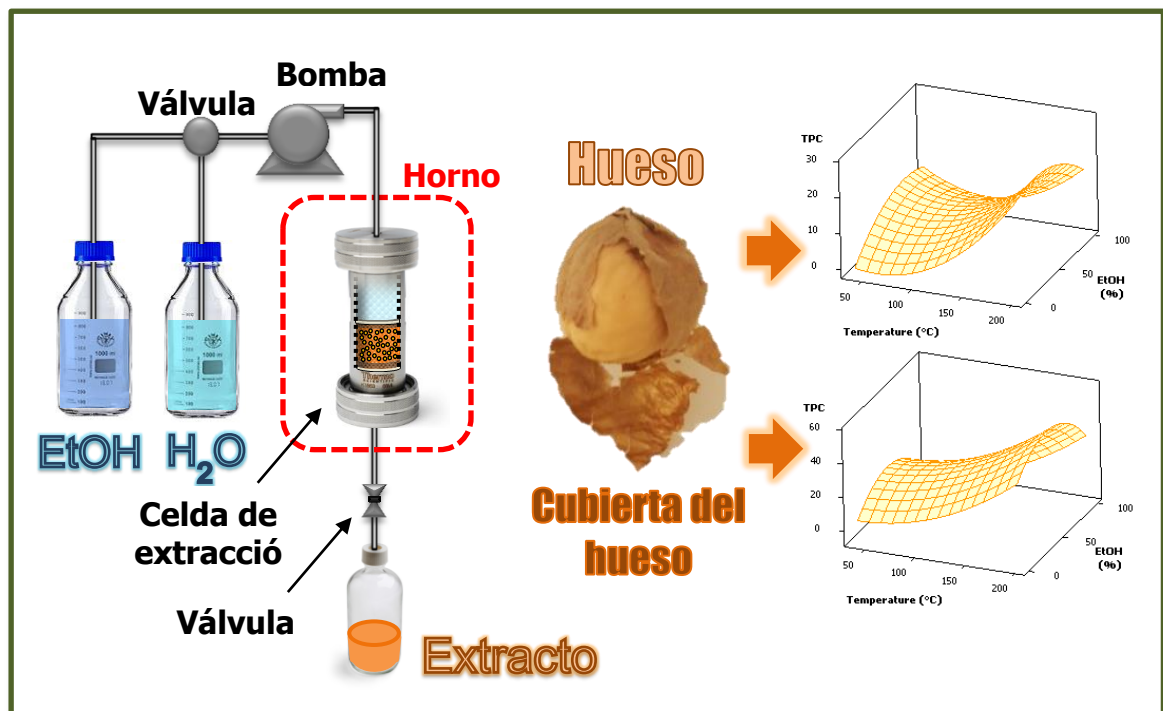
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Capítulo III



Potential use of drying and green extraction techniques for developing functional ingredients from avocado seed by-products

Abstract

Response surface methodology in a central composite design has been used to optimize the pressurized liquid extraction (PLE) of phenolic compounds from avocado seed and seed coat by-products. Extraction temperature (40–200 °C) and solvent mixture ethanol:water (0–100 % ethanol) were the selected independent variables, whereas total phenolic content (TPC) was the response variable. In addition, the optimization of the drying process was carried out in samples of seed at five different temperatures (45, 55, 65, 75 and 85 °C). The optimum extraction conditions for both avocado samples were obtained with a temperature of 200 °C and a solvent mixture 50:50 (v/v). According to the TPC, the best drying temperature for both samples was 85 °C. Under the optimal drying temperature and extraction conditions, the maximum TPC for avocado seed and seed coat were 29.5 ± 0.4 and 71.2 ± 1.7 mg EAG/g dm, respectively. To our knowledge, this is the first available study that optimized the phenolic compounds extraction from avocado seed and seed coat by-products using PLE with GRAS solvents. Besides, the changes of their phenolic profiles during drying processes were investigated by HPLC-DAD-ESI-TOF-MS. In this sense, were monitored 42 and 52 phenolic compounds were individually identified and quantified in avocado seed and seed coat, respectively. The optimal drying temperature for avocado seed was 85 °C. While for the seed coating each group of phenolic compounds had a different optimal drying temperature, in general 75 °C was the best temperature for most of the groups evaluated.

Keywords:

PLE, avocado seed, avocado seed coat, phenolic compounds, air drying

1. Introduction

Avocado (*Persea americana* Mill.) is a tropical fruit, native of Central America and Mexico. There are many varieties of avocado of which Hass is the most common worldwide (Kosińska, et al., 2012). This fruit is very nutritious, rich in unsaturated fatty acids (oleic, linoleic and linolenic acids), vitamins (B6, B12, C and E), minerals (potassium, phosphorus, calcium, iron and sodium) and essential amino acids (Barbosa-Martín, et al., 2016). Avocado is consumed worldwide as a fresh fruit or value-added products such as guacamole, avocado pulp and avocado oil. Nevertheless, the manufacture of these products produces a large amount of by-products, concretely around 21–30 % of the fruit weight are discarded (Rodríguez-Carpena, et al., 2011).

Nowadays, there is a special interest in using by-products as a source of bioactive compounds, especially considering that the content of phenolic compounds in the by-products sometimes is higher than in the edible part of fruits and vegetables. For instance, in avocado seed it has been reported that the total phenolic content (TPC) is several times higher than in the pulp (Rodríguez-Carpena, et al., 2011; Widsten, et al., 2014).

However, the avocado seed has a moisture content of around 55%, therefore, a drying process is necessary to prevent the development of some microorganisms responsible for deterioration. Convective air and freeze drying are the most thoroughly studied methods to reduce the water activity of foods. On the one side, freeze-drying is based on the dehydration by sublimation of a frozen product,

resulting in higher quality final products and it is more effective in preserving antioxidants. Nevertheless, its costs are 4–8 times higher than air-drying (Ratti, 2001), therefore, its cost limit its use on industrial scale. As a consequence, convective air drying is the most used drying method in the food industry, mainly due to its low cost and generally shorter drying times. It is commonly known that the effect of drying on the content of phenolic compounds in fruits depends basically on the temperature and time applied. Nevertheless, some studies revealed that the effect of drying on concentration of individual phenolic compounds is compound-dependent (Esparza-Martínez, et al., 2016; Joshi, et al., 2011; Wojdyło, et al., 2009). Therefore, for each matrix a drying optimization is necessary to maximize the concentration of the phenolic compounds present in this sample. A previous study analysed the effect of drying on phenolic compounds in avocado seed (Saavedra, et al., 2017). Nonetheless, solely four phenolic compounds were evaluated. Furthermore, as far as we are concerned, the drying effect on TPC and individual phenolic compounds has not been evaluated before in the avocado seed coat. Thus, considering the great wealth in phenolic compounds of the avocado seed, more research is needed in order to optimize the drying and extraction process, avoiding the degradation of these compounds.

In this sense, the extraction of bioactive compounds plays an important and crucial role in the final result and the subsequent use of the extract. Conventional extraction methods has been commonly used to evaluated the potential of avocado by-products as a source of bioactive compounds as phenolic compound (Calderón-Oliver, et al., 2016; Kosińska, et al., 2012; Pahua-Ramos, et al., 2012; Ramos-Jerz, et

al., 2013; Saavedra, et al., 2017; Torres, et al., 1987; Widsten, et al., 2014). Nevertheless, conventional solid–liquid extraction techniques such as Soxhlet extraction and dynamic maceration are time consuming and use high amounts of solvents (Wijngaard, et al., 2012). In addition, most of these researches have used solvents incompatible with the use in food, pharmaceutical or cosmetic industry. For this reason it is needed to the production of purified extracts rich in phenolic compounds with green extraction techniques based on the use of GRAS (Generally Recognized As Safe) solvents, such as pressurized liquid extraction (PLE).

The basic principles of PLE consist in the use of solvents at temperatures beyond their normal boiling points and pressures enough to keep the extracting fluid in the liquid state (Herrero, et al., 2013). The principal advantages of this technique are decreased use of synthetic and organic chemicals, reduced operational time and better yield and quality of the extract (Azmir, et al., 2013; Moraes, et al., 2013).

Moreover, there is a current consumer demand for non-synthetic and more natural food ingredients. Thus, the objective of the present research was to optimize the drying process and PLE of phenolic compounds from avocado seed and seed coat by-products, in order to obtain an extract rich in bioactive compounds that can be used as functional ingredient in the food, pharmaceutical or cosmetic industry.

2. Material and methods

2.1. Chemicals and reagents

In order to obtain an extract compatible with the food/pharmaceutical/cosmetic industry, only ethanol and water were used as extraction solvents. In addition,

Ottawa sand was used as a dispersing material. Ethanol and Ottawa sand were supplied from Fisher Scientific (Leicestershire, UK). Ultrapure water with conductivity lower than 18.2 M Ω generated by a Milli-Q system (Millipore, Bedford, MA, USA) was used for extraction and analysis. To measure the TPC, Folin–Ciocalteu reagent, sodium carbonate and gallic acid were purchased from Sigma-Aldrich (Steinheim, Germany). For mobile phase preparation acetonitrile (HPLC-MS grade) and formic acid were purchased from Fisher Scientific (Leicestershire, UK) and Fluka Chemika (Buchs, Switzerland), respectively.

For identification and quantification purposes the standards benzoic acid, (+)-catechin, chlorogenic acid, dihydrocaffeic acid, (-)-epicatechin, gentisic acid, 4-hydroxybenzoic acid, naringenin, neohesperedin, procyanidin dimer A2, procyanidin trimer B2, protocatechuic acid, quercetin, quercetin-3- β -glucoside and rutin were purchased either from Extrasynthese (Genay Cedex, France), Fluka Chemika (Buchs, Switzerland) or Sigma-Aldrich (Steinheim, Germany).

2.2. Samples

Fresh avocado fruits (*Persea americana* Mill. cv Hass) were kindly supplied by commercial group La Caña, Miguel García Sánchez e Hijos, S.A. (Motril, Spain). Avocados were allowed to ripen at room temperature. The complete avocado seeds were manually separated, cleaned with a continuous flow of tap water and stored at -20 ° C before drying.

2.3. Drying experiments

Avocado seeds were dried in an oven (Memmert UNE 200, Memmert, Germany) at different temperatures: 45, 55, 65, 75 and 85 °C. Due to the avocado seed hardness, the drying process was made in two steps. First, the whole avocado seed was dried until the seed coat was detached. Once the avocado seed coat was carefully separated, the seed were sliced and dried until a final moisture content of less than 8 %. The samples were turned periodically to ensure uniform dryness. Drying curves kinetics (kg of water/kg of dry matter) were obtained by periodically weighing avocado seed during dehydration. Then, the dried samples were milled in an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting avocado seed and seed coat powder had an average particle size of 0.5 mm. The obtained material was stored avoiding light exposure and kept at room temperature until extraction. All the drying experiments were done in triplicate.

2.4. Extraction

An ASE 350 System (Dionex Corp., Sunnyvale, CA) equipped with a solvent controller was used for pressurized liquid extraction. Extraction conditions were optimized using response surface methodology (RSM) in order to determine the relationship between the TPC and the independent variables such as temperature (40–200 °C) and percentage of ethanol of the extraction solvent (0–100 % EtOH). In this regard a central composite design (CCD) with two axial points was employed. The experimental conditions are summarized in Table 1. Experimental runs were

randomised, to minimise the effects of unexpected variability in the observed responses.

For each experiment, one gram of dried avocado seed or seed coat was mixed homogeneously with 5 g of sand and loaded into 34 mL stainless steel PLE vessel. Disposable cellulose filters were placed at each end of the cell in order to prevent clogging in the metal frits of the extraction cell. Furthermore, two portions of sand (5 g) were placed between sample and cellulose filters. Prior to use, solvents were previously degassed for 15 min to remove the dissolved oxygen in order to avoid any possible oxidation. The extraction was performed with a static extraction cycle of 20 min at 11 MPa. After that, the cell was flushed with solvent (60 % of the cell volume) and purged with nitrogen (100 s). The obtained extracts were immediately cooled on ice to attain room temperature and centrifuged at 12,000 rpm for 15 min at 4 °C in a centrifuge (Sorvall ST 16R, Thermo Scientific, Leicestershire, UK). Then, the supernatants were evaporated under vacuum (13 kPa) at 35 °C to dryness in a Scavan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). Finally the extracts were stored at -20 °C until further use. Two experiments were carried out at each experimental design point.

2.5. Determination of total phenolic content (TPC)

The total phenolic content (TPC) was quantified by the colorimetric assay Folin-Ciocalteu, as described by Rodríguez-Pérez, et al. (2016). Briefly, every dried extract was reconstituted in an appropriated volume of its correspondent extraction solvent for obtaining solutions at a concentration of 1 mg/mL. Then, 10 µL of diluted

extracted was mixed with 600 μL of water and 50 μL of undiluted Folin-Ciocalteu reagent. After 10 min, 150 μL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. This mixture was incubated for 2 h at room temperature in darkness. After that, 200 μL of the mixture was transferred into a 96-well polystyrene microplate. A Synergy Mx Monochromator Based Multi-Mode Micro plate reader (BioTek Instruments, Winooski, VT, USA) was used to measure the absorbance at 760 nm. The measurement was compared to a calibration curve of gallic acid (5–150 $\mu\text{g}/\text{mL}$) prepared in the same manner. The TPC was calculated as mean \pm SD ($n = 3$) and expressed as mg gallic acid equivalents (GAE)/g dry mass.

2.6. Quantification of phenolic compounds by HPLC-DAD-ESI-TOF-MS

The analyses were performed on an Agilent 1200 RRLC instrument (Agilent Technologies, Palo Alto, CA, USA) of the Series Rapid Resolution equipped with a binary pump, an online degasser, an autosampler, a thermostatically controlled column compartment and a diode array detector.

Every dried extract was re-dissolved in an appropriated volume of its correspondent extraction solvent for obtaining solutions at concentration of 10 mg/mL. Then, 10 μL of diluted extracted was injected at a flow rate of 0.8 mL/min. Pre-filters used as precolumn were RRLC in-line filters, 4.6 mm, 0.2 μm (Agilent Technologies, Palo Alto, CA, USA) and the analytes were separated on an Agilent Zorbax Eclipse Plus C18 column (4.6 \times 150 mm, 1.8 μm particle size) maintained at 25 $^\circ\text{C}$. The mobile phase was composed of solvent A (water plus 1% of formic acid, v/v) and solvent B (acetonitrile). The following multi-step linear gradient was used in order to achieve

efficient separation: 0.0 min [A:B 95/5], 30.0 min [A:B 75/25], 35.0 min [A:B 65/35] and 40.0 min [A:B 5/95]. Finally, initial conditions were kept for 5 min at the end of each analysis to equilibrate the system before the subsequent injection. The UV spectra were recorded from 190 to 950 nm.

The HPLC system was coupled to a microTOF™ mass analyser (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface operating in negative ionization mode. The effluent from the HPLC column was splitted using a T-type phase separator before being introduced into the mass spectrometer (split ratio 1:3) to provide a stable spray and, consequently, reproducible results. The ESI–TOF parameters were: capillary voltage of 4500 V; drying gas temperature, 190 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 2.0 Bar; and end plate offset –500 V. The values of transfer parameters were: capillary exit, –150 V; skimmer 1, –50 V; hexapole 1, –23 V, RF hexapole, 199 V; skimmer 2, –22.5 V; the trigger time was set to 53 μs (50 μs for setting transfer time and 3 μs for pre-pulse storage time). The detection of the compounds of interest was carried out considering a mass range of 50–1000 m/z.

During the analysis batch, external mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the instrument. A sodium formate cluster solution, containing 5 mM sodium hydroxide in water: 2-propanol 1/1 (v/v) with 0.1% (v/v) of formic acid was used. The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification. Due to the compensation of temperature drifts in the microTOF instrument, this external

calibration provided accurate mass values (better 5 ppm) for a complete run without the need of a dual sprayer setup for internal mass calibration.

The accurate mass data of the molecular ions were processed using the software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas using the Generate Molecular Formula™ Editor, and provide a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula.

In order to quantify the amount of each compound, seven calibration curves were calculated by using nine concentration levels in analysed in triplicate. The curves were prepared using catechin, 5-O-caffeoylquinic acid, epicatechin, procyanidin A2, procyanidin B2, quercetin-3- β -glucoside and rutin. Neohesperidin was used as internal standard at a concentration of 10 mg/L. All of them were obtained by plotting the standard concentration as a function of the relative peak area (defined as compound peak area divided by the peak area of internal standard) obtained from HPLC-ESI-TOF analyses. This method was validated in a previous study (Figuroa, et al., 2018). All calibration curves showed good linearity among different concentrations, and the determination coefficients (r^2) were higher than 0.99 in all cases.

The compound concentrations were determined using the relative peak area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. In this way, catechin, 5-O-caffeoylquinic acid, epicatechin, procyanidin A2, procyanidin B2, quercetin-3- β -glucoside and rutin were quantified

with the calibration curves of their own standards. The remaining compounds were tentatively quantified on the basis of calibration curves from other compounds with structural similarities. It should be taken into account that the response of the standards can differ from that of the analytes found in the extracts, and consequently the quantification of these compounds is only an estimation of their actual concentrations although it can be considered a useful approximation. Thus, the calibration curve of catechin was used to quantify catechin derivatives, 5-O-caffeoylquinic for phenolic acids and phenolic alcohol derivatives, procyanidin A2 for type A procyanidins, procyanidin B2 for type B procyanidins, and rutin for flavonols derivatives.

2.7. Statistical analysis

Statistical analysis was conducted using the statistical package MINITAB 16.0 software (PA, USA). All the data were expressed as means \pm standard deviation (SD) of three analyses of each chemical determination. The PLE optimization was performed with response surface methodology using a central composite design (CCD) with two axial points. Furthermore, the data collected for TPC and individual phenolic compound content were statistically analysed by one-way analysis of variance (ANOVA) to test the effects of the five drying temperatures applied (45, 55, 65, 75 and 85 °C). The Tukey's post hoc test was applied for comparisons of means, and differences were considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Optimization of pressurized liquid extraction

Pressurized liquid extraction (PLE) was used in order to optimize the extraction of phenolic compounds from avocado seed and seed coat. The main factors influencing the outcome of the PLE process are temperature and type of solvent (Azmir, et al., 2013; Mustafa & Turner, 2011). In the other hand, pressure and extraction time has been reported to possess a less critical effect in PLE (Herrero, et al., 2013; Moraes, et al., 2013; Mustafa & Turner, 2011). In this sense, in the present work the pressure was set at 11 MPa in order to maintain the solvent in a liquid state and the static extraction time was set at 20 min, value high enough to reach equilibrium between those sample components still bound to the matrix and those already solubilized (Luthria, et al., 2007; Miron, et al., 2011; Wijngaard, et al., 2012).

Extraction temperature is one of the most important factors. In this sense, raising the temperature increase the mass transfer rate and extraction rates, and decreases the viscosity and surface tension of the solvents (Ramos, et al., 2002). In these sense, the extraction yield could be higher when the extraction is performed with high temperatures (Herrero, et al., 2013). Furthermore, PLE offers a wide range of polarities by changing the temperature (Ramos, et al., 2002). For instance, water at 25 °C has a dielectric constant of ~80 whereas at 250 °C present a dielectric constant between 25–27, value similar to less polar solvents at room temperature (Ollanketo, Peltoketo, Hartonen, Hiltunen, & Riekkola, 2002). Nevertheless, high temperatures might have negative effects on the bioactivity of some thermo-labile compounds

(Azmir, et al., 2013; Herrero, et al., 2013; Moraes, et al., 2013). Thus, temperature extraction was selected as factor to optimize in PLE of bioactive compounds and varied from 40 to 200 °C (full operation range of accelerated solvent extractor ASE 350).

The type of solvent is also an important factor and it was one of the most thoroughly investigated factors (Rodríguez-Pérez, et al., 2016). The solvent must be able to solubilize the compounds of interest and minimize the co-extraction of others components (Azmir, et al., 2013; Skalicka-Woźniak & Głowniak, 2012). In addition, the solvent used should be Generally Recognized as Safe (GRAS) for use in the food, pharmaceutical and cosmetic industries (Wijngaard, et al., 2012). Thus, hydro-ethanolic mixtures are the most appropriate solvents used in PLE of bioactives. In the present study, a fully range of ratio EtOH:H₂O (0–100 % EtOH) was evaluated.

In order to evaluate the interactive effects among the variables studied, response surface was employed to maximize the TPC in the avocado seed and seed coat extract. In this sense, nine different conditions were tested (Table 1). The TPC was significantly affected by temperature extraction. Furthermore, for the same solvent ratio EtOH:H₂O higher TPC were found when the temperature extraction was increase.

Table 1.- Central composite design of the five-level and two-variable and observed response (TPC) under different experimental conditions

Run	Independent variables		Response –TPC (mg EAG/g)					
	Temperature (°C)	Solvent/Relation (%)	Seed					Seed coat
			45 °C	55 °C	65 °C	75 °C	85 °C	65 °C
1	176	85	4.5	11.2	9.1	9.6	9.1	25.3
2	120	50	4.3	9.2	6.5	5.0	9.5	27.1
3	63	85	0.9	1.9	1.6	2.1	8.2	9.1
4	176	15	8.2	10.8	6.5	8.1	14.7	31.8
5	63	15	0.8	4.1	3.7	0.7	3.9	16.1
6	63	15	0.8	3.4	3.9	0.7	4.6	17.2
7	120	0	0.3	1.5	0.9	8.7	4.0	15.2
8	40	50	0.9	4.0	3.0	4.3	7.8	13.9
9	120	50	4.4	11.1	7.7	5.9	8.3	28.1
10	40	50	1.0	4.4	3.3	4.5	7.9	14.4
11	120	100	1.1	1.1	1.4	2.0	6.1	5.7
12	120	50	4.4	10.2	7.2	5.5	9.0	27.9
13	176	15	8.1	11.5	7.4	8.1	16.3	33.9
14	120	50	4.4	10.4	7.3	5.6	9.1	28.4
15	200	50	9.3	26.0	23.0	24.2	29.3	65.7
16	120	50	4.3	10.0	7.1	5.4	8.8	27.3
17	120	50	4.4	10.3	7.2	5.5	9.0	28.0
18	120	0	0.4	1.6	1.2	9.2	4.0	17.2
19	63	85	1.1	2.1	1.6	1.8	7.2	9.1
20	176	85	4.7	10.9	8.8	9.5	10.4	24.1
21	120	50	4.4	10.4	7.3	5.6	9.1	28.3
22	120	50	4.2	9.9	7.0	5.3	8.7	26.9
23	120	100	1.1	1.0	1.6	2.3	5.7	5.6
24	120	50	4.4	10.4	7.3	5.6	9.1	28.3
25	120	50	4.4	10.3	7.3	5.5	9.1	28.1
26	200	50	10.8	24.8	22.3	25.2	29.8	62.2

Likewise, the solvent ratio EtOH:H₂O presented effect on the TPC, in both sample the higher result were found when a hydro-ethanolic mixtures were used. Moreover, as can be seen in Figure 1, in both samples the maximum point is outside the range of temperatures evaluated. Nevertheless, since the independent variables were evaluated in their fully operational range, the original design could not be modified to achieve optimal conditions.

The optimum PLE conditions given by the models for avocado seed were 200 °C and 28.5 % EtOH, whereas to avocado seed coat were 200 °C and 42.4 % EtOH. Notwithstanding, higher TPC were found for seed (29.55 ± 0.37 mg GAE/g dm) and seed coat (63.91 ± 2.49 mg GAE/g dm) with an axial point at 200 °C and 50 % EtOH.

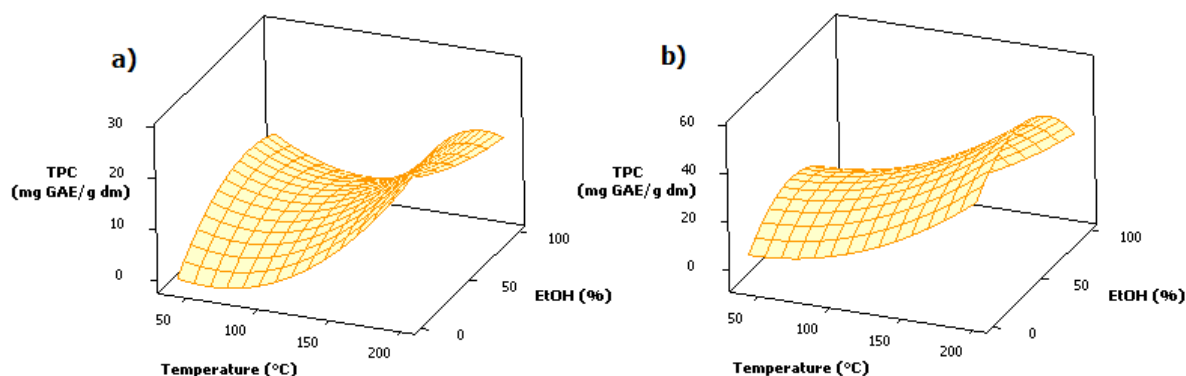


Figure 1.- Three dimensional plot of response surface for the effects of solvent ratio (percentage ethanol in the extraction mixture) and temperature on TPC in a) Seed and b) Seed coat.

