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Programa de Doctorado en Química

CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS EN PRODUCTOS Y SUBPRODUCTOS VEGETALES MEDIANTE TÉCNICAS CROMATOGRÁFICAS ACOPLADAS A ESPECTROMETRÍA DE MASAS

Presentada por

ANA LÓPEZ COBO

para optar al grado de

Doctora en Química por la Universidad de Granada

Dirigida por

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POR

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Granada, 26 de mayo de 2017

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El Dr. D. ALBERTO FERNÁNDEZ GUTIÉRREZ, Catedrático Emérito de Universidad en el Departamento de Química Analítica "Profesor Fermín Capitán" y en el Instituto de Nutrición y Tecnología de los Alimentos "José Mataix" y director del Centro de Investigación y Desarrollo del Alimento Funcional (CIDAF) de la Universidad de Granada y Dra. Dª ANA MARÍA GÓMEZ CARAVACA, Profesora Contratada Doctora del Departamento de Química Analítica de la Universidad de Granada,

CERTIFICAN

Que el trabajo que se presenta en esta tesis doctoral bajo el título: **"CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS EN PRODUCTOS Y SUBPRODUCTOS VEGETALES MEDIANTE TÉCNICAS CROMATOGRÁFICAS ACOPLADAS A ESPECTROMETRÍA DE MASAS",** ha sido realizado bajo nuestra dirección en los laboratorios del Departamento de Química Analítica de la Universidad de Granada y del Centro de Investigación y Desarrollo del Alimento Funcional (CIDAF) y que reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dª Ana López Cobo pueda optar al grado de Doctora en Química por la Universidad de Granada.

Y para que conste, expedimos y firmamos el presente certificado en Granada a 26 de mayo de 2017.

Fdo: D. Alberto Fernández Gutiérrez Fdo: Dª Ana María Gómez Caravaca

La doctoranda Ana López Cobo y los directores de la tesis, Dr. D. Alberto Fernández Gutiérrez y Dra. Dª Ana María Gómez Caravaca:

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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Objetivos

La importancia de la alimentación en la prevención de ciertas enfermedades ha promovido, en los últimos años, la investigación acerca de los compuestos bioactivos presentes en los vegetales. Estos compuestos bioactivos se encuentran tanto en las partes comestibles de los alimentos como en aquellas que habitualmente se desechan. Debido a que la gestión de los desechos producidos por la industria alimentaria puede suponer un problema ambiental y económico, su utilización eficaz y sostenible está cobrando cada día mayor importancia. Por estos motivos, existe un creciente interés en el uso de los subproductos como fuente de compuestos bioactivos que puedan emplearse en la producción de ingredientes funcionales que aumenten el valor nutricional de los alimentos, y que permitan una revalorización de estos subproductos.

En base a esto, el objetivo principal de la tesis doctoral que se presenta en esta memoria será la búsqueda de matrices vegetales y subproductos de los mismos que tengan interés en su empleo como posibles fuentes de compuestos bioactivos, y la posterior caracterización cualitativa y cuantitativa de los mismos mediante técnicas cromatográficas avanzadas que permitan el desarrollo de métodos rápidos, eficaces y fiables para la determinación de este tipo de compuestos.

Para el desarrollo de este objetivo principal establecemos los siguientes objetivos específicos:

- Seleccionar diferentes matrices vegetales de interés (patata, aguacate, mango y ajedrea) y los subproductos generados en el procesado industrial de las mismas, que puedan utilizarse como fuente de compuestos bioactivos: principalmente compuestos fenólicos, alquilresorcinoles y esteroles.

- Obtener extractos enriquecidos en los compuestos bioactivos de interés mediante técnicas de extracción adecuadas, como sólido-líquido (SLE) y líquido-líquido (LLE).

- Desarrollar metodologías para el análisis de los compuestos de interés encontrados utilizando técnicas separativas avanzadas como la cromatografía líquida de alta resolución (HPLC) y la cromatografía de gases (CG) acopladas a diferentes sistemas de detección: espectrofotometría ultravioleta-visible (UV-Vis), fluorimetría (FLD) y espectrometría de masas (MS) con analizadores de cuadrupolo, tiempo de vuelo y cuadrupolo-tiempo de vuelo.

- Identificar y cuantificar los compuestos bioactivos objeto de estudio mediante la información proporcionada por los detectores utilizados junto con la información disponible en la literatura.

- Evaluar la distribución de los compuestos bioactivos en los productos y subproductos de las matrices seleccionadas.

- Determinar y comparar la actividad antioxidante de las matrices seleccionadas mediante los métodos ABTS (ácido 2,2'-azino-bis (3-ethilbenzotiazolin-6-sulfónico)), FRAP (poder antioxidante reductor del hierro), DPPH (2,2-difenil-1-picrilhidrazilo) y ORAC (capacidad de absorción de radicales de oxígeno).

Resumen de esta memoria doctoral

La importancia que supone una correcta alimentación con el fin de conseguir y mantener una buena salud y prevenir la aparición de diversas patologías en el ser humano se encuentra ampliamente reconocida en todas las fases de la vida. Los compuestos bioactivos presentes en los vegetales y que se ingieren en la dieta, son capaces de disminuir el riesgo de padecer multitud de enfermedades crónico-degenerativas y atenuar sus efectos cuando estas ya están en curso. Además, los compuestos bioactivos también están presentes en aquellas partes de los vegetales que habitualmente no se destinan al consumo y son desechadas como desperdicios. A nivel industrial, el aprovechamiento de estos desechos abre nuevas posibilidades a potenciales fuentes de compuestos bioactivos, además, de esta manera se disminuyen los residuos lo que repercute a nivel ambiental y económico.

La presente memoria recoge los resultados obtenidos durante la realización de la tesis doctoral titulada: "CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS EN PRODUCTOS Y SUBPRODUCTOS VEGETALES MEDIANTE TÉCNICAS CROMATOGRÁFICAS ACOPLADAS A ESPECTROMETRÍA DE MASAS" en la que se aporta nuevo conocimiento científico acerca de vegetales ampliamente consumidos en España y en el mundo como: ajedrea, patata, aguacate y mango,. Concretamente, se aborda la identificación y la cuantificación de compuestos fenólicos, alquilresorcinoles y esteroles de las partes comestibles y los subproductos de estos. Además se hace un estudio de la actividad antioxidante de las diferentes especies y sus subproductos. La memoria se estructura en tres secciones principales: (1) Introducción, (2) Parte experimental, resultados y discusión y (3) Conclusiones.

La primera parte de la **Introducción** presenta los vegetales y sus subproductos como fuentes de compuestos bioactivos. Seguidamente se hace

una clasificación de los compuestos bioactivos profundizando en las familias estudiadas en esta tesis doctoral como los compuestos fenólicos, alquilresorcinoles y esteroles vegetales, y en sus beneficios para la salud. A continuación, se expone el estado actual de las matrices estudiadas (ajedrea, patata, aguacate y mango) y los subproductos de estas, así como su contenido en compuestos bioactivos. Más adelante se presentan los métodos de pretratamiento y tratamiento de las muestras, en concreto se resumen los tipos de secado más empleados para matrices vegetales, haciendo énfasis en el secado al aire y en la liofilización, y las técnicas de extracción de compuestos bioactivos, centrándose en la extracción sólido-líquido (SLE). Posteriormente, se detallan las técnicas cromatográficas empleadas para la separación (HPLC y GC), ionización (EI y ESI) y detección (DAD, FLD y MS con analizadores TOF y QTOF) de los compuestos bioactivos objeto de estudio.

La **Parte experimental, resultados y discusión** consta de 6 capítulos. El **Capítulo 1** recoge el trabajo realizado para la identificación y cuantificación de la fracción fenólica de la parte aérea de una variedad de ajedrea endémica del Este de Serbia (*Satureja montana* subsp. *kitaibelii*). La técnica analítica empleada fue HPLC-DAD-ESI-TOF-MS, siendo la primera vez que esta técnica se utilizaba para determinar los compuestos fenólicos en *Satureja montana* subsp. *kitaibelii*. Además, se llevó a cabo la determinación de la capacidad antioxidante de la muestra mediante los ensayos ABTS y DPPH.

El **Capítulo 2** describe el estudio de la distribución de los compuestos fenólicos y otros compuestos polares en el tubérculo de patata (*Solanum tuberosum* L.). Se llevó a cabo la identificación y cuantificación de estos compuestos en todo el tubérculo, así como en la piel y en la pulpa por

separado, en dos variedades de patata: 'Blue Bell', cuya piel está parcialmente pigmentada de azul, y 'Melody', una variedad de pulpa y piel blancas. La determinación se realizó mediante HPLC-DAD-ESI-QTOF-MS en los modos negativo y positivo. Los datos obtenidos mediante espectrofotometría UV-Vis, los espectros de masas (MS) y MS/MS, junto a la bibliografía previa permitió la identificación y cuantificación de numerosos compuestos. Además, el estudio se completó con la determinación de la actividad antioxidante de las dos fracciones y de su conjunto mediante los ensayos ORAC, FRAP y ABTS.

El **Capítulo 3** presenta el estudio comparativo de los compuestos fenólicos y otros compuestos polares de la parte comestible y subproductos (piel y hueso) del aguacate (*Persea americana* Miller), concretamente de la variedad 'Hass', una de las más consumidas en el mundo. Los análisis se realizaron con aguacates en el punto óptimo de maduración para el consumo y con aguacates con un exceso de maduración y que, por lo tanto, pueden suponer un desecho para la empresa. Para llevar a cabo los análisis se emplearon dos técnicas: 1) HPLC-DAD-ESI-QTOF-MS, que se utilizó por primera vez para la determinación de los compuestos fenólicos en piel y hueso de aguacate, y 2) HPLC-FLD-MS para la identificación y cuantificación de flavan-3-oles.

El **Capítulo 4** se centra en la identificación y cuantificación de compuestos fenólicos libres y enlazados, además de otros compuestos de naturaleza polar que se encuentran en la pulpa de mango de la variedad 'Keitt' (*Mangifera indica* L.), así como en sus subproductos, piel y hueso. Cabe destacar que es la primera vez que se ha realizado este tipo de análisis en la cáscara del hueso de mango (endocarpio). Además, también es la primera vez que se han determinado los compuestos fenólicos enlazados en estas cuatro fracciones

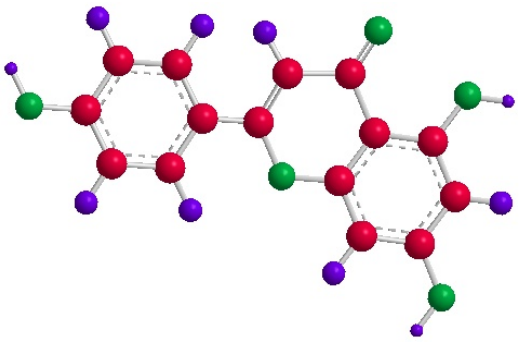
de mango. Los análisis se llevaron a cabo mediante HPLC-DAD-ESI-QTOF-MS demostrándose la eficacia de esta herramienta para la identificación y cuantificación de compuestos fenólicos en mango.

El **Capítulo 5** presenta la determinación de diferentes familias de derivados fenólicos (compuestos fenólicos libres y enlazados, incluyendo antocianinas) y alqu(en)ilresorcinoles, en la parte comestible y subproductos (piel, hueso y cáscara del hueso) de tres variedades de mango (*Mangifera indica* L.) de elevado interés comercial como 'Keitt', 'Osteen' y 'Sensación'. Los análisis fueron realizados con HPLC-DAD-ESI-QTOF-MS en los modos de ionización positivo para antocianinas, y negativo para el resto de compuestos fenólicos, y con GC-QTOF-MS para la determinación de alquilresorcinoles. Además se llevaron a cabo las medidas de la actividad antioxidante de las cuatro fracciones de mango de las tres variedades mediante los métodos FRAP y ABTS.

El **Capítulo 6** muestra el último trabajo llevado a cabo en la presente tesis doctoral y describe la caracterización y cuantificación de esteroles y tocoferoles en pulpa y subproductos (piel, hueso y cáscara del hueso) de tres variedades de mango ('Keitt', 'Osteen' y 'Sensación'). La técnica empleada para los análisis fue GC-QTOF-MS y, con ella, se desarrollaron dos metodologías con columnas cromatográficas de última generación: HP-5ms and Rtx-200MS. En este capítulo se comparan los parámetros de validación de ambos métodos, así como los resultados obtenidos con ambas columnas acerca de la cuantificación de los compuestos objeto de estudio.

La última sección de esta memoria recoge las **Conclusiones** donde se exponen los avances científicos más relevantes producidos en esta tesis doctoral.

Introducción



La introducción de esta memoria de tesis doctoral consta de 4 apartados principales. En el primero de ellos se presentan los vegetales y sus subproductos como fuentes ricas en compuestos bioactivos, y se hace una clasificación de estos profundizando en las familias estudiadas en esta tesis doctoral: los compuestos fenólicos, alquilresorcinoles y esteroles vegetales. Seguidamente, en el segundo apartado se describen las matrices estudiadas y se expone el estado actual de cada una de ellas: ajedrea, patata, aguacate y mango, así como sus subproductos. A continuación, se presentan los métodos de pretratamiento y tratamiento de las muestras, en concreto las técnicas de secado empleadas en esta tesis doctoral (secado al aire y liofilización), y las técnicas de extracción de compuestos bioactivos utilizadas. Finalmente, el último apartado de esta introducción detalla las técnicas cromatográficas empleadas para la separación (HPLC y GC), ionización (EI y ESI) y detección (DAD, FLD y MS con analizadores TOF y QTOF) de los compuestos bioactivos estudiados.

A. PRODUCTOS DE ORIGEN VEGETAL COMO FUENTE DE COMPUESTOS BIOACTIVOS

La utilización de las plantas para la prevención y tratamiento de enfermedades ha acompañado a la humanidad desde hace miles de años. Estas se han consumido directamente a través de la dieta, en forma de preparado para ingerir o aplicadas en forma de cataplasmas. Inicialmente, el hombre usó las plantas a imitación de los animales guiado por su instinto,

más tarde empíricamente, y por último de forma más racional, a partir del conocimiento progresivo de sus propiedades terapéuticas[1].

Estudios epidemiológicos han señalado que el consumo de frutas y vegetales proporciona beneficios para la salud, como la reducción del riesgo de padecer enfermedades coronarias e infarto o ciertos tipos de cáncer. Estos beneficios para la salud se les atribuyen principalmente a la fibra alimentaria y a micronutrientes orgánicos como carotenoides, compuestos fenólicos, tocoferoles, vitamina C, y otros. Por ello se recomienda un consumo mínimo diario de cinco frutas y verduras, particularmente verduras verdes y amarillas y frutas cítricas[2].

Aunque los consumidores están cada vez más informados acerca de la relación entre dieta y salud[3], un gran grupo de la población no alcanza el consumo mínimo recomendado de frutas y verduras. Por tanto, los suplementos alimenticios y los alimentos enriquecidos pueden ser una alternativa al bajo consumo de vegetales. Para obtener estos suplementos y enriquecer alimentos, los ingredientes funcionales provenientes de fuentes vegetales son una alternativa, ya que pueden sustituir al uso de aditivos sintéticos cada vez más rechazados por los consumidores. Esto es particularmente válido para los compuestos fenólicos que a diferencia de

[1] Muñoz López de Bustamente, F. Las plantas medicinales. In *Plantas Medicinales y Aromáticas. Estudio, Cultivo y Procesado.*; Muñoz López de Bustamente, F., Ed.; Mundi-Prensa, **2002**; pp 15–24.

[2] Heimendinger, J.; Chapelsky, D. The national 5 a day for better health program. In *Dietary Phytochemicals in Cancer Prevention and Treatment*; American Institute for Cancer Research, Ed.; Springer, **1996**; Vol. 401, pp 199–206

[3] Gilbert, L. The consumer market for functional foods. *J. Nutraceuticals, Funct. Med. Foods* **1997**, *1* (3), 5–21.

carotenoides y vitaminas no se sintetizan químicamente y tienen que ser extraídos de la materia vegetal[4].

Entre las fuentes vegetales más ricas en compuestos bioactivos encontramos productos y subproductos de la industria alimentaria, así como plantas medicinales. A continuación se resume el interés en la revalorización de estas matrices como fuente de ingredientes funcionales.

A.1. Productos y subproductos de la industria alimentaria

Muchos de los productos vegetales que son cosechados experimentan uno o más procesos de manipulación industrial para dar lugar a productos listos para el consumo. El procesado es una forma de reducir pérdidas en periodos donde hay picos de producción y de maximizar el potencial de la cosecha a través de productos variados, incluidos zumos, néctar y otros. Además, el procesado posibilita el suministro de los productos de la cosecha durante todo el año, y también permite el uso de la parte de la cosecha con peor aspecto que no puede ser vendida directamente en el mercado[5]. De esta transformación surgen subproductos como pieles, huesos, pulpas resultantes de la extracción del jugo, etc. Así por ejemplo, los zumos de fruta y productos derivados como los néctares y las bebidas han experimentado una creciente popularidad en los últimos años[4]. Entre otras razones, el aumento del consumo y exportación de zumos procesados de fruta, pulpas y

[4] Schieber, A.; Stintzing, F. C.; Carle, R. By-products of plant food processing as a source of functional compounds — recent developments. *Trends Food Sci. Technol.* **2002**, *12*, 401–413.

[5] Ribeiro, S. M. R.; Schieber, A. Bioactive compounds in mango (*Mangifera indica* L.). In *Bioactive Foods in Promoting Health*; Watson, R. R.; Preedy, V. R., Ed.; Academic Press, **2010**; pp 507–523.

concentrados se puede atribuir a la mejora en el transporte y sistemas de distribución, y a la mejora del cultivo y los métodos de procesado[4].

La producción en agricultura es cada vez mayor. Según los datos más recientes disponibles, en el año 2014 se produjeron, por ejemplo, más de 1.037 millones de toneladas de maíz, más de 740 millones de toneladas de arroz y más de 380 millones de toneladas de patata en el mundo según la FAOSTAT[6] (**Figura 1**).

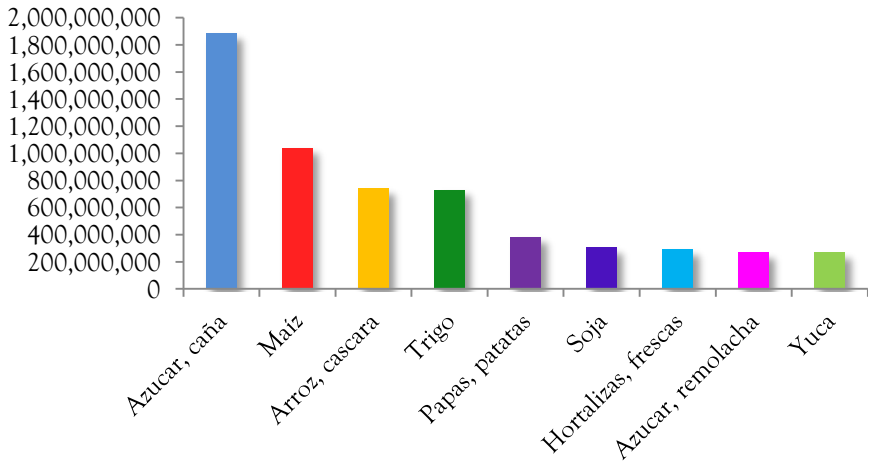


Figura 1. Producción de productos alimentarios y agrícolas en el mundo.

Debido al incremento en la producción, los materiales de desecho representan un creciente problema ya que el material vegetal normalmente es propenso a la degradación por microorganismos, limitando así una explotación mayor. Por otro lado, los costes de secado, almacenaje y transporte de los subproductos son factores limitantes desde el punto de vista económico. Por tanto, los desperdicios agroindustriales son a menudo

[6] FAOSTAT (Food and Agriculture Organization of the United Nations). Base de datos. Dirección de estadística. http://www.fao.org/faostat/es/#data/QC/visualize (acceso Mayo, 2017).

utilizados como alimento para animales o fertilizantes. Sin embargo, la demanda de alimento puede variar y depender de las cosechas. Además, el problema de deshacerse de los subproductos se ve agravado por las restricciones legales. Así, la utilización eficaz, económica y sostenible de los subproductos de la industria alimentaria desde el punto de vista ambiental está cobrando más importancia, especialmente debido a que la rentabilidad puede verse perjudicada[7].

A pesar de ello, existen ya estudios en los que los residuos de la agroindustria de frutas se utilizan para la generación de productos de valor añadido, lo que permite mitigar problemas ambientales como la propagación de plagas, malos olores, contaminación de suelos y tanques de agua, entre otros. Los residuos del procesado de la guayaba, por ejemplo, se han utilizado para la producción de etanol, compuestos antimicrobianos, biomasa ácido láctica y ácido láctico[8]. Las semillas de algunas frutas como el mango se emplean para la extracción de ácidos grasos[9]. Los residuos de plátano son útiles en las formulaciones para alimentación animal[10], y los de piña se utilizan como

[7] Lowe, E. D.; Buckmaster, D. R. Dewatering makes big difference in compost strategies. *Biocycle* **1995**, *36*, 74–77.

[8] Serna-Cock, L.; Mera-Ayala, J. D.; Angulo-López, J. E. Guava *Psidium guajava* seed flour and dry *Aspergillus niger* mycelium as nitrogen sources for the production of biomass and antimicrobial compounds produced by *Weissella confusa*. *Electron. J. Biotechnol.* **2013**, *16* (6), 1-9.

[9] Sogi, D. S.; Siddiq, M.; Greiby, I.; Dolan, K. D. Total phenolics, antioxidant activity, and functional properties of 'Tommy Atkins' mango peel and kernel as affected by drying methods. *Food Chem.* **2013**, *141* (3), 2649–2655.

[10] Dormond, H.; Rojas, A.; Boschini, C.; Mora, G.; Sibaja, G. Evaluación preliminar de la cáscara de banano maduro como material de ensilaje, en combinación con pasto King grass (*Pennisetum purpureum*). *Rev. electrónica las sedes Reg. la Univ. Costa Rica* **2011**, *23* (12), 17–31.

sustrato para la síntesis de ácido láctico[11]; las pieles de varias frutas como la sandía, también se emplean para la extracción de pectina[12]. Las semillas de uvas son utilizadas para extraer antioxidantes y aceites con altos contenidos de ácidos grasos insaturados[13]. Los estudios de Kim *y col.* (2012)[14] muestran que los extractos etanólicos de piel de mango de la variedad 'Irwin' ayudan a la prevención del cáncer de cuello uterino. Además, a partir de los subproductos vegetales es posible obtener fibras dietéticas de alto valor prebiótico[15], así como compuestos antioxidantes[16].

Hay un gran interés en la utilización de antioxidantes naturales como ingredientes funcionales en la formulación de alimentos debido a que estos garantizan la protección de los constituyentes celulares contra el daño oxidativo y limitan el riesgo de la aparición de enfermedades degenerativas relacionadas con el estrés oxidativo[17,18]. Por estas razones, el uso de los

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[11] Araya-Cloutier, C.; Rojas-Garbanzo, C.; Velázquez-Carillo, C. Síntesis de ácido láctico, a través de la hidrolisis enzimática simultanea a la fermentación de un medio a base de un desecho de piña (*Ananas comosus*), para su uso como materia prima en la elaboración de ácido poliláctico. *Rev. Iberoam. polímeros* **2010**, 11 (7), 407–416.

[12] Prakash Maran, J.; Sivakumar, V.; Thirugnanasambandham, K.; Sridhar, R. Microwave assisted extraction of pectin from waste *Citrullus lanatus* fruit rinds. *Carbohydr. Polym.* **2014**, *101* (1), 786–791.

[13] Maier, T.; Schieber, A.; Kammerer, D. R.; Carle, R. Residues of grape (*Vitis vinifera* L.) seed oil production as a valuable source of phenolic antioxidants. *Food Chem.* **2009**, *112* (3), 551–559.

[14] Kim, H. H.; Kim, H. H.; Mosaddik, A.; Gyawali, R.; Ahn, K. S.; Cho, S. K. Induction of apoptosis by ethanolic extract of mango peel and comparative analysis of the chemical constitutes of mango peel and flesh. *Food Chem.* **2012**, *133* (2), 416–422.

[15] O´Shea, N.; Arendt, E. K.; Gallagher, E. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. *Innov. Food Sci. Emerg. Technol.* **2012**, *16*, 1–10.

[16] Fernández-Ponce, M. T.; Casas, L.; Mantell, C.; Rodríguez, M.; Martínez De La Ossa, E. Extraction of antioxidant compounds from different varieties of *Mangifera indica* leaves using green technologies. *J. Supercrit. Fluids* **2012**, *72*, 168–175.

[17] Scalbert, A.; Manach, C.; Morand, C.; Rémésy, C.; Jiménez, L. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45* (4), 287–306.

subproductos de la industria alimentaria para la producción de ingredientes funcionales que aumenten el valor nutricional de los alimentos a los que se adicionan supone una revalorización de estos subproductos que resulta muy atractiva desde un punto de vista económico[19,20].

A.2. Plantas medicinales

Se definen como plantas medicinales a aquellos vegetales que sintetizan principios activos capaces de ejercer una acción farmacológica, beneficiosa o perjudicial, sobre el organismo. Su utilidad primordial, a veces específica, es servir como droga o medicamento que alivie la enfermedad o restablezca la salud perdida; es decir, que tiendan a disminuir o neutralizar el desequilibrio orgánico que es la enfermedad. Constituyen aproximadamente la séptima parte de las especies de plantas existentes. La repartición mundial de las especies medicinales depende de factores ecológicos, donde el suelo y sobre todo el clima son decisivos. Hay especies calcícolas y calcífugas y otras que soportan intervalos amplios del pH del suelo1.

En cuanto al clima, la temperatura media así como las temperaturas máximas y mínimas, son de gran importancia para la distribución de la flora medicinal. Algunas plantas no soportan las heladas (albahaca), mientras que otras bienales precisan haber estado sometidas al frío del invierno anterior para florecer (beleño). La humedad es primordial para algunas especies, por

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[18] Al-Weshahy, A.; El-Nokety, M.; Bakhete, M.; Rao, V. Effect of storage on antioxidant activity of freeze-dried potato peels. *Food Res. Int.* **2013**, *50* (2), 507–512.

[19] Mohdaly, A. A. A.; Sarhan, M. A.; Smetanska, I.; Mahmoud, A. Antioxidant properties of various solvent extracts of potato peel, sugar beet pulp and sesame cake. *J. Sci. Food Agric.* **2010**, *90* (2), 218–226.

[20] Akyol, H.; Riciputi, Y.; Capanoglu, E.; Caboni, M. F.; Verardo, V. Phenolic compounds in the potato and its byproducts: An overview. *Int. J. Mol. Sci.* **2016**, *17* (6), 835-853.

lo que viven al lado del agua (salicaria) o en prados húmedos (cólchico) mientras que otras son xerófilas (sen). Unas especies buscan el sol (lavanda), mientras que algunas requieren la sombra (vinca).

Tradicionalmente, los productos naturales de plantas han sido aportados a la industria farmacéutica como una de sus fuentes más importantes de compuestos líderes en la búsqueda de nuevas drogas y medicinas[21]. Según consta en diversos testimonios históricos pertenecientes a distintas civilizaciones y culturas que han ido sucediéndose en nuestro planeta, el aprovechamiento por parte del hombre de las plantas aromático-medicinales se remonta a la más remota antigüedad. Por ejemplo, en el Papiro de Ebers (2278 a. C.) y en el de Smith (2263 a. C.) se citan una serie de drogas, haciendo referencia desde el cultivo de la planta hasta cómo prepararlas (ej. adormidera), y también es sabido que los indios peruanos cultivaban el árbol de la coca[1]. En la historia más reciente, el uso de plantas como medicina llevó al aislamiento de compuestos bioactivos, comenzando con la morfina del opio a prinpicios del siglo XIX[22,23] hasta drogas como cocaína, codeína, digitoxina y quinina, algunas de las cuales se siguen usando en la actualidad[23-25]. Los análisis llevados a cabo por la revista *Annual Reports of Medicinal Chemistry* durante el periodo 1989-1995, concluyeron que el 60% de las drogas aprobadas y los candidatos pre-NDA (pre-New Drug

[21] Haslam, E. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J. Nat. Prod.* **1996**, *59* (2), 205–215.

[22] Kinghorn, A. D. Pharmacognosy in the 21st century. *J. Pharm. Pharmacol.* **2001**, *53* (2), 135–148.

[23] Samuelsson, G.; Bohlin, L. *Drugs of Natural Origin: A Treatise of Pharmacognosy*; Swedish Pharmaceutical Society, **2017**.

[24] Newman, D. J.; Cragg, G. M.; Snader, K. M. The influence of natural products upon drug discovery. *Nat. Prod. Rep.* **2000**, *17* (3), 215–234.

[25] Butler, M. S. The role of natural product chemistry in drug discovery. *J. Nat. Prod.* **2004**, *67* (12), 2141–2153.

Application) (excluyendo los de origen biológico) con aplicaciones anticancerígenas y antiinfecciosas, son de origen vegetal. Las drogas de origen natural son clasificadas como: productos naturales originarios, productos derivados de forma semisintética y productos sintéticos basados en modelos de productos naturales[26].

En los últimos años, las plantas están recibiendo cada vez mayor atención. Así, Farnsworth *y col.* han descrito que al menos 119 compuestos derivados de 90 especies de plantas se pueden considerar drogas importantes de uso actual, siendo el 77% de estos derivados de plantas usadas en medicina tradicional[27]. El hecho de que más de tres cuartas partes de la población de los países desarrollados use plantas medicinales para el cuidado básico de su salud y que el 80% de la población mundial dependa de la medicina tradicional como principal asistencia sanitaria según la OMS (Organización Mundial de la Salud) ofrece más evidencias de la importancia de estos productos.

A pesar de la existencia de otros métodos para descubrir drogas, los productos naturales siguen ofreciendo un número equivalente de nuevos candidatos a drogas. Esto fue demostrado por Newman *y col.* que analizaron el número de éstas derivadas de productos naturales presentes en las drogas lanzadas desde 1981 a 2002[26,28], concluyendo que los productos naturales seguían siendo una importante fuente de drogas, especialmente en áreas terapéuticas de cáncer e hipertensión[28].

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[26] Cragg, G. M.; Newman, D. J.; Snader, K. M. Natural products in drug discovery and development. *J. Nat. Prod.* **1997**, *60* (1), 52–60.

[27] Farnsworth, N. R.; Akerele, O.; Bingel, A. S.; Soejarto, D. D.; Guo, Z. Medicinal plants in therapy. *Bull. World Health Organ.* **1985**, *63* (6), 965–981.

[28] Newman, D. J.; Cragg, G. M.; Snader, K. M. Natural products as sources of new drugs over the period 1981-2002. *J. Nat. Prod.* **2003**, *66* (7), 1022–1037.

Entre las aplicaciones industriales actuales de las plantas encontramos[1]:

- Fitosanitaria: herbicidas, insecticidas, fungicidas, nematicidas, acaricidas, helícidas.
- Alimentaria: aditivos naturales, saborizantes, colorantes, antioxidantes y conservantes, dietética, licorería, nutrición animal.
- Farmacéutica: extractos, medicamentos, medicamentos de hemisínteis, herboristería, homeopatía, fármacos para veterinaria.
- Perfumero-cosmética: colonias, perfumes, cosméticos, lociones, jabones, dentífricos, sales de baño.
- Tintorera: azul, verde, amarillo, rosa, rojo, ocre, beige.

A.3. Compuestos bioactivos

Los cambios en la nutrición actual han repercutido considerablemente en la salud. Un mayor conocimiento del impacto de la dieta en la regulación a nivel genético y molecular está cambiando el modo en que consideramos el papel de la nutrición, resultando en nuevas estrategias de la dieta. Hasta hace relativamente poco tiempo, las vitaminas y otros micronutrientes se recomendaban sólo para evitar los síntomas de su deficiencia. Hoy en día, además de suministrar nutrientes de calidad en cantidades adecuadas, se cree que una dieta saludable debe tener características adicionales como contribuir a la protección contra enfermedades. Esta protección se consigue por la presencia de compuestos bioactivos en alimentos funcionales, los cuales se caracterizan por ser alimentos que pueden proporcionar un

beneficio para la salud además de los nutrientes que contienen[29]. Se definen los compuestos bioactivos como **"compuestos esenciales y no esenciales (ej. vitaminas o compuestos fenólicos) presentes en la naturaleza, que forman parte de la cadena alimenticia, y que haya sido demostrado tengan un efecto sobre la salud humana"**[30]. Las sustancias bioactivas presentes como constituyentes naturales en los alimentos proporcionan beneficios para la salud subyacentes al valor nutricional básico del producto[30].

A través de varios estudios se ha comprobado que un elevado consumo de frutas y verduras está asociado con una menor incidencia y tasa de varias enfermedades degenerativas como el cáncer [31, 32], las enfermedades cardiovasculares [33-35] y la disfunción inmunológica [36]. Los compuestos bioactivos tienen sobre todo alta capacidad antioxidante y pueden proteger a

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[29] Scrinis, G. Functional foods or functionally marketed foods? A critique of, and alternatives to, the category of "functional foods". *Public Health Nutr.* **2008**, *11* (5), 541–545.

[30] Biesalski, H. K.; Dragsted, L. O.; Elmadfa, I.; Grossklaus, R.; Müller, M.; Schrenk, D.; Walter, P.; Weber, P. Bioactive compounds: Definition and assessment of activity. *Nutrition* **2009**, *25* (11–12), 1202–1205.

[31] Doll, R. An overview of the epidemiological evidence linking diet and cancer. *Proc. Nutr. Soc.* **1990**, *49* (2), 119–131.

[32] Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56* (11), 317–333.

[33] Hertog, M. G. L.; Feskens, E. J. M.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007–1011.

[34] Hertog, M. G. L.; Kromhout, D.; Aravanis, C.; Blackburn, H.; Buzina, R.; Fidanza, F.; Giampaoli, S.; Jansen, A.; Menotti, A.; Nedeljkovic, S.; *et al.* Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. *Arch. Intern. Med.* **1995**, *155*, 381–386.

[35] Hertog, M. G. L.; Sweetnam, P. M.; Fehily, A. M.; Elwood, P. C.; Kromhout, D. Antioxidant flavonols and ischemic heart disease in a Welsh population of men: The Caerphilly Study. *Am. J. Clin. Nutr.* **1997**, *65* (5), 1489–1494.

[36] Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90* (17), 7915–7922.

las células contra el daño oxidativo causado por los radicales libres[37]. El estrés oxidativo puede causar daño a las grandes biomoléculas como las proteínas, ADN y lípidos, resultando en un incremento del riesgo de aparición de cáncer y enfermedades cardiovasculares[36]. Entre los efectos beneficiosos para la salud de los compuestos bioactivos podemos destacar los siguientes[38,39]:

- Actividad antioxidante
- Actividad anticancerígena
- Efecto antimutagénico en el ADN
- Modificación de la comunicación celular
- Modificación del perfil hormonal
- Modulación del perfil lipídico
- Estimulación del sistema inmunológico
- Efecto antiinflamatorio
- Actividad antimicrobiana

Los compuestos bioactivos pueden clasificarse en base a las diferentes estructuras y actividades biológicas de estos. En la **Figura 2** se muestran los principales grupos de compuestos bioactivos donde también se incluyen compuestos derivados y algunos micronutrientes que pueden influir en determinados procesos metabólicos relacionados estrechamente con la salud.

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[37] Wang, H.; Cao, G.; Prior, R. L. Total antioxidant capacity of fruits. *J. Agric. Food Chem.* **1996**, *44* (3), 701–705.

[38] Kris-Etherton, P. M.; Lefevre, M.; Beecher, G. R.; Gross, M. D.; Keen, C. L.; Etherton, T. D. Bioactive compounds in nutrition and health-research methodologies for establishing biological function: the antioxidant and anti-inflammatory effects of flavonoids on atherosclerosis. *Annu. Rev. Nutr.* **2004**, *24* (1), 511–538.

[39] Denny, A.; Buttriss, J. *Plant Foods and Health: Focus on Plant Bioactives*; EuroFir. Synthesis Report nº4, **2005**.

Entre estos compuestos cabe destacar los compuestos fenólicos, los alquilresorcinoles (ARs) y los esteroles, las tres familias de compuestos bioactivos estudiadas en esta tesis doctoral.

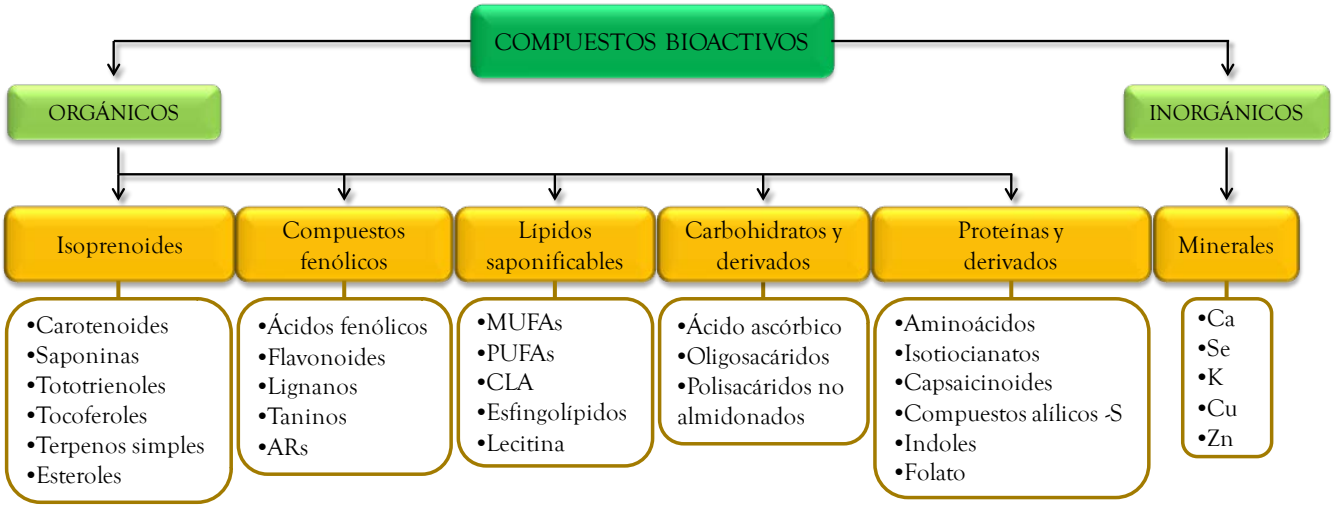

Figura 2. Principales compuestos bioactivos presentes en vegetales[40].

A.3.1. Compuestos fenólicos

Los compuestos fenólicos son metabolitos secundarios de plantas que se sintetizan a partir de dos rutas metabólicas: la vía del ácido shikímico, y la vía del ácido acético[32]. Su concentración puede estar condicionada al grado de estrés al que esté sometida la planta, bien sea biótico o abiótico[32]. El estrés abiótico engloba el estrés hídrico, temperaturas elevadas, bajas temperaturas y congelación, radiación UV-B, salinidad y metales pesados. Por el contrario, el estrés biótico incluye la acción de herbívoros, ataque de patógenos y

[40] Martí del Moral, A. A.; Martínez-Hernández, J. A. *¿Sabemos realmente lo que comemos?: Alimentos transgénicos, ecológicos y funcionales*; S.A. EUNSA. Ediciones Universidad de Navarra, **2005**.

parásitos y alelopatía[41,42]. El resultado más común de estrés en plantas es la formación de ROS (especies reactivas de oxígeno) a nivel celular y molecular, potentes moléculas oxidantes que hacen un importante daño a los sistemas de membrana celulares y al ADN. Las ROS incluyen al radical superóxido ($O_2^{\bullet-}$), peróxido de hidrógeno (H_2O_2), radical hidroxil ($OH^{\bullet}$), oxígeno singlete ($^1O_2$), etc[41,42].

Los compuestos fenólicos se componen de al menos un anillo aromático y presentan uno o más grupos hidroxilo. Normalmente se presentan como ésteres, metil ésteres o glicósidos[43] y, generalmente, se encuentran unidos a otras moléculas como azúcares o proteínas aunque también pueden unirse a lípidos, ácidos carboxílicos u otros compuestos fenólicos[32]. Lo más habitual en la naturaleza es encontrarlos unidos a uno o más residuos de azúcar que pueden presentarse como monosacáridos, disacáridos u oligosacáridos. Aunque la glucosa es el residuo más común también pueden unirse a galactosa, ramnosa, xilosa y arabinosa, así como a los ácidos glucurónico y galacturónico, entre otros[32].

Estos compuestos conforman uno de los grupos de fitoquímicos más ampliamente distribuidos en vegetales y predominan en frutas, verduras, hierbas, raíces, hojas y semillas, y juegan un papel fisiológico y morfológico importante en muchas funciones, como la estructura, sistema de defensa innato, estabilidad oxidativa, propiedades reproductivas y sensoriales. Son responsables de las principales características organolépticas de los vegetales

[41] Mittler, R. ROS are good. *Trends Plant Sci.* **2016**, *22* (1), 11–19.

[42] Redondo-Gómez, S. Abiotic and biotic stress tolerance in plants. In *Molecular Stress Physiology of Plants*; Ranjan Rout, G., Bandhu Das, A., Eds.; Springer Dordrecht Heidelberg, **2013**; pp 1–12.