The TPC obtained for avocado seed and seed coat was strongly affected by the solvent dielectric constant, similar behaviour was observed for both samples (Figure 2). The treatment with a dielectric constant of 26.0 (200 °C and 50 % EtOH) presented the highest values of TPC for both samples. These results agree with

those reported by Ciulu, et al. (2017), who optimized the extraction of the polyphenols from *Stevia rebaudiana* Bertoni leaves and found the highest TPC with the same dielectric constant (26.0). Moreover, a reduction on the TPC was observed with highest and lowest dielectric constants. Rodríguez-Carpena, et al. (2011) evaluated the effect of different solvents (ethyl acetate, acetone/water and methanol/water) on the TPC of avocado seed. They found that the mixture acetone/water with a dielectric constant of 35.70 (Akerlof, 1932) presented the highest TPC. Therefore, was confirmed that the use of solvents with dielectric constant range from 26 to 36 enhance the extraction of polyphenols compounds from avocado seed. For both samples, a point out of the tendency was observed (dielectric constant of 24), identical behaviour has been found by Ciulu, et al. (2017). This could be due to the low temperature used in this condition, according to Moraes, et al. (2013) the quantity of antioxidants extracted is high at elevated temperatures.

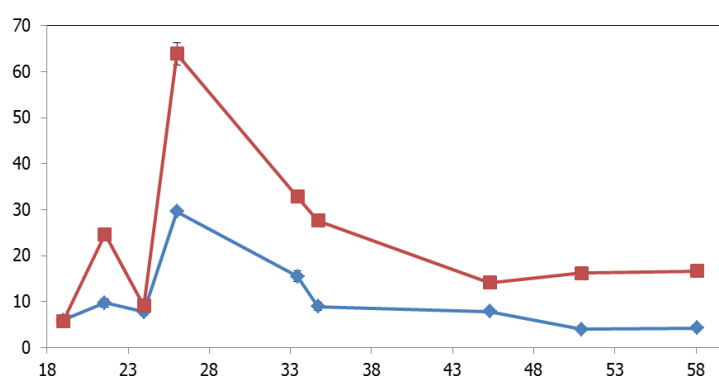


Figure 2.- Effect of solvent dielectric constant on the TPC in avocado seed and seed coat.

The extraction yields are presented in Table 2 (samples dried at 65 °C), for both samples the higher yields were obtained with dielectric constants of 26 and 33.4. On the other hand, the lowest yield was observed with a dielectric constant of 19.

Table 2.- Dielectric constant, extraction yield and TPC in the avocado seed and seed coat extracts under different experimental conditions.

Dielectric constant	Temperature (°C)	EtOH (%)	Yield	
			Seed	Seed coat
19.0	120.0	100.0	6.6 ± 0.1 ^{efB}	7.7 ± 0.0 ^{gA}
21.6	176.0	85.0	12.3 ± 0.1 ^{dB}	28.7 ± 1.6 ^{deA}
24.0	63.0	85.0	7.4 ± 0.0 ^{efB}	14.8 ± 0.1 ^{fA}
26.0	200.0	50.0	50.7 ± 1.4 ^{aB}	60.6 ± 0.7 ^{aA}
33.4	176.0	15.0	37.6 ± 2.4 ^{bB}	45.2 ± 0.6 ^{bA}
34.7	120.0	50.0	17.0 ± 0.1 ^{cB}	37.5 ± 0.4 ^{cA}
45.3	40.0	50.0	10.9 ± 0.0 ^{deB}	27.2 ± 0.4 ^{eA}
50.9	120.0	0.0	4.8 ± 0.0 ^{fB}	37.5 ± 0.3 ^{cA}
58.1	63.0	15.0	14.4 ± 0.2 ^{cdB}	30.4 ± 0.3 ^{dA}

For a same sample, values followed by different lower case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

For a same dielectric constant, values followed by different upper case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

Moreover, when was used the same mixture of solvent the extraction temperature showed a significant effect ($p \leq 0.05$) on extraction yield efficiency for both samples. This could be explained by the increase in solubility and mass transfer as a consequence of the increase in extraction temperature (Luthria, 2008). Moreover, avocado seed coat presented higher extraction yields ($p \leq 0.05$) than seed for all treatments.

3.2. Effect of temperature drying on TPC

Once optimum PLE conditions were obtained, the effect of drying temperature on TPC was investigated. The initial moisture content of avocado seed was 53.3 ± 0.7 g water/100 g, so in order to avoid microbiological decomposition during storage a drying process is necessary. This moisture content was in close agreement with that reported by Saavedra, et al. (2017) of 52.68 ± 1.00 g water/100 g. Figure 3 shows the drying kinetics of avocado seed obtained at 45, 55, 65, 75 and 85 °C. As expected, a clear influence of air-drying temperature on the drying rate was observed, as reported in the literature (López, et al., 2010; Mrad, et al., 2012). Due to the drying process was started with whole avocado seed, an induction stage was appreciated during the first hours. Then, a constant drying period rate was observed until the samples reached the critical moisture content (~ 0.5 g water/g solids). Finally, a falling rate period was observed due to bound water at the surface and shrinkage of the product (Mrad, et al., 2012). The equilibrium moisture contents were similar for all the evaluated treatments (~ 0.07 g water/g solids).

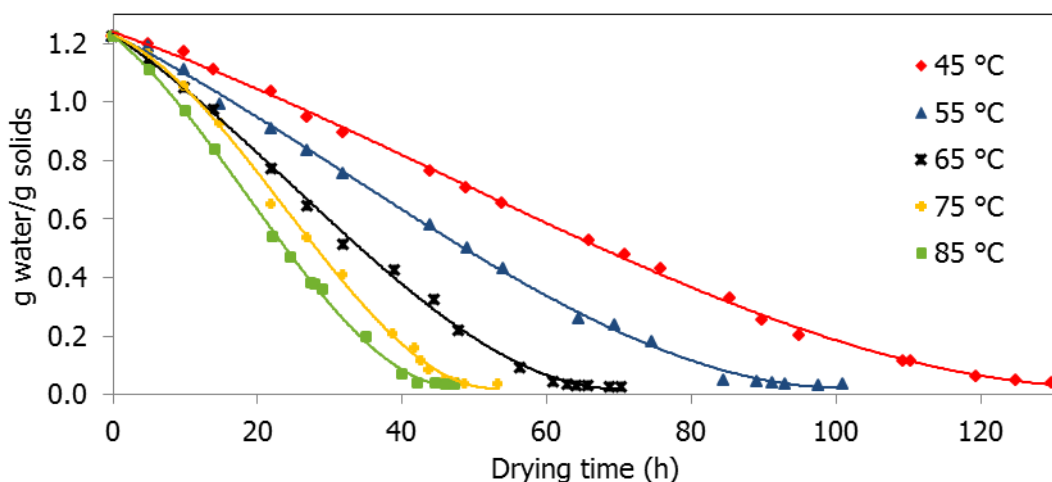


Figure 3.- Drying kinetics of avocado seed at different temperatures.

The time needed to reach a final moisture content of 0.07 g water/g solids decreased when the drying temperature increased. The drying times at 45, 55, 65, 75 and 85 °C were 114, 81, 57, 44 and 39 h, respectively. These values were lower than the findings reported by Avhad y Marchetti (2015), who found that at 80 °C the time required to reduce 45.59 % of the weight in non-pretreated and Hass avocado seed was 96 h. In addition, Avhad y Marchetti (2015) reported that the use of high drying temperature (80 °C) resulted in hardening and charring of the outer surface of avocado seeds. On the contrary, only a negligible visual colour change was observed in the present study. In our study, the reduction of the sample size provided a greater sample surface, which allowed the increase of the rate of drying in a faster process and contributed to avoid hardening or charring reactions.

The effect of drying temperature on extraction yield and TPC are show in Table 3 for extracts obtained with optimum PLE conditions (solvent dielectric constant of 26.0). These results suggested that drying temperature had negligible effects on the extraction yield in avocado seed samples. On the other hand, in avocado seed coat the drying temperature had a significant effect ($p \leq 0.05$) on extraction yield, where the highest value was found with a drying temperature of 65 °C.

Regarding TPC, the drying temperature showed a strongesr effect, being the highest and lowest values for both samples found with drying temperatures of 85 and 45 °C, respectively. This behaviour could be related to longer drying times observed in processes performed at lower temperatures. This slow drying may cause degradation of phenolic compounds which induce a decrease of TPC. Indeed, the

time necessary to dry avocado seeds at 45 °C was almost three folds higher than the one resulted at 85 °C.

Table 3.- Effect of drying temperature on extraction yield and TPC in the avocado seed and seed coat extracts.

Temp. (°C)	Rendimiento (%)		TPC (mg EAG/100g)	
	Seed	Seed coat	Seed	Seed coat
45	42.3 ± 5.4 ^{aA}	49.7 ± 1.4 ^{cA}	10.1 ± 1.1 ^{cB}	50.9 ± 0.5 ^{cA}
55	46.7 ± 1.3 ^{aB}	54.1 ± 0.5 ^{bA}	25.4 ± 0.8 ^{bB}	61.9 ± 0.7 ^{bA}
65	50.7 ± 1.4 ^{aB}	60.6 ± 0.7 ^{aA}	22.6 ± 0.5 ^{bB}	63.9 ± 2.5 ^{bA}
75	49.6 ± 5.9 ^{aA}	50.3 ± 0.3 ^{cA}	24.7 ± 0.7 ^{bB}	64.6 ± 0.6 ^{bA}
85	48.2 ± 0.2 ^{aA}	45.3 ± 0.1 ^{dB}	29.5 ± 0.4 ^{aB}	71.2 ± 1.7 ^{aA}

For a same sample, values followed by different lower case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

For a same temperature and parameter, values followed by different upper case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

Furthermore, for avocado seed the effect of drying temperature for all dielectric constant detailed in Section 3.1 were evaluated. As it can be observed in Figure 4, avocado seed showed a wide range of TPC from 0.3 to 29.5 depending on the solvent dielectric constant and drying temperature used. The highest TPC for either drying temperature was found when PLE extraction took place with a solvent dielectric constant of 26.0 (200 °C and 50 % EtOH), whereas the lower results were obtained with both higher and lower dielectric constants tested. Therefore, the results presented in Figure 4 confirmed than the better conditions in order to maximize the extraction of phenolic compounds were drying temperature performed at 85 °C and dielectric constant of 26.

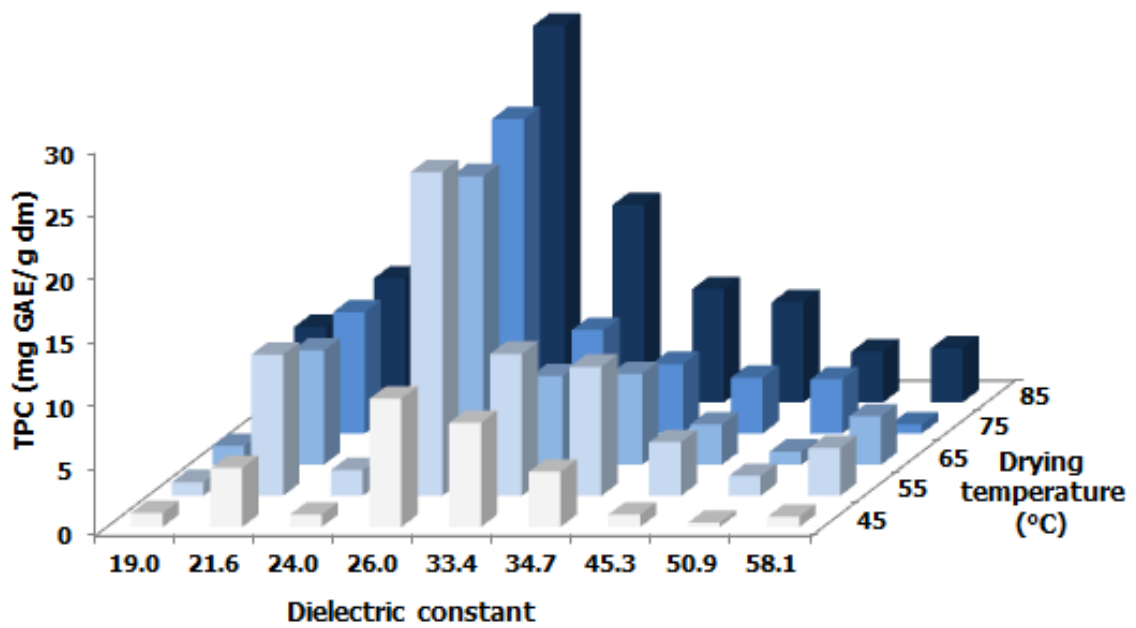


Figure 4.- Effect of solvent dielectric constant and drying temperature on TPC in avocado seed.

Avocado seed coat contained a significantly ($p \leq 0.05$) higher amount of TPC values than seed. The TPC found with the optimum conditions were 71.2 ± 1.7 and 29.5 ± 0.4 mg GAE/100 g dm for avocado seed coat and seed, respectively. The result obtained for avocado seed was higher than the values reported by Calderón-Oliver, et al. (2016) and Morais, et al. (2015), who used water and methanol as extraction solvents. On the other hand, some studies have found higher results using mixtures of water with organic solvents, specifically acetone (Rodríguez-Carpena, et al., 2011; Saavedra, et al., 2017). Nevertheless, avocado seed coat TPC levels were considerably higher than these values. To the best of our knowledge, this is the first study that quantified the TPC in avocado seed coat.

3.3. Phenolic compound composition by HPLC-ESI-TOF-MS analysis.

According to the TPC results described in section 3.2, the best drying temperature for seed and seed coat was 85 °C. Nevertheless, the TPC could be deviate due to presence of nonphenolic reducing substances in the extracts, such as reducing sugar and ascorbic acid (Zhao, et al., 2017). Therefore, in order to investigate the drying temperature effect on potential antioxidant phenolic compounds individual phenolic compound quantification have been performed by HPLC-ESI-TOF-MS analysis. In this sense, were monitored 42 and 52 phenolic compounds were individually identified and quantified in avocado seed and seed coat, respectively. Those compounds belong to six groups: phenolic acids, phenolic alcohol derivatives, flavonols, flavanones, catechins and procyanidins. The predominant phenolic compounds were procyanidins and phenolic acids, with sixteen and eleven compounds identified, respectively.

These compounds were tentatively identified using the information provided by the mass analyser comparing their mass spectra and elution order with standards when available, and after a thorough survey of the literature. The information of the identified compounds such as retention time, experimental and calculated m/z , molecular formula, error and $mSigma$ was listed in Supplementary Table 1.

The effects of drying temperature on the phenolic families were shown in Table 4. Regarding the individual phenolic compounds the information is recovered in Table 5 and Table 6. The results put into light that the drying conditions affected the contents of all phenolic compounds evaluated in both samples ($p \leq 0.05$).

Table 4.- Phenolic profile of avocado seed and seed coat.

Sample	T (°C)	Phenolic profile (mg/100 g dm)						
		Phenolic acids	Phenolic alcohol derivatives	Flavonols	Flavanones	Catechins	Procyanidin	
Seed	45	106 ± 2 ^e	11.2 ± 0.2 ^d	5.11 ± 0.03 ^d	2.55 ± 0.01 ^d	42.6 ± 0.3 ^e	39.7 ± 0.2 ^e	
	55	214 ± 2 ^c	31.1 ± 0.4 ^c	6.50 ± 0.01 ^c	2.60 ± 0.01 ^d	77 ± 2 ^b	77.4 ± 0.6 ^b	
	65	185 ± 0.3 ^d	33.4 ± 0.5 ^c	7.18 ± 0.02 ^b	3.46 ± 0.07 ^c	56.9 ± 0.7 ^d	73.2 ± 0.7 ^c	
	75	262 ± 5 ^b	65 ± 2 ^b	8.2 ± 0.01 ^a	3.77 ± 0.02 ^b	68.6 ± 0.7 ^c	69.1 ± 0.2 ^d	
	85	518 ± 8 ^a	169 ± 2 ^a	7.15 ± 0.03 ^b	4.16 ± 0.03 ^a	199 ± 2 ^a	227 ± 2 ^a	
Coat	45	540 ± 20 ^b	133 ± 4 ^b	8.24 ± 0.05 ^c	3.83 ± 0.05 ^e	117 ± 3 ^c	310 ± 3 ^c	
	55	640 ± 20 ^a	127 ± 3 ^{bc}	8.71 ± 0.1 ^b	4.40 ± 0.07 ^c	164 ± 8 ^a	356 ± 6 ^b	
	65	570 ± 20 ^b	190 ± 10 ^a	9.56 ± 0.02 ^a	4.19 ± 0.04 ^d	139 ± 2 ^b	322 ± 1 ^c	
	75	652 ± 6 ^a	115 ± 2 ^c	9.37 ± 0.1 ^a	4.56 ± 0.04 ^b	161 ± 5 ^a	394 ± 7 ^a	
	85	290 ± 10 ^c	63 ± 0.4 ^d	7.8 ± 0.1 ^d	5.17 ± 0.08 ^a	136 ± 7 ^b	320 ± 20 ^c	

*For a same sample, mean values in a column superscripted by different letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

The highest concentrations of phenolic acids were found when the dehydration was carried out at 85 °C. Solely, the 5-p-coumaroylquinic acid presented a slightly higher concentration at 75 °C than 85 °C. On the other hand, this kind of compound was highly affected with a temperature of 45 °C. Moreover, the isomers of caffeoylquinic acid were the phenolic acids more affected by temperature, with a reduction of up to 94 % with the lowest temperature. Similar results were found by Esparza-Martínez, et al. (2016), who evaluated the effect of air-drying temperature on phenolic acids of lime wastes and observed that chlorogenic acid was the compound more affected.

Additionally, three phenolic alcohol derivatives were identified in both samples. Avocado seed coat showed a higher concentration than seed, with values of 191.4 and 168.3 mg/100 g dry mater, respectively. In avocado seed the higher phenolic alcohol derivatives was hydroxytyrosol glucoside (77 mg/100 g dry mater), whereas in avocado seed coat was tyrosol-glucosyl-pentoside (110 mg/100 g dry mater). Moreover, the air drying temperature showed effect on the concentration of these compounds. In avocado seed the highest values were found at 85 °C, where the concentrations were up to five folds higher than in sample dried at 45 °C. Meanwhile in seed coat the highest concentrations for all the phenolic alcohol derivatives were found with a temperature of 65 °C.

Table 5.- Concentration of phenolic compounds in avocado seed after drying process a different temperatures.

Analyte	RT (min)	m/z	Concentration (mg/100 g, dry mater)				
			45 °C	55 °C	65 °C	75 °C	85 °C
Perseitol	1.87	211.082	83 ± 7	111 ± 5	99 ± 3	117 ± 3	151 ± 1
Quinic acid	1.99	191.056	298 ± 2	359 ± 5	425 ± 3	386 ± 8	384 ± 7
Citric acid	2.49	191.019	46 ± 1	49 ± 1	46.7 ± 0.3	36.4 ± 0.6	47 ± 2
3,4-dihydroxyphenylacetic acid	8.05	167.037	13.3 ± 0.7	15.8 ± 0.9	14.9 ± 0.3	17.8 ± 0.8	18 ± 0.5
Hydroxytyrosol glucoside	8.80	315.109	11.2 ± 0.2	20.6 ± 0.1	18.1 ± 0.8	21.3 ± 0.7	77 ± 1
Pyrocatechol	9.64	109.031	7.48 ± 0.03	8.92 ± 0.03	9.4 ± 0.2	10.1 ± 0.1	9 ± 0.2
3-O-caffeoylquinic acid	10.77	353.089	n.d.	29.9 ± 0.5	19.8 ± 0.4	27.2 ± 0.8	107 ± 3
Tyrosol-glucoside	11.45	299.117	n.d.	n.d.	n.d.	19 ± 0.9	49.3 ± 0.5
Pensternide	12.55	443.195	4.69 ± 0.08	5.8 ± 0.2	5.8 ± 0.1	4.92 ± 0.05	5.2 ± 0.1
Proc. dimer B isomer 1	13.17	577.142	n.d.	n.d.	n.d.	n.d.	9.8 ± 0.9
Tyrosol-glucosyl-pentoside	13.30	431.157	n.d.	10.4 ± 0.3	15.4 ± 0.3	24.6 ± 0.9	42 ± 2
4-hydroxybenzoic acid	13.37	137.027	31 ± 2	36 ± 1	35 ± 1	45.6 ± 0.9	51 ± 1
3-p-coumaroylquinic acid	13.70	337.097	12.8 ± 0.4	22.5 ± 0.2	21.6 ± 0.3	19.5 ± 0.5	22.6 ± 0.3
p-Coumaric acid	13.94	163.041	19.4 ± 0.2	22.1 ± 0.6	29.7 ± 0.8	25.6 ± 0.7	30.7 ± 0.5
Proc. dimer B isomer 2	14.24	577.144	n.d.	n.d.	n.d.	n.d.	18.8 ± 0.5
5-O-caffeoylquinic acid	14.53	353.089	n.d.	n.d.	n.d.	19.1 ± 0.4	41.7 ± 0.7
(+)-Catechin	15.53	289.074	20.1 ± 0.2	46 ± 2	26.9 ± 0.6	36.1 ± 0.5	137 ± 2
4-O-caffeoylquinic acid	15.84	353.090	10 ± 0.1	44 ± 2	26.3 ± 0.4	53 ± 2	179 ± 8
Proc. dimer B isomer 4	17.70	577.143	n.d.	n.d.	n.d.	n.d.	16.3 ± 0.7
5-p-coumaroylquinic acid	18.58	337.097	7.72 ± 0.07	14.7 ± 0.1	12.5 ± 0.1	23.6 ± 0.9	22.2 ± 0.3
4-p-coumaroylquinic acid	19.21	337.102	11.6 ± 0.4	29 ± 1	25.1 ± 0.8	31 ± 1	46 ± 2

Analyte	RT (min)	m/z	Concentration (mg/100 g, dry matter)				
			45 °C	55 °C	65 °C	75 °C	85 °C
(-)-Epicatechin	19.52	289.073	8.24 ± 0.03	14.3 ± 0.3	11.3 ± 0.1	13.1 ± 0.2	44.2 ± 0.7
Proc. trimer A isomer 1	20.43	863.175	4.21 ± 0.05	15.2 ± 0.5	11.5 ± 0.4	6.25 ± 0.08	29.4 ± 0.2
Proc. trimer A isomer 2	20.70	863.177	3.79 ± 0.03	13.3 ± 0.3	11.3 ± 0.1	6.2 ± 0.1	26.6 ± 0.3
Quercetin-diglucoside isomer 1	21.73	625.145	0.986 ± 0.002	1.309 ± 0.004	1.379 ± 0.006	1.365 ± 0.003	1.315 ± 0.003
Quercetin-diglucoside isomer 2	21.91	625.143	0.973 ± 0.008	1.257 ± 0.006	1.321 ± 0.006	1.403 ± 0.004	1.398 ± 0.003
Proc. dimer B isomer 5	22.00	577.138	n.d.	n.d.	n.d.	n.d.	7.9 ± 0.2
Proc. trimer A isomer 3	23.13	863.175	2.99 ± 0.04	5.1 ± 0.2	7.2 ± 0.1	4.56 ± 0.05	31.1 ± 0.5
Proc. trimer A isomer 4	24.18	863.176	3.29 ± 0.07	5.9 ± 0.2	7.83 ± 0.04	5.7 ± 0.1	37.4 ± 0.5
Proc. dimer B isomer 6	24.58	577.138	n.d.	n.d.	n.d.	n.d.	7.2 ± 0.3
Hydroxyabscisic acid glucoside	24.62	441.181	330 ± 10	410 ± 40	368 ± 9	407 ± 6	413 ± 9
Proc. dimer A isomer 1	25.28	575.118	6.65 ± 0.05	10.1 ± 0.1	10.3 ± 0.1	9.6 ± 0.1	12.5 ± 0.1
Quercetin-3-β-glucoside	26.84	463.089	0.962 ± 0.002	1.222 ± 0.003	1.382 ± 0.005	1.461 ± 0.003	1.46 ± 0.005
Proc. dimer A isomer 2	28.22	575.118	6.4 ± 0.1	8.81 ± 0.04	8.24 ± 0.04	9.14 ± 0.02	9.7 ± 0.2
Proc. dimer A isomer 3	28.28	575.117	6.12 ± 0.03	8.46 ± 0.05	8.53 ± 0.09	8.61 ± 0.01	9.1 ± 0.2
Proc. dimer A isomer 4 (A2)	29.42	575.114	6.27 ± 0.03	10.5 ± 0.1	8.38 ± 0.02	10.4 ± 0.1	10.8 ± 0.03
(Epi)gallicocatechin	30.28	305.067	14.2 ± 0.1	17 ± 0.03	18.7 ± 0.1	19.32 ± 0.05	18.2 ± 0.1
Proc. dimer A isomer 6	31.09	575.114	n.d.	n.d.	n.d.	8.58 ± 0.02	n.d.
Quercetin	37.84	301.036	1.2 ± 0.02	1.43 ± 0.01	1.68 ± 0.02	1.98 ± 0.01	1.58 ± 0.04
Kaempferol	38.97	285.042	0.995 ± 0.004	1.284 ± 0.005	1.43 ± 0.02	1.995 ± 0.003	1.4 ± 0.01
(±)-Naringenin	39.08	271.060	1.32 ± 0.01	1.328 ± 0.002	1.81 ± 0.03	1.94 ± 0.04	2.25 ± 0.04
Sakuranetin	40.10	285.074	1.23 ± 0.02	1.272 ± 0.007	1.65 ± 0.04	1.83 ± 0.04	1.91 ± 0.03

For a same row, values followed by different lower case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

Table 6.- Concentration of phenolic compounds in avocado seed coat after drying process a different temperatures.