[43] Shahidi, F.; Naczk, M. *Food phenolics, sources, chemistry, effects, applications.*; Lancaster, P. A., Ed.; Technomic Publishing Co Inc, **1995**.

como el color (incluyen pigmentos amarillos, naranjas, rojos y azules), sabor (pricipalmente son responsables del amargor y la astringencia, que se da por la interacción de los taninos y las proteínas de la saliva) y aroma[44,45]. Ciertos compuestos fenólicos, como la quercetina, se encuentran ampliamente distribuidos en los productos vegetales: frutas, verduras, cereales, zumos de frutas, té, vino, infusiones, etc., mientras que otros, como las flavanonas e isoflavonas, son específicos de alimentos concretos. En la mayoría de los casos, los alimentos contienen mezclas complejas de compuestos fenólicos. La distribución de los compuestos fenólicos a nivel tisular, celular y subcelular no es uniforme. Los compuestos fenólicos insolubles se encuentran en las paredes celulares, mientras que los solubles están presentes dentro de las vacuolas celulares[46]. Además, las capas externas de los vegetales contienen niveles más altos de fenoles que aquellas localizadas en las partes internas[47]. Por ejemplo, las raíces contienen niveles más bajos de compuestos fenólicos que la zona de la corteza o la piel, y las hojas presentan la mayor concentración de compuestos fenólicos[48].

El contenido de compuestos fenólicos varía en función del vegetal y de otros factores entre los que se incluyen las técnicas de cultivo utilizadas, la

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[44] Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99* (1), 191–203.

[45] Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *J. Pharm. Biomed. Anal.* **2006**, *41* (5), 1523–1542.

[46] Wink, M. Compartmentation of secondary metabolites and xenobiotics in plant vacuoles. *Adv. Bot. Res.* **1997**, *25* (C), 141–169.

[47] De Simon, B. F.; Perez-Ilzarbe, J.; Hernasndez, T.; Gómez-Cordovés, C.; Estrella, I. Importance of phenolic compounds for the characterization of fruit juices. *J. Agricuhural Food Chem.* **1992**, *40* (9), 1531–1535.

[48] Klepacka, J.; Gujska, E.; Michalak, J. Phenolic compounds as cultivar- and variety-distinguishing factors in some plant products. *Plant Foods Hum. Nutr.* **2011**, *66* (1), 64–69.

variedad, las condiciones de crecimiento, el proceso de maduración, las condiciones de procesado y almacenaje, factores ambientales y factores edáficos como el tipo de suelo, la exposición al sol, la precipitación, etc[45,49]. Su contenido puede aumentar bajo condiciones de estrés como la radiación ultravioleta, infección por patógenos y parásitos, lesiones, aire contaminado y exposición a temperaturas extremas [50]. Entre los factores descritos anteriormente, el grado de maduración afecta considerablemente a la concentración y proporción de algunos compuestos fenólicos[51]. En general, se ha observado que el contenido de ácidos fenólicos disminuye con la maduración, mientras que la concentración de antocianinas aumenta[52]. El almacenaje de los alimentos, mencionado anteriormente, es otro factor que afecta directamente al contenido de compuestos fenólicos debido a la rápida oxidación de estos[51]. Las reacciones de oxidación resultan en la formación de sustancias más o menos polimerizadas, que llevan a cambios en la calidad de los alimentos, concretamente en el color y características organolépticas. Estos cambios pueden ser beneficiosos como en el caso del té negro, o dañinos como en la oxidación de la fruta. El almacenamiento en frio

[49] Spencer, J. P. E.; Abd El Mohsen, M. M.; Minihane, A.-M.; Mathers, J. C. Biomarkers of the intake of dietary polyphenols: Strengths, limitations and application in nutrition research. *Br. J. Nutr.* **2008**, *99* (1), 12–22.

[50] Tomás-Barberán, F. A.; Espín, J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81* (9), 853–876.

[51] Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr.* **2004**, *79* (5), 727–747.

[52] Beckman, C. H. Phenolic-storing cells: Keys to programmed cell death and periderm formation in wilt disease resistance and in general defence responses in plants? *Physiol. Mol. Plant Pathol.* **2000**, *57* (3), 101–110.

conserva mejor el contenido de estos compuestos[53]. En el caso de la harina, por ejemplo, su almacenamiento resulta en una importante pérdida de ácidos fenólicos[54]. Parr *y col.* comprobaron que tras seis meses de almacenamiento, la harina contenía los mismos ácidos fenólicos desde el punto de vista cualitativo, pero su concentración era 70% más baja comparada con la harina fresca. Además de los factores mencionados, el cocinado del vegetal también puede tener un gran efecto en la concentración de los compuestos fenólicos. Por ejemplo, Parr *y col.* comprobaron que cebollas y tomates perdían entre un 75% y un 80% del contenido inicial de quercetina después de hervirlos durante 15 min, el 65% después de cocinarlos en un horno microondas y el 30% después de freirlos[55].

[53] Price, K. R.; Bacon, J. R.; Rhodes, M. J. C. Effect of storage and domestic processing on the content and composition of flavonol glucosides in onion (*Allium cepa*). *J. Agric. Food Chem.* **1997**, *45* (3), 938–942.

[54] Sosulski, F.; Krygier, K.; Hogge, L. Free, esterified, and insoluble-bound phenolic acids. 3. Composition of phenolic acids in cereal and potato flours. *J. Agric. Food Chem.* **1982**, *30* (2), 337–340.

[55] Crozier, A.; Lean, M. E. J.; McDonald, M. S.; Black, C. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *J. Agric. Food Chem.* **1997**, *45* (3), 590–595.

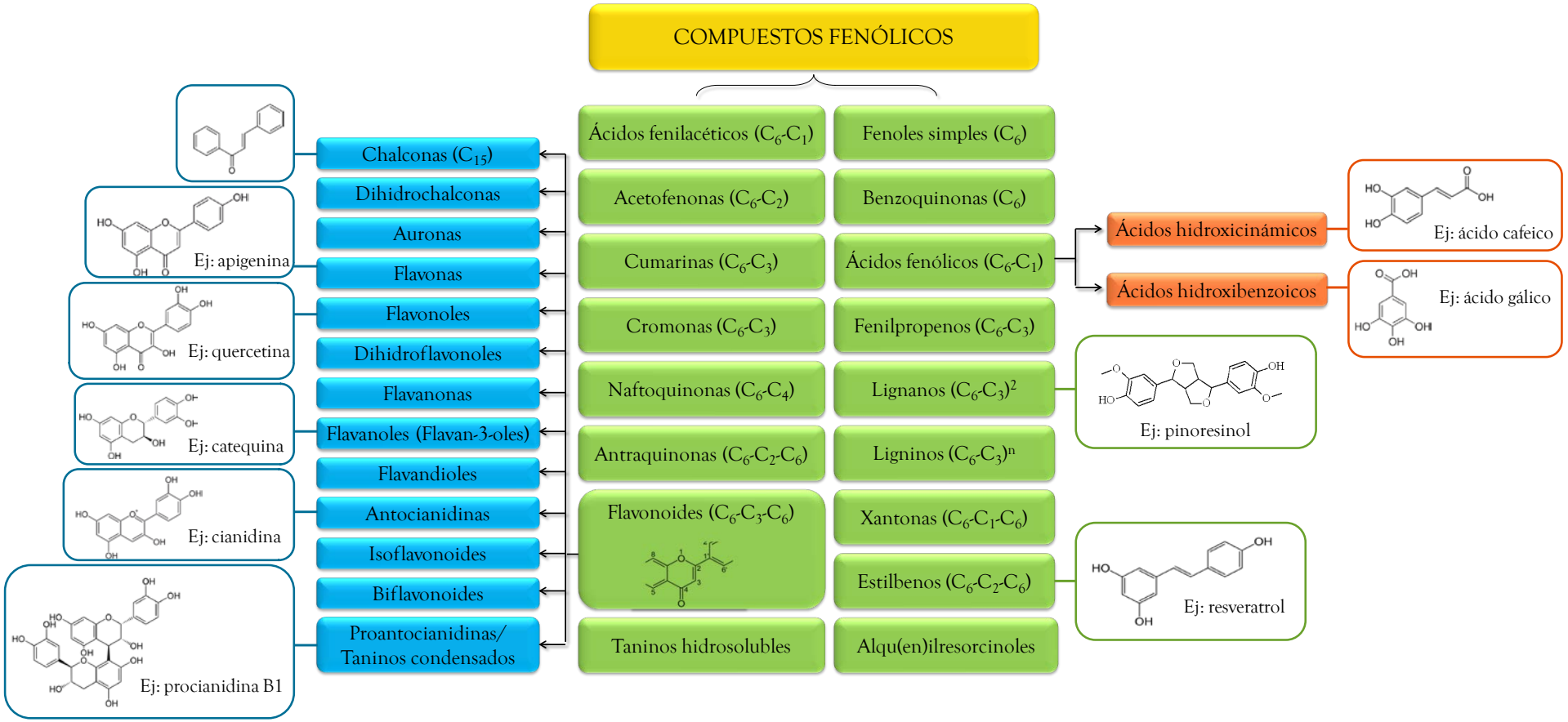

Figura 3. Clasificación de las principales familias de compuestos fenólicos[32,56,57].

[56] Soto, M.; Falqué, E.; Domínguez, H. Relevance of natural phenolics from grape and derivative products in the formulation of cosmetics. *Cosmetics* **2015**, *2* (3), 259–276.

[57] Crozier, A.; Jaganath, I. B.; Clifford, M. N. Phenols, polyphenols and tannins: an overviw. In *Plant Secondary Metabolites. Occurrence, Structures and Role in the Human Diet*; Crozier, A., Clifford, M. N., Ashihara, H., Eds.; Blackwell Publishing, **2008**; pp 1–14.

Los compuestos fenólicos se dividen en diferentes familias en función de su estructura química básica **(Figura 3).** A continuación se describen las más relevantes.

Los **flavonoides** y sus derivados son el grupo más grande e importante de los compuestos fenólicos presentes en las plantas. Este grupo tiene una estructura básica común que consta de dos anillos aromáticos unidos por tres átomos de carbono que forman un heterociclo oxigenado **(Figura 4)**. Han sido identificados más de 5.000 variedades de flavonoides, muchos de los cuales son responsables del atractivo color de las flores, frutas y hojas[58,59]. En base a la variación en el tipo de heterociclo implicado, han sido descritas 13 subclases de flavonoides, las seis más importantes son: flavonoles, flavonas, flavanonas, flavanoles, antocianinas e isoflavonas **(Figura 3)**[56,60]. Las diferencias individuales dentro de cada grupo surgen de la variación en el número y disposición de los grupos hidroxilo y su grado de alquilación y glucosilación[49]. Algunos de los flavonoides más comunes son quercetina, miricetina o catequina. Entre sus efectos biológicos se incluyen la inhibición de la oxidación de la lipoproteína de baja densidad (LDL), la inhibición de la proteasa del virus de inmunodeficiencia humana tipo 1 y las capacidades antimicrobianas y anticarcinogénicas[61-64].

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[58] De Groot, H.; Rauen, U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. *Fundam. Clin. Pharmacol.* **1998**, *12* (3), 249–255.

[59] Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomás-Barberán, F. A.; Datta, N.; Singanusong, R.; Chen, S. S. Flavonoids in food and their health benefits. *Plant Foods Hum. Nutr.* **2004**, *59* (3), 113–122.

[60] Pandey, K. B.; Rizvi, S. I. Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Med. Cell. Longev.* **2009**, *2* (5), 270–278.

[61] Muldoon, M. F.; Kritchevsky, S. B. Flavonoids and heart disease - Evidence of benefit still fragmentary. *Br. Med. J.* **1996**, *312* (7029), 458–459.

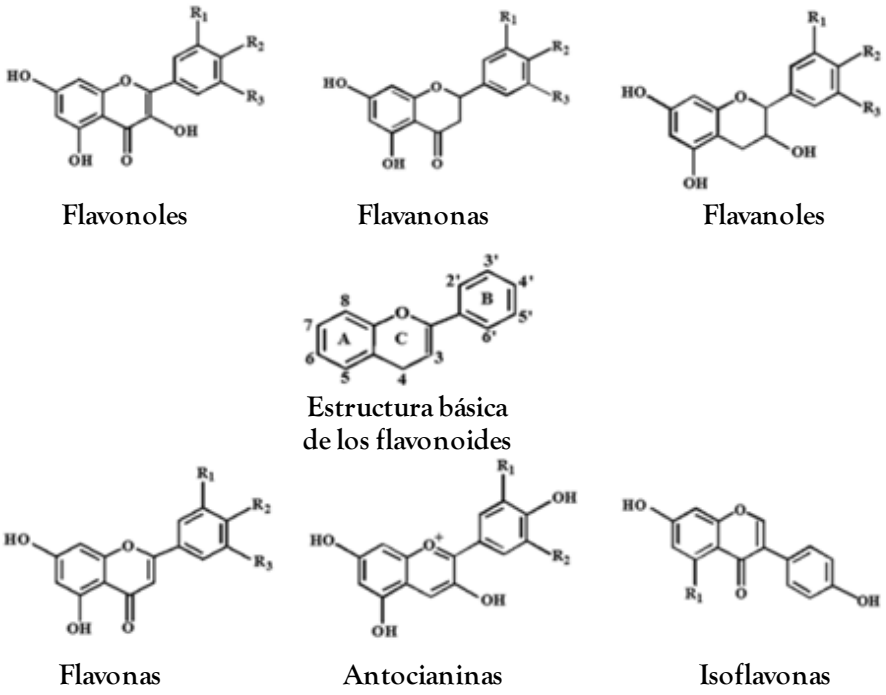

Figura 4. Estructura química de las subclases de flavonoides.

Otro importante grupo de compuestos fenólicos son los **ácidos fenólicos**. Estos se encuentran abundantemente en alimentos y están divididos en dos subclases: derivados del ácido hidroxibenzoico y derivados del ácido hidroxicinámico[60]. El contenido de ácido hidroxibenzoico presente en las plantas comestibles es generalmente bajo, con excepción de ciertas frutas, rábano negro y cebollas que pueden llegar a tener algunas decenas de miligramos por kilogramo de peso fresco[43]. En cambio, los ácidos

[62] Agullo, G.; Gamet-Payrastre, L.; Manenti, S.; Viala, C.; Rémésy, C.; Chap, H.; Payrastre, B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: A comparison with tyrosine kinase and protein kinase C inhibition. *Biochem. Pharmacol.* **1997**, *53* (11), 1649–1657.

[63] Meyer, A. S.; Yi, O.-S.; Pearson, D. A.; Waterhouse, A. L.; Frankel, E. N. Inhibition of human low-density lipoprotein oxidation in relation to composition of phenolic antioxidants in grapes (*Vitis vinifera*). *J. Agric. Food Chem.* **1997**, *45* (5), 1638–1643.

[64] Chun, O. K.; Kim, D.-O.; Smith, N.; Schroeder, D.; Han, J. T.; Chang, Y. L. Daily consumption of phenolics and total antioxidant capacity from fruit and vegetables in the American diet. *J. Sci. Food Agric.* **2005**, *85* (10), 1715–1724.

hidroxicinámicos son más comunes y, entre ellos, cabe destacar los ácidos *p*-cumárico, cafeico, ferúlico y sinápico[56,60].

Los **estilbenos** contienen dos fragmentos fenilo unidos por un puente de metileno de dos carbonos. La presencia de estilbenos en la dieta humana es bastante baja, se encuentran, por ejemplo, en uvas, vino, soja y cacahuete[57]. Muchos estilbenos actúan como fitoalexinas antifúngicas en plantas, compuestos que se sintetizan únicamente en respuesta a una infección o lesión. Uno de los estilbenos mejor estudiados es el resveratrol (3,4',5-trihidroxiestilbeno), que se encuentra de forma abundante en uvas. Un producto de las uvas, el vino tinto, también contiene cantidades significativas de resveratrol[60].

Los **lignanos** son compuestos difenólicos que contienen una estructura 2,3-dibencilbutano que está formada por la dimerización de dos residuos de ácido hidroxicinámico **(Figura 3)**. Los primeros lignanos identificados en plantas fueron secoisolariciresinol y matairesinol, más adelante se describieron el pinoresinol y lariciresinol, entre otros[65]. Algunos lignanos como el secoisolariciresinol se consideran fitoestrógenos. La fuente más rica es la semilla de lino, que contiene secoisolariciresinol (hasta 3.7 g/kg peso seco) y pequeñas cantidades de matairesinol [66], aunque también se encuentran en otras frutas, semillas, granos, árboles y verduras[56].

Los **taninos,** tras los flavonoides y ácidos fenólicos, son el tercer grupo más abundante de compuestos fenólicos[44]. Presentan un peso molecular

[65] Holmbom, B.; Eckerman, C.; Eklund, P.; Hemming, J.; Nisula, L.; Reunanen, M.; Sjöholm, R.; Sundberg, A.; Sundberg, K.; Willför, S. Knots in trees - A new rich source of lignans. *Phytochem. Rev.* **2003**, *2* (3), 331–340.

[66] Adlercreutz, H.; Mazur, W. Phytoestrogens and Western diseases. *Ann. Med.* **1997**, *29* (2), 95–120.

relativamente alto, entre 500-3000 Da y pueden subdividirse en (1) hidrolizables, ésteres del ácido gálico (galo y elagitaninos) y (2) taninos condensados, conocidos como proantocianidinas, polímeros de polihidroxiflavan-3-oles que se incluyen dentro de los flavonoides. Existe una tercera subdivisión compuesta únicamente por florotaninos que se encuentran en varios géneros de alga parda[56], pero su presencia no es significativa en la dieta humana. Los taninos condensados e hidrolizables son capaces de unirse y precipitar las proteínas de colágeno de los cueros animales. Esto permite que la piel se transforme en cuero ya que la hace resistente a la putrefacción. Además son los responsables de la astringencia en el sabor y textura de algunos alimentos y bebidas, los más representativos son el té y el vino tinto. Se encuentran en mayor concentración en frutas inmaduras y en las partes más externas[57].

Otras clases de compuestos fenólicos son las **cumarinas** y las **cromonas**, estas derivan del ácido cinámico por ciclación de la cadena lateral del ácido *o*-cumárico[67]. Normalmente están presentes como glucósidos[56].

Los compuestos fenólicos han sido asociados a los beneficios derivados del consumo de grandes cantidades de frutas y verduras[33, 68]. Los efectos beneficiosos derivados de los compuestos fenólicos han sido atribuidos a su

[67] Matern, V.; Lüer, P.; Kreusch, D. Biosynthesis of coumains. In *Comprehensive Natural Products Chemistry: Polyketides and other Secondary Metabolites Including Fatty Acids and Their Derivatives*; Barton, D., Nakanishi, K., Meth-Cohn, O., Sankawa, U., Eds.; Pergamon Press, **1999**; pp 623–637.

[68] Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80* (7), 985–1012.

actividad antioxidante[69]. Esta actividad está relacionada con la capacidad de estos compuestos de reducir el daño oxidativo capturando radicales libres, donando átomos de hidrógeno o electrones, o actuando como agentes quelantes de cationes metálicos [70, 71] y esta capacidad está directamente relacionada con su estructura. Se define como daño oxidativo al desequilibrio entre la producción de ROS y las defensas antioxidantes[72]. El organismo presenta mecanismos de defensa frente a las ROS como las enzimas superóxido dismutasa (SOD) y catalasa [73]. Sin embargo, estos mecanismos no siempre son suficientes para hacer frente a la elevada producción de ROS y deben ser ingeridos en la dieta elementos antioxidantes como los compuestos fenólicos, entre otros[74].

Los compuestos fenólicos presentan un amplio rango de propiedades fisiológicas como[75-77]:

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[69] Heim, K. E.; Tagliaferro, A. R.; Bobilya, D. J. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.* **2002**, *13* (10), 572–584.

[70] Afanas'ev, I. B.; Dcrozhko, A. I.; Brodskii, A. V.; Kostyuk, V. A.; Potapovitch, A. I. Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.* **1989**, *38* (11), 1763–1769.

[71] Amarowicz, R.; Pegg, R. B.; Rahimi-Moghaddam, P.; Barl, B.; Weil, J. A. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* **2004**, *84* (4), 551–562.

[72] Betteridge, D. J. What is oxidative stress? *Metabolism.* **2000**, *49* (2 SUPPL. 1), 3–8.

[73] Rahal, A.; Kumar, A.; Singh, V.; Yadav, B.; Tiwari, R.; Chakraborty, S.; Dhama, K. Oxidative stress, prooxidants, and antioxidants: The interplay. *Biomed Res. Int.* **2014**, *2014*, 1–19.

[74] De Roos, B.; Duthie, G. G. Role of dietary pro-oxidants in the maintenance of health and resilience to oxidative stress. *Mol. Nutr. Food Res.* **2015**, *59* (7), 1229–1248.

[75] Benavente-García, O.; Castillo, J.; Lorente, J.; Ortuño, A.; Del Rio, J. A. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem.* **2000**, *68* (4), 457–462.

[76] Graf, B. A.; Milbury, P. E.; Blumberg, J. B. Flavonols, flavones, flavanones, and human health: Epidemiological evidence. *J. Med. Food* **2005**, *8* (3), 281–290.