Analyte	RT (min)	m/z	Concentration (mg/100 g, dry mater)				
			45 °C	55 °C	65 °C	75 °C	85 °C
Persitol	1.87	211.082	238 ± 6	243 ± 4	255 ± 2	240 ± 10	107 ± 2
Quinic acid	1.99	191.056	627 ± 3	730 ± 50	800 ± 20	750 ± 30	260 ± 10
Citric acid	2.49	191.019	68 ± 2	62 ± 2	68.9 ± 0.9	63 ± 5	33.2 ± 0.7
3,4-dihydroxyphenylacetic acid	8.05	167.037	19.5 ± 0.7	18.3 ± 0.4	19.7 ± 0.2	22.5 ± 0.5	24.9 ± 0.3
Hydroxytyrosol glucoside	8.80	315.109	40 ± 1	29 ± 1	40 ± 1	34.6 ± 0.9	16 ± 0.7
Pyrocatechol	9.64	109.031	13.8 ± 0.3	12.85 ± 0.09	14.9 ± 0.2	14.56 ± 0.05	16.1 ± 0.6
3-O-caffeoylquinic acid	10.77	353.089	150 ± 10	145 ± 4	111 ± 9	108 ± 7	26.5 ± 0.4
Tyrosol-glucoside	11.45	299.117	31 ± 2	31 ± 1	41.4 ± 0.3	34.5 ± 0.6	17.9 ± 0.5
Pensternide	12.55	443.195	11.1 ± 0.3	10.2 ± 0.5	12.4 ± 0.2	14.1 ± 0.4	4.2 ± 0.3
Proc. dimer B isomer 1	13.17	577.142	7.5 ± 0.2	11.9 ± 0.4	8.5 ± 0.2	8.7 ± 0.3	5.9 ± 0.3
Tyrosol-glucosyl-pentoside	13.30	431.157	63 ± 1	66 ± 3	110 ± 10	45 ± 2	29 ± 1
4-hydroxybenzoic acid	13.37	137.027	66 ± 4	67 ± 2	66 ± 2	96.2 ± 0.9	67 ± 2
3-p-coumaroylquinic acid	13.70	337.097	46.3 ± 0.6	40.3 ± 0.6	41.4 ± 0.5	44 ± 1	11.5 ± 0.2
p-Coumaric acid	13.94	163.041	87 ± 3	75 ± 3	74 ± 0.9	86 ± 2	95 ± 9
Proc. dimer B isomer 2	14.24	577.144	9.8 ± 0.4	15 ± 0.4	9.7 ± 0.2	11.5 ± 0.2	8.7 ± 0.1
5-O-caffeoylquinic acid	14.53	353.089	n.d.	15 ± 0.2	15.5 ± 0.3	16.7 ± 0.5	n.d.
Vanillic acid	15.19	167.034	11.5 ± 0.3	11.8 ± 0.3	13.6 ± 0.4	12.1 ± 0.4	n.d.
3-feruloylquinic acid	15.24	367.104	14.2 ± 0.3	15.7 ± 0.2	14.8 ± 0.2	13.9 ± 0.3	n.d.
(+)-Catechin	15.53	289.074	63 ± 3	102 ± 7	82 ± 1	96 ± 4	69 ± 4

Analyte	RT (min)	m/z	Concentration (mg/100 g, dry matter)				
			45 °C	55 °C	65 °C	75 °C	85 °C
4-O-caffeoylquinic acid	15.84	353.090	76.8 ± 0.6 ^c	142 ± 8 ^a	120 ± 1 ^b	141 ± 3 ^a	36.3 ± 0.4 ^d
Proc. dimer B isomer 3	16.12	577.143	5.6 ± 0.3 ^c	7.9 ± 0.2 ^a	5.8 ± 0.06 ^c	5.7 ± 0.4 ^c	6.9 ± 0.2 ^b
Proc. dimer B isomer 4	17.70	577.143	15.6 ± 0.3 ^c	20.1 ± 0.5 ^b	11.4 ± 0.1 ^d	27 ± 1 ^a	28 ± 3 ^a
5-p-coumaroylquinic acid	18.58	337.097	8.47 ± 0.06 ^b	12.8 ± 0.5 ^a	12.8 ± 0.2 ^a	13.6 ± 0.3 ^a	n.d.
4-p-coumaroylquinic acid	19.21	337.102	27.1 ± 0.5 ^c	41 ± 3 ^b	44 ± 1 ^b	48 ± 1 ^a	15.4 ± 0.6 ^d
(-)-Epicatechin	19.52	289.073	32 ± 0.1 ^c	39 ± 1 ^b	30.5 ± 0.8 ^c	39 ± 1 ^b	44 ± 3 ^a
Vanillin	19.72	151.039	28 ± 1 ^b	28 ± 2 ^b	31.8 ± 0.7 ^a	29 ± 1 ^b	22.69 ± 0.06 ^c
Proc. trimer A isomer 1	20.43	863.175	57 ± 2 ^a	46 ± 2 ^b	49.1 ± 0.1 ^b	57 ± 1 ^a	32 ± 4 ^c
4-feruloylquinic acid	20.51	367.104	9.3 ± 0.2 ^c	10.9 ± 0.3 ^b	13.5 ± 0.3 ^a	11.02 ± 0.05 ^b	n.d.
Proc. trimer A isomer 2	20.70	863.177	62 ± 1 ^b	53.7 ± 0.5 ^c	52.5 ± 0.6 ^c	86 ± 3 ^a	84 ± 4 ^a
Proc. trimer B isomer 1	20.95	865.191	n.d.	n.d.	n.d.	9.4 ± 0.3 ^b	14.2 ± 0.2 ^a
Quercetin-diglucoside isomer 1	21.73	625.145	1.68 ± 0.01 ^c	1.82 ± 0.02 ^b	1.96 ± 0.01 ^a	1.75 ± 0.03 ^{bc}	1.49 ± 0.04 ^d
Quercetin-diglucoside isomer 2	21.91	625.143	1.56 ± 0.02 ^c	1.71 ± 0.01 ^b	1.93 ± 0.01 ^a	1.74 ± 0.03 ^b	1.68 ± 0.05 ^b
5-feruloylquinic acid	22.13	367.101	20.1 ± 0.9 ^d	42 ± 1 ^a	25 ± 1 ^c	38 ± 1 ^b	9.57 ± 0.04 ^e
Proc. trimer A isomer 3	23.13	863.175	21.8 ± 0.5 ^c	38 ± 1 ^a	31 ± 0.5 ^b	32 ± 1 ^b	11.4 ± 0.5 ^d
Proc. trimer A isomer 4	24.18	863.176	38 ± 1 ^c	59 ± 2 ^a	44.5 ± 0.5 ^b	60 ± 2 ^a	35 ± 1 ^c
Hydroxyabscisic acid glucoside	24.62	441.181	900 ± 40 ^{bc}	790 ± 30 ^c	1050 ± 40 ^a	1000 ± 100 ^{ab}	280 ± 20 ^d
Proc. dimer A isomer 1	25.28	575.118	14.76 ± 0.04 ^d	16.6 ± 0.2 ^c	17.57 ± 0.09 ^b	18.5 ± 0.1 ^a	14.2 ± 0.3 ^e
Proc. dimer B isomer 7	26.29	577.138	6.2 ± 0.2 ^c	7.9 ± 0.1 ^b	6.1 ± 0.1 ^c	7.9 ± 0.4 ^b	10 ± 1 ^a

Analyte	RT (min)	m/z	Concentration (mg/100 g, dry mater)				
			45 °C	55 °C	65 °C	75 °C	85 °C
Quercetin-3-β-glucoside	26.84	463.089	1.79 ± 0.05 c	1.92 ± 0.04 b	2.128 ± 0.003 a	1.98 ± 0.03 b	1.39 ± 0.03 d
Proc. dimer A isomer 2	28.22	575.118	8.79 ± 0.07 d	9.8 ± 0.1 b	10.7 ± 0.1 a	9.4 ± 0.1 c	9 ± 0.1 d
Proc. dimer A isomer 3	28.28	575.117	12.9 ± 0.2 bc	12.5 ± 0.2 c	13.2 ± 0.1 b	13.46 ± 0.09 b	14.5 ± 0.4 a
Proc. dimer A isomer 4 (A2)	29.42	575.114	9.7 ± 0.1 b	11.6 ± 0.5 a	11.3 ± 0.1 a	11.5 ± 0.1 a	10.1 ± 0.2 b
(Epi)galocatechin	30.28	305.067	21.4 ± 0.2 c	23.7 ± 0.3 b	26 ± 0.6 a	27.2 ± 0.9 a	22.3 ± 0.6 bc
Proc. dimer A isomer 5	30.85	575.113	8.19 ± 0.08 d	9.2 ± 0.2 b	10.05 ± 0.03 a	8.5 ± 0.09 c	8.5 ± 0.1 c
Proc. dimer A isomer 6	31.09	575.114	8.94 ± 0.06 c	9.9 ± 0.2 b	10.71 ± 0.04 a	9.5 ± 0.1 bc	9.5 ± 0.5 bc
Proc. dimer A isomer 7	32.57	575.115	8.53 ± 0.05 c	9.05 ± 0.04 b	10.54 ± 0.08 a	9.3 ± 0.1 b	7.84 ± 0.03 d
Proc. dimer A isomer 8	34.68	575.116	7.61 ± 0.05 c	8.18 ± 0.07 b	9.4 ± 0.2 a	n.d.	n.d.
Proc. dimer A isomer 9	35.87	575.117	8.09 ± 0.03 c	8.69 ± 0.06 b	9.62 ± 0.02 a	8.5 ± 0.1 b	8 ± 0.1 c
Quercetin	37.84	301.036	1.82 ± 0.03 d	1.79 ± 0.02 d	1.89 ± 0.01 c	2.21 ± 0.01 a	1.95 ± 0.01 b
Kaempferol	38.97	285.042	1.4 ± 0.01 d	1.463 ± 0.009 c	1.656 ± 0.006 b	1.7 ± 0.02 a	1.332 ± 0.004 e
(±)-Naringenin	39.08	271.060	1.75 ± 0.03 e	2.08 ± 0.01 c	1.88 ± 0.03 d	2.24 ± 0.02 b	2.64 ± 0.07 a
Sakuranetin	40.10	285.074	2.08 ± 0.05 c	2.32 ± 0.06 b	2.31 ± 0.01 b	2.32 ± 0.03 b	2.54 ± 0.07 a

For a same row, values followed by different lower case letter are significantly different ($p \leq 0.05$) according to Tukey's Multiple Range Test.

On the other hand, five flavonols were identified and quantified in both samples: kaempferol, quercetin and its glucoside derivative. Contrary to the others five groups of phenolic compounds identified, the highest concentration of flavonols was found at 75 °C in both samples. This behaviour is derived mainly because of quercetin, which presented a concentration 25 % higher at 75 °C than 85 °C. Meanwhile, in avocado seed coat, quercetin-3- β -glucoside and quercetin showed the highest concentration at 65 and 75 °C, respectively. This could be explained because the time required to dehydrate and remove the coat from the seed was similar in the different treatments, so the main factor to reduce the concentration of phenolic compounds was the temperature.

The flavanones, (\pm)-naringenin and sakuranetin showed an increase in the concentration when higher temperatures were used. Therefore, the highest concentrations in both samples were at 85 °C. Esparza-Martínez, et al. (2016) reported a gradually increase in concentration of non-extractable (\pm)-naringenin according to an increase in the temperature from 60 °C to 120 °C in the drying of lime wastes. Nevertheless, Esparza-Martínez, et al. (2016) also found that levels of extractable (\pm)-naringenin remains almost the same at different drying temperatures.

The (+)-catechin and (-)-epicatechin were identified in both samples. The (+)-catechin was the predominant flavanols in both samples. In avocado seed the highest concentration of flavanols was found at 85 °C (199 mg/100 g dry mater), whereas in seed coat was at 75 °C (161 mg/100 g dry mater). In the seed, both flavanols were thermally affected, with a reduction of approximately 78 % between

the concentrations found at 85 and 45 °C. Meanwhile, in the seed coat, the highest concentration of (+)-catechin and (-)-epicatechin were found at 55 and 85 °C, respectively.

According to the obtained results, procyanidins type A were more thermostable than type B. In fact, type B dimers were full degraded at 45 °C in the avocado seed. Moreover, the concentration of type A dimers was around two folds lower at 45 °C than at 85 °C. In addition, in avocado seed coat, the degradation of type B procyanidins was higher than type A in the different treatments, with values of around 51 and 20 %, respectively. This could be due to the fact that type A procyanidins are flavan-3-ol units doubly linked through C4-C8 bond and an additional ether bond between C2-O7; whereas type B solely presented a C4-C8 bond (Gu, et al., 2003). Besides, trimers showed higher degradation than dimers with a main reduction of 88 and 39 %, respectively.

Summarizing, the elevation of the dehydration temperature positively impacted the phenolic compounds content in the avocado seed (Table 4). Indeed, the total contents of phenolic acids, phenolic alcohol derivatives, flavanols and procyanidins were more than five folds higher at 85 °C than 45 °C. Similar results were found by Qin, et al. (2017), who evaluated the effect of drying temperature on phenolic compounds in *Cimicifuga dahurica* (Turcz.). They found that the concentration of phenolic acids increased gradually with drying temperature. This fact might be explained by the fact that long drying times at lower temperatures contributed to a notable thermal degradation of phenolic compounds, as mentioned before (Garau, et al., 2007).

On the other hand, as detailed above, the time necessary to dried seed coat was lower than for the avocado seed. In this sense, the effect of dehydration leads to a lesser pronounced degradation in the seed coat than seed. Besides, in seed coat each group of phenolic compounds had a different optimal drying temperature. For phenolic acids, flavonols, catechins and procyanidin the best drying temperatures was 75 °C, whereas for phenolic alcohol derivatives and flavanones were 65 and 85 °C, respectively (Table 4).

It is worth noting that in contrast to the results obtained for the avocado seed, seed coat had higher ($p \leq 0.05$) amounts of phenolic acids, phenolic alcohol derivatives, flavanones, flavonols and procyanidins. Solely flavanols presented a slight higher concentration in seed than seed coat, with values of 181 and 140 mg/100 g dry mater, respectively.

Respect to data previously reported, the phenolic acids and flavonols contents in seed were higher than those reported by Rodríguez-Carpena, et al. (2011) in the same avocado variety. Opposite to that, the procyanidin content was lower than the value reported by López-Cobo, et al. (2016) and Rodríguez-Carpena, et al. (2011), which could be due to a thermal degradation. Indeed, the total content of procyanidins was reduced around 81 % when the sample was dried at 45 °C compared with the value at 85 °C, which could indicate a thermal instability of this kind of compounds (Di Mattia, et al., 2013).

According to our best knowledge, this is the first time that the effect of drying temperature on the concentration of more than forty phenolic compounds has been performed in avocado seed and seed coat.

4. Conclusions

In present work, the drying pre-treatment step and the PLE of phenolic compounds from avocado seed and seed coat have been optimized. The influence of two pressurized liquid extraction parameters (temperature and solvent concentration) on the extraction of phenolic compounds from avocado seed and seed coat were examined. Moreover, in the avocado seed, the optimization of the PLE was carried out in samples dried at five different temperatures. PLE conditions resulting in the highest TPC were 200 °C and a solvent mixture of ethanol:water 50:50 (v/v) for both sample matrices. According to the TPC, the best drying temperature for both samples was 85 °C. Under the optimal drying temperature and extraction conditions, the maximum TPC for avocado seed and seed coat were 29.5 ± 0.4 and 71.2 ± 1.7 mg EAG/g dm, respectively. Besides, more than forty phenolic compounds were simultaneously identified and quantified by HPLC-DAD-ESI-TOF-MS to assess the effect of drying temperature on individual concentration. The optimal drying temperature for avocado seed was 85 °C. Nevertheless, in seed coat each group of phenolic compounds had a different optimal drying temperature, in this sense, phenolic acids, flavonols, catechins and procyanidin the best drying temperatures was 75 °C, whereas for phenolic alcohol derivatives and flavanones were 65 and 85 °C, respectively.

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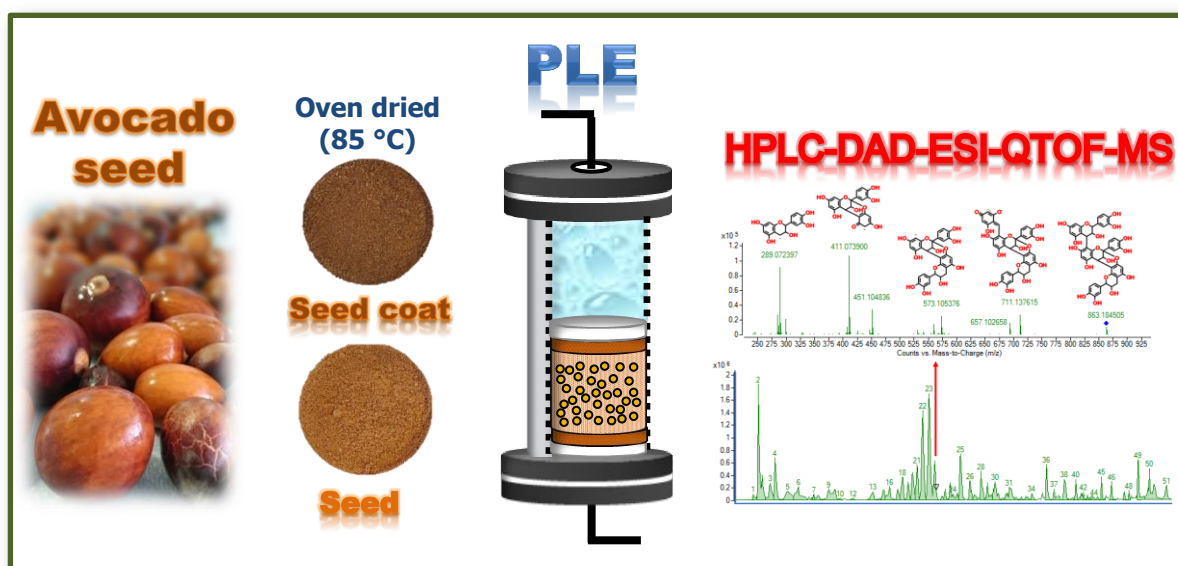
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Supplementary Table 1.- Identification of phenolic compounds in avocado seed and seed coat extracts by HPLC-DAD-ESI-TOF-MS.

Analyte	RT (min)	m/z experimental (M-H)-	m/z calculated (M-H)-	Molecular formula	Error (ppm)	mSigma	Seed	Seed coat
Perseitol	1.85	211.082	211.0823	C ₇ H ₁₆ O ₇	1.6	9	+	+
Quinic acid	1.97	191.0563	191.0561	C ₇ H ₁₂ O ₆	-0.9	14.7	+	+
Citric acid	2.36	191.0192	191.0197	C ₆ H ₈ O ₇	2.9	8.2	+	+
3,4-dihydroxyphenylacetic acid	9.1	167.0366	167.035	C ₈ H ₈ O ₄	-9.9	14.7	+	+
Hydroxytyrosol glucoside	8.16	315.1085	315.1085	C ₁₄ H ₂₀ O ₈	0.1	9.4	+	+
Pyrocatechol	10.7	109.0309	109.0295	C ₆ H ₆ O ₂	-12.4	37.5	+	+
3-O-caffeoylquinic acid	10.07	353.0886	353.0878	C ₁₆ H ₁₈ O ₉	-2.3	1.6	+	+
Tyrosol-glucoside	10.8	299.1169	299.1136	C ₁₄ H ₂₀ O ₇	-11	9.1	+	+
Penstemide	11.79	443.1951	443.1923	C ₂₁ H ₃₂ O ₁₀	-6.3	3.2	+	+
Proc. dimer B isomer 1	15	577.1418	577.1351	C ₃₀ H ₂₆ O ₁₂	-11.6	42.8	+	+
Tyrosol-glucosyl-pentoside	12.46	431.1569	431.1559	C ₁₉ H ₂₈ O ₁₁	-2.3	18.7	+	+
4-hydroxybenzoic acid	14.6	137.0266	137.0244	C ₇ H ₆ O ₃	-15.6	6.9	+	+
3-p-coumaroylquinic acid	15.6	337.0969	337.0929	C ₁₆ H ₁₇ O ₈	-11.9	19.3	+	+
p-Coumaric acid	15.5	163.0414	163.0401	C ₉ H ₈ O ₃	-8.2	28.1	+	+
Proc. dimer B isomer 2	16.3	577.144	577.1351	C ₃₀ H ₂₆ O ₁₂	-15.3	12	+	+
5-O-caffeoylquinic acid	13.82	353.0886	353.0878	C ₁₆ H ₁₈ O ₉	-2.3	15.7	+	+
Vanillic acid	7.37	167.0335	167.035	C ₈ H ₇ O ₄	9	18.8		+
3-feruloylquinic acid	14.65	367.1035	367.1035	C ₁₇ H ₂₀ O ₉	-0.2	45.6		+
(+)-Catechin	14.42	289.0737	289.0718	C ₁₅ H ₁₄ O ₆	-6.6	7	+	+
4-O-caffeoylquinic acid	14.9	353.0898	353.0878	C ₁₆ H ₁₈ O ₉	-5.7	8	+	+
Proc. dimer B isomer 3	20.1	577.143	577.1351	C ₃₀ H ₂₆ O ₁₂	-13.6	25.9		+
Proc. dimer B isomer 4	23	577.1431	577.1351	C ₃₀ H ₂₆ O ₁₂	-13.8	32.6	+	+
5-p-coumaroylquinic acid	21.1	337.0966	337.0929	C ₁₆ H ₁₈ O ₈	-11.1	18.5	+	+
4-p-coumaroylquinic acid	21.7	337.1018	337.0929	C ₁₆ H ₁₈ O ₈	-8.9	13.4	+	+
(-)-Epicatechin	18.22	289.0728	289.0718	C ₁₅ H ₁₄ O ₆	-3.6	6.1	+	+
Vanillin	21.3	151.0393	151.0401	C ₈ H ₈ O ₃	4.9	4.6		+
Proc. trimer A isomer 1	20.9	863.1753	863.1829	C ₄₅ H ₃₆ O ₁₈	8.8	8.2	+	+
4-feruloylquinic acid	19.81	367.1043	367.1035	C ₁₇ H ₂₀ O ₉	-2.4	49.3		+
Proc. trimer A isomer 2	22.2	863.1766	863.1829	C ₄₅ H ₃₆ O ₁₈	7.3	15.1	+	+
Proc. trimer B isomer 1	22.8	865.1912	865.1985	C ₄₅ H ₃₈ O ₁₈	8.5	22.1		+
Quercetin-diglucoside isomer 1	20.3	625.145	625.141	C ₂₇ H ₃₀ O ₁₇	-6.3	33.3	+	+

Analyte	RT (min)	m/z experimental (M-H)-	m/z calculated (M-H)-	Molecular formula	Error (ppm)	mSigma	Seed	Seed coat
Quercetin-diglucoside isomer 2	20.59	625.1431	625.141	C ₂₇ H ₃₀ O ₁₇	-3.3	13.7	+	+
Proc. dimer B isomer 5	25.26	577.1379	577.1351	C ₃₀ H ₂₆ O ₁₂	-4.8	8.7	+	
5-feruloylquinic acid	21.9	367.1011	367.1035	C ₁₇ H ₂₀ O ₉	6.4	108		+
Proc. trimer A isomer 3	24.3	863.1747	863.1829	C ₄₅ H ₃₆ O ₁₈	9.5	34.2	+	+
Proc. trimer A isomer 4	25.4	863.1763	863.1829	C ₄₅ H ₃₆ O ₁₈	7.6	38.5	+	+
Proc. dimer B isomer 6	27.4	577.1379	577.1351	C ₃₀ H ₂₆ O ₁₂	-4.8	44.2	+	
Hydroxyabscisic acid glucoside	23.51	441.1811	441.1766	C ₂₁ H ₃₀ O ₁₀	-10.2	18.7	+	+
Proc. dimer A isomer 1	25.7	575.1182	575.1195	C ₃₀ H ₂₄ O ₁₂	2.2	138.6	+	+
Proc. dimer B isomer 7	28.5	577.1375	577.1351	C ₃₀ H ₂₆ O ₁₂	-4	54.2		+
Quercetin-3-β-glucoside	25.57	463.0888	463.0882	C ₂₁ H ₂₀ O ₁₂	-1.2	30	+	+
Proc. dimer A isomer 2	26.5	575.1178	575.1195	C ₃₀ H ₂₄ O ₁₂	2.9	43.8	+	+
Proc. dimer A isomer 3	28.9	575.1172	575.1195	C ₃₀ H ₂₄ O ₁₂	4.1	63.4	+	+
Proc. dimer A isomer 4 (A2)	29.5	575.114	575.1195	C ₃₀ H ₂₄ O ₁₂	9.6	33.1	+	+
(Epi)gallocatechin	30.6	305.067	305.0667	C ₁₅ H ₁₄ O ₇	-1.1	22	+	+
Proc. dimer A isomer 5	30.6	575.1133	575.1195	C ₃₀ H ₂₄ O ₁₂	10.8	33.1		+
Proc. dimer A isomer 6	32	575.114	575.1195	C ₃₀ H ₂₄ O ₁₂	9.6	40.3	+	+
Proc. dimer A isomer 7	32.2	575.1153	575.1195	C ₃₀ H ₂₄ O ₁₂	7.3	85.2		+
Proc. dimer A isomer 8	33.8	575.1162	575.1195	C ₃₀ H ₂₄ O ₁₂	5.8	90.9		+
Proc. dimer A isomer 9	36.5	575.1172	575.1195	C ₃₀ H ₂₄ O ₁₂	3.9	47.7		+
Quercetin	37.14	301.0356	301.0354	C ₁₅ H ₁₀ O ₇	-0.6	16.8	+	+
Kaempferol	39.6	285.0424	285.0405	C ₁₅ H ₁₀ O ₆	-6.7	17.3	+	+
(±)-Naringenin	39.1	271.0597	271.0553	C ₁₅ H ₁₂ O ₅	5.4	19.5	+	+
Sakuranetin	40.1	285.0744	285.0768	C ₁₆ H ₁₄ O ₅	8.7	38.3	+	+

Capítulo IV



Comprehensive characterization of phenolic and other polar compounds in the seed and seed coat of avocado by HPLC-DAD-ESI-QTOF-MS

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Sánchez and Antonio Segura-Carretero



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Comprehensive characterization of phenolic and other polar compounds in the seed and seed coat of avocado by HPLC-DAD-ESI-QTOF-MS

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Highlights

- Avocado seed coat phenolic profile has been studied for the first time.
- Accelerated solvent extraction was performed with green solvents (water/ethanol).
- Eighty four compounds from eight families were identified.
- Forty five phenolic compounds were identified for the first time in avocado seed.
- Avocado seed and seed coat have potential as a source of phenolic compounds.