- Antiaterogénica
- Antiinflamatoria
- Antimicrobiana
- Antioxidante
- Efectos cardioprotectores y vasodilatadores
- Anticancerígena
- Antidiabética

Por último, dentro de los compuestos fenólicos se encuentra la familia de los comúnmente denominados alquilresorcinoles, dentro de la cual se engloban los alquil- y alquenilresorcinoles (alqu(en)ilresorcinoles). A ellos se les dedica un apartado independiente debido a que su especial estructura les confiere características físico-químicas diferentes a los anteriores. Además, los alqu(en)ilresorcinoles no están tan ampliamente presentes en vegetales como el resto de compuestos fenólicos, sino que únicamente se encuentran en algunas especies vegetales.

A.3.1.1. Alqu(en)ilresorcinoles

Los alqu(en)ilresorcinoles (ARs), cuyo descubrimiento data de la década de los 30, son un grupo de metabolitos secundarios que estructuralmente están definidos por una molécula con características anfipáticas compuesta por un anillo aromático con dos grupos hidroxilo en las posiciones 1 y 3 (parte hidrofilica) y una cadena carbonada en la posición 5 del anillo (parte hidrofóbica). Esta cadena alifática varía su longitud entre 1 y 29 átomos de

[77] Arts, I. C.; Hollman, P. C. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl).

carbono **(Figura 5)**[78-80] y puede ser saturada o insaturada con 1-4 dobles enlaces en configuración *cis*. La localización de los dobles enlaces es diferente y depende de la longitud de la cadena alifática[81, 82]. Son lípidos no-isoprenoides que pertenecen a la familia de los fenoles, los cuales se consideran poco comunes debido a su simplicidad comparable al de un ácido graso, en donde el grupo carboxilo es reemplazado por el anillo dihidroxibenceno[83]. Los ARs pueden ser incorporados a las membranas biológicas, como las membranas de fosfolípidos y pueden formar membranas monocapa por sí mismos[84]. Dependiendo de la concentración, los ARs pueden aumentar o inhibir la actividad de ciertas enzimas de membrana, se piensa que es debido a su efecto en la movilidad de los fosfolípidos de membrana[85]. Esto les confiere numerosas propiedades, asociadas a la

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[78] Kozubek, A.; Pietr, S.; Czerwonka, A. Alkylresorcinols are abundant lipid components in different strains of *Azotobacter chroococcum* and *Pseudomonas* spp. *J. Bacteriol.* **1996**, *178* (14), 4027–4030.

[79] Zarnowski, R.; Suzuki, Y.; Yamaguchi, I.; Pietr, S. J. Alkylresorcinols in barley (*Hordeum vulgare* L. *distichon*) grains. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* **2002**, *57* (1–2), 57–62.

[80] Zarnowski, R.; Suzuki, Y.; Zarnowska, E. D.; Esumi, Y.; Kozubek, A.; Pietr, S. J. 5-n-alkylresorcinols from the nitrogen-fixing soil bacterium *Azotobacter chroococcum* Az12. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* **2004**, *59* (5–6), 318–320.

[81] Tyman, J. H. Determination of the component phenols in natural and technical cashew nut-shell liquid by gas-liquid chromatography. *Anal. Chem.* **1976**, *48* (1), 30–34.

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[83] Kozubek, A.; Tyman, J. H. P. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. *Chem. Rev.* **1999**, *99*.

[84] Kozubek, A. The effect of 5-(n-alk(en)yl)resorcinols on membranes. I. Characterization of the permeability increase induced by 5-(n-heptadecenyl)resorcinol. *Acta Biochim. Pol.* **1987**, *34* (4), 357–367.

[85] Kozubek, A.; Sikorski, A. F. Modulation of the activities of membrane enzymes by cereal grain resorcinolic lipids. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* **1992**, *47* (1–2), 41–46.

conformación de la bicapa lipídica, respiración mitocondrial, bioquímica celular y estructura de la membrana, afectando a las propiedades físico-químicas de microorganismos patógenos[79,83,86].

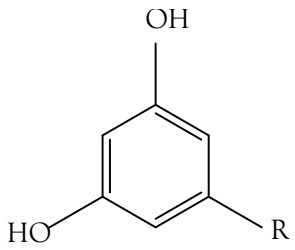

Figura 5. Estructura general de un alqu(en)ilresorcinol.
R=Grupo alquilo o alquenilo (C_1-C_{29}).

Los ARs se han identificado en muchas plantas y en algunas bacterias y hongos[83]. Estos se sintetizan en los diferentes organismos para cumplir funciones importantes; por ejemplo, en algunas plantas los ARs son sintetizados para la protección de la semilla contra algunos depredadores así como forma de defensa frente a hongos y bacterias debido a las propiedades antimicrobianas[83,87] y antifungicas que algunos ARs exhiben[79]. Otro tipo de funciones que desempeñan están relacionadas con su actividad antiparasitaria, anticancerígena[88,89], citotóxica[90], y antioxidante (evidenciada

[86] Bitkov, V. V; Nenashev, V. A.; Pridachina, N. N.; Batrakov, S. G. Membrane-structuring properties of bacterial long-chain alkylresorcinols. *BBA - Biomembr.* **1992**, *1108* (2), 224–232.

[87] Alonso, E.; Ramón, D. J.; Yus, M. Simple synthesis of 5-substituted resorcinols: A revisited family of interesting bioactive molecules. *J. Org. Chem.* **1997**, *62* (2), 417–421.

[88] Zhu, Y.; Conklin, D. R.; Chen, H.; Wang, L.; Sang, S. 5-Alk(en)ylresorcinols as the major active components in wheat bran inhibit human colon cancer cell growth. *Bioorganic Med. Chem.* **2011**, *19* (13), 3973–3982.

[89] Liu, L.; Winter, K. M.; Stevenson, L.; Morris, C.; Leach, D. N. Wheat bran lipophilic compounds with in vitro anticancer effects. *Food Chem.* **2012**, *130* (1), 156–164.

por la habilidad que tienen los ARs para donar un hidrógeno)[91 - 93] y antimutagénica disminuyendo hasta en un 70% el efecto de productos mutagénicos[94]. Por otro lado, los ARs juegan un papel importante en las plantas por ser parte del gran grupo de los fenoles, dado que están involucrados en protección vegetal frente a diferentes tipos de estrés, ya sea por radiación UV, radicales libres, heridas, patógenos o herbívoros[95].

Su actividad biológica abarca desde la interacción con enzimas metabólicas (por ejemplo, la inhibición de la 3-fosfoglicerato deshidrogenasa, enzima clave en adipocitos)[96] hasta la disminución del colesterol en hígado de rata[97],

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[90] Chaturvedula, V. S. P.; Schilling, J. K.; Miller, J. S.; Andriantsiferana, R.; Rasamison, V. E.; Kingston, D. G. I. New cytotoxic bis 5-alkylresorcinol derivatives from the leaves of *Oncostemon bojerianum* from the Madagascar rainforest. *J. Nat. Prod.* **2002**, *65* (11), 1627–1632.

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[92] Gliwa, J.; Gunenc, A.; Ames, N.; Willmore, W. G.; Hosseinian, F. S. Antioxidant activity of alkylresorcinols from rye bran and their protective effects on cell viability of PC-12 AC cells. *J. Agric. Food Chem.* **2011**, *59* (21), 11473–11482.

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[94] Gąsiorowski, K.; Szyba, K.; Brokos, B.; Kozubek, A. Antimutagenic activity of alkylresorcinols from cereal grains. *Cancer Lett.* **1996**, *106* (1), 109–115.

[95] Magnucka, E. G.; Suzuki, Y.; Pietr, S. J.; Kozubek, A.; Zarnowski, R. Action of benzimidazole fungicides on resorcinolic lipid metabolism in rye seedlings depends on thermal and light growth conditions. *Pestic. Biochem. Physiol.* **2007**, *88* (2), 219–225.

[96] Tsuge, N.; Mizokami, M.; Imai, S.; Shimazu, A.; Seto, H. Adipostatins a and b, new inhibitors of glycerol-3-phosphate dehydrogenase. *J. Antibiot. (Tokyo).* **1992**, *45* (6), 886–891.

[97] Ross, A. B.; Chen, Y.; Frank, J.; Swanson, J. E.; Parker, R. S.; Kozubek, A.; Lundh, T.; Vessby, B.; Åman, P.; Kamal-Eldin, A. Cereal alkylresorcinols elevate ϒ-tocopherol levels in rats and inhibit ϒ-tocopherol tetabolism *in vitro*. *J. Nutr.* **2004**, *134* (3), 506–510.

o efectos anticancerígenos y citotóxicos *in vitro*[83,98]. Los ARs son capaces de inducir cambios en la permeabilidad de los eritrocitos, con efectos diferentes dependiendo de la concentración, la longitud de la cadena y modificación de la cadena lateral. La longitud de la cadena lateral es inversamente proporcional a la actividad hemolítica[99]. Los ARs monoinsaturados causan mayor cambio en la permeabilidad de las membranas que los ARs saturados, y a una concentración de 15 μM pueden causar la lisis de los eritrocitos[100]. Los ARs de membrana son prácticamente insolubles en agua debido a la baja polaridad de su parte hidrofilica pero son capaces de formar capas monomoleculares estables en la interfase aire-agua[83] e incorporar otros componentes lipofílicos en la membrana como el colesterol o los ácidos grasos[101]. Por otro lado, los ARs han sido reconocidos por sus efectos alergénicos al contacto con la piel[79,102].

En plantas usadas habitualmente para alimentación, los ARs están presentes en cantidades significativas sólo en el grano integral de trigo y centeno y en sus subproductos, en los cuales la longitud de las cadenas alquil saturadas

[98] Ross, J. A.; Kasum, C. M. Dietary flavonoids: Bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **2002**, *22*, 19–34.

[99] Kozubek, A. The effect of 5-(n-alk(en)yl)resorcinols on membranes. II. Dependence on the aliphatic chain length and unsaturation. *Acta Biochim. Pol.* **1987**, *34* (4), 387–394.

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[101] Przeworska, E.; Gubernator, J.; Kozubek, A. Formation of liposomes by resorcinolic lipids, single-chain phenolic amphiphiles from *Anacardium occidentale* L. *Biochim. Biophys. Acta - Biomembr.* **2001**, *1513* (1), 75–81.

[102] Anderson, H. H.; David, N. A.; Leake, C. D. Oral toxicity of certain alkylresorcinols in Guinea pigs and rabbits. *Proc. Soc. Exp. Biol. Med.* **1931**, *28* (6), 609–612.

varían entre 15-27 carbonos[103]. Por ejemplo, el grano de centeno *(Scale cereale)* presenta 360-3200 μg ARs/g materia seca, el grano de trigo *(Triticum aestivum)* presenta 317-1430 μg ARs/g materia seca y el grano de trigo candeal *(Triticum durum)* presenta 54-1080 μg ARs/g materia seca. La cebada y el triticale también presentan ARs, pero el hombre no consume estos cereales en grandes cantidades. El maíz contiene pequeñas cantidades de orcinol (C1:0) en la cera del pericarpio[104]. Los ARs también se encuentran en los brotes de arroz[105,106], en el látex, la piel del mango[107] y en el líquido de la cáscara del anacardo (C15:0, C15:1, C15:2, C15:3)[108]. Zarnowski y Kozubek[109] publicaron la presencia de pequeñas cantidades de ARs concentrados en el pericarpio y cotiledones de diferentes variedades de guisante de jardín *(Pisum sativum)*, aunque las cantidades en los guisantes blancos comestibles era muy pequeña (0.5-0.15 μg/g materia seca). También se han identificado ARs como C15:0 y C17:0 en extractos de pulpa y hojas

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[103] Ross, A. B.; Kamal-Eldin, A.; Åman, P. Dietary alkylresorcinols: absorption, bioactivities, and possible use as biomarkers of whole-grain wheat- and rye-rich foods. *Nutr. Rev.* **2004**, *62* (3), 81–95.

[104] Gembeh, S. V; Brown, R. L.; Grimm, C.; Cleveland, T. E. Identification of chemical components of corn kernel pericarp wax associated with resistance to *Aspergillus flavus* infection and aflatoxin production. *J. Agric. Food Chem.* **2001**, *49* (10), 4635–4641.

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[106] Suzuki, Y.; Esumi, Y.; Hyakutake, H.; Kono, Y.; Sakurai, A. Isolation of 5-(8'Z-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. *Phytochemistry* **1996**, *41* (6), 1485–1489.

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[108] Kubo, I.; Komatsu, S.; Ochi, M. Molluscicides from the cashew *Anacardium occidentale* and their large-scale isolation. *J. Agric. Food Chem.* **1986**, *34* (6), 970–973.

[109] Zarnowski, R.; Kozubek, A. Alkylresorcinol homologs in *Pisum sativum* L. Varieties. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* **1999**, *54* (1–2), 44–48.

de *Ginkgo biloba* L., una planta frecuentemente usada en medicina alternativa pero no como alimento[110]. Se han encontrado recientemente derivados de ARs en un vegetal cretense de ensalada, *Cichorium spinosum*[111], que aumenta la posibilidad de que los ARs y/o sus derivados estén más extendidos en la naturaleza de lo que en un principio se pensaba.

A.3.2. Esteroles

Los esteroles vegetales o fitosteroles son compuestos bioactivos con estructuras similares y funciones análogas al colesterol de los vertebrados[112] **(Figura 6)**[113]. Son de la familia de los triterpenos y difieren del colesterol en que incluyen un grupo metilo o etilo en el carbono 24. En general estas sustancias actúan como componentes estructurales de las membranas vegetales regulando la fluidez y permeabilidad, a la vez que sirven de intermediarios para la biosíntesis de celulosa y numerosos productos vegetales secundarios como los alcaloides, entre otros[114,115].

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[110] Zarnowska, E. D.; Zarnowski, R.; Kozubek, A. Alkylresorcinols in fruit pulp and leaves of *Ginkgo biloba* L. *Zeitschrift fur Naturforsch. - Sect. C J. Biosci.* **2000**, *55* (11–12), 881–885.

[111] Melliou, E.; Magiatis, P.; Skaltsounis, A.-L. Alkylresorcinol derivatives and sesquiterpene lactones from *Cichorium spinosum*. *J. Agric. Food Chem.* **2003**, *51* (5), 1289–1292.

[112] Moreau, R. A.; Whitaker, B. D.; Hicks, K. B. Phytosterols, phytostanols, and their conjugates in foods: Structural diversity, quantitative analysis, and health-promoting uses. *Prog. Lipid Res.* **2002**, *41* (6), 457–500.

[113] Brzeska, M.; Szymczyk, K.; Szterk, A. Current knowledge about oxysterols: A review. *J. Food Sci.* **2016**, *81* (10), R2299–R2308.

[114] Peng, L.; Kawagoe, Y.; Hogan, P.; Delmer, D. Sitosterol-β-glucoside as primer for cellulose synthesis in plants. *Science.* **2002**, *295* (5552), 147–150.

[115] Read, S. M.; Bacic, T. Plant biology: Prime time for cellulose. *Science.* **2002**, *295* (5552), 59–60.

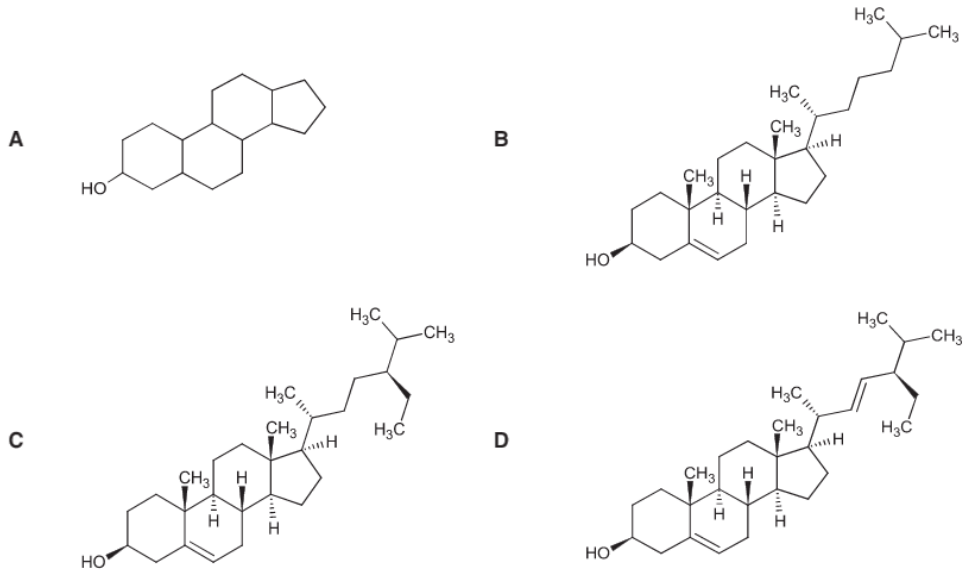

Figura 6. Esqueleto esterólico (A), fórmula estructural del colesterol (B), β-sitosterol (C) y estigmasterol (D).

Dentro del grupo de los esteroles vegetales encontramos dos categorías, (1) los esteroles, con un doble enlace en posición 5 y, por tanto, compuestos insaturados, y (2) los estanoles que no presentan ese doble enlace, es decir, con una reducción-5α, y, por ello, compuestos saturados[116,117]. Los esteroles pueden convertirse en estanoles por hidrogenación química. Dado que los estanoles son mucho menos abundantes que los esteroles, cuando se habla en términos generales de esteroles vegetales se hace referencia a estos últimos. Se han descrito alrededor de 200 tipos diferentes de esteroles vegetales en distintas especies de plantas, pero los más frecuentes en la naturaleza son β-sitosterol, seguido de campesterol y estigmasterol[117], y

[116] Ogbe, R. J.; Ochalefu, D. O.; Mafulul, S. G.; Olaniru, O. B. A review on dietary phytosterols: Their occurrence , metabolism and health benefits. *Asian J. Plant Sci. Res.* **2015**, *5* (4), 10–21.

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formas de fitosteroles saturados con 5α-hidrogenación como campestanol y sitostanol[118].

En la naturaleza, además de en la forma libre, los fitosteroles pueden aparecer como compuestos conjugados, en los cuales el grupo 3β-OH del esterol se encuentra esterificado por ácidos grasos, ácido ferúlico (potente antioxidante semejante a las vitaminas E y C), o bien glicosilados (**Figura 7**). Los esteroles con ésteres de ácidos grasos están presentes en la mayoría de las plantas y constituyen cerca del 50% del total de esteroles en algunos alimentos como el aceite de maíz. Por su parte, los ésteres de ferulato también aparecen en cantidades apreciables en muchos alimentos (ej. salvado de arroz), mientras que los esteroles vegetales glicosilados son un componente minoritario en los alimentos vegetales, salvo algunas excepciones como la patata[117,119-121]. Al contrario que los esteroles o estanoles libres, que son cristalinos y muy poco liposolubles, las formas esterificadas se disuelven fácilmente en alimentos que contengan grasa[122]. Las fuentes principales de esteroles son maíz, judías y sobre todo aceites vegetales, margarinas y otras cremas para untar, panes, cereales y vegetales como coles

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de Bruselas, brócoli o coliflor[118,123]. Estos constituyen el 50-80% de la ingesta diaria de esteroles vegetales[123-125]. En la dieta occidental típica, la ingesta media diaria de esteroles vegetales está sobre los 300 mg[124,125], pero puede llegar a 600 mg en vegetarianos[126]. Los más abundantes son sitosterol y campesterol que suponen el 60% y el 20%, respectivamente, de la ingesta total de esteroles vegetales [124,125].

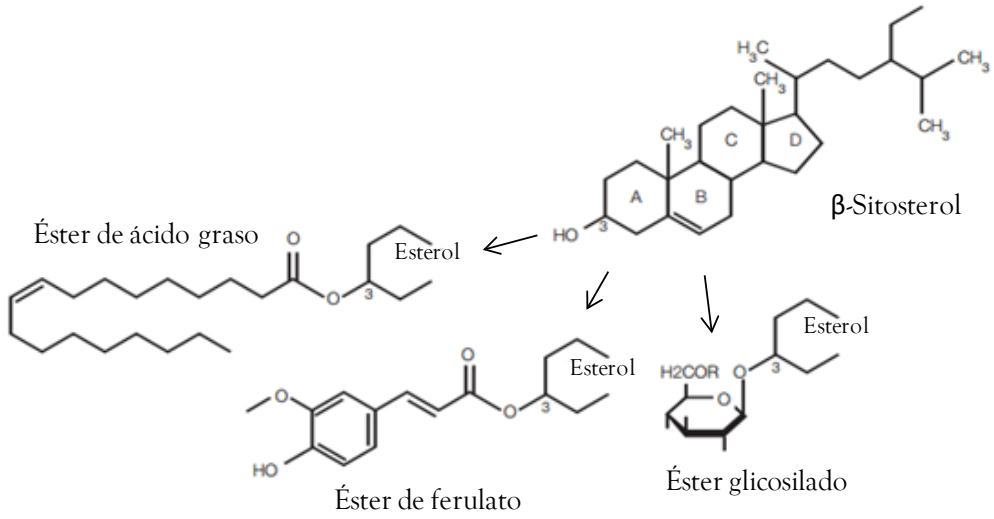

Figura 7. Modificaciones del grupo 3β-hidroxilo de los esteroles vegetales.