Abstract

Avocado seed and seed coat are important by-products from avocado industrialization, with important functional properties. The aim of the present study was to determine the phenolic profile and other polar compounds of avocado seed and seed coat using pressurized liquid extraction (PLE) and liquid chromatography coupled to Ultra-High-Definition Accurate-Mass Q-TOF. In this research 83 compounds were identified, within eight subclass group, among these 45 phenolic compounds were identified for first time in avocado seed. Condensed tannins, phenolic acids and flavonoids were the most representative groups in both samples. As far as we are concerned, this is the first time that avocado seed coat has been studied regarding its phenolic compounds using such a powerful instrumental technique. In addition, the radical-scavenging activities were analysed in order to estimate the antioxidant potential of extracts. These results point out that avocado seed and seed coat constitute a source of bioactive ingredients for its use in the food, cosmetic or pharmaceutical sector.

Keywords

Avocado seed; Avocado seed coat; green solvent; PLE; HPLC-DAD-ESI-QTOF-MS; Polyphenols

1. Introduction

The Avocado (*Persea americana*) is an important oleaginous fruit, source of bioactive compounds with health benefits (Calderón-Oliver et al., 2016). This fruit is native to Central America, nevertheless are now cultivated around the world (Dabas, Shegog, Ziegler, & Lambert, 2013). The 'Hass' variety is the most common worldwide (Kosińska et al., 2012). In 2014 the world production was 5 million tons, and the Spanish production was 77 thousand tons (FAO, 2017). Moreover, the European Union and North America are the largest importers in the world (María del Refugio Ramos-Jerz, 2007).

Regarding its processing, guacamole and oil are the main industrialized products from avocado (Rodríguez-Carpena, Morcuende, Andrade, Kylli, & Estévez, 2011). This manufacturing process generates a large amount of by-products, peel and seed, which nowadays are discarded with no further applications. The seed represented up of 16% of the weight of the fruit (Dabas et al., 2013), but this by-product currently have no commercial uses. Besides, the avocado seed has a little brown seed coat, which is used as an internal fruit characteristic indicative of harvest maturity (Blumenfeld & Gazit, 1971). Despite some studies suggested the potential of avocado seed as anticancer, anti-inflammatory, antidiabetic, antihypertensive, hypocholesterolemic, antimicrobial and insecticidal (Calderón-Oliver et al., 2016; Dabas et al., 2013; María del Refugio Ramos-Jerz, 2007), this sample matrix has not been characterized in depth.

In this sense, polyphenols are one the most important bioactive compounds present in avocado seed. Indeed, its antioxidant, anti-inflammatory or antimicrobial properties could be attributed to phenolic compounds. Thus, several studies reported the presence of phenolic compounds in avocado seed; nevertheless, these researchers had identified no > 16 compounds (Kosińska et al., 2012; López-Cobo et al., 2016; Torres, Mau-Lastovicka, & Rezaaiyan, 1987; Widsten, Cruz, Fletcher, Pajak, & McGhie, 2014). Moreover, in all studies mentioned incompatible solvents (methanol and acetone) with pharmaceutical, cosmetic and food industries were used, limiting the use of these by-products as natural additives (Kosińska et al., 2012). In these sense, the use of extraction procedures compatibles with solvents generally recognized as safe or GRAS such as ethanol, water or their mixtures, is necessary. Therefore, Accelerated Solvent Extraction is a “green” technology that obtains similar or greater yields than traditional procedures totally friendly with this kind of solvents (Sun, Ge, Lv, & Wang, 2012).

Furthermore, as far as we are concerned, avocado seed coat has been only analysed by Prabha and Patwardhan (1980), who reported the presence of catechin, (-)-epicatechin, 3-leucoanthocyanidins and isoflavone; however, the identification was made by paper chromatography. Thus, comprehensive identification on avocado seed and seed coat phenolic compound profiles is still scarce. In addition, the possible use of these waste materials in other sectors can minimize cost and environmental impact, meanwhile adding value to the avocado industry (Rodríguez-Carpa et al., 2011; Wang, Bostic, & Gu, 2010).

Therefore, avocado seed represent a potential inexpensive source of phenolic compounds with opportunity in the food industry, cosmetic or pharmaceutical sector. The aim of the present study was to determine the phenolic profile and other polar compounds of avocado seed and seed coat using pressurized liquid extraction (PLE) and liquid chromatography coupled to Ultra-High-Definition Accurate-Mass Q-TOF.

2. Material and methods

2.1. Chemicals and reagents

All reagents and solvents were of analytical or MS grade. For extraction, ethanol and Ottawa sand were supplied from Fisher Scientific (Leicestershire, UK), and ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). The reagents used to measure the antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS), fluorescein, 2,2'-azobis(2-methyl-propionamide) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich (Steinheim, Germany). Regarding HPLC analysis, acetonitrile (HPLC-MS-grade) were purchased from Fisher Scientific (Leicestershire, UK), and formic acid was supplied from Fluka Chemika (Buchs, Switzerland). For identification purposes, sixteen standards were used: dihydrocaffeic acid, procyanidin dimer A2 and trimer B2 were purchased from Extrasynthese (Genay Cedex, France); benzoic acid, (+)-catechin, chlorogenic acid, citric acid, p-coumaric acid, (-)-epicatechin, gentisic acid, (±)-naringenin, protocatechuic acid, quercetin and quercetin-3-β-

glucoside were supplied from Sigma-Aldrich (Steinheim, Germany); 4-hydroxybenzoic acid was acquired from Fluka Chemika (Buchs, Switzerland) and quinic acid was supplied from Acros Organics (Geel, Belgium).

2.2. Samples

Around 80 kg of avocado fruit variety 'Hass' were kindly supplied by the commercial group La Caña, Miguel García Sánchez e Hijos, S.A. (Motril, Spain). All fruits were store at room temperature until full maturity. Complete avocado seed was manually separated and cleaned under continuous flow of tap water. Then, these by-products were oven-dried at 85 °C during 24 h, moment at which the seed coat was separated manually and the seeds were sliced in order to accelerate the drying process. After that, the obtained by-product was chopped and oven-dried at 85 °C while turned periodically to ensure uniform dryness until a percentage moisture content of < 10%. Then, the dried samples were milled in an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting avocado seed and seed coat had an average particle size of 0.5 mm. The material was stored at room temperature and protected from light until their extraction and analysis.

2.3. Pressurized liquid extraction (PLE)

The extractions of bioactive compounds were performed using a pressurized liquid extractor model ASE 350 (Dionex Corporation, Sunnyvale, CA), according to the method of Herrero, Plaza, Cifuentes, and Ibáñez (2010) with some modifications. Briefly, 1.5 g of dry sample (seed or seed coat) was extracted with ethanol-water (1:1, v/v) at 200 °C and 11 MPa. The solvent was previously sonicated for 15 min to

remove the dissolved oxygen in order to avoid any possible oxidation. Moreover, in order to prevent clogging of the metal frits, disposable cellulose filters were placed at each end of the cell. The obtained extracts were immediately cooled in ice to attain a temperature of 20–25 °C and centrifuged for 15 min at a RCF of 12,902 and 4 °C in a Sorvall ST 16 R centrifuge (Thermo Scientific, Leicestershire, UK). Finally, the supernatants were evaporated under vacuum (13 kPa) at 35 °C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The resulting extracts were stored at – 20 °C until further analyses. All experiments were performed in duplicate.

2.4. Determination of phenolic and other polar compounds by HPLC-DAD-ESI-QTOF-MS

Prior to analysis, the extracts were reconstituted in ethanol-water (1:1, v/v) at a concentration of 10,000 mg/L and filtered with regenerated cellulose syringe-filters of 0.2 µm pore size (Millipore, Bedford, MA, USA). Avocado seed and seed coat were analysed using High Performance Liquid Chromatography coupled to electrospray ionization quadrupole-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS).

An Agilent 1260 series Rapid Resolution Liquid Chromatographer coupled to a diode-array detector (DAD) were used for the chromatographic determination. The chromatographic separation was performed in a Zorbax Eclipse Plus C18 column (4.6 × 150 mm, 1.8 µm) at a flow rate of 0.5 mL/min using an injection volume of 10 µL. The mobile phases were acidified water (0.1% formic acid, v/v) and acetonitrile as solvent A and B, respectively. The following multi-step linear gradient was used in

order to achieve efficient separation: 0.0 min [A:B 95/5], 25.0 min [A:B 50/50], 33.0 min [A:B 0/100], and 36.0 min [A:B 95/5]. Finally, initial conditions were kept for 4 min at the end of each analysis to equilibrate the system before the subsequent injection. The column temperature and auto-sampler compartment were set at 25 °C and 4 °C, respectively.

Detection was performed in negative ionization mode over a mass range from 100 to 1700 m/z, and the detection window was set to 100 ppm. Ultrahigh pure nitrogen was used as drying and nebulizing gas. The operating parameters were as follows: drying gas temperature, 325 °C; drying gas flow rate, 10 L/min; nebulizer pressure, 172 kPa; nebulizer gas temperature, 400 °C; nebulizer gas flow, 12 L/min; capillary, 4000 V; fragmentor, 130 V; nozzle voltage, 500 V; skimmer, 45 V and octopole radiofrequency voltages, 750 V. In order to maintain mass accuracy during the runtime, all spectra were corrected by means of continuous infusion of Agilent TOF mixture containing two reference masses: m/z 112.985587 (trifluoroacetate anion) and 1033.988109 (adduct of hexakis (1H,1H, 3H-tetrafluoropropoxy) phosphazine or HP-921). Both reference ions provided accurate mass measurements typically better than 2 ppm. All operations were processed through MassHunter Qualitative Analysis B.06.00 (Agilent Technologies, Palo Alto, CA, USA). Literature search for published spectral information was carried out by using SciFinder®.

2.5. Antioxidant activity assays

Trolox equivalent antioxidant capacity (TEAC) assay is based on the reduction of the radical cation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by

antioxidants. TEAC assay was carried out following a previously described method using Trolox as standard in the range from 1 to 40 μM (Cádiz-Gurrea, Fernández-Arroyo, Joven, & Segura-Carretero, 2013). The absorbance was measured at 734 nm and the results were expressed in $\mu\text{mol TE/g}$ dry weight of the sample (DW).

DPPH radical scavenging assay was performed by using the procedure described by Brand-Williams, Cuvelier, and Berset (1995). DPPH values were calculated using a DPPH standard solution and measuring the absorbance at 516 nm. The results were expressed in terms of the concentration of avocado and seed extracts ($\mu\text{g/mL}$) needed to decrease by 50% the initial DPPH concentration (IC50 value) as well as antioxidant activity index (AAI) proposed by Scherer and Teixeira Godoy (2009).

Oxygen radical absorbance capacity (ORAC) assay was performed according to Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002). Briefly, Trolox was used as standard and fluorescence at 494/521 nm (excitation and emission wavelengths respectively) was measured. ORAC values were calculated using a regression equation between the trolox concentration and the net area of the fluorescence decay curve (area under curve, AUC). The final results were expressed as $\mu\text{mol TE/g}$ DW.

Absorbance (DPPH and TEAC) and fluorescence (ORAC) measurements were carried out on a Synergy Mx Monochromator Based Multi-Mode Micro plate reader (BioTek Instruments, Winooski, VT, USA) using 96-well microplates. All the experiments were performed in triplicate.

2.6. Statistical analysis

Statistical analysis was carried out using the statistical package MINITAB 14.0 software (PA, USA). The compositional and antioxidant activity variation between seed and seed coat were evaluated in terms of phenolic compounds peak area. Data were analysed by Student's t-test in order to evaluate differences between avocado seed and seed coat. Differences were considered significant when the p value was < 0.05.

3. Results and discussion

3.1. Characterization of avocado seed and seed coat by HPLC-DAD-ESI-QTOF-MS

A comprehensive analytical characterization of phenolic and other polar compounds were performed in avocado seed and seed coat. The use of PLE and HPLC-DAD-ESI-QTOF-MS enable the identification of a total of 83 compounds (Fig. 1). Among these, 46 compounds have been identified for first time in avocado seed. To the best of our knowledge, no other publication covers such a number of phenolic compounds in avocado seed and seed coat. The identification was performed on basis their retention time values, UV-Vis spectra, by interpreting the accurate mass information determined, together with information previously reported in literature. The identified compounds were classified in different families described below, such as organic acids, phenolic acids, phenolic alcohol derivatives, flavonoids, catechins, condensed tannins and other polar compounds.

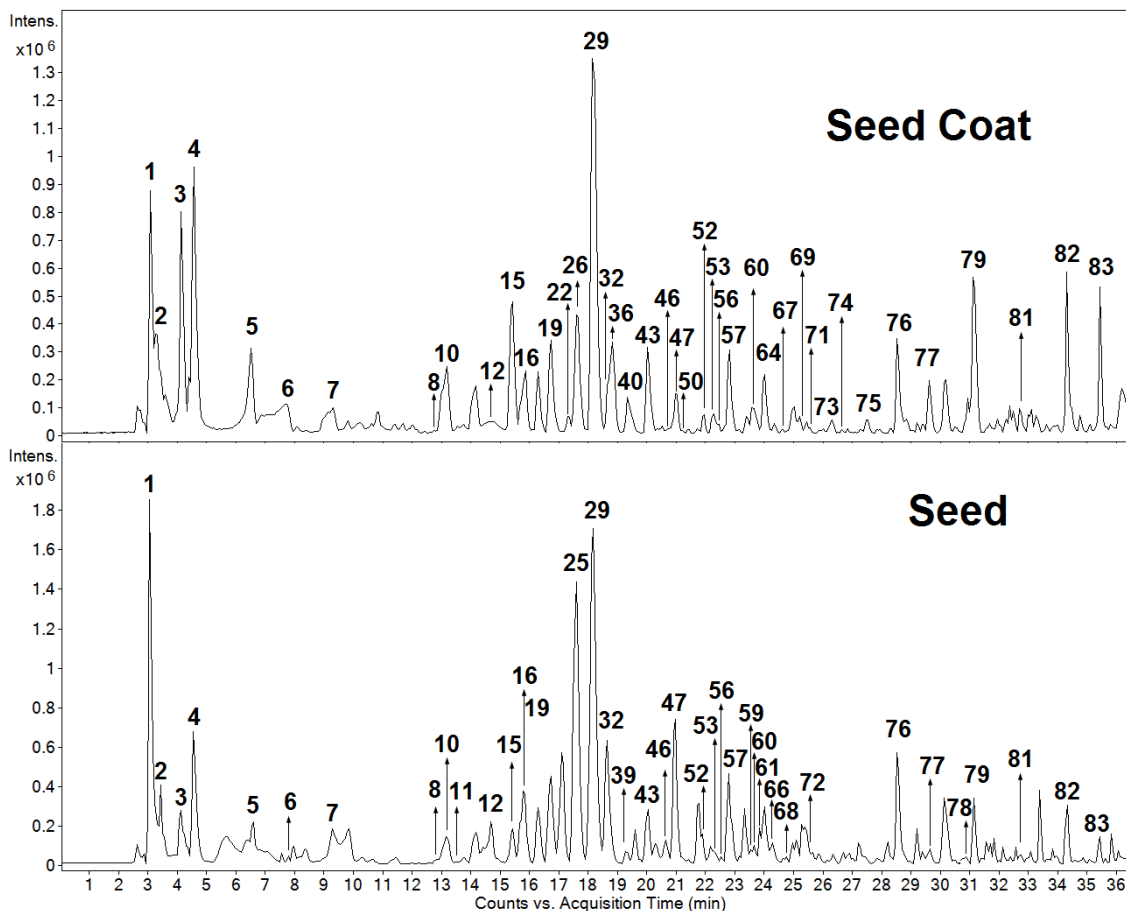


Fig. 1. Base peak chromatograms of avocado seed and seed coat extracts obtained by HPLC-ESI-QTOF-MS.

3.1.1. Organic acids

A total of five organic acids (peaks 2–6) were detected in avocado seed and seed coat. The use of ultra-high-definition accurate-mass spectrometry (UHR-MS) was useful for the identification of quinic and citric acids (Friščić, Bucar, & Hazler Pilepić, 2016), according their accurate-mass with at m/z 191.057 and 191.021, respectively. In addition, the identification of these organic acids was confirmed by comparing their spectrum and retention time with those of reference substances. Besides, the

MS spectra showed the presence of a citric acid isomer at RT 6.51 min. The elution order of both, citric acid and was in agreement with previous report (Abu-Reidah, Contreras, Arráez-Román, Segura-Carretero, & Fernández-Gutiérrez, 2013).

Furthermore, Malic acid (peak 3) was identified according its fragmentation patter. It showed a precursor ion in negative ionization mode of m/z 133.015 $[M - H]^-$ and a daughter ion peak at m/z 115.003 corresponding to $[M - H - H_2O]^-$ (Mata et al., 2016). Peak 6 was tentatively identified as succinic acid (RT = 7.77, m/z = 117.019), according to the molecular formula provided for its accurate mass. As far as we are concerned, all organic acids described above have never been reported before in avocado seed coat.

3.1.2. Phenolic acids

Respect to phenolic acids, different sub-classes of these compounds have been characterized in both sample matrix, mainly hydroxybenzoic acids, hydroxyphenylacetic acids and hydroxycinnamic acids.

A total of five and six hydroxybenzoic acids have been characterized in avocado seed and seed coat, respectively. Furthermore, one hydroxyphenylacetic acid has been characterized in both samples. In this sense, two isomers (peaks 15 and 35) with molecular formula $C_7H_6O_4$ were found in both samples and were unambiguously identified as protocatechuic acid (RT = 15.4 min and m/z = 153.020) and gentisic acid (RT = 18.84 min and m/z = 153.02) by comparison of their retention time, UV and MS/MS spectra to those of commercial standards. Moreover, their mass

fragmentation patterns are in agreement with the data reported by Sun, Liang, Bin, Li, and Duan (2007).

In addition, 4-hydroxybenzoic acid (peak 29) was confirmed by comparing its spectrum and retention time with those of the standard. This compound was present in both samples with RT 18.2 min and m/z 137.025. Their major fragment were found at m/z 108.022 and 119.014, in accordance with the value reported by Gómez-Caravaca, López-Cobo, Verardo, Segura-Carretero, and Fernández-Gutiérrez (2016).

Also the presence of benzoic acid (peak 53) was confirmed by comparing its spectrum and retention time with those of a standard. This compound was detected in avocado seed and seed coat. Related to it, methoxybenzoic acid was detected solely in avocado seed coat at 17.3 min and m/z 197.046, which showed two main fragments at m/z 135.045 and 179.035, in agreement with the information reported by Hossain, Rai, Brunton, Martin-Diana, and Barry-Ryan (2010). Based on the fragmentation pathway, this compound was tentatively identified as syringic acid.

Concerning hydroxyphenylacetic acids, peaks 10 and 41, detected in both samples, displayed the same precursor ions $[M - H]^-$ at m/z 167.035 and MS/MS product ions at m/z 123.045 $[M - H - CO_2]^-$ and 108.022 $[M - H - CO_2 - CH_3]^-$ (Fig. 2). Therefore, the identification was made based on the elution order (Bianco et al., 2003), thus 3,4-dihydroxy-phenylacetic acid was found at RT 13.2 min, while vanillic acid at RT 19.4 min. Furthermore, differences in the relative abundances of the fragment ions were observed (Table 1).

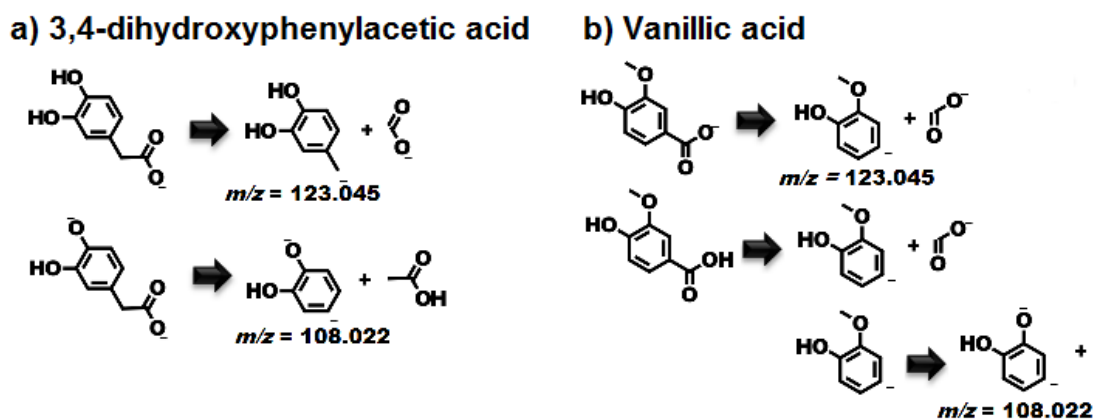


Fig. 2. The fragmentation pathway of 3,4-dihydroxy-phenylacetic acid and vanillic acid.

Respect to the presence of this kind of compounds in avocado seed, the compounds 3,4-dihydroxyphenylacetic acid and benzoic acid have never been reported before in avocado seed. Furthermore, all these hydroxybenzoic and hydroxyphenylacetic acids have never been reported before in avocado seed coat.

In addition, three isomers of caffeoylquinic acid (CQA) were detected in avocado seed and seed coat. The identification was made based on the relative intensities of fragment ions in MS/MS spectra (Clifford et al., 2003). The 4-CQA (RT = 21.0 min and $m/z = 353.09$), yielded a MS/MS base peak at $m/z 173.046$. On the contrary, the isomers 3-CQA and 5-CQA present as MS/MS base peak at $m/z 191.056$ and a minor fragment at $m/z 179.034$. In spite of that, they could be differentiated on the basis of their relative fragment ion abundances, being more intense the fragment $m/z 179.034$ in the isomer 3-CQA (around 56%) compared to 5-CQA (with abundance less than 3%). Moreover, the 5-CQA was confirmed by comparing its spectrum and retention time with those of a commercial standard. These isomers have been

characterized before in avocado seed (Calderón-Oliver et al., 2016; Pahua-Ramos et al., 2012; Maria del R Ramos-Jerz et al., 2013; Rodríguez-Carpena et al., 2011; Saavedra et al., 2017; Widsten et al., 2014). On the contrary, to the best of our knowledge, these isomers of CQA have never been reported in avocado seed coat.

Table 1. Identification of phenolic and other polar compounds in the avocado seed and seed coat extract by HPLC-DAD-ESI-QTOF-MS.