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Los fitosteroles producen un amplio espectro de actividades biológicas en animales y humanos. Ciertos esteroles vegetales son precursores de los brasinoesteroides, hormonas vegetales implicadas en la división celular, el desarrollo embionario, la fertilidad y el crecimiento de las plantas y juegan un papel positivo por la disminución de la incidencia de enfermedades cardiovasculares. Concretamente los fitosteroles son considerados eficientes agentes para bajar el colesterol[118]. Los esteroles vegetales afectan a la absorción intestinal del colesterol, a su síntesis y a los sistemas de eliminación[127]. Al ser más hidrofóbicos que el colesterol, pueden desplazarlo de las micelas de absorción[128] y se ha demostrado que tanto *in vitro* como *in vivo* se produce una disminución por competición de la incorporación del colesterol de las micelas y, en consecuencia, disminuye su absorción intestinal [129 , 130] . Tras la ingesta de esteroles vegetales, descienden las concentraciones de colesterol LDL y de colesterol total, debido a la menor absorción a pesar del incremento compensatorio en su síntesis endógena[118]. Este efecto se observa tanto con esteroles vegetales libres como con esterificados [131] . Se ha demostrado que una ingesta diaria de esteroles vegetales de 2 a 2.5 g produce la mayor tasa de absorción de colesterol, provocando así la expresión más elevada del receptor LDL, compensando el

[127] De Jong, A.; Plat, J.; Mensink, R. P. Metabolic effects of plant sterols and stanols (Review). *J. Nutr. Biochem.* **2003**, *14* (7), 362–369.

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incremento de la síntesis de colesterol endógena que se produce tras la ingesta de estos, lo cual da como resultado, en conjunto, a un descenso de colesterol LDL en torno a un 14%. Dosis mayores de 2-2.5 g no han demostrado producir un descenso mayor de colesterol LDL[132]. Además, los fitosteroles tienen efecto protector frente a enfermedades crónicas como enfermedades cardiovasculares [133, 134], hepáticas [135], diabetes [136] y cáncer[134,137,138].

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B. MATRICES ESTUDIADAS

B.1. Patata (*Solamun tuberosum* L.)

La patata (*Solanum tuberosum* L.) (**Figura 8**) es una planta herbácea que puede alcanzar hasta 1 m de altura, perteneciente a la familia Solanaceae. Sus tubérculos son perennes mientras que sus hojas y tallos son caducos. Las hojas están compuestas por 7-9 foliolos, de forma lanceolada y se disponen en forma espirilada en los tallos. Presenta 3 tipos de tallos: uno aéreo erecto sobre el que se disponen las hojas y 2 tallos subterráneos: tubérculo y rizoma. Los rizomas se originan a partir de brotes laterales que surgen de la base del tallo aéreo. El rizoma a través de un engrosamiento en el extremo distal da lugar a los tubérculos. Estos son órganos de almacenamiento de nutrientes y de reproducción asexual independiente de semillas. Así en su superficie se forman brotes que crecen y se desarrollan como tallos aéreos obteniendo una nueva planta. Este modo de reproducción es habitual entre las variedades cultivadas, mientras que las variedades salvajes se reproducen de forma sexual a través de semillas. Las flores se agrupan en inflorescencias y constan de 5 pétalos unidos por sus bordes; los colores van desde el blanco hasta el azul-violáceo dependiendo de la cantidad de antocianinas presentes. Las anteras se hallan unidas formando

Figura 8. Planta de patata con flor fruto y tubérculo.

un tubo alrededor del pistilo. Sus frutos son bayas redondeadas semejantes al tomate, de 1 a 3 cm de diámetro de colores verde-amarillo o violeta-rojizo[139].

Este vegetal es originario de Sudamérica donde ha sido usado durante más de 10.000 años[140]. Fue domesticado durante el periodo precolombino hace más de 8.000 años [141] y traído a Europa tras la conquista del nuevo continente. En un primer lugar fue utilizado como planta ornamental pero más tarde, en tiempos de hambruna, se comenzó su uso como alimento entre la población rural más pobre. Poco a poco este tubérculo se fue introduciendo en la dieta de la aristocracia y la burguesía europea convirtiéndose en un elemento muy apreciado de la cocina[139].

Según los datos más recientes de los que se dispone, la patata es actualmente el cuarto cultivo más importante para el ser humano, con una producción anual de más de 381 millones de toneladas a nivel mundial y más de 2.5 millones en España[6] en el año 2014. Es el alimento básico más consumido en el mundo occidental. Su inmensa popularidad se debe a sus características organolépticas, a su valor como alimento saludable, a su relativo bajo coste y a su atractivo gastronómico[142].

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La **Tabla 1** muestra la composición nutricional básica de la patata, teniendo en cuenta que se consume principalmente sin piel y cocinada[143]. La patata ha sido tradicionalmente consumida como una fuente de carbohidratos debido a sus altos niveles de almidón. Hay estudios que demuestran que las patatas hervidas tienen el mayor índice de saciedad (SI) entre 38 alimentos agrupados en 6 clases de alimentos (frutas, productos de panadería, snacks, alimentos ricos en carbohidratos, alimentos ricos en proteínas y cereales de desayuno)[144]. También sabemos que aporta otros beneficios nutricionales si se compara con cereales básicos como son una proteína de alta calidad, bajo contenido en grasa, fibra dietética y altos niveles de vitamina C, calcio, potasio, hierro y zinc[145]. Por otro lado, la patata contiene otros fitoquímicos como los compuestos fenólicos y glicoalcaloides (α-solanina, α-chaconina) [32,146-149].

[143] USDA (U.S. Department of Agriculture).Potatoes, boiled, cooked without skin, flesh, without salt. Nutrient Data Laboratory. USDA National Nutrient Database for Standard Reference, Release 28. U.S. Department of Agriculture. https://ndb.nal.usda.gov/ndb/foods/show/3095?n1=%7Bn1%3D%7BQv%3D1%7D%2C+Qv%3D1%7D&fgcd=&man=&lfacet=&count=&max=50&sort=default&qlookup=potato+flesh+boiled&offset=&format=Abridged&new=&measureby=&ds=&Qv=1&qt=&qp=&qa=&qn=&q=&ing= (acceso Mayo, 2017).

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[148] Hale, A. L.; Reddivari, L.; Nzaramba, M. N.; Bamberg, J. B.; Miller Jr., J. C. Interspecific variability for antioxidant activity and phenolic content among *Solanum* species. *Am. J. Potato Res.* **2008**, *85* (5), 332–341.

Tabla 1. Características nutricionales por 100 g de patata hervida.

Composición nutricional general	Vitaminas	Lípidos	Minerales
Energía: 86 kcal	Vitamina C: 7,4 mg	Ácidos grasos saturados (SFA): 0,026 g	Calcio (Ca): 8 mg
Agua: 77,46 g	Tiamina (Vit. B1): 0,098 mg	Ácidos grasos monoinsaturados (MUFA): 0,002 g	Hierro (Fe): 0,31 mg
Proteínas: 1,71 g	Riboflavina (Vit. B2): 0,019 mg	Ácidos grasos poliinsaturados (PUFA): 0,043 g	Magnesio (Mg): 20 mg
Lípidos Totales: 0,10 g	Niacin (Vit. B3): 1,312 mg		Fósforo (P): 40 mg
Carbohidratos: 20,01 g	Vitamina B6: 0,269 mg		Potasio (K): 328 mg
Fibra Dietética Total: 1,8 g	Folato (Vit. B9) (EFD): 9 µg		Sodio (Na): 5 mg
Azúcares totales: 0,89 g	Vitamina A (UI): 2 IU		Zinc (Zn): 0,27 mg
	Vitamina E: 0,1 mg		
	Vitamina K1 (Filoquinona): 2,2 µg		

UI: Unidad Internacional.

El germoplasma de esta planta muestra una sorprendente variedad de compuestos fenólicos[150]. Los niveles de estos compuestos en patata pueden variar considerablemente dependiendo del color y la variedad de patata[150,151] **(Figura 9)**. En la mayoría de los casos la patata presenta un contenido total en compuestos fenólicos mayor que otras frutas y vegetales ampliamente consumidos como la zanahoria, cebolla o tomate[64], aunque es cierto que no se encuentra entre los cultivos que más destacan por su alto contenido en

[149] López-Cobo, A.; Gómez-Caravaca, A. M.; Cerretani, L.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Distribution of phenolic compounds and other polar compounds in the tuber of *Solanum tuberosum* L. by HPLC-DAD-q-TOF and study of their antioxidant activity. *J. Food Compos. Anal.* **2014**, *36* (1–2), 1–11.

[150] Andre, C. M.; Ghislain, M.; Bertin, P.; Oufir, M.; Herrera, M. D. R.; Hoffmann, L.; Hausman, J.-F.; Larondelle, Y.; Evers, D. Andean potato cultivars (*Solarium tuberosum* L.) as a source of antioxidant and mineral micronutrients. *J. Agric. Food Chem.* **2007**, *55* (2), 366–378.

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compuestos fenólicos. Estos compuestos sirven como defensa a la planta, por ello es probable que debido a la selección natural que ha experimentado la patata durante cientos de años, algunas especies salvajes contengan niveles más altos de compuestos fenólicos que las variedades cultivadas.

Figura 9. Diferentes variedades de patata.

Los compuestos fenólicos presentes en la patata son principalmente ácidos fenólicos y flavonoides[152]. Los ácidos fenólicos son los compuestos fenólicos más abundantes[153-155], entre todos ellos el ácido cafeoilquínico, éster de los

[152] Deußer, H.; Guignard, C.; Hoffmann, L.; Evers, D. Polyphenol and glycoalkaloid contents in potato cultivars grown in Luxembourg. *Food Chem.* **2012**, *135* (4), 2814–2824.

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ácidos cafeico y quínico, ha sido el más descrito en patata[149,156-160] (hasta 2193 mg/100 g peso seco) [152] y existe en forma de tres isómeros: ácido clorogénico (ácido 5-*O*-cafeoilquínico), ácido neoclorogénico (ácido 3-*O*-cafeoilquínico) y el ácido criptoclorogénico (ácido 4-*O*-cafeoilquínico)[161]. El ácido cafeico es también otro de los compuestos fenólicos mayoritarios en la patata (hasta 172,4 mg/100 g peso seco) [154]. Además, otros ácidos fenólicos como el ácido ferúlico, el ácido gálico o el ácido *p*-cumárico también han sido cuantificados en patata en un rango de 0-5 mg/100 g peso seco[154,162-164].

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[154] Mäder, J.; Rawel, H.; Kroh, L. W. Composition of phenolic compounds and glycoalkaloids α-solanine and α-chaconine during commercial potato processing. *J. Agric. Food Chem.* **2009**, *57* (14), 6292–6297.

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[162] Albishi, T.; John, J. a.; Al-Khalifa, A. S.; Shahidi, F. Phenolic content and antioxidant activities of selected potato varieties and their processing by-products. *J. Funct. Foods* **2013**, *5* (2), 590–600.

[163] Sabeena Farvin, K. H.; Grejsen, H. D.; Jacobsen, C. Potato peel extract as a natural antioxidant in chilled storage of minced horse mackerel (*Trachurus trachurus*): Effect on lipid and protein oxidation. *Food Chem.* **2012**, *131* (3), 843–851.

Asimismo, hay evidencia de la presencia en menor concentración de los ácidos siríngico, vanílico, sinapíco y salicílico[152,155,163,165].

En cuanto a las familias de flavonoides más comunes en patata se encuentran flavonoles, flavanoles y antocianinas[152]. La patata de pulpa blanca presenta concentraciones de flavonoides entorno a 30 mg /100 g de peso fresco, siendo esta concentración casi el doble en patata de pulpa roja y/o púrpura, como resultado de la presencia de antocianinas[166] **(Figura 9)** Uno de los flavonoides más abundantes en la patata es la catequina, (1.1-204 mg/100 g peso seco) [154,167-169]. Flavonoles como la quercetina, el kaempferol rutinósido[152,161,170-172] o la rutina[152,161,170,173] también se encuentran en este

[164] Xu, X.; Li, W.; Lu, Z.; Beta, T.; Hydamaka, A. W. Phenolic content, composition, antioxidant activity, and their changes during domestic cooking of potatoes. *J. Agric. Food Chem.* **2009**, *57* (21), 10231–10236.

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[171] Andre, C. M.; Oufir, M.; Guignard, C.; Hoffmann, L.; Hausman, J.-F.; Evers, D.; Larondelle, Y. Antioxidant profiling of native Andean potato tubers (*Solanum tuberosum* L.) reveals cultivars with high levels of β-carotene, α-tocopherol, chlorogenic acid, and petanin. *J. Agric. Food Chem.* **2007**, *55* (26), 10839–10849.

tubérculo. Entre las antocianidinas más comunes (formas agliconas de las antocianinas), en la patata se encuentran malvidina, petunidina, delfinidina y peonidina en los tubérculos púrpura y pelargonidina en los rojos[174,175]. Además de estas antocianidinas, también se ha descrito la presencia de cianidina y petanina[171,176].

B.1.1 Subproductos procedentes del procesado de la patata

Existe una gran variedad de formas de utilizar la patata que van desde la patata fresca a productos procesados como puré, patatas fritas, snacks y comidas precocinadas[163, 177]. El procesado industrial de la patata genera grandes cantidades de subproductos, principalmente piel. La cantidad de desechos producida es definida como la masa de desechos acumulada dividida entre la masa de producto que puede ser vendida, llamado Índice

[172] Navarre, D. A.; Pillai, S. S.; Shakya, R.; Holden, M. J. HPLC profiling of phenolics in diverse potato genotypes. *Food Chem.* **2011**, *127* (1), 34–41.

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[177] Sirisakulwat, S.; Nagel, A.; Sruamsiri, P.; Carle, R.; Neidhart, S. Yield and quality of pectins extractable from the peels of Thai mango cultivars depending on fruit ripeness. *J. Agric. Food Chem.* **2008**, *56* (22), 10727–10738.

Específico de Desechos[178]. Este índice en la piel de la patata es de 0,3-0,5 [179]. Sin embargo, la piel de patata es una gran fuente de compuestos fenólicos entre los que encontramos principalmente, al igual que ocurre en la pulpa, ácidos fenólicos y sus derivados. El ácido cafeoilquínico constituye el 90% de los compuestos fenólicos presentes en la piel de la patata[180]. Además en la piel se pueden encontrar los ácidos protocatecuico, cafeico, *p*-cumárico, ferúlico y quínico[146,157,161,181].

Numerosos estudios destacan los efectos antioxidantes de los compuestos fenólicos de la patata para preservar alimentos, concretamente para evitar la oxidación de las grasas (ej. aceite de soja o de girasol) [153,158,182-184]. Además, algunos estudios han investigado los efectos antimicrobianos, antioxidantes, antiproliferativos y anticancer de los compuestos fenólicos de la piel de la

[178] Russ, W.; Schnappinger, M. Waste related to the food industry: a challenge in material loops. In *Utilization of By-products and Treatment of Waste in the Food Industry*; Oreopoulou, V., Russ, W., Eds.; Springer, **2007**.

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[183] Onyeneho, S. N.; Hettiarachchy, N. S. Antioxidant activity, fatty acids and phenolic acids compositions of potato peels. *J. Sci. Food Agric.* **1993**, *62* (4), 345–350.

[184] Zia-Ur-Rehman; Habib, F.; Shah, W. H. Utilization of potato peels extract as a natural antioxidant in soy bean oil. *Food Chem.* **2004**, *85* (2), 215–220.

patata en la salud humana[168,185-187]. Sin embargo, el extracto de patata debe usarse con precaución debido a la presencia de glicoalcaloides tóxicos. Estos compuestos pueden causar la muerte a concentraciones mayores de 330 mg/kg muestra[188], si bien a concentraciones más bajas pueden tener efectos beneficiosos para la salud (ej. efecto anticarcinogénico frente a determinadas células cancerosas humanas)[189]. Por otro lado, el uso de la piel de la patata en la industria podría tener otras aplicaciones, ya que un estudio reciente muestra que la incorporación de harina de piel de patata mejora la textura y propiedades sensoriales de bizcochos[190].

[185] Singh, N.; Rajini, P. S. Antioxidant-mediated protective effect of potato peel extract in erythrocytes against oxidative damage. *Chem. Biol. Interact.* **2008**, *173* (2), 97–104.

[186] Ji, X.; Rivers, L.; Zielinski, Z.; Xu, M.; MacDougall, E.; Stephen, J.; Zhang, S.; Wang, Y.; Chapman, R. G.; Keddy, P.; *et al.* Quantitative analysis of phenolic components and glycoalkaloids from 20 potato clones and *in vitro* evaluation of antioxidant, cholesterol uptake, and neuroprotective activities. *Food Chem.* **2012**, *133* (4), 1177–1187.

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B.2. Aguacate (*Persea americana* Mill.)

El aguacate *(Persea americana* Mill.*)* **(Figura 10)** es un árbol originario de Centroamérica y México donde ha sido un alimento básico desde hace más de 9.000 años[191]. Pertenece a la familia Lauraceae y se caracteriza por un rápido crecimiento tanto en grosor como en altura, llegando hasta los 20 m. Es un árbol perenne aunque sus hojas tienen una vida media corta, de hasta 12 meses. Sus hojas son alternas, coriáceas y con limbo generalmente glauco por el envés. Son escasamente pubescentes en el haz pero muy densamente por el envés. La producción de flores es alta, aunque no más de un 0.1% de estas llegan a formar fruto. Este es una gran drupa de color verde amarillento o marrón rojizo, con forma de pera u ovoide. El aguacate es un fruto climatérico cuya maduración ocurre una vez es recogido del árbol y que alcanza su momento óptimo para el consumo aproximadamente pasados 10 días a temperatura ambiente[192]. El

Figura 10. Árbol de aguacate junto a flor y fruto.

[191] Chen, H.; Morrell, P. L.; Ashworth, V. E. T. M.; De La Cruz, M.; Clegg, M. T. Tracing the geographic origins of major avocado cultivars. *J. Hered.* **2009**, *100* (1), 56–65.

[192] Ozdemir, F.; Topuz, A. Changes in dry matter, oil content and fatty acids composition of avocado during harvesting time and post-harvesting ripening period. *Food Chem.* **2004**, *86* (1), 79–83.

mesocarpio es carnoso y envuelve una gran semilla redondeada **(Figura 11)** que en la mayoría de los casos ocupa un 15-16% del peso total del fruto[193].

El aguacate presenta diversas variedades adaptadas a los distintos ambientes según su geografía. Esta especie incluye tres grandes variedades salvajes no cultivadas (var. *floccosa* Mez., var. *steyermarkii* Allen y var. *nubigena* (Williams) Kopp) y tres variedades hortícolas importantes (var. *americana* Mill. 'antillana', var. *guatemalensis* Williams 'guatemalteca' y var. *drymifolia* (Schlecht. y Cham.) Blake 'mexicana'[194]. Cada una de ellas presenta características propias en las hojas, frutos, periodo de floración, etc[195]. No existen barreras reproductivas entre las variedades por lo que son comunes los híbridos[196]. Por esta razón, la mayoría de las variedades comerciales son híbridos, desarrollados a partir de plántulas casuales con diferentes grados de hibridación[197,198]. Los híbridos 'guatemalteco-mexicano', por ejemplo 'Hass', 'Bacon' y 'Fuerte' son cultivados en climas subtropicales mientras que 'antillana' o los híbridos 'antillano-guatemalteco', por ejemplo 'Simmonds', 'Tonnage', 'Choquette' y 'Monroe' se cultivan en climas tropicales.

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[193] García-Fajardo, J. a; Del, ; M; Ramos-Godínez, R.; Mora-Galindo, ; J. Estructura de la semilla de aguacate y cuantificación de la grasa extraída por diferentes técnicas. *Rev. Chapingo Ser. Hortic.* **1999**, *5*, 123–128.

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[198] Rajalakshmi, S.; Narasimhan, S. Food antioxidants: Sources and methods of evalucation. In *Food Antioxidants: Technological, Toxicological and Health Perspectives*; Madhavi, D. L., Deshpande, S. S., Salunkhe, D. K., Eds.; Mercel Dekker, Inc., **1996**; pp 65–158.

La producción de aguacate en el mundo, según los datos más recientes de que se dispone, fue de más de 5 millones de toneladas en el año 2014, ocupando México el primer puesto con más de 1,5 millones[6]. Entre las variedades, 'Hass' es la variedad predominante cultivada en todo el mundo[199]. La producción de aguacate en España no supone más del 1,5% de la producción mundial, siendo en el año 2014 de más de 77.400 toneladas, aunque hay que resaltar que es el primer país productor en Europa, llegando al 79% de la producción europea[6]. Las condiciones climáticas características de España favorecen el cultivo de este tipo de frutos tropicales y subtropicales que requieren condiciones edafoclimáticas específicas como temperaturas de 20-21 ºC, humedad relativa durante la floración mayor del 70%, zonas sin heladas, etc. Por ello, es un cultivo que crece fácilmente en el sur y este de la península ibérica así como en el archipiélago canario. Andalucía es el principal productor de aguacate de España con 68.763 toneladas, y concretamente las áreas costeras de Granada y Málaga ocupan las primeras posiciones en áreas de producción de aguacate[200].

Tradicionalmente el aguacate ha sido muy utilizado, aparte de como alimento por sus cualidades nutricionales, con propósitos medicinales. Entre los usos más comunes del aguacate encontramos como hipotensivo, hipoglucémico, antiviral, analgésico, afrodisiaco y emenagogo y se utiliza para el tratamiento de úlceras, disentería y diarrea. Otro de los usos más

[199] Ashworth, V. E. T. M.; Chen, H.; Clegg, M. T. Avocado. In *Genome Mapping and Molecular Breeding in Plants, Fruits and Nuts, vol 4*; Kole, C., Ed.; Springer, **2007**; pp 325–329.

[200] Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente. Gobierno de España. Anuario de estadística 2015 http://www.mapama.gob.es/es/estadistica/temas/publicaciones/anuario-de-estadistica/2015/default.aspx?parte=3&capitulo=13&grupo=9&seccion=15 (acceso Mayo, 2017).

extendido del aguacate es en diversas formulaciones dermatológicas para curar heridas, estimular el crecimiento del cabello y en emulsiones para el tratamiento de la piel seca, como agente protector contra la radiación ultravioleta y agentes antiedad[201- 208].

Sus componentes nutricionales varían dependiendo, entre otros factores, de la variedad, grado de maduración y condiciones de cultivo[209]. En la **Tabla** 2 se muestran los componentes nutricionales del aguacate.

[201] Korać, R. R.; Khambholja, K. M. Potential of herbs in skin protection from ultraviolet radiation. *Pharmacogn. Rev.* **2011**, *5* (10), 164–173.

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[207] Kosińska, A.; Karamać, M.; Estrella, I.; Hernández, T.; Bartolomé, B.; Dykes, G. a. Phenolic compound profiles and antioxidant capacity of *Persea americana* Mill. peels and seeds of two varieties. *J. Agric. Food Chem.* **2012**, *60* (18), 4613–4619.

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[209] Whiley, A. W.; Schaffer, B.; Wilstenholme, B. N. *The Avocado Botany, Production and Uses*; CAB International Inc., **2002**.

Tabla 2. Contenido nutricional en 100 g de pulpa de aguacate maduro[211].

Composición nutricional general	Vitaminas	Lípidos	Minerales
Energía: 160 kcal	Vitamina C: 10 mg	Ácidos grasos saturados (SFA): 2,126 g	Calcio (Ca): 12 mg
Agua: 73,23 g	Tiamina (Vit. B1): 0,067 mg	Ácidos grasos monoinsaturados (MUFA): 9,799 g	Hierro (Fe): 0,55 mg
Proteínas: 2,0 g	Riboflavina (Vit. B2): 0,130 mg	Ácidos grasos poliinsaturados (PUFA): 1,816 g	Magnesio (Mg): 29 mg
Lípidos Totales: 14,66 g	Niacin (Vit. B3): 1,738 mg		Fósforo (P): 52 mg
Carbohidratos: 8,53	Vitamina B6: 0,257 mg		Potasio (K): 485 mg
Fibra Dietética Total: 6,7 g	Folato (Vit. B9) (EFD): 81 µg		Sodio (Na): 7 mg
Azúcares Totales: 0.66 g	Vitamina A (EAR): 7 µg		Zinc (Zn): 0,64 mg
	Vitamina E: 2,07 mg		
	Vitamina K1 (Filoquinona): 21 µg		

EFD:Equivalentes de Folato Dietético
EAR: Equivalentes de Actividad de Retinol

El nivel de proteínas en el aguacate es mayor que en otras frutas, alcanzando valores del 2%, mientras que la mayoría de las frutas presentan un contenido de proteínas de alrededor del 1%[196]. Entre los nutrientes mayoritarios se encuentran los lípidos, el aguacate contiene el mayor contenido de grasa de todas las frutas (14,66%), a excepción quizás de la aceituna[210, 211]. Su contenido aumenta con el proceso de maduración[192]. Los ácidos grasos monoinsaturados (MUFA) son los predominantes, lo que parece que ayude a mejorar la biodisponibilidad de nutrientes y compuestos bioactivos, además

[210] Borrone, J. W.; Brown, J. S.; Tondo, C. L.; Mauro-Herrera, M.; Kuhn, D. N.; Violi, H. A.; Sautter, R. T.; Schnell, R. J. An EST-SSR-based linkage map for *Persea americana* Mill. (avocado). *Tree Genet. Genomes* **2009**, *5* (4), 553–560.

[211] USDA (U.S. Department of Agriculture). Avocado, raw, all comercial varieties Composition. Nutrient Data Laboratory. USDA National Nutrient Database for Standard Reference, Release 28. U.S. Department of Agriculture. https://ndb.nal.usda.gov/ndb/foods/show/2156?fgcd=&manu=&lfacet=&format=&count=&max=50&offset=&sort=default&order=asc&qlookup=avocado&ds=&qt=&qp=&qa=&qn=&q=&ing= (acceso Mayo, 2017).

de enmascarar el sabor y la textura de la fibra alimentaria[211,212]. Entre ellos destaca el ácido oleico (C18:1n-9) como el más representativo (sobre el 57% del contenido total) [213]. Además, el aguacate contiene ácidos grasos poliinsaturados (PUFA) entre los que destaca el ácido linoleico (C18:2n-6), y ácidos grasos saturados (SFA) entre los que se encuentra el ácido palmítico (C16:0). A medida que el aguacate madura va disminuyendo su contenido en SFA y aumenta el ácido oleico monoinsaturado[192,214,215]. Un aguacate (136 g) tiene un perfil de nutrientes y compuestos bioactivos similar a 42,5 g de frutos secos de cáscara (almendras, pistachos o nueces), a los que se les han atribuido beneficios saludables para el corazón [216, 217]. Entre los componentes que destacan del aguacate también encontramos fitosteroles concretamente estigmasterol, campesterol y β-sitosterol. El aguacate es la

[212] Unlu, N. Z.; Bohn, T.; Clinton, S. K.; Schwartz, S. J. Carotenoid absorption from salad and salsa by humans is enhanced by the addition of avocado or avocado oil. *J. Nutr.* **2005**, *135* (3), 431–436.

[213] Plaza, L.; Sánchez-Moreno, C.; de Pascual-Teresa, S.; de Ancos, B.; Cano, M. P. Fatty acids, sterols, and antioxidant activity in minimally processed avocados during refrigerated storage. *J. Agric. Food Chem.* **2009**, *57* (8), 3204–3209.

[214] Lu, Q.-Y.; Zhang, Y.; Wang, Y.; Wang, D.; Lee, R.; Gao, K.; Byrns, R.; Heber, D. California Hass avocado: Profiling of carotenoids, tocopherol, fatty acid, and fat content during maturation and from different growing areas. *J. Agric. Food Chem.* **2009**, *57* (21), 10408–10413.

[215] Carvalho, C. P.; Velásquez, M. A. Fatty acid content of avocados (*Persea americana* Mill. cv. Hass) in relation to orchard altitude and fruit maturity stage. *Agron. Colomb.* **2015**, *33* (2), 220–227.

[216] FDA (Food and Drug Administration). Qualified Health Claims: Letter of Enforcement Discretion - Nuts and Coronary Heart Disease (Docket No 02P-0505) http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ucm072926.htm (acceso Mayo, 2017).

[217] FAO (Food and Agriculture Organization of the United Nations). Qualified Health Claims: Letter of Enforcement Discretion - Walnuts and Coronary Heart Disease (Docket No 02P-0292) http://www.fda.gov/Food/IngredientsPackagingLabeling/LabelingNutrition/ucm072910.htm (acceso Mayo, 2017).

fuente frutal conocida más rica en fitosteroles[218] con más de 113 mg por fruto (136 g) de fruta[219]. Aunque su contenido en el aguacate es menor que alimentos enriquecidos y suplementos dietéticos su matriz única de grasa emulsionada y fitosteroles glucósidos naturales puede ayudar más al bloqueo intestinal del colesterol que los alimentos enriquecidos y los suplementos[220].

El aguacate es además una importante fuente de vitaminas especialmente vitaminas E, C y B_6 minerales como potasio, pigmentos (antocianinas, clorofilas y carotenoides como β-caroteno)[221-223], compuestos fenólicos[224] y azúcares de 7 carbonos y sus alcoholes correspondientes (D-mannoheptulosa y perseitol), los cuales no son azúcares muy comunes[225].

En el aguacate se han identificado diversos grupos de compuestos fenólicos como son los ácidos fenólicos (gálico, protocatecuico, gentísico, 4-hidroxibenzoico, clorogénico, etc), flavonoles (rutina, quercetina,

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[225] Meyer, M. D.; Terry, L. A. Fatty acid and sugar composition of avocado, cv. Hass, in response to treatment with an ethylene scavenger or 1-methylcyclopropene to extend storage life. *Food Chem.* **2010**, *121* (4), 1203–1210.

kaempferol, etc...), flavanonas (narirutina, naringina, naringerina, etc...), flavonas (apigenina, laricitrina, luteolina, pinocembrina, etc...) y flavanoles (catequina y epicatequina)[226- 234].

[226] Hurtado-Fernández, E.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Profiling LC-DAD-ESI-TOF MS method for the determination of phenolic metabolites from avocado (*Persea americana*). *J. Agric. Food Chem.* **2011**, *59* (6), 2255–2267.

[227] Hurtado-Fernández, E.; González-Fernández, J. J.; Hormaza, J. I.; Bajoub, A.; Fernández-Gutiérrez, A.; Carrasco-Pancorbo, A. Targeted LC-MS approach to study the evolution over the harvesting season of six important metabolites in fruits from different avocado cultivars. *Food Anal. Methods* **2016**, *9* (12), 3479–3491.

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[230] Hurtado-Fernández, E.; Pacchiarotta, T.; Mayboroda, O. A.; Fernández-Gutiérrez, A.; Carrasco-Pancorbo, A. Quantitative characterization of important metabolites of avocado fruit by gas chromatography coupled to different detectors (APCI-TOF MS and FID). *Food Res. Int.* **2014**, *62*, 801–811.

[231] Hurtado-Fernández, E.; Pacchiarotta, T.; Longueira-Suárez, E.; Mayboroda, O. A.; Fernández-Gutiérrez, A.; Carrasco-Pancorbo, A. Evaluation of gas chromatography-atmospheric pressure chemical ionization-mass spectrometry as an alternative to gas chromatography-electron ionization-mass spectrometry: Avocado fruit as example. *J. Chromatogr. A* **2013**, *1313*, 228–244.

[232] Contreras-Gutiérrez, P. K.; Hurtado-Fernández, E.; Gómez-Romero, M.; Hormaza, J. I.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Determination of changes in the metabolic profile of avocado fruits (*Persea americana*) by two CE-MS approaches (targeted and non-targeted). *Electrophoresis* **2013**, *34* (19), 2928–2942.

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[234] López-Cobo, A.; Gómez-Caravaca, A. M.; Pasini, F.; Caboni, M. F.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado. *LWT - Food Sci. Technol.* **2016**, *73*, 505–513.

Estudios más recientes han relacionado los compuestos bioactivos del aguacate con actividades beneficiosas para la salud humana como efectos antiinflamatorios, cardioprotectores debido a que su alto contenido en fitosteroles mantiene reducidos los niveles de colesterol [235] y la quimioprevención[236-239], (ej. la pulpa de aguacate posee efectos ihnibitorios *in vitro* de crecimiento contra el cáncer) [214]. También son conocidos sus efectos beneficiosos para la piel, por ejemplo, la fracción insaponificable de la pulpa combinada con aceite insaponificable de soja es usado para el tratamiento de osteoartritis[238].

B.2.1. Subproductos procedentes del procesado del aguacate

El aguacate es manufacturado sobre todo para producir guacamole, puré y aceite de aguacate [240]. Los subproductos de esta acción son el orujo de aguacate, el hueso y la piel principalmente. Actualmente, estos subproductos no tienen un uso comercial[240]; sin embargo, presentan mayor cantidad de

[235] Grant, W. C. Influence of avocados on serum cholesterol. *Proc. Soc. Exp. Biol. Med.* **1960**, *104* (1), 45–47.

[236] Ding, H.; Chin, Y.-W.; Kinghorn, a D.; D'Ambrosio, S. M. Chemopreventive characteristics of avocado fruit. *Semin. Cancer Biol.* **2007**, *17* (5), 386–394.

[237] Altinel, L.; Saritas, Z. K.; Kose, K. C.; Pamuk, K.; Aksoy, Y.; Serteser, M. Treatment with unsaponifiable extracts of avocado and soybean increases TGF-β1 and TGF-β2 levels in canine joint fluid. *Tohoku J. Exp. Med.* **2007**, *211* (2), 181–186.

[238] Boileau, C.; Martel-Pelletier, J.; Caron, J.; Msika, P.; Guillou, G. B.; Baudouin, C.; Pelletier, J.-P. Protective effects of total fraction of avocado/soybean unsaponifiables on the structural changes in experimental dog osteoarthritis: inhibition of nitric oxide synthase and matrix metalloproteinase-13. *Arthritis Res. Ther.* **2009**, *11* (2), R41.

[239] Lu, Q.-Y.; Arteaga, J. R.; Zhang, Q.; Huerta, S.; Go, V. L. W.; Heber, D. Inhibition of prostate cancer cell growth by an avocado extract: Role of lipid-soluble bioactive substances. *J. Nutr. Biochem.* **2005**, *16* (1), 23–30.

[240] Barbosa-Martín, E.; Chel-Guerrero, L.; González-Mondragón, E.; Betancur-Ancona, D. Chemical and technological properties of avocado (*Persea americana* Mill.) seed fibrous residues. *Food Bioprod. Process.* **2016**, *100*, 457–463.

compuestos fenólicos y actividad antioxidante que la pulpa[241-243]. De hecho, el contenido fenólico y actividad antoxidante de la piel y el hueso del aguacate son varias veces superior a los que han sido atribuidos a los arándanos, que son conocidos por su alta capacidad antioxidante[242,244].

B.2.1.1 Hueso

Figura 11. Hueso de aguacate.

El hueso del aguacate **(Figura 11)** constituye hasta el 18,3% del peso total del fruto[242]. Tiene un perfil rico en compuestos bioactivos y una larga historia de uso etnobotánico[245,246]. A pesar de esto es considerado en su gran mayoría como un producto de desecho y, hoy en día es un recurso al que se le podría sacar mucho más provecho[247].

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[241] Soong, Y.-Y.; Barlow, P. J. Antioxidant activity and phenolic content of selected fruit seeds. *Food Chem.* **2004**, *88* (3), 411–417.

[242] Wang, W.; Bostic, T. R.; Gu, L. Antioxidant capacities, procyanidins and pigments in avocados of different strains and cultivars. *Food Chem.* **2010**, *122* (4), 1193–1198.

[243] Rodríguez-Carpena, J.-G.; Morcuende, D.; Andrade, M.-J.; Kylli, P.; Estévez, M. Avocado (*Persea americana* Mill.) phenolics, in vitro antioxidant and antimicrobial activities, and inhibition of lipid and protein oxidation in porcine patties. *J. Agric. Food Chem.* **2011**, *59* (10), 5625–5635.

[244] Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52* (12), 4026–4037.

[245] Ramos-Jerz, M. del R. *Phytochemical Analysis of Avocado Seeds (Persea americana Mill., c.v. Hass)*; Cuvillier Verlag Göttingen, **2007**.

[246] Olaeta, J. A.; Schwartz, M.; Undurraga, P.; Contreras, S. *Use of Hass Avocado (Persea americana Mill.) Seed as a Processed Product*; Proceedings VI World Avocado Congress, **2007**.

[247] Dabas, D.; Shegog, R. M.; Ziegler, G. R.; Lambert, J. D. Avocado (*Persea americana*) seed as a source of bioactive phytochemicals. *Curr. Pharm. Des.* **2013**, *19* (34), 6133–6140.

En cuanto a su composición el contenido de proteína es de 9,6% del peso seco del hueso, el de fibra supone el 10,7% y el total de lípidos del 3,9% del peso seco. El hueso presenta una menor proporción de lípidos que la pulpa y un porcentaje menor de estos corresponden a lípidos neutros, 77-80% frente al 95-99% de la pulpa. Entre estos se encuentran principalmente monoacilgliceroles, diacilgliceroles, esteroles libres y ácidos grasos libres, y en menor medida triacilgliceroles. El porcentaje de las otras dos fracciones lipídicas, glucolípidos (12-13%) y fosfolípidos (7,4-10,9%) es mayor en hueso que en pulpa (2,5-3,2% en hueso frente a 0,7-2,1% en pulpa)[248].

Además, en el hueso de aguacate se encuentran otras familias de compuestos bioactivos como son los fitosteroles, alkaloides, triterpenos, saponinas, ácidos furanoicos, ácido abscísico, y compuestos fenólicos. Entre estos últimos destacan las procianidinas tipo A y en menor medida las de tipo B, al contrario de lo que ocurre en pulpa y piel. La cuantificación de las procianidinas se ha llevado a cabo para los monómeros (catequina/epicatequina), los oligómeros hasta un grado de polimerización mayor de 10 y para el polímero[234,242,243]. También se han identificado y cuantificado compuestos fenólicos de diferentes familias como ácidos hidroxibenzoicos, ácidos hidroxicinámicos, y flavonoles[234,243]. Al igual que ocurre en la pulpa, los niveles de estos compuestos en el hueso varían en función de la variedad de aguacate, de las condiciones de cultivo y el estado de maduración[247].

Estudios etnofarmacológicos de las culturas azteca y maya muestran los usos de la decocción del hueso de aguacate en el tratamiento de infecciones

[248] Takenaga, F.; Matsuyama, K.; Abe, S.; Torii, Y.; Itoh, S. Lipid and fatty acid composition of mesocarp and seed of avocado fruits harvested at northern range in Japan. *J. Oleo Sci.* **2008**, *57* (11), 591–597.

micóticas y parasitarias [249]. Preparados de hueso también han sido mencionados para el uso contra la diabetes, la hipertensión [250], irregularidades gastrointestinales, anemia, como diurético e incluso como agente abortivo [251]. En la tradición africana, los huesos molidos eran consumidos para tratar panadizo y disentería. Aplicaciones tópicas de preparados de hueso de aguacate han sido usadas para tratar la artritis, erupciones cutáneas, mejorar la caspa o calmar el dolor de muelas [252]. Históricamente, los exudados coloreados de las semillas de aguacate fueron usados como tinta permanente por los conquistadores en el siglo XVI[252].

Las investigaciones recientes sobre la bioactividad potencial del hueso de aguacate están todavía en estados iniciales pero podemos decir que presenta actividad anticancerígena [253,254], antiinflamatoria [255,256], antidiabética [257,258],

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[249] Appel Kunow, M. *Maya Medicine: Traditional Healing in Yucatan.*; Appel Kunow, M., Ed.; University of New Mexico Press, **2003**.

[250] Ozolua, R. I.; Anaka, O. N.; Okpo, S. O.; Idogun, S. E. Acute and sub-acute toxicological assessment of the aqueous seed extract of *Persea americana* Mill (Lauraceae) in rats. *African J. Tradit. Complement. Altern. Med.* **2009**, *6* (4), 573–578.

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[257] Edem, D. O. Hypoglycemic effects of ethanolic extracts of alligator pear seed (*Persea americana* Mill) in rats. *Eur. J. Sci. Res.* **2009**, *33* (4), 669–678.

antihipertensiva[206, 259, 260], hipocolesterolémica[261-263], antimicrobiana[264-267], insecticida[265,268] y efectos beneficiosos para la piel[256,269-271].

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[258] Edem, D. O.; Ekanem, I. S.; Ebong, P. E. Effect of aqueous extracts of alligator pear seed (*Persea americana* Mill) on blood glucose and histopathology of pancreas in alloxan-induced diabetic rats. *Pak. J. Pharm. Sci.* **2009**, *22* (3), 272–276.

[259] Imafidon, K. E.; Igbinaduwa, P. The effect of aqueous extract of *P. americana* Mill. (Avocado) seeds on blood pressures and electrolytes in hypertensive rats. *Biosci. Biotechnol. Res. Asia* **2010**, *7* (1), 133–137.