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS frag. (m/z, relt abun)	Peak Area x E+4		Proposed compound	Reference
							Seed	Seed coat		
1	3.08	C ₇ H ₁₆ O ₇	211.0833	99.6	4.51	101.025 (100)	1445 ± 18 ^a	793 ± 20 ^b	Perseitol	(López-Cobo et al., 2016)
2	3.39	C ₇ H ₁₂ O ₆	191.0571	99.8	5.33	127.040 (100)	426 ± 10 ^a	300 ± 0.27 ^b	Quinic acid	(Friščić et al., 2016)
3	4.14	C ₄ H ₆ O ₅	133.0150	99.7	5.53	115.003 (100)	292 ± 4 ^b	616 ± 22 ^a	Malic acid	(Mata et al., 2016)
4	4.58	C ₆ H ₈ O ₇	191.0200	100.0	1.35	111.009 (100)	707 ± 26 ^b	1064 ± 73 ^a	Citric acid	(Abu-Reidah et al., 2013)
5	6.51	C ₆ H ₈ O ₇	191.0210	99.3	6.29	111.009 (100)	404 ± 12 ^b	624 ± 16 ^a	Isocitric acid	(Abu-Reidah et al., 2013)
6	7.69	C ₄ H ₆ O ₄	117.0195	100.0	1.39	-	97 ± 4 ^b	630 ± 3 ^a	Succinic acid	(López-Cobo et al., 2016)
7	9.32	C ₆ H ₆ O ₃	125.0245	100.0	1.09	124.016 (100), 108.021 (11)	161 ± 4 ^a	175 ± 3 ^a	Pyrogallol	
8	12.68	C ₆₀ H ₄₈ O ₂₄	1151.2450	99.9	-0.58	-	7 ± 1 ^b	12 ± 0.02 ^a	Proc. tetramer A isomer 1	
9	13.06	C ₆₀ H ₄₈ O ₂₄	1151.2447	99.9	-0.43	863.183 (100), 573.104 (87), 411.074 (85), 711.136 (72), 451.104 (55), 289.072 (32)	4 ± 0.05 ^b	8 ± 0.07 ^a	Proc. tetramer A isomer 2	
10	13.18	C ₈ H ₈ O ₄	167.0358	99.6	5.25	108.022 (100), 123.045 (41)	259 ± 25 ^b	440 ± 14 ^a	3,4-dihydroxyphenylacetic acid	(Bianco et al., 2003)
11	13.93	C ₆₀ H ₄₈ O ₂₄	1151.2452	99.7	-1.51	-	10 ± 0.48 ^a	3 ± 0.02 ^b	Proc. tetramer A isomer 3	
12	14.68	C ₁₄ H ₂₀ O ₈	315.1092	99.9	1.83	135.045 (100), 153.055 (9)	407 ± 32 ^a	69 ± 1 ^b	Hydroxytyrosol glucoside	(López-Cobo et al., 2016)
13	14.80	C ₇₅ H ₆₀ O ₃₀	1439.3076	99.4	-2.04	Nd	-	3 ± 1	Proc. pentamer A	
14	15.43	C ₃₀ H ₂₆ O ₁₂	577.1369	99.3	3.12	125.024 (100), 289.072 (100), 407.078 (76)	104 ± 10 ^a	61 ± 2 ^b	Proc. dimer B isomer 1	(Sandhu & Gu, 2010)
15	15.43	C ₇ H ₆ O ₄	153.0203	99.5	5.92	109.030 (100)	233 ± 16 ^b	635 ± 3 ^a	Protocatechuic acid	(Sun et al., 2007)

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS frag. (m/z, relt abun)	Peak Area x E+4			Proposed compound	Reference
							Seed	Seed coat			
16	15.86	C ₁₆ H ₁₈ O ₉	353.0901	98.3	6.27	191.056 (100), 135.045 (74), 179.035 (56)	603 ± 45 ^a	327 ± 5 ^b		3-O-caffeoylquinic acid	(Clifford et al., 2003)
17	15.86	C ₃₀ H ₂₆ O ₁₂	577.1367	99.4	2.88	125.024 (100), 289.073 (82), 407.078 (58)	68 ± 3 ^a	25 ± 1 ^b		Proc. dimer B isomer 2	(Sandhu & Gu, 2010)
18	16.32	C ₂₁ H ₂₄ O ₁₁	451.1256	99.7	2.29	289.072 (100)	–	42 ± 1		(Epi)catechin glucopyranoside (isomer 1)	(Cádiz-Gurrea et al., 2014)
19	16.74	C ₆ H ₆ O ₂	109.0303	99.6	6.87	–	599 ± 32 ^a	410 ± 8 ^b		Pyrocatechol	(Gasperotti et al., 2014)
20	16.93	C ₄₅ H ₃₆ O ₁₈	863.1832	99.6	-0.56	411.074 (100), 289.073 (82), 711.137 (26), 573.105 (24)	58 ± 5 ^a	6 ± 1 ^b		Proc. trimer A isomer 1	
21	17.05	C ₃₀ H ₂₆ O ₁₂	577.1363	99.7	-2.17	125.024 (100), 289.072 (84), 407.078 (58)	24 ± 0.48 ^a	22 ± 1 ^a		Proc. dimer B isomer 3	(Sandhu & Gu, 2010)
22	17.30	C ₉ H ₁₀ O ₅	197.0462	99.6	1.37	135.045 (100), 179.035 (1)	–	106 ± 2		Syringic acid	(Hossain et al., 2010)
23	17.42	C ₃₀ H ₂₆ O ₁₂	577.1367	99.4	2.79	289.073 (100), 125.025 (84), 407.079 (62)	51 ± 2 ^a	49 ± 0.29 ^a		Proc. dimer B isomer 4	(Sandhu & Gu, 2010)
24	17.49	C ₄₅ H ₃₈ O ₁₈	865.1986	99.4	1.64	287.057 (100), 407.077 (58), 289.072 (57), 577.136 (44), 451.105 (32), 695.140 (27), 739.162 (16)	21 ± 2 ^a	26 ± 1 ^a		Proc. trimer B isomer 1	(Rockenbach et al., 2012)
25	17.61	C ₁₄ H ₂₀ O ₇	299.1152	99.2	4.91	119.035 (100), 137.024 (20)	393 ± 5 ^a	114 ± 2 ^b		Tyrosol-glucoside	(López-Cobo et al., 2016)
26	17.67	C ₁₀ H ₁₀ O ₄	193.0514	99.6	4.51	149.060 (100), 177.046 (18)	128 ± 11 ^a	153 ± 7 ^a		Ferulic acid	(Hossain et al., 2010)
27	17.86	C ₄₅ H ₃₆ O ₁₈	863.1824	99.2	-2.69	411.073 (100), 289.073 (81), 711.136 (26), 573.104 (24)	–	10 ± 1		Proc. trimer A isomer 2	
28	18.01	C ₂₁ H ₂₄ O ₁₁	451.1270	98.6	5.07	289.072 (100)	1789 ± 133	–		(Epi)catechin glucopyranoside (isomer 2)	(Cádiz-Gurrea et al., 2014)
29	18.16	C ₇ H ₆ O ₃	137.0253	99.6	6.17	108.022 (100), 119.014 (8)	2486 ± 123 ^a	1789 ± 133 ^b		4-hydroxybenzoic acid	(Gómez-Caravaca et al., 2016)
30	18.28	C ₆₀ H ₄₈ O ₂₄	1151.2448	99.6	1.44	863.182 (100), 573.105 (82), 711.136 (60), 411.074 (54), 451.105 (43), 289.072 (24)	12 ± 1 ^b	17 ± 0.29 ^a		Proc. tetramer A isomer 4	
31	18.47	C ₃₀ H ₂₆ O ₁₂	577.1358	88.9	1.93	289.072 (100), 125.024 (91), 407.078 (73)	23 ± 1 ^a	5 ± 0.24 ^b		Proc. dimer B isomer 5	(Sandhu & Gu, 2010)
32	18.72	C ₁₅ H ₁₄ O ₆	289.0734	98.7	6.18	109.029 (100), 125.024 (53), 151.040 (35), 137.024 (29), 221.082 (18), 205.051 (10)	711 ± 42 ^a	214 ± 3 ^b		(+)-Catechin	(Rojas-Garbanzo et al., 2016)

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS frag. (m/z, relt abun)	Peak Area x E+4				Proposed compound	Reference	
							Seed	Seed coat					
33	18.72	C ₁₀ H ₁₀ O ₅	209.0462	99.6	4.05	137.061 (100), 165.055 (36)	69 ± 3	109 ± 4	3 ^b	109 ± 4	3 ^a	Hydroxyferulic acid	(Cichon et al., 2017)
34	18.78	C ₁₆ H ₁₈ O ₈	337.0941	99.3	4.07	163.040 (100)	126 ± 5	59 ± 1	5 ^a	59 ± 1	1 ^b	3- <i>p</i> -coumaroylquinic acid	(Clifford et al., 2003)
35	18.84	C ₇ H ₆ O ₄	153.0199	99.4	-3.59	-	14 ± 1	23 ± 1	1 ^b	23 ± 1	1 ^a	Gentisic acid	(Sun et al., 2007)
36	18.84	C ₄₅ H ₃₆ O ₁₈	863.1859	99.0	3.19	411.074 (100), 289.072 (85), 711.138 (25), 573.105 (24)	401 ± 6	624 ± 6	6 ^b	624 ± 6	6 ^a	Proc. trimer A isomer 3	
37	18.97	C ₁₉ H ₂₈ O ₁₁	431.1575	99.3	-1.02	119.049 (100), 137.060 (17), 299.114 (15)	52 ± 2	38 ± 1	2 ^a	38 ± 1	1 ^b	Tyrosol-glucosyl-pentoside	(López-Cobo et al., 2016)
38	19.15	C ₆₀ H ₄₈ O ₂₄	1151.2474	99.4	1.92	-	15 ± 0.46	16 ± 0.34	0.46 ^a	16 ± 0.34	0.34 ^a	Proc. tetramer A isomer 5	
39	19.34	C ₂₁ H ₃₂ O ₁₀	443.1943	99.0	4.29	101.024 (100), 113.025 (51)	81 ± 2	40 ± 0.04	2 ^a	40 ± 0.04	0.04 ^b	Penstemide	(López-Cobo et al., 2016)
40	19.34	C ₃₀ H ₂₆ O ₁₂	577.1382	98.2	5.10	289.073 (100), 125.025 (90), 407.079 (73)	72 ± 5	185 ± 2	5 ^b	185 ± 2	2 ^a	Proc. dimer B isomer 6 (B2)	(Sandhu & Gu, 2010)
41	19.47	C ₈ H ₈ O ₄	167.0354	99.9	-2.37	108.022 (100), 123.045 (54)	22 ± 0.26	68 ± 0.14	0.26 ^b	68 ± 0.14	0.14 ^a	Vanillic acid	(Bianco et al., 2003)
42	20.09	C ₁₇ H ₂₀ O ₉	367.1045	99.9	-0.27	193.051 (100)	9 ± 0.07	16 ± 1	0.07 ^b	16 ± 1	1 ^a	3-feruloylquinic acid	(Clifford et al., 2003)
43	20.09	C ₁₆ H ₁₈ O ₉	353.0891	99.6	3.06	191.056 (100), 179.034 (1), 135.044 (1)	300 ± 9	22 ± 0.13	9 ^a	22 ± 0.13	0.13 ^b	5-O-caffeoylquinic acid	(Clifford et al., 2003)
44	20.65	C ₃₀ H ₂₆ O ₁₂	577.1360	90.9	-1.51	125.024 (100), 289.072 (91), 407.078 (15)	49 ± 2	9 ± 1	2 ^a	9 ± 1	1 ^b	Proc. dimer B isomer 7	(Sandhu & Gu, 2010)
45	20.71	C ₇₅ H ₆₂ O ₃₀	1441.3229	99.5	-1.94	Nd	-	1 ± 0.07	-	1 ± 0.07	-	Proc. pentamer B isomer 1	
46	20.71	C ₄₅ H ₃₆ O ₁₈	863.1849	98.2	-4.15	411.073 (100), 289.072 (95), 711.135 (31), 573.105 (26)	239 ± 19	35 ± 2	19 ^a	35 ± 2	2 ^b	Proc. trimer A isomer 4	
47	20.96	C ₁₆ H ₁₈ O ₉	353.0896	99.0	4.75	173.045 (100), 191.056 (88), 179.035 (63)	805 ± 7	163 ± 3	7 ^a	163 ± 3	3 ^b	4-O-caffeoylquinic acid	(Clifford et al., 2003)
48	20.96	C ₄₅ H ₃₈ O ₁₈	865.1998	99.8	1.52	287.056 (100), 289.072 (64), 407.077 (53), 577.135 (40), 451.104 (30), 695.140 (22), 739.167 (10)	68 ± 4	65 ± 3	4 ^a	65 ± 3	3 ^a	Proc. trimer B isomer 2	(Rockenbach et al., 2012)
49	21.15	C ₆₀ H ₅₀ O ₂₄	1153.2632	99.9	1.06	287.056 (100), 575.119 (87), 577.136 (50), 413.087 (43), 863.180 (23), 865.194 (22), 739.167 (16), 1001.211 (14)	3 ± 0.05	16 ± ###	0.05 ^b	16 ± ###	### ^a	Proc. tetramer B isomer 1	
50	21.21	C ₃₀ H ₂₆ O ₁₂	577.1364	99.7	2.16	289.072 (100), 125.024 (96), 407.079 (23)	74 ± 3	21 ± 1	3 ^a	21 ± 1	1 ^b	Proc. dimer B isomer 8	(Sandhu & Gu, 2010)
51	21.59	C ₇₅ H ₆₂ O ₃₀	1441.3243	99.9	-0.27	Nd	-	3 ± 0.07	-	3 ± 0.07	-	Proc. pentamer B isomer 2	

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS frag. (m/z, rel abun)	Peak Area x E+4				Proposed compound	Reference
							Seed		Seed coat			
52	21.96	C ₄₅ H ₃₆ O ₁₈	863.1853	99.4	-2.49	411.073 (100), 289.073 (86), 573.105 (29), 711.137 (28)	198 ± 8	^a	75 ± 3	^b	Proc. trimer A isomer 5	
53	22.27	C ₇ H ₆ O ₂	121.0296	100.0	2.25	121.030 (100)	49 ± 3	^b	89 ± 8	^a	Benzoic acid	
54	22.27	C ₃₀ H ₂₆ O ₁₂	577.1371	77.3	-2.04	125.024 (100), 289.072 (60), 407.077 (13)	12 ± 1	^a	3 ± 0.09	^b	Proc. dimer B isomer 9	(Sandhu & Gu, 2010)
55	22.33	C ₉ H ₈ O ₃	163.0399	99.7	0.87	163.039 (100), 135.044 (36), 134.037 (31), 107.050 (14), 117.034 (13), 119.050 (9)	56 ± 2	^a	36 ± 0.12	^b	p-Coumaric acid	(Hossain et al., 2010)
56	22.46	C ₃₀ H ₂₄ O ₁₂	575.1211	98.2	4.99	285.041 (100), 289.072 (59), 449.088 (26), 407.078 (18)	26 ± 2	^a	18 ± 2	^b	Proc. dimer A isomer 1	
57	22.83	C ₁₅ H ₁₄ O ₆	289.0740	98.2	7.41	109.030 (100), 125.025 (50), 151.040 (36), 137.025 (28), 221.082 (19), 205.051 (12)	403 ± 12	^a	305 ± 2	^b	(-)-Epicatechin	(Rojas-Garbanzo et al., 2016)
58	23.33	C ₂₂ H ₁₈ O ₁₀	441.0846	98.6	4.60	289.072 (100), 125.025 (5)	–		4 ± 0.32		(Epi)catechin gallate	(Sandhu & Gu, 2010)
59	23.56	C ₁₆ H ₁₈ O ₈	337.0944	98.8	5.49	191.057 (100)	133 ± 13		–		5-p-coumaroylquinic acid	(Clifford et al., 2003)
60	23.58	C ₈ H ₈ O ₃	151.0406	99.8	3.79	122.038 (100), 108.022 (74), 136.017 (48)	83 ± 5	^b	133 ± 13	^a	Vanillin	(de la Torre-Carbot et al., 2005)
61	23.89	C ₁₆ H ₁₈ O ₈	337.0942	99.1	4.69	173.046 (100)	185 ± 2	^a	36 ± 1	^b	4-p-coumaroylquinic acid	(Clifford et al., 2003)
62	23.89	C ₃₀ H ₂₆ O ₁₂	577.1365	86.3	3.16	289.072 (100), 125.024 (73), 407.078 (21)	25 ± 1	^a	14 ± 1	^b	Proc. dimer B isomer 10	(Sandhu & Gu, 2010)
63	23.89	C ₄₅ H ₃₆ O ₁₈	863.1833	99.2	-2.68	411.073 (100), 289.073 (92), 711.135 (32), 573.105 (27)	34 ± 0.09	^a	6 ± 0.33	^b	Proc. trimer A isomer 6	
64	24.02	C ₉ H ₁₀ O ₄	181.0513	99.8	3.76	137.061 (100)	24 ± 2	^b	239 ± 8	^a	Dihydrocaffeic acid	(Farrell et al., 2011)
65	24.02	C ₃₀ H ₂₄ O ₁₂	575.1215	91.6	10.86	285.041 (100), 289.073 (63), 449.089 (27), 407.079 (24)	15 ± 0.31	^a	7 ± 1	^b	Proc. dimer A isomer 2	
66	24.27	C ₂₄ H ₂₀ O ₉	451.1059	97.9	6.10	341.069 (100), 109.029 (38), 189.020 (35), 231.030 (25)	22 ± 0.12	^a	10 ± 1	^b	Cinchonain (isomer 1)	(Beltrame et al., 2006)
67	24.64	C ₉ H ₈ O ₄	179.0353	99.7	2.06	108.022 (100), 122.037 (18), 137.061 (8), 135.045 (3)	34 ± 2	^b	42 ± 1	^a	Caffeic acid	(Gonthier et al., 2003)
68	24.70	C ₁₇ H ₂₀ O ₉	367.1047	99.8	1.78	173.046 (100)	20 ± 0.33	^a	11 ± 0.27	^b	4-feruloylquinic acid	(Clifford et al., 2003)
69	25.20	C ₃₀ H ₂₄ O ₁₂	575.1208	98.9	3.96	285.041 (100), 289.072 (55), 407.078 (23), 449.089 (23)	12 ± 0.23	^a	8 ± 1	^b	Proc. dimer A isomer 3	

Cp	RT (min)	Molecular Formula	m/z	Score	Error (ppm)	MS/MS frag. (m/z, relt abun)	Peak Area x E+4		Proposed compound	Reference
							Seed	Seed coat		
70	25.45	C ₃₅ H ₃₆ O ₁₈	863.1802	99.9	0.38	411.073 (100), 289.073 (65), 711.135 (18), 573.105 (11)	-	3 ± 0.07	Proc. trimer A isomer 7	
71	25.52	C ₃₀ H ₂₄ O ₁₂	575.1224	96.5	7.09	285.044 (100), 289.075 (70), 449.096 (25), 407.084 (18)	7 ± 1 ^b	16 ± 2 ^a	Proc. dimer A isomer 4	
72	26.01	C ₃₀ H ₂₆ O ₁₂	577.1372	98.9	3.88	125.024 (100), 289.072 (77), 407.079 (42)	14 ± 0.43 ^a	13 ± 0.11 ^a	Proc. dimer B isomer 11	(Sandhu & Gu, 2010)
73	26.33	C ₂₇ H ₃₀ O ₁₇	625.1429	99.3	3.03	300.029 (100), 301.035 (32)	-	6 ± 0.14	Quercetin-diglucoside (isomer 1)	(López-Cobo et al., 2016)
74	26.64	C ₂₇ H ₃₀ O ₁₇	625.1432	98.7	3.61	300.028 (100), 301.034 (24)	-	8 ± 0.46	Quercetin-diglucoside (isomer 2)	(López-Cobo et al., 2016)
75	27.46	C ₃₀ H ₂₄ O ₁₂	575.1210	97.1	6.36	285.041 (100), 289.072 (57), 407.079 (31), 449.089 (29)	33 ± 2 ^a	20 ± 3 ^b	Proc. dimer A isomer 5	
76	28.52	C ₂₁ H ₃₀ O ₁₀	441.1800	97.2	7.17	139.077 (100), 330.133 (91), 161.046 (22), 397.187 (22)	543 ± 15 ^a	323 ± 3 ^b	Hydroxyabscisic acid glucoside	(Maria del R Ramos-Jerz et al., 2013)
77	29.63	C ₁₅ H ₁₄ O ₇	305.0685	98.7	6.13	179.035 (100), 125.024 (38)	99 ± 1 ^b	206 ± 4 ^a	(Epi)gallocatechin	(Sun et al., 2007)
78	30.87	C ₂₁ H ₂₀ O ₁₂	463.0926	93.4	10.90	300.029 (100), 301.035 (44)	11 ± 0.12 ^a	11 ± 0.02 ^a	Quercetin-3-β-glucoside	(Rojas-Garbanzo et al., 2016)
79	31.12	C ₉ H ₁₀ O ₄	181.0517	99.4	5.68	108.022 (100), 109.029 (32), 153.020 (2)	278 ± 6 ^b	522 ± 31 ^a	Ethyl Protocatechuate	(Baderschnaider & Winterhalter, 2001)
80	31.87	C ₂₄ H ₂₀ O ₉	451.1060	88.8	14.27	341.067 (100), 189.020 (26), 231.030 (26), 109.029 (24)	36 ± 4 ^a	15 ± 1 ^b	Cinchonain (isomer 2)	(Beltrame et al., 2006)
81	33.62	C ₁₅ H ₁₀ O ₇	301.0369	98.9	4.99	151.004 (100), 121.030 (45), 107.014 (38), 178.999 (19), 273.042 (3)	19 ± 1 ^a	29 ± 4 ^a	Quercetin	(Rojas-Garbanzo et al., 2016)
82	34.30	C ₁₅ H ₁₂ O ₅	271.0631	98.5	7.10	199.041 (100), 200.048 (70), 228.044 (47), 271.062 (20)	220 ± 26 ^b	356 ± 30 ^a	(±)-Naringenin	
83	35.43	C ₁₆ H ₁₄ O ₅	285.0789	98.4	7.04	199.041 (100), 241.051 (2), 267.067 (2)	109 ± 12 ^b	320 ± 52 ^a	Sakuranetin	(Ristivojević et al., 2015)

¹ Compounds checked with a commercially available authentic standard. Means within a row followed by the same lower case letter are not significantly different ($p > 0.05$) according to Student's t-test.

Furthermore, peaks 34, 59 and 61 were detected with a $[M - H]^-$ ion at m/z 337.094, according with their accurate mass were assigned as isomers of

coumaroylquinic acid (CoQA). It was possible to differentiate these three isomers based on MS/MS base peak (Clifford et al., 2003). The 3-p-CoQA (peak 34), 5-p-CoQA (peak 59) and 4-p-CoQA (peak 61) yielded MS/MS base peaks at m/z 163.040, 191.057 and 173.046, respectively. The isomers 3-p-CoQA and 4-p-CoQA were detected in both samples, while 5-p-CoQA was found only in avocado seed. Respect to the data found in literature, the 3-p-CoQA has been found before in avocado seed (Calderón-Oliver et al., 2016; Kosińska et al., 2012; López-Cobo et al., 2016; Widsten et al., 2014). Nevertheless, the 5-p-CoQA and 4-p-CoQA have never been reported in avocado seed and seed coat.

Moreover, *p*-coumaric acid (RT = 22.3 min and m/z = 163.04) was unambiguously identified since it matched its retention time and MS data with the commercial standard. Peak 67, detected at 24.6 min and with m/z 179.035, was tentatively assigned as caffeic acid according to the molecular formula provided for its accurate mass. The fragmentation pattern was similar with the data reported by Gonthier et al. (2003). Both compounds have been previously described in avocado seed (Saavedra et al., 2017; Torres et al., 1987). On the other hand, these compounds were reported here for the first time in avocado seed coat.

Besides, a total of four methoxycinnamic acids were identified in both samples. Peak 26 (RT = 17.7 min) had $[M - H]^-$ 193.051 which gave daughter ions at m/z 149.060 and 177.046. The fragmentation pattern was consistent with a previous report (Hossain et al., 2010). This compound was tentatively assigned as ferulic acid, which has been previously reported in avocado seed (Saavedra et al., 2017; Torres et al., 1987), but not in avocado seed coat. Peak 33 was tentatively identified as

hydroxyferulic acid (RT = 18.7 min and m/z = 209.046). The main MS/MS ions were 137.061 and 165.055, in agreement with the information reported by Cichon et al. (2017). Belonging to methoxycinnamic acids, two isomers of feruloylquinic acid (FQA) were found in both samples. The identification was made based on MS/MS base peak (Clifford et al., 2003). The peaks 42 (RT = 20.1 min) and 68 (RT = 24.7 min) yielded MS/MS base peaks at m/z 193.051 and 173.046, respectively. Therefore, peak 42 was assigned as 3-FQA and peak 68 as 4-FQA. To the best of our knowledge, the compounds hydroxyferulic acid, 3-feruloylquinic acid and 4-feruloylquinic acid have never been reported in avocado seed and seed coat.

Finally, peaks 64 and 79, were detected in both samples at RT 24.0 and 31.1 min showing the same precursor ion of m/z 181.051. As can be seen in Fig. 3 their product ions were different: peak 64 showed the main daughter ion at m/z 137.061 corresponding to $[M - H - CO_2]^-$ (Farrell et al., 2011), meanwhile, peak 79 yielded the main daughter ions at m/z 108.022 $[M - H - CO_2 - C_2H_5]^-$, 109.029 $[M - H - CO_2 - C_2H_4]^-$ and 153.019 $[M - H - C_2H_4]^-$, in agreement with the information reported by Baderschneider and Winterhalter (2001). Therefore, peaks 64 and 79 were assigned as dihydrocaffeic acid and ethyl protocatechuate, respectively. Moreover, dihydrocaffeic acid was confirmed by comparing its spectrum and retention time with those of a standard. To the best of our knowledge, both compounds have never been reported in avocado seed and seed coat.