[260] Kate, I. E.; Lucky, O. O. Biochemical evaluation of the tradomedicinal uses of the seeds of *Persea americana* Mill., (family: Lauraceae). *World J. Med. Sci.* **2009**, *4* (2), 143–146.

[261] Pahua-Ramos, M. E.; Ortiz-Moreno, A.; Chamorro-Cevallos, G.; Hernández-Navarro, M. D.; Garduño-Siciliano, L.; Necoechea-Mondragón, H.; Hernández-Ortega, M. Hypolipidemic effect of avocado (*Persea americana* Mill) seed in a hypercholesterolemic mouse model. *Plant Foods Hum. Nutr.* **2012**, *67* (1), 10–16.

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[263] Nwaoguikpe, R. N.; Braide, W. The effect of aqueous seed extract of *Persea americana* (avocado pear) on serum lipid and cholesterol levels in rabbits. *African J. Pharm. Pharmacol. Res.* **2011**, *1* (2), 23–29.

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[266] Nagaraj, M.; Sandhya, V.; Supriya, G.; Manju, R.; Kumari, P.; Bole, S.; Lalitha, V.; Kiran, B. Antioxidant and antibacterial activity of Avocado (*Persea gratissima* Gaertner.) Seed extract. *World Appl. Sci. J.* **2010**, *9* (6), 695–698.

[267] Ruiseco, M. G. Oil based scalp treatment composition. US 4849214 A, 1989.

[268] Torres, R. C.; Garbo, A. G.; Walde, R. Z. M. L. Larvicidal activity of *Persea americana* Mill. against *Aedes aegypti*. *Asian Pac. J. Trop. Med.* **2014**, *7* (S1), S167–S170.

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[270] Ramos-Jerz, M. del R.; Villanueva, S.; Deters, A. M. Influence of avocado seed fractions (*Persea americana* Mill.) obtained by HSCCC on human skin keratinocytes and fibroblasts: Differences of effects in regard of tested cell types. *Planta Med.* **2007**, *73*, P_485.

Una de las recientes aplicaciones del hueso de aguacate ha demostrado como este puede tener uso como agente antioxidante en la industria. Un estudio ha demostrado su capacidad antioxidante para evitar el oscurecimiento de las gambas[272].

B.2.1.2. Piel

Figura 12. Piel de aguacate.

La piel es la fracción del aguacate que ha sido menos estudiada (**Figura 12**). Puede representar hasta un 20,9% del peso total del fruto dependiendo de la variedad[242]. En cuanto a su composición, la piel presenta 6,3% de proteína, 3,5% de lípidos totales y 46,9% de fibra. Además entre los minerales destacan potasio (899 mg/100 g piel seca) y calcio (679,3 mg/100 g piel seca), pero además contiene Mg, Na, Cu, Fe, Mn y Zn. Entre los ácidos grasos más destacados se encuentran ácido oleico (C18:1n-9) (822,7 mg/100 g piel seca), ácido palmítico (C16:0) (588,5 mg/100 g piel seca) y ácido linoleico (C18:2n-6) (466,4 mg/100 g piel seca), pero también presenta ácido mirístico (C14:0), ácido esteárico (C18:0), ácido cis-7 hexadecenoico C16:1n-9, ácido vaccénico (C18:1n-7), ácido eicosenoico

[271] Counts, D. F.; Huber, R. Lipidic furans and retinol palmitate composition useful for skin therapeutics. US 5514709 A, **1996**.

[272] Dao, P. T. A.; An, N. T. H.; Thuy, N. T. T.; Tuyet, N. T. A.; Truc, T. T. M. Screening on antioxidant activities of by-products from vegetables and fruits in Tay Nguyen region and applying for shrimp cold storage. In *Proceedings- 3rd International Conference on Green Technology and Sustainable Development (GTSD) 2016*; IEEE, **2016**; pp 93–97.

(C20:1n-9), ácido α-linolénico (C18:3n-3) y ácido eicosadienoico (C20:2n-6)[273].

Entre los compuestos bioactivos de la piel de aguacate, al igual que en el hueso, se encuentran los compuestos fenólicos, entre los que destacan las procianidinas tipo A y B. En este caso la cuantificación de las procianidinas también se ha llevado a cabo para los monómeros (catequina/epicatequina), los oligómeros hasta un grado de polimerización mayor de 10 y para el polímero[234,242]. También han sido identificados compuestos fenólicos de diferentes familias como ácidos hidroxibenzoicos, ácidos hidroxicinámicos y flavonoles[234,243].

La presencia de este tipo compuestos bioactivos le da a la piel cualidades beneficiosas para la salud humana. Se han descrito, entre otros beneficios, actividad larvicida frente a *Aedes aegypti*, mosquito transmisor de la fiebre amarilla, actividad antimicrobiana[243], y la presencia, en piel del aguacate inmaduro, de compuestos con actividad antifúngica[274].

[273] Morais, D. R.; Rotta, E. M.; Sargi, S. C.; Bonafe, E. G.; Suzuki, R. M.; Souza, N. E.; Matsushita, M.; Visentainer, J. V. Proximate composition, mineral contents and fatty acid composition of the different parts and dried peels of tropical fruits cultivated in Brazil. *J. Braz. Chem. Soc.* **2017**, *28* (2), 308–318.

[274] Adikaram, N. K. B.; Ewing, D. F.; Karunaratne, A. M.; Wijeratne, E. M. K. Antifungal compounds from immature avocado fruit peel. *Phytochemistry* **1992**, *31* (1), 93–96.

B.3. Mango (*Mangifera indica* L.)

El mango (*Mangifera indica* L.) **(Figura 13)** pertenece a la familia Anarcadiaceae. Es un árbol perenne, con hojas alternas lanceoladas y ásperas que tienen unos 15-35 cm de longitud y 6-16 cm de ancho y cambian su color con el tiempo, siendo naranjas rosadas cuando son jóvenes, rápidamente cambian a rojo intenso y cuando son maduras se transforman a verde oscuro[275]. La forma y el tamaño del árbol dependen, sin tener en cuenta la variedad, del tipo de reproducción. Árboles con reproducción sexual por semilla crecen erectos y altos, dando frutos generalmente después del sexto año, y llegan a medir hasta 25-35 m de altura. Sin embargo, los árboles provenientes de reproducción asexual (ej. injerto), son de menor tamaño, hasta 5-10 m de altura y de ramificación escasa y abierta, y comienzan a producir fruto a partir del tercer año de sembrados[276]. Las inflorescencias miden 25-30 cm de largo y contienen flores pentámeras hermafroditas dispuestas en su mayoría en el ápice de la inflorescencia y flores masculinas mezcladas en la misma. Produce gran cantidad de flores por árbol, lo que conduce a una eliminación natural de las mismas a fin de proteger al árbol del exceso de frutos, que por su peso y

Figura 13. Árbol de mango con flor y fruto.

[275] Kittiphoom, S. Utilization of mango seed. *Int. Food Res. J.* **2012**, *19* (4), 1325–1335.

[276] Sergent, E. *El Cultivo del Mango (Mangifera indica L.). Botánica, Manejo y Comercialización*; Universidad Central de Venezuela. Consejo de Desarrollo Científico y Humanístico, **1999**; pp 53–58.

actividad metabólica le afectaría negativamente. Generalmente se considera que un 1% de formación de frutos produce cosechas extraordinarias[276]. El fruto es una drupa grande de forma oval y de color verde al comienzo y amarillo, naranja e incluso rojo granate cuando está maduro. Tarda en madurar 3-6 meses. De piel lisa y grosor moderado a grueso. La pulpa pudiendo ser fibrosa o no, es jugosa, firme, amarilla anaranjada, muy dulce, aromática, y tiene una buena cantidad comestible. A comienzos del periodo de domesticación los árboles de mango probablemente dieran frutos pequeños, pero la selección del hombre de plantas de mejor calidad durante cientos de años ha resultado en la producción de mayores frutos, pudiendo llegar a 2 kg en algunas variedades[275,277,278] **(Figura 14)**. El hueso es aplanado y con forma arriñonada **(Figura 16)**, presenta en su exterior un tejido duro (endocarpio), que en su lado externo se prolonga con fibras que penetran al mesocarpio, y que encierra una almendra[276].

[277] Mukherjee, S. K.; Litz, R. E. *Introduction: Botany and Importance*; **2009**.

[278] Sergent, E. *El Cultivo del Mango (Mangifera indica L.). Botánica, manejo y comercialización*; Universidad Central de Venezuela. Consejo de Desarrollo Científico y Humanístico, **1999**; p 41.

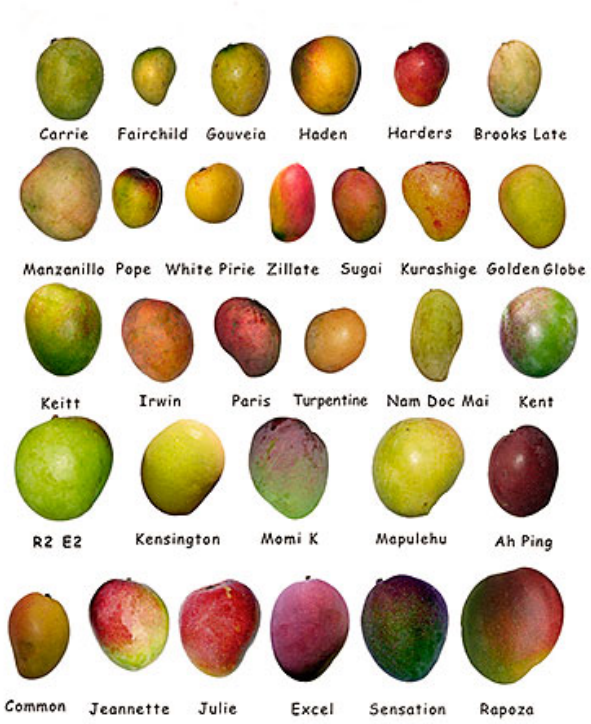

Figura 14. Diferentes variedades de mango.

Este árbol es originario de India, aunque hoy se cultiva en países de clima cálido y algunos de clima templado. Registros históricos sugieren que el cultivo de este árbol se remonta hasta hace unos 4000 años[277]. Hasta 1970 los mangos eran poco conocidos por los consumidores fuera de los trópicos y la comercialización de la fruta fresca no existía. Sin embargo, en los años sucesivos hubo una rápida expansión de la producción de mango en áreas donde no era tradicional y se estableció el comercio de mango fresco y derivados procesados[5]. Con el crecimiento mundial de la producción, el mango representa una de las frutas tropicales más importantes, gracias a su agradable sabor y aroma y a su alto valor nutricional[279]. La producción

[279] Ibarra-Garza, I. P.; Ramos-Parra, P. A.; Hernández-Brenes, C.; Jacobo-Velázquez, D. A. Effects of postharvest ripening on the nutraceutical and physicochemical properties of mango (*Mangifera indica* L. cv Keitt). *Postharvest Biol. Technol.* **2015**, *103*, 45–54.

mundial de mango está estimada en 42 millones de toneladas al año. Sin embargo, esta está muy concentrada, ya que según los últimos datos disponibles, en 2014 Asia sostenía más del 76% de la producción total de mango, y América Central y del Sur junto con África sostenían más del 21%. No obstante, India sigue siendo el mayor productor mundial de mango con 1.525.000 toneladas al año seguido de China, Kenia, Tailandia, Indonesia, Pakistán y México[6] **(Figura 15)**.

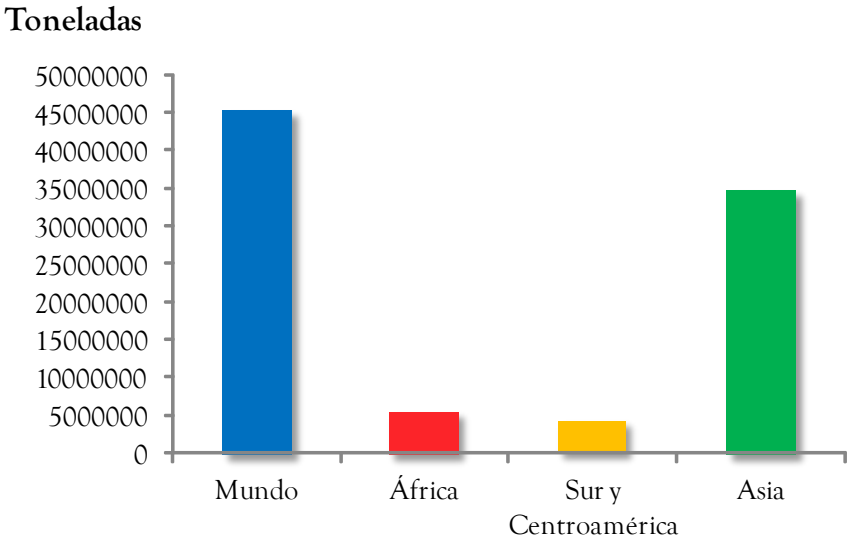

Figura 15. Producción mundial de mango[6].

Su composición en macronutrientes no varía mucho entre variedades. Contiene bajos niveles de lípidos y proteínas, y aproximadamente el 15% de carbohidratos totales. Como muchas frutas, la pulpa de mango contribuye poco en el aporte calórico de una dieta[5].

A continuación se muestra en la **Tabla 3** el contenido nutricional de la pulpa de mango.

Tabla 3. Contenido nutricional en 100 g de pulpa de mango[280].

Composición nutricional general	Vitaminas	Lípidos	Minerales
Energía: 60 kcal	Vitamina C: 36,4 mg	Ácidos grasos saturados (SFA): 0,092 g	Calcio (Ca): 11 mg
Agua: 83,46 g	Tiamina (Vit. B1): 0,028 mg	Ácidos grasos monoinsaturados (MUFA): 0,140 g	Hierro (Fe): 0,16 mg
Proteínas: 0,82 g	Riboflavina (Vit. B2): 0,038 mg	Ácidos grasos poliinsaturados (PUFA): 0,071 g	Magnesio (Mg): 10 mg
Lípidos Totales: 0,38 g	Niacin (Vit. B3): 0,669 mg		Fósforo (P): 14 mg
Carbohidratos: 14,98	Vitamina B6: 0,119 mg		Potasio (K): 168 mg
Fibra Dietética Total: 1,6 g	Folato (Vit. B9) (EFD): 43 µg		Sodio (Na): 1 mg
Azúcares totales: 13,66 g	Vitamina A (EAR): 54 µg		Zinc (Zn): 0,09 mg
	Vitamina E: 0,90 mg		
	Vitamina K1 (Filoquinona): 4,2 µg		

EFD:Equivalentes de Folato Dietético
EAR: Equivalentes de Actividad de Retinol

Con respecto a los micronutrientes, el contenido de minerales no es muy elevado[5,281], y por tanto la pulpa no es considerada como fuente de estos nutrientes. Contiene carotenoides como β-caroteno que es el carotenoide más abundante en muchas variedades[282,283]. Las vitaminas presentes en mayor porcentaje son las vitaminas A y C. Otro elemento muy representativo

[280] USDA (U.S. Department of Agriculture). Mango, raw Composition. Nutrient Data Laboratory. USDA National Nutrient Database for Standard Reference, Release 28. U.S. Department of Agriculture. https://ndb.nal.usda.gov/ndb/foods/show/2271?manu=&fgcd=&ds= (acceso Mayo, 2017).

[281] Leterme, P.; Buldgen, A.; Estrada, F.; Londoño, A. M. Mineral content of tropical fruits and unconventional foods of the Andes and the rain forest of Colombia. *Food Chem.* **2006**, *95* (4), 644–652.

[282] Mercadante, A. Z.; Rodriguez-Amaya, D. B. Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango. *J. Agric. Food Chem.* **1998**, *46* (1), 128–130.

[283] Ornelas-Paz, J. D. J.; Yahia, E. M.; Gardea-Bejar, A. Identification and quantification of xanthophyll esters, carotenes, and tocopherols in the fruit of seven Mexican mango cultivars by liquid chromatography-atmospheric pressure chemical ionization-time-of-flight mass spectrometry [LC-(APcI+)-MS]. *J. Agric. Food Chem.* **2007**, *55* (16), 6628–6635.

de esta fruta es la fibra dietética, su contenido es diferente en cantidad y calidad entre variedades y es una de las principales características que influyen en las preferencias del consumidor de las variedades en fresco, siendo las de pulpa con bajo contenido en fibra o sin fibra las preferidas[284]. En cuanto a los compuestos fenólicos mayoritarios en esta fruta destacan los ácidos fenólicos (sobre todo ácido gálico y derivados), gallotaninos, flavonas, flavonoles (quercetina y kaempferol y sus derivados), derivados de la benzofenona (maclurin e iriflofenona glicósidos) y xantonas (manguiferina y derivados)[234, 285 - 288]. Las propiedades saludables del mango radican principalmente en su elevada capacidad antioxidante de la que son responsables en gran medida los compuestos fenólicos. Por ejemplo, existen estudios que relacionan los compuestos fenólicos de la pulpa del mango con actividad anticancerígena en mama[289]. Los fitosteroles son un grupo de

[284] Mahattanatawee, K.; Manthey, J. A.; Luzio, G.; Talcott, S. T.; Goodner, K.; Baldwin, E. A. Total antioxidant activity and fiber content of select Florida-grown tropical fruits. *J. Agric. Food Chem.* **2006**, *54* (19), 7355–7363.

[285] Berardini, N.; Fezer, R.; Conrad, J.; Beifuss, U.; Carle, R.; Schieber, A. Screening of mango (*Mangifera indica* L.) cultivars for their contents of flavonol O- and xanthone C-glycosides, anthocyanins, and pectin. *J. Agric. Food Chem.* **2005**, *53*, 1563–1570.

[286] Dorta, E.; González, M.; Lobo, M. G.; Sánchez-Moreno, C.; de Ancos, B. Screening of phenolic compounds in by-product extracts from mangoes (*Mangifera indica* L.) by HPLC-ESI-QTOF-MS and multivariate analysis for use as a food ingredient. *Food Res. Int.* **2014**, *57*, 51–60.

[287] Barreto, J. C.; Trevisan, M. T. S.; Hull, W. E.; Erben, G.; de Brito, E. S.; Pfundstein, B.; Würtele, G.; Spiegelhalder, B.; Owen, R. W. Characterization and quantitation of polyphenolic compounds in bark, kernel, leaves, and peel of mango (*Mangifera indica* L.). *J. Agric. Food Chem.* **2008**, *56*, 5599–5610.

[288] Schieber, A.; Berardini, N.; Carle, R. Identification of flavonol and xanthone glycosides from mango (*Mangifera indica* L. Cv. 'Tommy Atkins') peels by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 5006–5011.

[289] Nemec, M. J.; Kim, H.; Marciante, A. B.; Barnes, R. C.; Hendrick, E. D.; Bisson, W. H.; Talcott, S. T.; Mertens-Talcott, S. U. Polyphenolics from mango (*Mangifera indica* L.) suppress breast cancer ductal carcinoma in situ proliferation through

compuestos muy representativo en mango y que también contribuyen a sus propiedades beneficiosas para la salud, como ayudar a reducir los niveles de colesterol LDL y colesterol total. Los fitosteroles mayoritarios que han sido identificados en la pulpa de mango son β-sitosterol, campesterol y estigmasterol[234,290].

B.3.1. Subproductos procedentes del procesado del mango

El mango puede ser consumido maduro o inmaduro, y aunque lo más habitual es comerlo fresco, hay una amplia variedad de comidas que se preparan a base de pulpa de mango[5]. Entre las formas procesadas en que podemos encontrar el mango se encuentra: enlatado, congelado como concentrado, hecho puré, deshidratado o en preparados de zumos y mermeladas[291]. Se estima que el 35-60% de la fruta es desechado como desperdicio tras el procesado[15].

activation of AMPK pathway and suppression of mTOR in athymic nude mice. *J. Nutr. Biochem.* **2017**, *41*, 12–19.

[290] Vilela, C.; Santos, S. A. O.; Oliveira, L.; Camacho, J. F.; Cordeiro, N.; Freire, C. S. R.; Silvestre, A. J. D. The ripe pulp of *Mangifera indica* L.: A rich source of phytosterols and other lipophilic phytochemicals. *Food Res. Int.* **2013**, *54* (2), 1535–1540.

[291] Martin, M.; He, Q. Mango bioactive compounds and related nutraceutical properties-A review. *Food Rev. Int.* **2009**, *25* (4), 346–370.

B.3.1.1. Hueso

Figura 16. Hueso de mango.

En el caso del hueso de mango **(Figura 16)**, anualmente se producen más de 1 millón de toneladas como desecho, y actualmente no se utiliza con ningún fin comercial más allá de la alimentación animal [292] . Dependiendo de la variedad, el hueso representa entre el 10% y el 25% del peso total del fruto [293] y, concretamente, la almendra de su interior ocupa el 45,7-72,8% del total del hueso[293,294]. El hueso del mango es una buena fuente de carbohidratos (58-80%) y aunque tiene bajo contenido de proteínas (6-13%), presenta la mayoría de los aminoácidos esenciales con altos valores de lisina, valina y leucina, por lo que se consideran proteínas de alta calidad[295- 301]. Sin embargo, no hay que

[292] Leanpolchareanchai, J.; Padois, K.; Falson, F.; Bavovada, R.; Pithayanukul, P. Microemulsion system for topical delivery of Thai mango seed kernel extract: Development, physicochemical characterisation and *ex vivo* skin permeation studies. *Molecules* **2014**, *19* (11), 17107–17129.