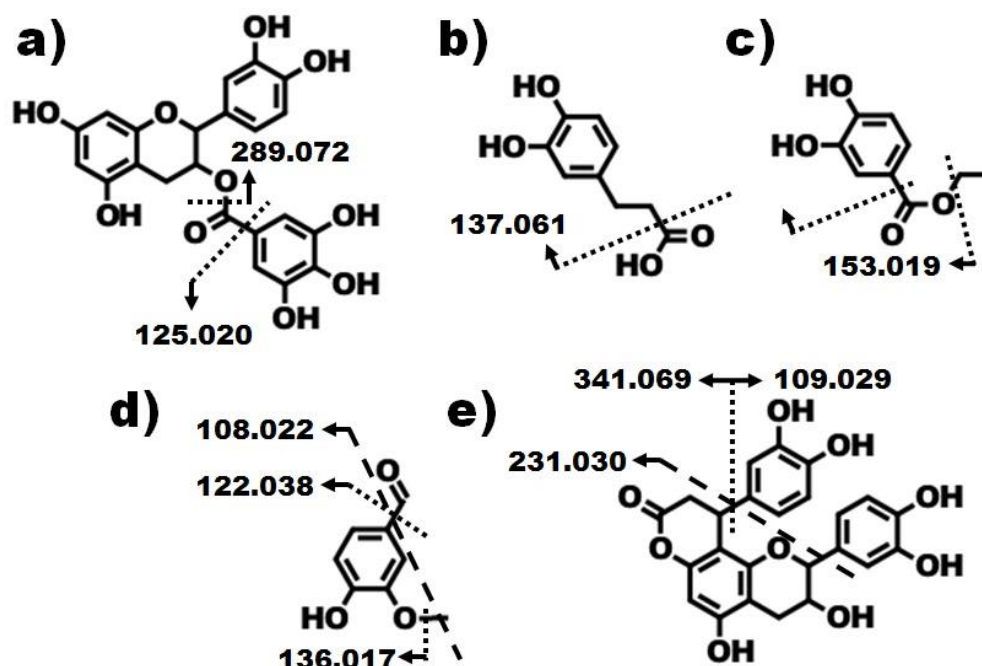


Fig. 3. The fragmentation pathway of a) (epi)catechin gallate, b) dihydrocaffeic acid c) ethyl protocatechuate, d) vanillin and e) cinchonain I.

3.1.3. Phenolic alcohol derivatives

Peaks 12 ($m/z = 315.110$), 25 ($m/z = 299.115$) and 37 ($m/z = 431.158$) were identified as phenolic alcohol derivatives in both samples. The fragmentation patterns were similar between these compounds, the MS/MS base peak of compound 12 was $m/z = 135.045$, while the one of peaks 25 and 37 showed a $m/z = 119.050$ resulting of an O-glycosidic cleavage. Moreover, the other product ions revealed the presence of hydroxytyrosol and tyrosol moieties (peaks 25 and 37) and the daughter ion $m/z = 299.114$ result of the loss of pentose for the compound 37. Consequently, these compounds were tentatively identified as hydroxytyrosol glucoside, tyrosol-glucoside, and tyrosol-glucosyl-pentoside. Hydroxytyrosol glucoside and tyrosol-glucoside have been reported before in avocado seed (López-Cobo et al., 2016;

María del Refugio Ramos-Jerz, 2007; Maria del R Ramos-Jerz et al., 2013). On the contrary, this is the first time that the tyrosol-glucosyl-pentoside has been reported in avocado seed and seed coat.

3.1.4. Flavonoids

Quercetin (RT = 33.6 min and m/z = 301.037) was unambiguously identified since it matched its retention time and MS data with the commercial standard. The main MS/MS fragments were m/z 151.004, 121.030, 107.014, 178.999 and 273.042. The fragmentation pattern was consistent with previous report (Rojas-Garbanzo et al., 2016). This compound had previously been characterized in avocado peel by Wong et al. (2016), nevertheless, these compounds have been identified here for the first time in avocado seed and seed coat.

Peak 78, found in avocado seed and seed coat, was unambiguously identified as quercetin-3- β -glucoside with a [M – H][–] ion at m/z 463.092 and a RT 30.9 min, since it matched its retention times and MS data with the commercial standard. The fragmentation profile showed the odd ion at m/z 301.035 and the even ion at m/z 300.029, which corresponded to O-glycosidic cleavage. The fragmentation pattern was consistent with a previous report (Rojas-Garbanzo et al., 2016). Compound 78 is reported for the first time in avocado seed and seed coat.

Furthermore, two isomers of quercetin-diglucoside (peaks 73 and 74) were tentatively identified in avocado seed coat. The mass spectrum of peak 74 and 75 showed a precursor ion at m/z 625.143 and daughter ions peaks at m/z 300.029 and 301.035. These fragment ions were derived from an O-glycosidic cleavage. The

fragmentation pattern was consistent with a previous report (López-Cobo et al., 2016). To the best of our knowledge, both compounds have never been reported in avocado seed and seed coat.

In addition, two flavanones were found in the studied avocado extracts. The ion found at m/z 271.063 and at RT 34.3 min was unambiguously identified as (\pm)-naringenin by comparison of its retention time, UV and MS/MS spectra to those of commercial standard. Furthermore, sakuranetin, a methoxyflavanone was found at m/z 285.078 and RT 35.4 min. The main MS/MS ions were m/z 267 $[M - H - H_2O]^-$, 241 $[M - H - CO_2]^-$ and 199 $[M - H - C_2H_2O - CO_2]^-$, in agreement with data previously reported by Ristivojević et al. (2015). To the best of our knowledge, both compounds have never been reported in avocado seed.

3.1.5. Catechins

Three monomers of catechin and three derivatives were found in avocado seed and seed coat. The isomers (+)-catechin (RT = 18.7 min) and (-)-epicatechin (RT = 22.8 min) were unambiguously identified since they matched their retention time and MS data with the commercial standards. Furthermore, the MS/MS fragmentation pattern showed several fragment ions at m/z 109.029, 125.024, 151.040, 137.024, 221.082 and 205.051. These fragments were in agreement with those reported by Rojas-Garbanzo et al. (2016). (+)-Catechin has been reported before in avocado seed by Rodríguez-Carpena et al. (2011) and Saavedra et al. (2017); while (-)-epicatechin has been previously reported in avocado seed by María del Refugio Ramos-Jerz (2007), Rodríguez-Carpena et al. (2011), Widsten et al. (2014) and Calderón-Oliver et

al. (2016). Peak 77, corresponding to (epi)gallocatechin (RT = 29.6 min) had $[M - H]^-$ at m/z 305.069, which gave daughter ions at m/z 179.035 and 125.025. These fragment ions were derived from the heterocyclic ring fission (HRF). Moreover, the fragmentation pattern was consistent with a previous report (Sun et al., 2007).

In addition, the mass spectrum of peaks 18 (RT = 16.32 min) and 28 (RT = 18.01 min) showed a precursor ion in the negative ionization mode at m/z 315.1093 $[M - H]^-$ and daughter ion peak at m/z 289.072, as a result of an O-glycosidic cleavage. Therefore, it was tentatively assigned as (epi)catechin glucopyranoside according to the molecular formula provided for its accurate mass and fragmentation pattern (Cádiz-Gurrea et al., 2014).

Finally, peak 58 (RT = 23.33 min) had $[M - H]^-$ at m/z 441.085, which gave daughter ions at m/z 289.072 and 125.025, as a result of deprotonated ion of (epi)catechin and decarboxylated gallic acid, respectively (Fig. 3). Furthermore, the fragmentation pattern was consistent with a previous report (Sandhu & Gu, 2010). It compound was tentatively assigned as (epi)catechin gallate.

To the best of our knowledge, this is the first report of (epi)gallocatechin and the isomers (epi)catechin glucopyranoside in avocado seed and seed coat. Besides, (epi)catechin gallate has been tentatively identified here for the first time in avocado seed coat.

3.1.6. Condensed tannins

3.1.6.1. Procyanidins dimers

The peaks 56, 65, 69, 71 and 75 were found in both samples and tentatively identified as procyanidins type A dimers with m/z 575.121 and RT of 22.4, 24.0, 25.2, 25.5 and 27.5 min. The main MS/MS fragments were m/z 285.041, 289.073, 449.089 and 407.079. The fragmentation pattern was consistent with previous report (Cádiz-Gurrea et al., 2014). Moreover, these fragments were suitable for a fragmentation pathway resulting of fission of the heterocyclic ring system (RDA), quinone methide (QM) cleavage and HRF, as can be observed in Fig. 4.

Moreover, eleven procyanidins type B dimers were tentatively identified in avocado seed and seed coat, with m/z 577.137 and RT 15.4, 15.9, 17.1, 17.4, 18.5, 19.3, 20.7, 21.2, 22.3, 23.9 and 26.0 min. The main MS/MS fragments were m/z 289.072, 125.024 and 407.079. The fragmentation pattern was consistent with previous report (Sandhu & Gu, 2010). The ions at m/z 289.072 corresponding to a loss of an (epi)-catechin unit, while ion at m/z 125.024 is formed after HRF on the top unit of the dimer and the ion 407.079 respond to a consecutive RDA reaction with a loss of a molecule of water. To the best of our knowledge, this is the first time that procyanidins type A dimers have been reported in avocado seed and seed coat in addition to this high amount of procyanidin type B dimers, due to previous work found only two type B dimers (Maria del R Ramos-Jerz et al., 2013).

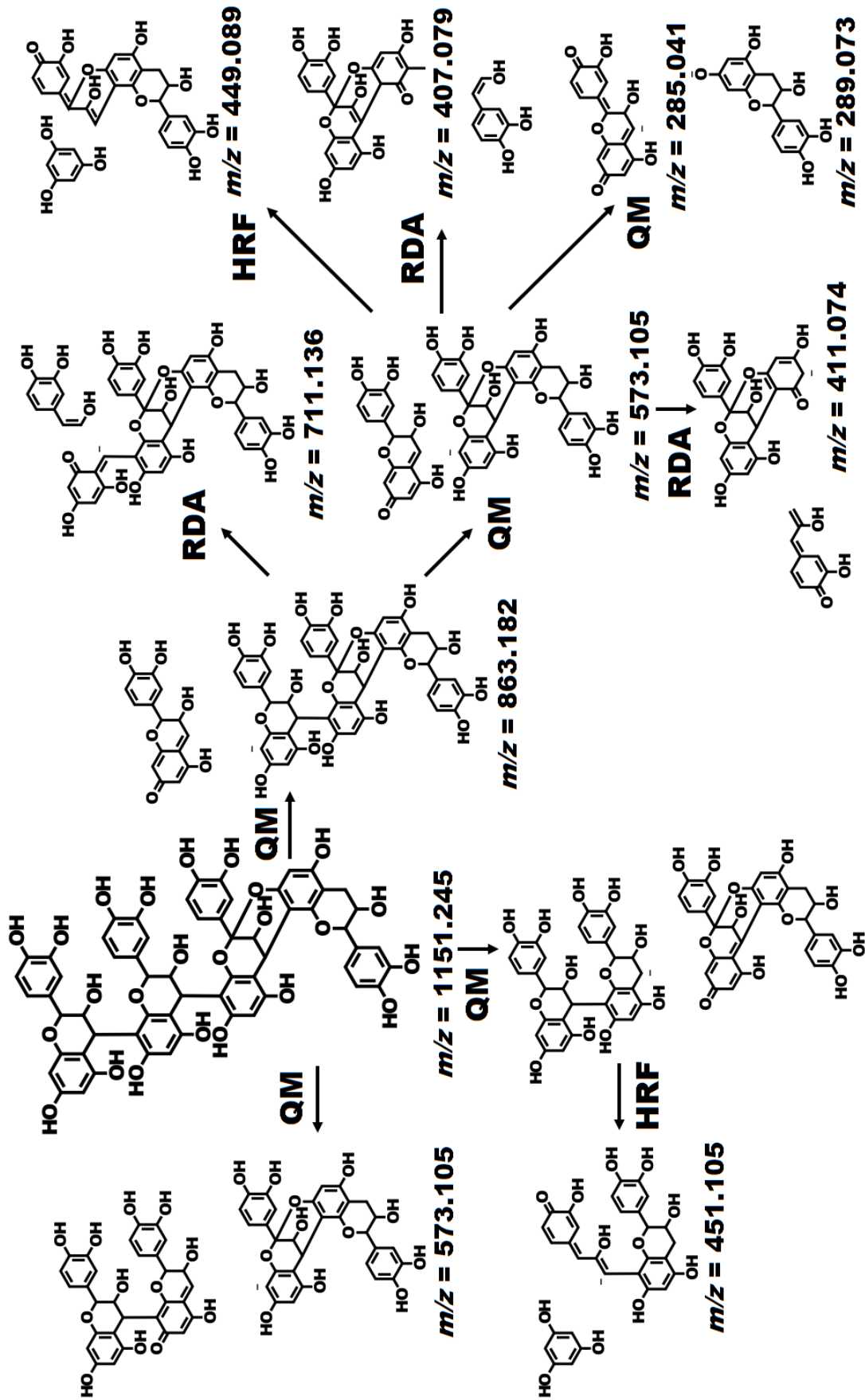


Fig. 4. The fragmentation pathway and MS/MS spectra of procyanidin type A dimer, trimer, and tetramer.

3.1.6.2. Procyanidin trimers

Five and seven isomers of procyanidin type A trimers were found in avocado seed and seed coat, respectively. Peaks 20, 36, 46, 52 and 63 were found in both samples, while the peaks 27 and 70 were found only in the seed coat. The main MS/MS fragments correspond to m/z 411.074, 289.072, 711.138 and 573.1056, in consistency with previous researches (Jayaprakasha, Ohnishi-Kameyama, Ono, Yoshida, & Rao, 2006). The ions at m/z 711.074 respond to RDA reaction, meanwhile the ions at m/z 573 and 289 were originated from QM cleavage. Moreover, the ion at m/z 573 indicates that the A-type interflavan linkage of the trimer was between the top and middle units (Wang et al., 2010). Finally, the observed m/z 411 ion was formed after the cleavage of the middle unit through the HRF (Fig. 4).

In addition, two procyanidins type B trimers (peaks 24 and 48) were found in each sample, with a RT of 17.4 and 20.9 min, respectively. The mass spectrum of these peaks showed a precursor ion with m/z 865.198 [M – H][–] and daughter ions peaks at m/z , 407.077, 289.072, 577.136, 451.105, 695.140 and 739.162, in agreement with those reported by Rockenbach et al. (2012). The fragment ions at m/z 695.140 and 407.077 are a result of water elimination after a RDA reaction. Moreover, the observed fragments m/z 739 and 451 were a result of HRF. Finally, fragments 577.136, 287.057 and 289.072 were obtained after QM cleavages, being this last fragment an indicative that the monomeric unit is (epi)catechin (Rockenbach et al., 2012).

In researches conducted by Kosińska et al. (2012), Maria del R Ramos-Jerz et al. (2013), Widsten et al. (2014), a lower number of this kind of compounds were identified compared to ours. Solely two procyanidins type A trimers were found in those publications, whereas, in the present study five and seven procyanidins type A trimers were characterized in avocado seed and seed coat respectively, along with two trimers type B found in avocado seed coat.

3.1.6.3. Procyanidins tetramers

Peaks 8, 9, 11, 30 and 38 were tentatively identified as procyanidin type A tetramers. These peaks showed a precursor ion $[M - H]^-$ 1151.24 and the main MS/MS fragments were m/z 863.183, 573.104, 411.074, 711.136, 451.104 and 289.072. These fragments were suitable for a RDA reaction, QM cleavage and HRF, as can be observed in Fig. 4. These compounds were found in both samples, seed and seed coat.

Furthermore, peak 49, found also in both samples, was detected at 21.1 min with m/z 1153.26. This peak was tentatively assigned to a procyanidin type B tetramer according to the molecular formula provided for its accurate mass. The MS/MS fragmentation pattern showed several fragment ions at m/z 287.056, 575.119, 577.136, 863.180 and 865.194, as a result of QM cleavages. Moreover, the ions 739.167 and 413.087 were a result of HRF and the ion 1001.211 was a result of RDA reactions. Respect to the data found in literature, Widsten et al. (2014) found only one tetramer type A. Nevertheless, in the present study five procyanidin type A

tetramers were detected. Furthermore, to the best of our knowledge, procyanidin type B tetramer has never been previously described in avocado seed and seed coat.

3.1.6.4. Procyanidins pentamers

Three procyanidins pentamers were found only in avocado seed coat. One type A (peak 13) and two type B (peaks 45 and 51), with $[M - H]^-$ ions at m/z 1439.308 and 1441.323, respectively. The identification was tentatively made according with the molecular formula provided for its accurate mass. This kind of compounds have been described before in avocado seed by López-Cobo et al. (2016); Rodríguez-Carpena et al. (2011); Wang et al. (2010). Nevertheless, their results were reported as a sum of pentamers or procyanidins total content. As far as we are concerned, this is the first report of procyanidin pentamers in avocado seed and seed coat as individual compounds.

3.1.7. Others polar compounds

Five other polar compounds were found in both avocado by-product samples. Peak 7 with a RT 9.3 min had $[M - H]^-$ of m/z 125.024 and product ion at m/z 108.021 corresponding to $[M - H - OH]^-$. It was tentatively assigned to pyrogallol or 1,2,3-trihydroxybenzene. Another benzene derivative was found at RT 16.7 min and at $m/z = 579.1384$. It was tentatively assigned to pyrocatechol or 1,2-dihydroxybenzene, according to the molecular formula provided for its accurate mass (Gasperotti et al., 2014). Moreover, vanillin ($m/z = 151.04$) was detected at RT 23.58 min. The MS/MS fragmentation pattern showed several fragment ions at m/z 122.038, 108.022, and 136.017 (de la Torre-Carbot et al., 2005). The fragmentation

pathway can be observed in Fig. 3. Furthermore, two flavalignan isomers were found with RT 24.2 and 31.9 min. These isomers presented a product ion at m/z 451.106, and yielded the main fragments ions at m/z 341.071, 231.032 and 109.030 (see Fig. 3). The fragmentation pattern was in agreement with that reported by Beltrame et al. (2006). As far as we are concerned, these compounds have never been reported before in avocado seed and seed coat.

In addition, two abscisic acid derivatives (peaks 39 and 76) were detected. Peak 39 with RT 19.3 min had $[M-H]^-$ of 443.194, which gave daughter ions at m/z 101.024 and 113.025, in accordance with the fragmentation pattern reported by López-Cobo et al. (2016). Thus, this compound was tentatively assigned as penstemide. Moreover, peak 76 was tentatively assigned to hydroxyabscisic acid glucoside (RT = 28.5 min and m/z = 441.18). Its MS/MS spectrum showed fragments at m/z 330.133, 397.188 and 161.046, corresponding to α -fragmentation with cleavage of the olefinic side chain of abscisic acid backbone, decarboxylation reaction and loss of hexose, respectively (María del Refugio Ramos-Jerz, 2007).

Furthermore, the sugar perseitol was detected with RT 3.1 min and precursor ion at m/z 211.083. Its main MS/MS product ion at m/z 101.025 was in agreement with data previously reported by López-Cobo et al. (2016). All these hydrophilic compounds were identified for the first time in avocado seed coat.

3.2. Comparison of phenolic and other polar compounds in avocado seed and seed coat

Once the chemical composition of the avocado by-product extracts was established, the differences between seed and seed coat were evaluated in terms of compositional variations. These differences were illustrated in Figs. 5 and 6 by means of comparisons of the area of the different compounds in the seed and seed coat hydro-alcoholic extracts. A total of 83 compounds were found within the two avocado samples, in the seed coat a sum of 80 compounds were identified, 5 more than in the seed. In this sense, compounds 5-p-coumaroylquinic acid, (epi)catechin glucopyranoside isomers 1 and 2 were found only in seed, and compounds proc. pentamer A, syringic acid, proc. trimer A isomer 2, proc. pentamer B isomers 1 and 2, (epi)catechin gallate, proc. trimer A isomers 7, quercetin-diglucoside isomers 1 and 2 were found only in seed coat. The base peak area of each signal in HPLC-MS chromatograms was used to provide semi-quantitative information for comparison purposes. The total content for all compounds families in avocado seed and seed coat was tentatively calculated as the sum of the individual compound areas. Statistically significant differences were detected between seed and seed coat for all groups, except for hydroxybenzoic acids family (Fig. 5). As a resume, solely flavonoids, other phenolic acids and organic acids presented higher content in avocado seed coat compared to pure seed.

Tannins were the most numerous group, with 34 compounds identified in seed coat and 29 in seed. It has been found that the content of tannins in seed is higher than in the seed coat, which could be explained due to the existent differences in the

contents of dimers type B and trimers type A (Fig. 6). The higher levels of catechin subclass in seed correspond mainly to the content of catechin and the presence of one extra compound compared to the coat. The proportion between catechin and epicatechin was almost two-fold greater in seed, which was higher than the one reported previously by Rodríguez-Carpena et al. (2011).

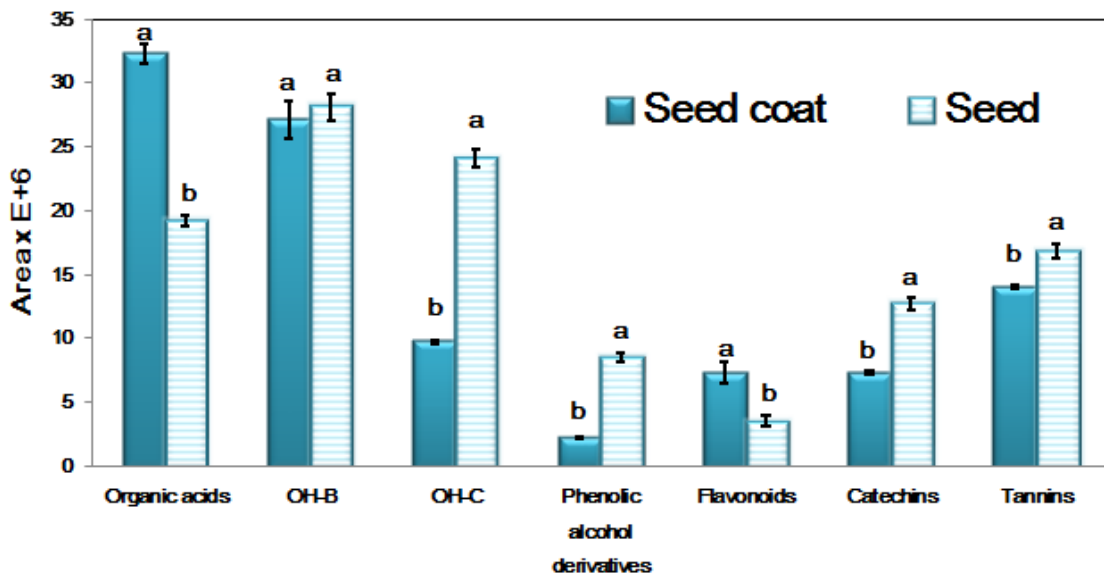


Fig. 5. Comparison of different families of compounds found in seed and seed coat. (OH-B: hydroxybenzoic acids; OH-C: hydroxycinnamic acids). Values with the same letter show that there is no statistically significant difference between the area of these compounds at the 95% confidence level ($p > 0.05$).

Flavonols, flavanones and their derivatives were grouped into flavonoids sub-class, and the higher content in seed coat than seed could be justified due to the content of flavanones as (\pm)-naringenin and the presence of isomers of quercetin-diglucoside.

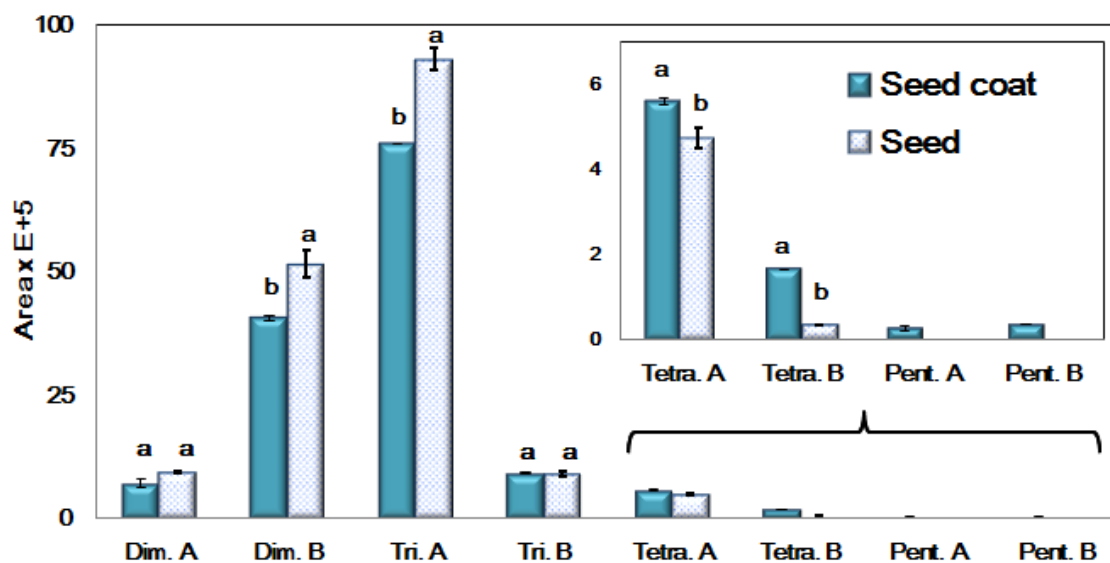


Fig. 6. Content of condensed tannins in avocado seed and seed coat. (Dim: dimers; Tri: trimers; Tetra: tetramers; Pent: pentamers). Values with the same letter show that there is no statistically significant difference between the area of these compounds at the 95% confidence level ($p > 0.05$).

Although that in the seed coat were identified more hydroxybenzoic acids, not statistically significant differences were found in this kind of compounds for both samples (Fig. 5).

The second more representative group of acids was hydroxycinnamic acids, with 12 and 11 compounds identified in seed and seed coat, respectively. The content was about twice times higher in seed than seed coat. This may be due to differences in the concentration of isomers of caffeoylquinic acid (Table 1). On the contrary, the 3,4-dihydroxyphenylacetic acid, dihydrocaffeic acid and ethyl protocatechuate, identified in both samples exhibit higher contents in seed coat compared to seed.

On the other hand, the content of all phenolic alcohol derivatives in seed were higher when compared to that of seed coat. On the contrary, the organic acid content is around two-fold greater in seed coat than seed, only the quinic acid content was higher in seed than seed coat (Table 1).

A detailed analysis of the condensed tannins group revealed further differences. For instance, in general the content of procyanidin type A is extremely higher than the one for type B, which should be highlighted due to some researchers suggest that the presence of procyanidin type A might possess additional health benefits, such as aid in maintaining urinary tract health (Kosińska et al., 2012). The avocado seed coat presented a higher content of procyanidins with mayor polymerization degree (Fig. 6), which could be conferred a higher antioxidant activity compared to the seed (Cádiz-Gurrea et al., 2014). Furthermore, as mention before, both types of procyanidin pentamers were found only in the seed coat.

3.3. Antioxidant properties

Considering the complexity of oxidative reactions, any single antioxidant activity assay could probably not predict all the oxidative details in every system. For this reason, antioxidant assays based on single electron transfer (DPPH and TEAC) and hydrogen atom transfer (ORAC) were performed in order to estimate the antioxidant potential of avocado seed and seed coat extracts.

With regard to single-electron transfer-based methods, avocado seed and seed coat extracts were found to have significant antioxidant activities (Table 2). Previous studies have reported that avocado seed showed higher antioxidant activities than

avocado pulp (Dabas et al., 2013; Rodríguez-Carpena et al., 2011). In this work, TEAC value for avocado seed extract was higher than the results reported by Kosińska et al. (2012) (94 $\mu\text{mol TE/g DW}$). These authors determined the TEAC value of an avocado seed extract obtained after a dynamic maceration with 80% methanol at 60 °C for 15 min of avocado seed. The difference among these values could be associated with the use of different extraction methods. PLE extraction has been reported to improve the extraction of antioxidant compounds compared to traditional techniques. Moreover, in this research the highest TEAC value was recorded in avocado seed coat (432 \pm 3 $\mu\text{mol TE/g DW}$). To the best of our knowledge, there is no information about the antioxidant activity of this by-product. The comparison of present results with those obtained in previous studies has pointed out that both samples presented higher antioxidant activity than other vegetables and food by-products (mango, guava and cocoa by-products) (Martínez et al., 2012a, 2012b).

Table 2. Antioxidant activity by TEAC, DPPH and ORAC assays of avocado seed and seed coat.

Sample	TEAC ($\mu\text{mol TE/g DW}$)	DPPH IC ₅₀ ($\mu\text{g/mL}$)	DPPH AAI	ORAC ($\mu\text{mol TE/g DW}$)
Seed coat	432 \pm 3 ^a	10.4 \pm 0.9 ^b	1.9 \pm 0.2 ^a	460 \pm 30 ^a
Seed	300 \pm 20 ^b	15 \pm 2 ^a	1.4 \pm 0.2 ^b	310 \pm 30 ^b

^a Means within a column followed by the different letter are significantly different ($p \leq 0.05$) according to Student's t-test.

Concerning DPPH assay, the concentration of avocado extracts required to decrease by 50% (IC50) absorbance were 10.4 and 15 µg extract/mL for seed coat and seed, respectively. Morais et al. (2015) determined the antioxidant capacity of seed extract obtained by using dynamic maceration and methanol as extraction solvent and reported IC50 value of 370.22 µg/mL. As mentioned before, the best antioxidant capacity of the extracts prepared in this research could be related to extraction method.

Concerning hydrogen atom transfer reaction methods, the results showed ORAC values ranging from 460 to 310 µmol TE/g DW. In literature, lower content of scavenge peroxy radicals in avocado seeds amounting to 210 µmol TE/g DW and 1.6 µg TE/g extract were reported by Kosińska et al. (2012) and Calderón-Oliver et al. (2016), respectively.

Despite many variables may influence the content of antioxidant components, extraction technique is one of the most important factors that affect the recovering of bioactive compounds. These results confirmed the potential use PLE with GRAS solvents as green technique to extract phenolic compound from avocado seed and seed coat for its use as ingredient in the food, cosmetic or pharmaceutical sector.

4. Conclusions

The combined use of PLE and HPLC-DAD-ESI-QTOF-MS enable to identify a total of 83 compounds in avocado seed and seed coat. Among these characterized compounds, forty six have been determined for the first time in avocado seed by-products. It is also important to highlight that this is the first time that avocado seed

coat have been studied individually regarding phenolic compound composition using HPLC with high resolution mass spectrometry. Condensed tannins, phenolic acids and flavonoids were the most representative groups in both samples. Furthermore, statistical differences were found within the phenolic profile of seed and seed coat, the organic acids and flavonoids presented higher content in seed coat, on the contrary, hydroxycinnamic acids, phenolic alcohol derivatives, catechins and condensed tannins showed higher content in seed. The presence of procyanidins type A, with potential health benefits, was found in both samples. In addition, the antioxidant properties of extracts were evaluated in term of single electron transfer (DPPH and TEAC) and hydrogen atom transfer (ORAC). Avocado seed and seed coat PLE extracts were found to have significant antioxidant activity in comparison with the previously reported data. Avocado seed coat exhibited significantly higher antioxidant activity than the seed for all three assays. The results obtained in this work encourage the use of avocado seed and seed coat for its use as ingredient in cosmetic, pharmaceutical and food industries due to the huge content of bioactive compounds. Future investigations are warranted to develop purified antioxidant extracts, with the application of more selective extraction techniques.

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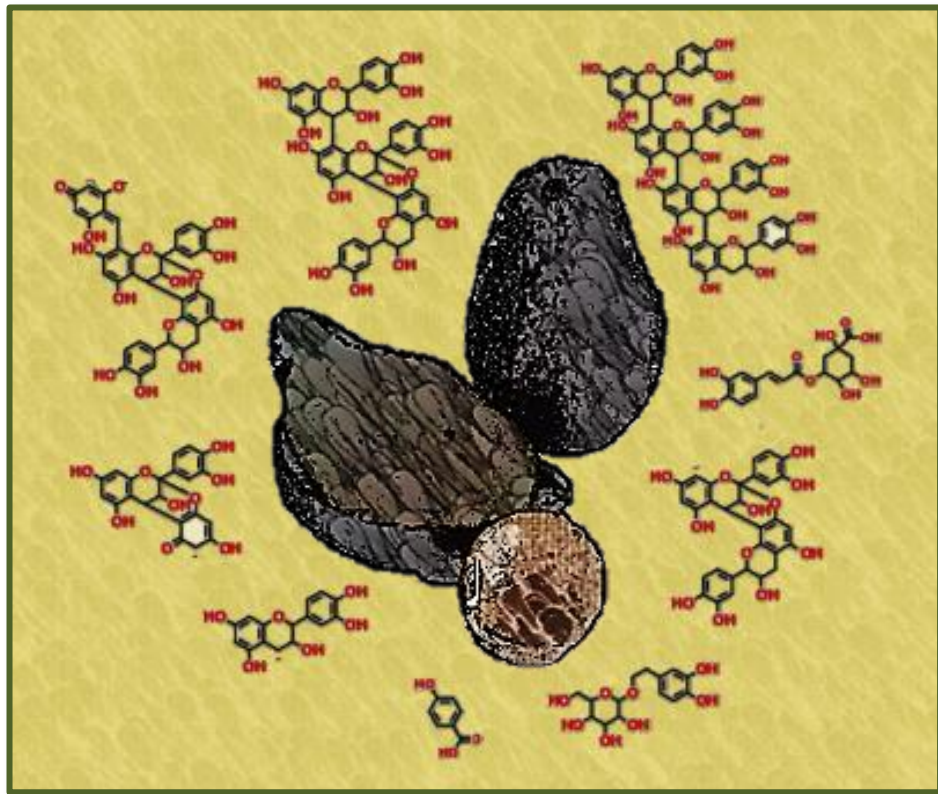
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Conclusiones

Conclusions

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Como resultado del trabajo realizado durante la presente Tesis Doctoral, se han obtenido las siguientes conclusiones:

- El secado es un paso esencial en la revalorización de los subproductos de aguacate con el fin de reducir la cantidad de agua y así inhibir el desarrollo microbiano y minimizar las reacciones de degradación de compuestos bioactivos. En este sentido, se evaluó y optimizó el efecto de la temperatura de secado sobre el TPC y las concentraciones de compuestos fenólicos individuales presentes en piel, hueso y cubierta del hueso.
- En cuanto al secado de la piel de aguacate, los mejores resultados se obtuvieron con el proceso de secado más corto a la temperatura más elevada, realizado a 85 °C durante 12 horas, que mostró el mayor valor de TPC (58 ± 4 mg GAE / g dm) y mayor contenido para casi todos los compuestos fenólicos. Puede deducirse que el tiempo de la etapa de secado es un parámetro crítico para el contenido de compuestos bioactivos. De manera global, los ácidos fenólicos fueron los compuestos más estables durante el proceso de secado, independientemente de la temperatura aplicada, aunque sus niveles disminuyen con un proceso de secado más prolongado a temperaturas más bajas. Por el contrario, las procianidinas fue el grupo fenólico más termolábil, mostrando una disminución en su contenido de alrededor del 75%.

- Similares resultados se obtuvieron para las muestras de hueso y cubierta de hueso de aguacate. De acuerdo con el TPC, la mejor temperatura de secado fue de 85 °C, con valores de 29.5 ± 0.4 y 71.2 ± 1.7 mg EAG / g dm, para el hueso y cubierta del hueso. Además, se cuantificaron los compuestos individuales por HPLC-DAD-ESI-TOF-MS, evaluando el efecto de la temperatura de secado sobre más de 40 compuestos fenólicos. Estudiando los contenidos individuales no se observa la existencia de una misma temperatura óptima para todos ellos. Sin embargo, con respecto a las procianidinas, se observó que el tipo A fue más termoestable que el tipo B, y la degradación aumentó con el grado de polimerización.
- De manera general para las tres matrices estudiadas se puede concluir que dentro de las condiciones evaluadas un secado realizado a mayor temperatura y por consiguiente menor tiempo de secado minimiza la degradación de compuestos bioactivos. En consecuencia, el proceso de secado optimizado con convección forzada a 85 °C parece ser adecuado para su escalado a nivel industrial para secar los sub-productos de aguacate antes de su transporte o procesamiento.
- Se optimizó la extracción de compuestos fenólicos presentes en la piel, hueso y cubierta del hueso de aguacate mediante PLE utilizando para ello disolventes GRAS. Este proceso se realizó utilizando un diseño experimental superficie de respuesta, considerando como variables independientes la composición de disolvente y la temperatura de extracción, y como variable dependiente el

contenido fenólico total de los extractos. Las condiciones de extracción óptimas para el proceso PLE para las tres matrices fueron una mezcla hidroalcohólica de EtOH / H₂O 50:50 (v / v) a una temperatura de 200 °C.

- Se caracterizó de forma exhaustiva el perfil fenólico de los extractos de piel de aguacate obtenidos por PLE mediante el uso de cromatografía líquida de alta resolución acoplada a espectrometría de masas con un analizador cuadrupolo-tiempo de vuelo (HPLC-ESI-Q-TOF-MS). Se lograron identificar tentativamente un total de 61 compuestos pertenecientes a once familias. Los grupos más representativos fueron procianidinas, flavonoles, ácidos hidroxibenzoicos y ácidos hidroxicinámicos. Cabe resaltar, que se identificaron 35 compuestos por primera vez en piel de aguacate, entre ellos 17 procianidinas con diferente grado de polimerización. Estos resultados destacan la potencial combinación de PLE y HPLC-DAD-ESI-QTOF-MS para extraer e identificar compuestos bioactivos en el subproducto de piel de aguacate.
- De igual forma, el uso combinado de la poderosa técnica de extracción verde (PLE) con disolventes GRAS y una potente plataforma analítica de separación y detección (HPLC-ESI-QTOF-MS) permitió identificar un total de 83 compuestos en el hueso y cubierta del hueso de aguacate. Entre estos compuestos caracterizados, 46 se han determinado por primera vez en esta matriz vegetal. También es importante destacar que es la primera vez que se ha estudiado el perfil fenólico del hueso de aguacate usando una técnica instrumental tan poderosa. En este sentido, las procianidinas, ácidos fenólicos y flavonoides

fueron los grupos más representativos en ambas muestras. Además, se encontraron diferencias estadísticas dentro del perfil fenólico del hueso y su cubierta, siendo los ácidos orgánicos y flavonoides los que estuvieron presentes en mayor concentración en la cubierta del hueso. Por el contrario, los ácidos hidroxicinámicos, derivados de alcoholes fenólicos, catequinas y procianidinas se encontraron en mayor contenido en el hueso. Además cabe destacar la presencia de procianidinas de tipo A en ambas muestras, compuestos con conocidos beneficios para la salud. Adicionalmente merece una mención especial el hecho de que se lograron separar e identificar por primera vez tetrámeros y pentámeros de procianidinas en el hueso y cubierta de aguacate.

- Se evaluó la capacidad antioxidante de los extractos obtenidos mediante los mecanismos de transferencia de electrones (métodos DPPH y TEAC) y transferencia de átomos de hidrógeno (método ORAC). Se encontró que los extractos de hueso de aguacate obtenidos mediante PLE, presentaron mayor actividad antioxidante comparados con resultados reportados por otros estudios. La cubierta del hueso de aguacate exhibió una actividad antioxidante significativamente mayor que el hueso para los tres ensayos realizados.
- Se desarrolló y optimizó un método por HPTLC para la separación e identificación de compuestos con actividad inhibitoria de la acetilcolinesterasa presentes en extractos de subproductos de aguacate. Además, el método de Ellman se puede usar eficientemente para estudios cualitativos de compuestos

con actividades inhibitoras de AChE en extractos ricos en polifenoles. En el hueso de aguacate se encontraron cuatro compuestos con actividad AChE.

- En general, los resultados obtenidos en este trabajo fomentan el uso de los subproductos del aguacate como ingredientes bioactivos para su uso en el sector alimentario, cosmético o farmacéutico debido a su alto contenido de compuestos con actividades antioxidante y antiaceticolinesterasa.

Conclusions

The following conclusions can be drawn from the work conducted in this PhD thesis:

- Drying is an essential step in the revalorization of avocado by-products in order to decrease water content to a level at which microbial spoilage and deterioration reactions are minimized. In this sense, the effect of air drying temperature on TPC and individual phenolic compound concentrations presents in avocado peel, seed and seed coat was evaluated and optimized.
- Regarding avocado peel drying, the best results were obtained with the shortest drying process, performed at 85°C for 12 hours, which showed the highest TPC value (58 ± 4 mg GAE/g dm) and in general the highest concentrations of all the individual compounds identified in the extracts. Respect to individual concentrations, phenolic acids were the more stable compounds during the drying process regardless of the applied temperature, although its levels decrease with longer drying periods performed at lower temperatures. On the opposite, procyanidins suffered an important degradation with longer drying steps, showing a decrease in their content of around 75%.
- Concerning avocado seed and seed coat drying, the best temperature was also 85 °C. Under this optimal drying temperature the maximum TPC for avocado seed and seed coat were 29.5 ± 0.4 and 71.2 ± 1.7 mg EAG/g dm, respectively. Besides, more than 40 phenolic compounds were simultaneously quantified by

HPLC-DAD-ESI-TOF-MS to assess the effect of drying temperature on individual compounds. Nevertheless, different optimum drying temperatures were obtained for the evaluated phenolic compounds. Regarding procyanidins, type A were more thermostable than type B, and the degradation increased with the polymerization degree.

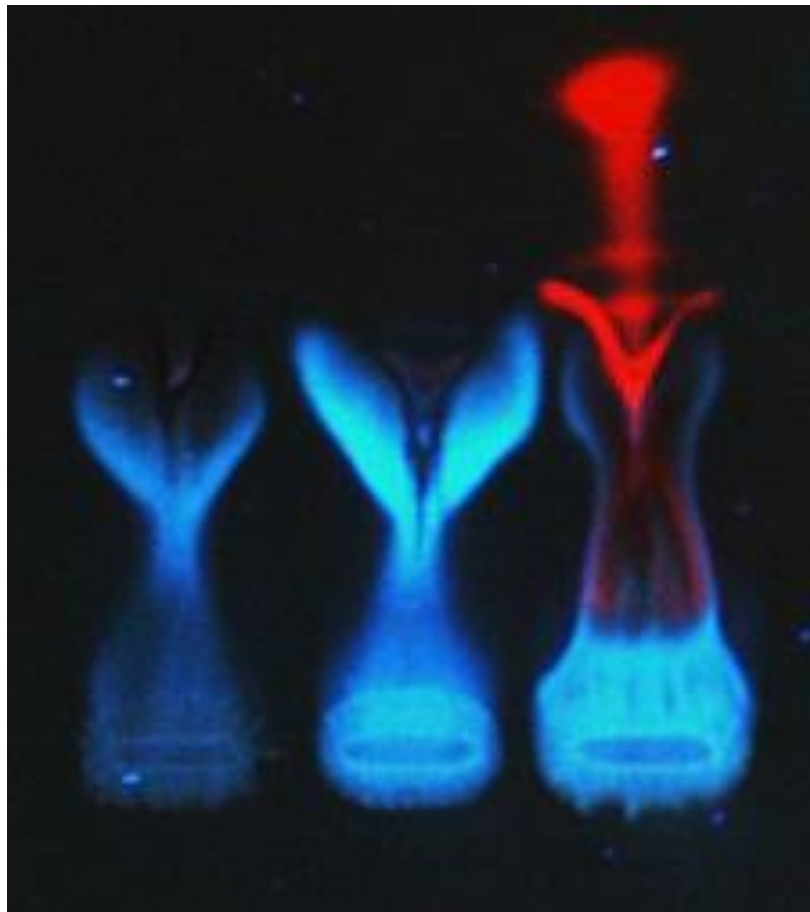
- It can be concluded that in general for these avocado by-products shorter drying step times at higher temperatures maintained the content of bioactive compounds in a better way. In this research, the optimized drying process performed at 85°C in a forced air oven seems to be a suitable process for industrial scale in order to dry the avocado peel, seed and seed coat by-products before its transport or processing.
- As far as we are concerned, the present Thesis is the first available study that optimized the phenolic compounds extraction from avocado peel, seed and seed coat using PLE with GRAS solvents. A response surface methodology (RSM) was used in order to optimize the extraction of phenolic compounds considering solvent composition and temperature as independent variables and Total Phenolic Compound as response variable. The optimum extraction conditions for the avocado matrices were established for the PLE process with a mixture of EtOH/H₂O 50:50 (v/v) at a temperature of 200 °C.
- The phenolic profile of avocado peel was comprehensively characterized by PLE in combination with liquid chromatography coupled to ultra-high-

definition accurate-mass Q-TOF analyzer. A total of 61 compounds belonging to eleven families were identified. Procyanidins, flavonols, hydroxybenzoic and hydroxycinnamic acids were the most common compounds. On this sense, 17 procyanidins with different polymerization degree, as well as 35 compounds have been identified for the first time in avocado peel. These results highlight the potential combination of PLE and HPLC-DAD-ESI-QTOF-MS to extract and identify bioactive compounds in avocado peel by-product.

- The combined use of a powerful green extraction technique (PLE) with GRAS solvents and a potent analytical platform (HPLC-ESI-QTOF-MS) enable to identify a total of 83 compounds in avocado seed and seed coat. Among these characterized compounds, 46 have been determined for the first time in avocado seed. It is also important to highlight that this is the first time that avocado seed coat have been studied individually regarding phenolic compound composition using such a powerful instrumental technique. Condensed tannins, phenolic acids and flavonoids were the most representative groups in both samples. Furthermore, statistical differences were found within the phenolic profile of seed and seed coat. Organic acids and flavonoids were present in higher content in seed coat; on the contrary, hydroxycinnamic acids, phenolic alcohol derivatives, catechins and condensed tannins showed higher content in seed. The presence of procyanidins type A, with potential health benefits, was found in both samples. Moreover, this is

the first report of procyanidin tetramers and pentamers in avocado seed and seed coat as individual compounds.

- The antioxidant properties of extracts were evaluated in term of single electron transfer (DPPH and TEAC methods) and hydrogen atom transfer (ORAC method) mechanisms. Avocado seed and seed coat PLE extracts were found to have significant antioxidant activity in comparison with the data previously reported. Avocado seed coat exhibited significantly higher antioxidant activity than the seed for all three assays.
- A HPTLC method was development and optimized to separate and identify compounds with AChE activity from avocado by-products. Furthermore, Ellman's method can be used efficiently for qualitative studies of compounds with AChE inhibitory activities in polyphenols-rich extracts. In avocado seed four compounds presented AChE activity.
- Overall, the results obtained in this work encourage the use of avocado byproducts as ingredient in cosmetic, pharmaceutical and food industries due to the huge content of bioactive compounds with antioxidant and AChE activities.



Annex

Annex: Avocado by-products as inhibitors of acetylcholinesterase enzyme

This work was carried out in collaboration with the “Department of Food Science and Technology” from the University of Concepcion (Chile) directed by Prof. Dr. Mario Aranda, and it is currently in progress.

1. Introduction

Alzheimer’s disease (AD) is the most popular neurodegenerative disease of this century and the most common reason of dementia between the older humans. This immutable neurological disturbance is determined by cognitive impairment, memory disorders and behavioural deficits in daily activities (Türkan, *et al.*, 2018). AD is a common type of progressive neurodegenerative disease characterized by low levels of acetylcholine in the cortex and hippocampus of the brain, oxidative stress and neuroinflammation in brain stream (Kocyigit, *et al.*, 2017). Acetylcholine (ACh) plays an important role in brain memory function, and elevation of ACh levels with acetylcholinesterase (AChE) inhibitors is currently one of the most effective treatment strategies against Alzheimer's disease (AD) (Kocyigit, *et al.*, 2017).

Anticholinesterase drugs such as donepezil, galantamine and rivastigmine are available for the symptomatic treatment of patients with mild-to moderate Alzheimer’s disease (Noridayu, *et al.*, 2011). Nevertheless, these drugs are known to have limitations due to their short-half-lives and/or unfavourable side effects such as hepatotoxicity, gastrointestinal disturbance, dizziness, diarrhoea, vomiting and

nausea, and this has led to the search for molecules from natural sources with fewer side effects (Abou-Donia, *et al.*, 2014; Noridayu, *et al.*, 2011).

Determination of AChE activity has become an important tool in drug design and discovery as well as in medicine and toxicology. A broad variety of methods have been developed over the past decades for AChE inhibitory activity quantification. The most common assays are Ellman's method and diazotization method (Abou-Donia, *et al.*, 2014; Ali-Shtayeh, *et al.*, 2014; Bräm & Wolfram, 2017; Ellman, *et al.*, 1961; Marston, *et al.*, 2002). Both methods could be adapted to a direct HPTLC assay or to quantitative measurements using spectrophotometric techniques.

In this scenario, HPTLC-methods combined with biological detection provides simultaneous chromatographic separation of a complex multicomponent matrix and the localisation of active constituents directly on a plate (Bräm & Wolfram, 2017). Furthermore, HPTLC has the advantage of simultaneous analysis of large numbers of samples, thus reducing time and cost per analysis (Abou-Donia, *et al.*, 2014; Alajmi & Alam, 2014).

The objective of the present study was to develop an optimized methodology for maximum separation of avocado by-products extracts in HPTLC using the PRISMA mobile phase optimization method and identification of contributing compounds responsible for the inhibitory AChE enzyme activity.

2. Materials and methods

2.1. Plates and reagents

The optimization of the mobile phase was performed on 20 × 20 cm glass backed TLC plates: silica gel 60 (Merck, Art. No. 1.05641) and the bioassays were performed on 20 × 10 cm glass backed HPTLC plates: silica gel 60 MS grade (Merck, Art. No. 1.05642).

For HPTLC separation the solvents: diethyl ether, ethanol, tetrahydrofuran, acetic acid, dichloromethane, ethyl acetate, toluene, chloroform, acetone, acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Purified water was produced by a Millipore-Milli-Q integral water purification system (Merck KGaA, Darmstadt, Germany).

Regarding AChE assay, fast Blue B salt, 1-naphthyl acetate, acetylthiocholine iodide (ATCI) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). The 2,2'-Dinitro-5,5'-dithiodibenzoic acid (DTNB) also referred to as Ellman's reagent, tris(hydroxymethyl)aminomethane (TRIS), hydrochloric acid, sodium chloride and magnesium chloride hexahydrate were obtained from Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade or higher purity.

The following buffers were used in the AChE assays. Buffer A: 50 mM Tris-HCl, pH 8; buffer B: 50 mM Tris-HCl, pH 8, containing 0.1 % BSA and buffer C: 50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M MgCl₂•6H₂O.

2.2. Extraction of samples

The phenolic compounds were extracted from avocado by-products (peel, seed and seed coat) according to the optimized PLE methods described in chapter 1 and 2. Furthermore, extracts obtained using ultrasonic assisted extraction (UAE) were used in order to optimize the HPTLC phenolic compound separation. The UAE was performed using an ultrasonic bath (Getidy KDC – 200B, China). Briefly, 5 g of dried avocado by-products (peel, seed and seed coat) were extracted with 25 mL of EtOH:H₂O (1:1, v/v) at 50 °C for 30 min. The obtained extracts were immediately cooled in ice to attain a temperature of 20–25 °C and centrifuged at 9,000 rpm for 12 min at 4 °C in a Universal 32 R centrifuge (Hettich-Zentrifugen, Germany). The extracts were stored at –20 °C until further use. All experiments were performed in triplicate.

2.3. HPTLC assay

The PLE extracts were reconstituted with a mixture of EtOH:H₂O (1:1, v/v) at a concentration of 17 mg/mL, whereas the UAE extracts were used with neither dilution. Then, the extracts were filtered for avoiding possible solid particles using PTFE syringe-filters with a pore size < 0.2 µm (Millipore, Bedford, MA, USA). After that, a volume of 15 µL of extract was applied on the plate with an automatic TLC sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland) as 5 mm band and the distance between two spots was fixed as 10 mm. A very low dosage speed of 40 nL/s was used in order to evaporate the solvent used to reconstitute the extract (EtOH:H₂O 1:1, v/v). From the bottom, left and right of the plate, 10 mm was excluded. The plates

were developed up to 8 cm using 10 mL of mobile phase, which was added only in one trough of an unsaturated twin-trough chamber (CAMAG, Muttenz, Switzerland) for 10 × 10 cm plates. After the mobile phase reached the required distance the plate was moved from the chamber to the fume hood for solvent evaporation. The TLC plate was photographed by Camag Reprostar 3 (Camag, Muttenz, Switzerland) at 254 and 365 nm. At each stage, the angle, exposure and the flux of light were maintained at a fixed level. The HPTLC plate with separated bands was scanned. All instruments were controlled via WinCats software.

In order to obtain the best separation of phenolic compounds, the composition of the mobile phase was optimized through the “PRISMA” method described by Nyiredy (2002). Briefly, the procedure consists of three stages: selection of individual solvents, evaluation different solvent combinations according the upper part of the prism model and fine-tuning of the better solvents combinations.

2.4. Determination of acetylcholinesterase inhibition properties using HPTLC method

The potential of avocado by-products as acetylcholinesterase inhibitors were evaluated with two direct HPTLC methods:

2.4.1. Diazotization method

After developing the HPTLC plate, enzyme inhibitory activities of the developed spots were detected by spraying the substrate, enzyme AchE and dye based on method described by Marston, *et al.* (2002). Firstly, the developed HPTLC plate was sprayed with 1-naphthyl acetate (1mg/mL in MeOH), then the HPTLC plate was dried

with a hair dryer for complete removal of MeOH. After that, the plate was sprayed with acetylcholinesterase enzyme (1.5 U/mL in buffer B) and thoroughly dried again. For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing a little water; by this means, water did not come directly into contact with the plate but the atmosphere was kept humid. The cover was placed on the tank and incubation was performed at 37°C for 20 min. The enzyme had satisfactory stability under these conditions. Finally, for detection of the enzyme a solution of fast blue B salt (1.5 mg/mL in distilled water) was sprayed onto the plate to give a purple coloration after 1–2 min.

2.4.2. Ellman's assay

Enzyme inhibitory activity was also evaluated with Ellman's method (Rhee, *et al.*, 2001). Briefly, the developed HPTLC plate was sprayed with 10 µL of a mixture of the substrate (1 mM ATCI in buffer A) and the dye (1 mM DTNB in buffer A). Nevertheless, the spots ran off must be avoided. After that, the plate was allowed to dry for 3–5 min. Finally, the plate was sprayed with AChE at a concentration of 1.5 U/mL in buffer B. A yellow background appeared, with white spots for inhibiting compounds becoming visible after ~5 min. Nonetheless, the plate must be photographed within 15 min before the reaction colour disappeared (Abou-Donia, *et al.*, 2014). Donepezil at 100 nM was used as positive control.

2.5. HPTLC–QqQ–MS analysis

After scanning the plate, the positions of compounds with AChE activity were marked with a soft pencil. For register the mass spectra of analytes, an HPLC pump

(Waters Assoc., Milford, CT, USA) and TLC-MS interface (Camag, Muttenz, Switzerland) were used. The zone of interest on the HPTLC plate was directly eluted into the electrospray ionization (ESI) source of the LCMS-8030 triple quadrupole mass spectrometer (Shimadzu Corporation, Japan). A mixture of ethanol and water 1:1 (v/v) was used as mobile phase at a flow rate of 0.2 mL/min.

The MS system was operated in full scan mode with the following parameters for ESI in negative ionization mode: the nebulising and drying gas were nitrogen at a flow rate of 3.0 and 15.0 L/min, respectively. The desolvation line temperature was set at 300°C, the spray voltage was fixed at 3500 V and the heat block temperature was 400 °C. Data were processed with LabSolutions LCMS Ver. 5.6 software.

2.6. Microplate assay

The acetylcholinesterase inhibition assay was performed according to Ellman, *et al.* (1961) with some modifications. The principle of the method is the measurement of the hydrolyzing rate of the acetylthiocholine (substrate) resulting in the product thiocholine which reacts with 5:5-dithiobis-2-nitrobenzoate ion (Ellman's reagent) to produce the yellow anion 5-thio-2-nitro-benzoic acid which can be detected at 405 nm (Ellman, *et al.*, 1961; Rhee, *et al.*, 2001). Firstly, Ellman's solution was prepared by mixing 2.5 mL of 15 mM ATCl in water, 12.5 mL of 3 mM DTNB in buffer C and 5 mL of buffer B. Then, 25 µL of sample solution and 200 µL Ellman solution were placed in a well, and the absorbance was measured at 405 nm every 30 s for 10 min. After that, 25 µL AChE solution (0.22 U/mL) were added and the absorbance was again read every 30 s for 10 min. Any increase in absorbance due to the

spontaneous hydrolysis of substrate was corrected by subtracting the rate of the reaction before adding the enzyme from the rate after adding the enzyme. Donepezil was used as reference and distilled water was used as control. The percent inhibition was calculated using following formula:

$$AChE \text{ activity (\%)} = \left(1 - \frac{\text{Reaction rate of sample at 405 nm}}{\text{Reaction rate of control at 405 nm}} \right) * 100$$

3. Results and discussion

3.1. Optimization of the mobile phase for avocado compounds separation

In order to select the appropriate composition of solvents for the separation of compounds present in the avocado extracts, ten experiments were run in TLC using eight groups of solvent as described by Snyder solvent classification (Snyder, 1978). On the basis of the preliminary experiments, toluene, acetone and water were selected based on the observed separation (Figure 1). In this sense, water was chosen because the phenolic compounds are highly polar compounds. Furthermore, toluene was selected since to separate the polar compounds it is necessary to include a solvent in which the compounds do not migrate (Nyiredy, 2002).

The upper part of the prism, which becomes a triangle, represents three solvent mixtures and coordinates as 333 (Sharif, et al., 2015). A total of 36 different compositions (selectivity points) were evaluated and optimized mobile phase was determined based on the number of bands per sample. Finally, a fine adjust was carried out modifying the combination of solvents between two selectivity points

chosen the previous stage. In order to avoid any interference on the enzyme assays in the optimization process no modifier was included into mobile phase composition.

Since the solvent strengths of the selected single solvents were very different, small changes in the selectivity points had a large effect on the separation (Dallenbach-Toelke, et al., 1986). Nevertheless, as a full separation seems to be not achievable with just one combination of solvents, three combinations of solvents were selected in order to separate different type of compounds. The combinations toluene:acetone (2:3, v/v) and toluene:acetone:water (0.75:3.85:0.4, v/v/v) were selected for medium polar and polar compounds, respectively. Furthermore, for high polar compounds two sequential development were necessary, the first development to 8 cm with the mixture toluene:acetone:water (0.75:3.85:0.4, v/v/v) and a second development to 2 cm with the mixture toluene:acetone:water (0.4:3.85:0.5, v/v/v) (Figure 2). For medium polar, polar and high polar compounds the optimized mobile phase were denoted as MP, P and HP, respectively.

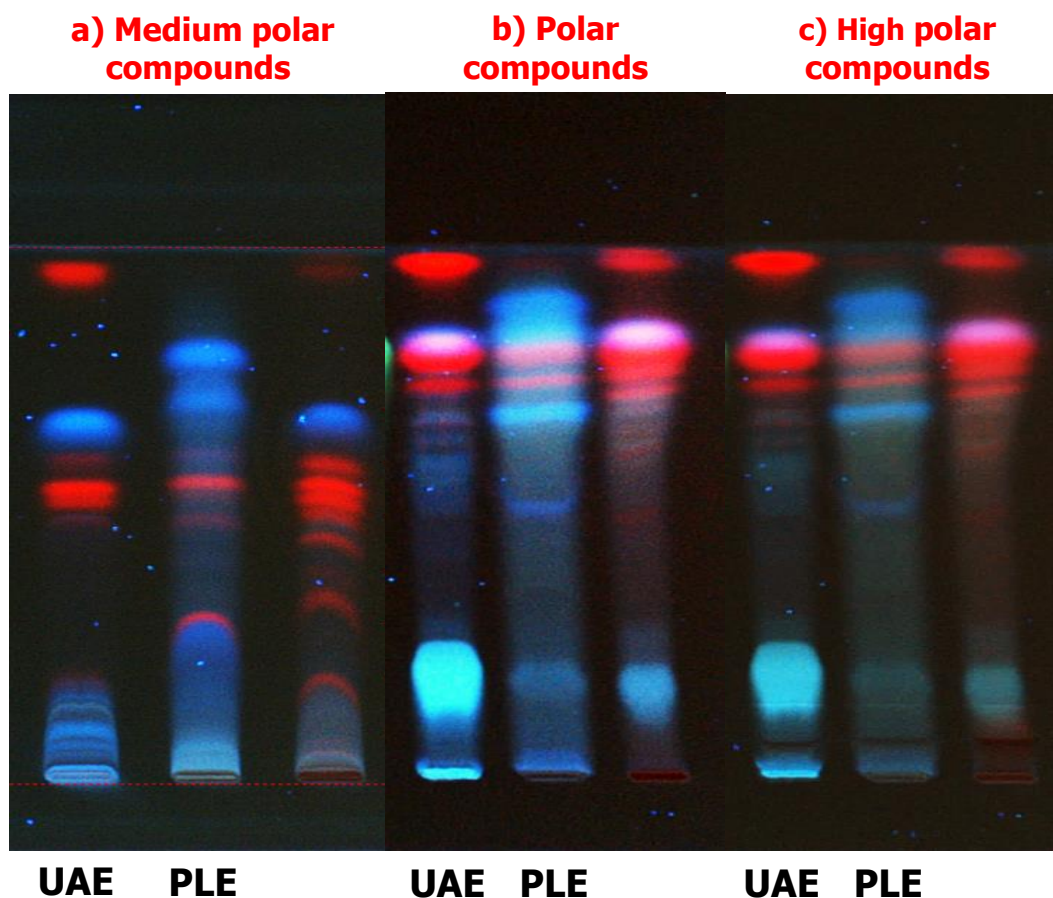


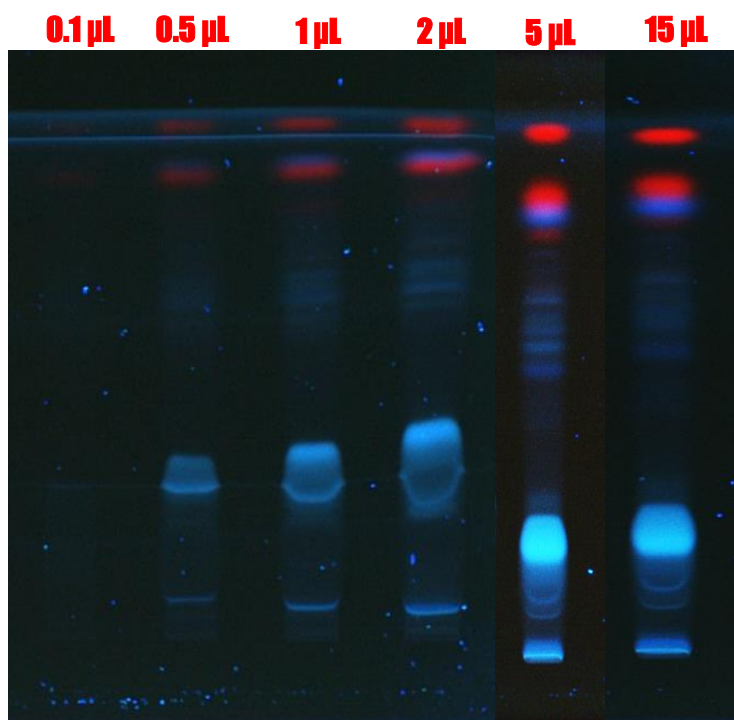
Figure 2.- Optimized mobile phase composition for a) medium polar compounds, toluene:acetone (2:3, v/v); b) polar compounds, toluene:acetone:water (0.75:3.85:0.4, v/v/v) and c) high polar compounds, first development with MP mobile phase to 8 cm and then a second development with toluene:acetone:water (0.4:3.85:0.5, v/v/v) to 2 cm.

Moreover, five different volumes of sample were applied in order to optimize the phenolic compounds separation. As showed in Figure 3a, the amount of sample has an evident effect on the number of bands observed. In this sense, different number of bands were revealed (2, 7, 10, 11, 17 bands) when 0.1, 0.5, 1, 2 and 5 μL of UAE peel extract were applied, respectively.

Furthermore, in order to achieve a best separation ethylenediaminetetraacetic acid (EDTA) at 5 % and phosphate buffer at 0.1M were used to modify the stationary

phase. This optimization was carried out using the mobile phase P. with phosphate buffer, EDTA and without modifier. In the separation a total of 14, 19 and 13 bands were distinguished in avocado peel UAE extracts. Nevertheless, both modifiers were discarded since EDTA presented interference in the AchE assay and a reduction in the bands was observed with buffer phosphate, as can be observed in Figure 3b.

a)



b)

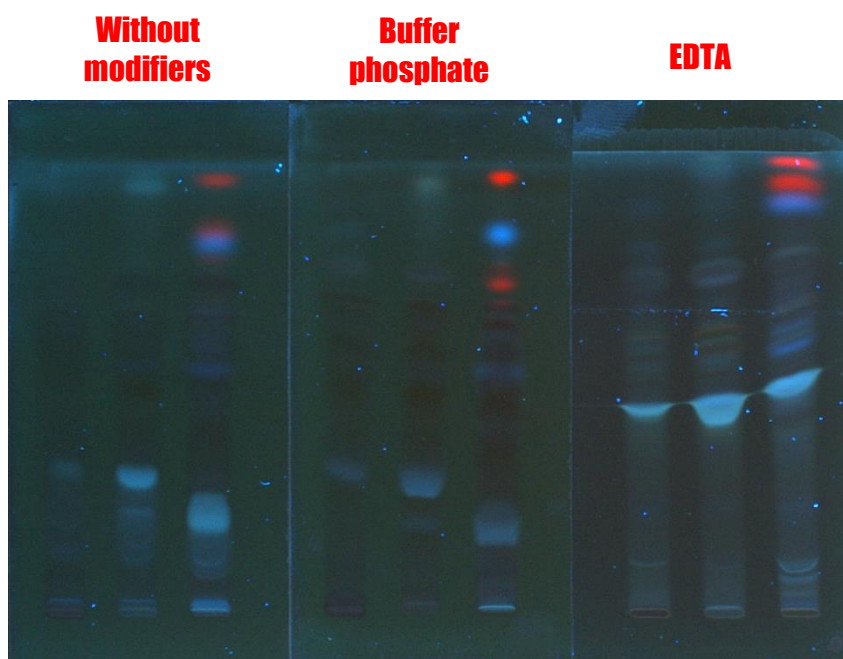


Figure 3.- Optimization of phenolic compounds separation by HPTLC: a) Different volume of sample applied, b) modified stationary phase.

3.2. Screening of avocado by-products as AchE inhibitors

Two methods were used in order to evaluate the antiacetylcholinesterase (AAChE) activity of the avocado by-products extracts obtained with different extraction methods.

Regarding diazotization method, when MP mobile phase was used, AAChE activity was found for seed, seed coat and peel UAE extracts (Figure 4a). Unfortunately, the compounds responsible of this activity could not be identified as the white bands were observed in the spotting line. Besides, when the mobile phase HP was use, intense purple bands were observed (Figure 4b). This could be explained due to polyphenolic compounds react with Fast Blue B salt to form diazonium dyes (Cieśla,

et al., 2015). For that reason false negative results could be observed with this assay when polyphenols are presents in the sample.

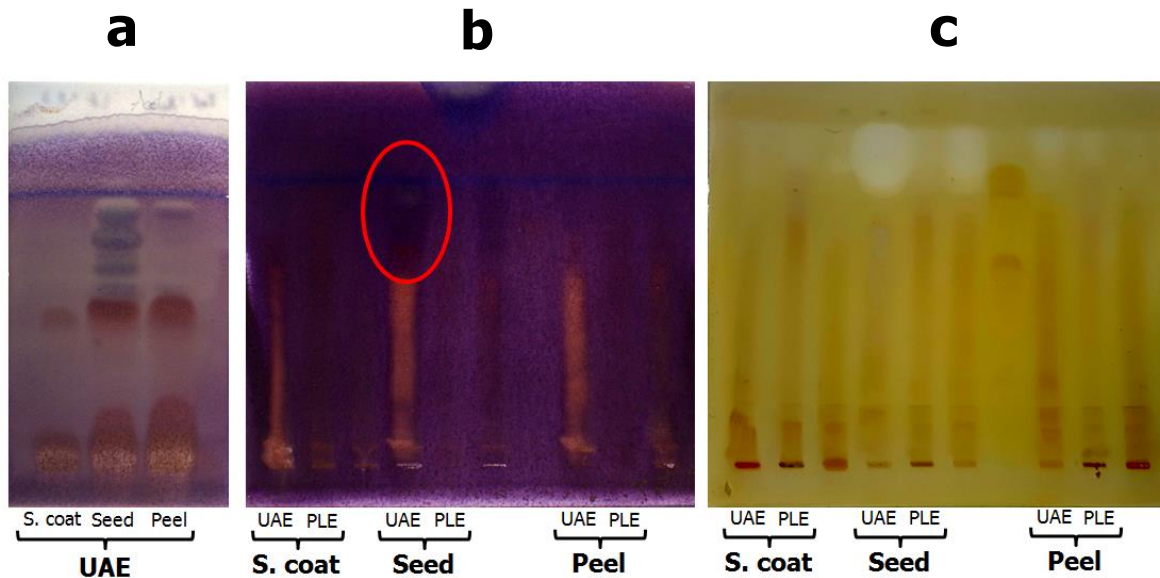


Figure 4.- Comparison of diazotization (a and b) and Ellma'n (c) assays for screening AChE activity from avocado by-products extracts.

On the other hand, AChE activity was found in seed and peel samples when Ellma'n method was used. In addition, those results were obtained for all the extraction methods (UAE and PLE). Nonetheless, the white spots were found in the solvent front when HP mobile phase was used, as can be observed in Figure 4c. Meanwhile, four white bands were seen after the development with MP mobile phase. The white band was more evident in samples obtained with PLE.



Figure 5.- Compounds with AChE activity.

Nevertheless, a possible co-elution was observed in avocado seed extract obtained with UAE (Figure 5). Therefore, a fine-tuning of MP mobile phase was carried out with different ratios of toluene–acetone (v:v). In this sense, when a ratio of 1.7:3.3 (v/v) of toluene–acetone was used, the compounds with AChE activity co-eluted. In the other hand, a new band was observed with a ratio of 2.5:2.5 (v/v) of these solvents (Figure 6).

The identification of avocado compounds with AChE was carried out using mass spectrometry, concretely a single quadrupole mass analyser (Q-MS).

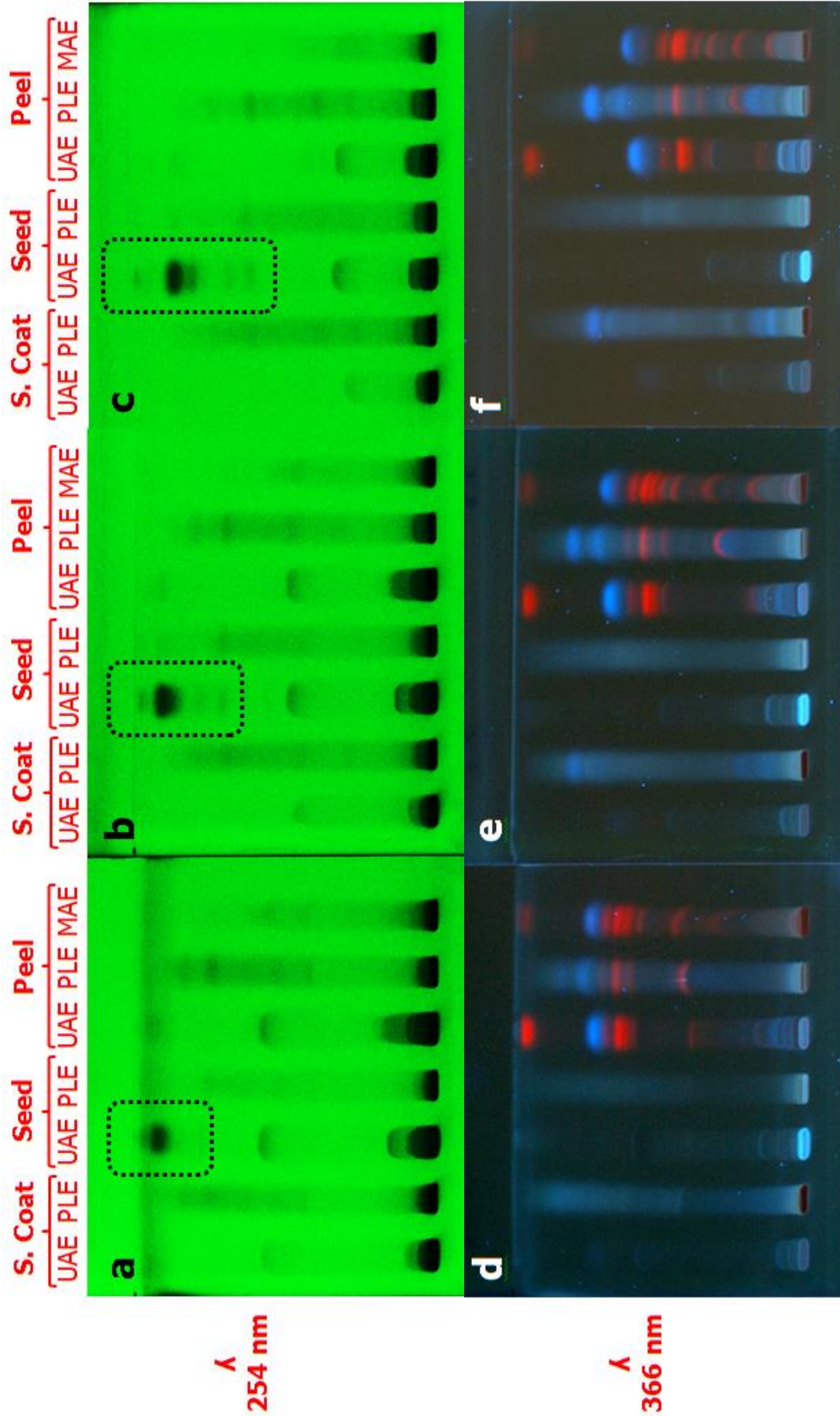


Figure 6.- Identification of compounds with AChE activity. All experiment used a mixture of toluene-acetone (v/v): ratio 1.7:3.3 (a and d); ratio 2:3 (b and e); ratio 2.5:2.5 (c and f).

4. Conclusions:

In this work, a HPTLC method was optimized to separate and identify compounds with AChE activity from avocado by-products. Furthermore, Ellman's method can be used efficiently for qualitative studies of compounds with AChE inhibitory activities in polyphenols-rich extracts. In avocado seed four compounds presented AChE activity.

The identification of compounds by Q-MS with AChE activity is still in progress and it will not be included in this thesis.

5. References:

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