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[294] Thammarat, N. S. Mango seed kernel oil and its physicochemical properties. *Int. Food Res. J.* **2013**, *20* (3), 1145–1149.

[295] Siaka, D. S. Potential of mango (*Mangifera indica* L.) seed kernel as a feed ingredient for poultry: A review. *Worlds. Poult. Sci. J.* **2014**, *70* (2), 279–288.

[296] Abdalla, A. E. M.; Darwish, S. M.; Ayad, E. H. E.; El-Hamahmy, R. M. Egyptian mango by-product 2: Antioxidant and antimicrobial activities of extract and oil from mango seed kernel. *Food Chem.* **2007**, *103* (4), 1141–1152.

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pasar por alto que la presencia de taninos[302] puede reducir el valor biológico de la proteína[5]. La composición lipídica de la semilla del mango ha atraído la atención de los científicos en los últimos años debido a sus particulares características físicas y químicas. Presenta un atractivo perfil lípidico (6-16%) formado por ácidos grasos saturados (44-48%), principalmente esteárico y palmítico, y ácidos grasos insaturados (52-56%), en su mayoría oleico y linolénico [295-300, 303, 304]. Además en menor medida, presenta también los ácidos grasos linoleico, araquidónico, behénico y lignocérico[305-307]. Su grasa presenta propiedades físicas y químicas muy similares a las de las mantecas de cacao comercial; pero además es similar a las mantecas de illipe, karité,

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[299] Gunstone, F. D. *Vegetable Oils in Food Technology: Composition, Properties and Uses*; Wiley-Blackwell; **2011.**.

[300] Akanda, M. J. H.; Sarker, M. Z. I.; Norulaini, N.; Ferdosh, S.; Rahman, M. M.; Omar, A. K. M. Optimization of supercritical carbon dioxide extraction parameters of cocoa butter analogy fat from mango seed kernel oil using response surface methodology. *J. Food Sci. Technol.* **2015**, *52* (1), 319–326.

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[303] Hemavathy, J.; Prabhakar, J. . V.; Sen, D. P. Composition of polar lipids of 'Alphonso' mango (*Mangifera Indica*) kernel. *J. Food Sci.* **1987**, *52* (3), 833–834.

[304] Mohamed, E. M.; Girgis, A. Y. Utilization of mango seed kernels for improving stability of some oils and biscuit production. *J. Agric. Sci. Mansoura Univ.* **2005**, *30* (8), 4625–4636.

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[306] Jahurul, M. H. A.; Zaidul, I. S. M.; Norulaini, N. N. A.; Sahena, F.; Jaffri, J. M.; Omar, A. K. M. Supercritical carbon dioxide extraction and studies of mango seed kernel for cocoa butter analogy fats. *Cyta-Journal Food* **2014**, *12* (1), 97–103.

[307] Sonwai, S.; Kaphueakngam, P.; Flood, A. Blending of mango kernel fat and palm oil mid-fraction to obtain cocoa butter equivalent. *J. Food Sci. Technol.* **2012**, *51* (10), 2357–2369.

kokum o sal[293,305,306,308,309], adecuadas para el uso en alimentación. De hecho, la grasa de esta semilla ha sido aprobada por las autoridades de la Unión Europea como sustituto de la manteca de cacao, muy utilizada en alimentación[5].

Por otro lado, esta semilla ha sido objeto de estudio debido a su contenido en compuestos fenólicos, esteroles, carotenoides, vitamina C y fibra dietética, que mejoran la salud humana[308]. Entre los compuestos fenólicos descritos en hueso de mango destacan en primer lugar los gallotaninos, ácidos fenólicos (principalmente derivados del ácido gálico), xantona glicósidos (mangiferina y derivados), flavonoides (quercetina y derivados). Los esteroles más comunes en hueso de mango son β-sitosterol, Δ-avenasterol, campesterol, estigmasterol. Además presenta tocoferoles α y ϒ[284,310-314].

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[308] Jahurul, M. H. A.; Zaidul, I. S. M.; Ghafoor, K.; Al-Juhaimi, F. Y.; Nyam, K. L.; Norulaini, N. A. N.; Sahena, F.; Mohd Omar, A. K. Mango (*Mangifera indica* L.) by-products and their valuable components: A review. *Food Chem.* **2015**, *183*, 173–180.

[309] Jahurul, M. H. A.; Zaidul, I. S. M.; Norulaini, N. A. N.; Sahena, F.; Jinap, S.; Azmir, J.; Sharif, K. M.; Omar, A. K. M. Cocoa butter fats and possibilities of substitution in food products concerning cocoa varieties, alternative sources, extraction methods, composition, and characteristics. *J. Food Eng.* **2013**, *117* (4), 467–476.

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[313] Gómez-Caravaca, A. M.; López-Cobo, A.; Verardo, V.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC-DAD-Q-TOF-MS as a powerful platform for the determination of phenolic and other polar compounds in the edible part of mango and its by-products (peel, seed and seed husk). *Electrophoresis* **2015**, *37*, 1072-1084.

El hueso de mango puede ser utilizado con diferentes fines, por ejemplo Zein *y col.* (2005) estudiaron la utilización de la harina de hueso de mango, tras ser remojada o hervida para la eliminación de elementos tóxicos, como principal ingrediente para la fabricación de pan, pasteles y galletas[315,316]. El aceite de hueso de mango también es usado como ingrediente en cosméticos[275] y para tratar lombrices intestinales, vómitos y diarrea[317]. Además, el alto contenido en taninos y flavonoides le dan propiedades antimicrobianas a esta semilla[288].

El hueso de mango también ha despertado especial interés científico por sus efectos beneficiosos para la salud humana, por ejemplo, su actividad contra cáncer de mama y colon[318], su actividad antimicrobiana contra bacterias Gram-positivas y Gram-negativas[288, 319 - 321] debido a su elevada capacidad antioxidante, efectos antidiarreicos que han sido atribuidos a su alto

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[314] López-Cobo, A.; Verardo, V.; Diaz-de-Cerio, E.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Gómez-Caravaca, A. M. Use of HPLC- and GC-QTOF to determine hydrophilic and lipophilic phenols in mango fruit (*Mangifera indica* L.) and its by-products. *Food Res. Int.* **2017** (in press).

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[316] Okpala, L. C.; Gibson-Umeh, G. I. Physicochemical Properties of Mango Seed Flour. *Niger. Food J.* **2013**, *31* (1), 23–27.

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[318] Torres-León, C.; Rojas, R.; Contreras-Esquivel, J. C.; Serna-Cock, L.; Belmares-Cerda, R. E.; Aguilar, C. N. Mango seed: Functional and nutritional properties. *Trends Food Sci. Technol.* **2016**, *55*, 109–117.

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contenido en taninos[322] o la capacidad de sus flavonoides para reducir eficazmente los niveles de lípidos en el suero y los tejidos en ratas[323].

B.3.1.2. Piel

Figura 17. Piel de mango.

Aunque un porcentaje mínimo de la piel generada en el procesado del mango se utiliza actualmente para la fabricación de concentrados, la mayor parte es considerada como un residuo y termina siendo una fuente de contaminación ambiental **(Figura 17)**[324]. La piel comprende entre el 7-24% del peso total del fruto[14,325]. Recientemente, este producto ha recibido considerable atención por parte de la comunidad científica debido a su alto contenido en compuestos valiosos y con beneficios para la salud como compuestos fenólicos, carotenoides, enzimas, vitaminas E y C, fibra dietética, celulosa, hemicelulosa, lípidos, proteínas y pectina[9,326].

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[322] Rajan, S.; Suganya, H.; Thirunalasundari, T.; Jeeva, S. Antidiarrhoeal efficacy of *Mangifera indica* seed kernel on Swiss albino mice. *Asian Pac. J. Trop. Med.* **2012**, *5* (8), 630–633.

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[326] Ajila, C. M.; Naidu, K. A.; Bhat, S. G.; Prasada Rao, U. J. S. Bioactive compounds and antioxidant potential of mango peel extract. *Food Chem.* **2007**, *105* (3), 982–988.

Algunos estudios se han centrado en el análisis de la piel del mango porque esta puede considerarse una fuente de fibra dietética de excelente calidad. Su contenido en la piel oscila entre 40,6-72,5%[286]. Además, esta fibra tiene niveles más altos que la pulpa de ciertos minerales importantes para la nutrición humana, como el calcio, potasio, magnesio, hierro y zinc[327].

Los compuestos de la piel del mango con actividad antioxidante son, entre otros, los compuestos fenólicos. Entre estos los más descritos en la piel del mango son las antocianinas y los ácidos fenólicos. Las antocianinas están presentes en mayor cantidad en pieles de fruta madura y las más comunes son derivados de cianidina, peonidina y pelargonidina[308,314]. Entre los ácidos fenólicos los más destacados son el ácido gálico, protocatecuico, elágico, siríngico y sus derivados. Además, han sido descritos en la piel flavonoides como el kaempferol, la quercetina y sus derivados, taninos (galotaninos), xantonas principalmente mangiferina y benzofenonas como derivados del maclurin[286,314,328].

La harina de piel de mango es usada como ingrediente funcional en muchos productos alimenticios, como fideos, pan, bizcochos, galletas y otros productos de panadería [329, 330], y puede ser utilizada para producir

[327] Larrauri, J. A. A.; Rupérez, P.; Borroto, B.; Saura-Calixto, F. Mango peels as a new tropical fibre: Preparation and characterization. *LWT - Food Sci. Technol.* **1996**, *29* (8), 729–733.

[328] Ajila, C. M.; Bhat, S. G.; Prasada Rao, U. J. S. Valuable components of raw and ripe peels from two Indian mango varieties. *Food Chem.* **2007**, *102* (4), 1006–1011.

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[330] Pathak, D.; Majumdar, J.; Raychaudhuri, U.; Chakraborty, R. Study on enrichment of whole wheat bread quality with the incorporation of tropical fruit by-product. *Int. Food Res. J.* **2017**, *24* (1), 238–246.

ingredientes de alto valor añadido, como fibra dietética o compuestos fenólicos, para diversas aplicaciones en alimentación[326,329,331].

Entre las propiedades beneficiosas de la piel del mango para la salud, se encuentran la inhibición de la formación de cataratas asociada a diabetes en ratas[332], inhibición del daño en el ADN de células cancerosas hepáticas[333] y moderada actividad hipoglucémica[334].

[331] Ajila, C. M.; Rao, L. J.; Rao, U. J. S. P. Characterization of bioactive compounds from raw and ripe *Mangifera indica* L. peel extracts. *Food Chem. Toxicol.* **2010**, *48* (12), 3406–3411.

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B.4. Ajedrea (*Satureja montana* subsp. *kitaibelii*)

La ajedrea (*Satureja montana* L.) (**Figura 18**) pertenece a la familia Lamiaceae, subfamilia Nepetoideae y a la tribu Menthae[335]. Es una planta aromática perenne, subarbustiva que alcanza 50 cm de altura. Sus hojas son opuestas y ovaladas y sus flores blancas. El género *Satureja* L. abarca más de 30 especies que se localizan principalmente en la parte este del Mediterráneo, ocupando hábitats áridos, soleados, pedregosos y rocosos[336]. A pesar de ello, actualmente se puede encontrar por toda Europa, Rusia y Turquía[337].

Figura 18. Ajedrea (*Satureja montana* L.) en flor.

En la región del Mediterráneo ha sido empleada como hierba culinaria desde la antigüedad. Sus hojas, flores y tallos son utilizados en infusión, y en medicina tradicional y homeopática para tratar enfermedades debido a su actividad bactericida, carminativa, digestiva, expectorante, fungicida, laxante,

[335] Cantino, P. D.; Harley, R. M.; Wagstaff, S. J. Genera of Labiatae: status and classification. In *Advances in Labiate Science*; Harley, M. R., Reynolds, T., Eds.; Royal Botanic Gardens, Kew, **1992**; pp 511–522.

[336] Slavkovska, V.; Jancic, R.; Bojovic, S.; Milosavljevic, S.; Djokovic, D. Variability of essential oils of *Satureja montana* L. and *Satureja kitaibelii* Wierzb. ex Heuff. from the central part of the Balkan peninsula. *Phytochemistry* **2001**, *57* (1), 71–76.

[337] Ćetković, G. S.; Čanadanović-Brunet, J. M.; Djilas, S. M.; Tumbas, V. T.; Markov, S. L.; Cvetković, D. D. Antioxidant potential, lipid peroxidation inhibition and antimicrobial activities of *Satureja Montana* L. subsp. *kitaibelii* extracts. *Int. J. Mol. Sci.* **2007**, *8* (10), 1013–1027.

antidiurética, etc[338]. *Satureja montana* y especies relacionadas como *S. hortensis* y *S. subspicata* se emplean, además de cómo especias en cocina, en el tratamiento de enfermedades respiratorias (resfriado, bronquitis) por sus acciones expectorante y anticatarral, para afecciones urinarias y digestivas como cólicos y diarrea, como astringente, afrodisiaco y para mejorar la actividad del estómago. Externamente son usadas contra la inflamación en piel y mucosas[338,339].

Satureja montana L. es una especie confusa desde el punto de vista taxonómico y por tanto corológico. Su alta variabilidad es evidente, incluso dentro de un único polimorfismo poblacional y, especialmente, en poblaciones de hábitats muy distantes. El taxón *S. montana* contiene numerosas subespecies entre las que se ha incluido por parte de muchos autores a *Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P. W. Ball[340,341]. Más recientemente, en el año 2001, Slavkovska *y col.* atendiendo a la composición de sus aceites esenciales clasificaron a *Satureja montana* y *Satureja kitaibelii* como dos especies separadas. En esta tesis se hace referencia a esta planta como *Satureja montana* subsp. *kitaibelii*, ya que es la nomenclatura más reciente (1972) acuñada por el Real Jardín Botánico de

[338] Lawless, J. *The Ilustrated Encyclopedia of Essential Oils*; Element, **1999**.

[339] Stanojković, T.; Kolundžija, B.; Ćirić, A.; Soković, M.; Nikolić, D.; Kundaković, T. Cytotoxicity and antimicrobial activity of *Satureja kitaibelii* wierzb. Ex heuff (Lamiaceae). *Dig. J. Nanomater. Biostructures* **2013**, *8* (2), 845–854.

[340] Ball, P. W.; Getliffe, F. M. Satureja L. In *Flora Europeae*; Tutin, G. R., Heywood, H. V., Burges, A. N., Moore, D. M., Valentine, H. D., Walters, M. S., Webb, A. D., Eds.; Cambridge University Press, **1972**; Vol. 3, pp 163–165.

[341] Diklic, N. *Satureja* L. In *Flore de la République Socialiste de Serbie*; Josifovic, M., Ed.; Académie Serbe des Sciences et des Arts, **1974**; pp 455–458.

Kew (Londres, Reino Unido), aunque *Satureja kitaibelii* también es reconocido como basiónimo[342].

Satureja montana subsp. *kitaibelii* no ha recibido mucha atención, lo que puede deberse a su restringida distribución que se limita a la parte norte de la península balcánica y que se extiende hacia el norte y sur de Rumanía[340]. Principalmente, su estudio se ha limitado a la composición de su aceite esencial. Una de las características diferenciales de la subfamilia *Nepetoideae* a la que pertenece la ajedrea es que sus representantes contienen más del 0.5% de aceite esencial[343]. El aceite esencial tiene ciertas funciones tanto biológicas como fisiológicas en el metabolismo de la planta, y funciones ecológicas en interacciones de la planta con el ambiente[344]. La composición del aceite esencial es también significativo para explicar las relaciones filogenéticas tan complejas en muchos casos. Entre los compuestos descritos en el aceite esencial de *Satureja montana* subsp. *kitaibelii* encontramos *p*-cimeno, geraniol, β-elemeno y limoneno como compuestos mayoritarios dependiendo de la ubicación de las muestras[339,345-349]. Tanto el aceite esencial como el extracto

[342] Royal Botanic Garden. World Checklist of Selected Plant Families http://apps.kew.org/wcsp/namedetail.do?name_id=185800 (acceso Mayo, 2017).

[343] El-Gazzar, A.; Watson, L. A taxonomic study of labiatae and related genera. *New Phytol.* **1970**, *69* (2), 451–486.

[344] Jančić, R.; Stošić, D.; Mimica-Dukić, N.; Lakušić, B. *Aromatic Plants of Serbia*; NIP Decije novine, **1995**.

[345] Kundaković, T.; Milenković, M.; Zlatković, S.; Kovacĕvić, N.; Goranc, N. Composition of *Satureja kitaibelii* essential oil and its antimicrobial activity. *Nat. Prod. Commun.* **2011**, *6* (9), 1353–1356.

[346] Pavlovic, S.; Z˘ivanovic, P.; Jancic, R.; Todorovic, B.; Sevarda, A. L.; Kuznjecova, G. A. The qualitative composition of the essential oil in species genus *Satureja* L. (Lamiaceae) distribution in Yugoslavia. *Biosistematika* **1987**, *13* (19).

[347] Ševarda, A. L.; Kuznjecova, G. A.; Živanović, P.; Pavlović, S.; Vujčić, S. The composition of essential oils of some population of the species *Satureja kitaibelii* Wierzb. ex Heuf. *Arch. Pharm. (Weinheim).* **1989**, *39*, 159–162.

etanólico de la ajedrea presentan actividad antioxidante y antimicrobiana frente a un amplio espectro de microorganismos[337,345,349-353].

Sin embargo, *Satureja montana* ha sido más estudiada y se conoce que, además de aceite esencial, contiene varios constituyentes con actividad biológica entre los que se encuentran triterpenos [354] y compuestos fenólicos [355,356]. Entre estos últimos cabe destacar la presencia de ácidos fenólicos como los ácidos gálico, protocatecuico, vanílico, cafeico, siríngico, cumárico, ferúlico, cinámico, elágico, clorogénico, *p*-hidroxibenzoico, 3,5-dimetoxi-4-hydroxicinámico, 2,3-dihidroxifenilacético, α-hidroxidihidrocafeico, cafeico metil ester, 3,4-dihidroxifenilacético metil

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[348] Konakchiev, A.; Tsankova, E. The essential oils of *Satureja montana* ssp. *kitaibelii* Wierzb. and *Satureja pilosa* var. *pilosa* velen from Bulgaria. *J. Essent. Oil Res.* **2002**, *14* (2), 120–121.

[349] Mihajilov-Krstev, T.; Kitić, D.; Radnović, D.; Ristić, M.; Mihajlović-Ukropina, M.; Zlatković, B. Chemical composition and antimicrobial activity of *Satureja kitaibelii* essential oil against pathogenic microbial strains. *Nat. Prod. Commun.* **2011**, *6* (8), 1167–1172.

[350] Pepeljnjak, S.; Stanić, G.; Potoćki, P. Antimicrobial activity of the ethanolic extract of *Satureja montana* ssp. *montana* [Antimikrobna djelotvornost etanolnog ekstrakta vrste *Satureja montana* ssp. *montana*]. *Acta Pharm.* **1999**, *49* (1), 65–69.

[351] Valnet, J. *The Practice of Aromatherapy*; Tisserand, R., Ed.; Healing Arts Press, 1990.

[352] Miladinović, D. Antimicrobial activity of some medicinal plants from Serbia. *Pharmacia* **2005**, *52* (1–2), 46–49.

[353] Madsen, H. L.; Nielsen, B. R.; Bertelsen, G.; Skibsted, L. H. Screening of antioxidative activity of spices. A comparison between assays based on ESR spin trapping and electrochemical measurement of oxygen consumption. *Food Chem.* **1996**, *57* (2), 331–337.

[354] Escudero, J.; López, J. C.; Rabanal, R. M.; Valverde, S. Secondary metabolites from *Satureja* species. New triterpenoid from *Satureja acinos*. *J. Nat. Prod.* **1985**, *48* (1), 128–131.

[355] Dorman, H. J. D.; Bachmayer, O.; Kosar, M.; Hiltunen, R. Antioxidant properties of aqueous extracts from selected Lamiaceae species grown in Turkey. *J. Agric. Food Chem.* **2004**, *52* (4), 762–770.

[356] Ćetković, G. S.; Mandić, A. I.; Čanadanović-Brunet, J. M.; Djilas, S. M.; Tumbas, V. T. HPLC screening of phenolic compounds in winter savory (*Satureja montana* L.) extracts. *J. Liq. Chromatogr. Relat. Technol.* **2007**, *30* (2), 293–306.

ester e hidroxifenilpropioico, y flavanoles como catequina y epicatequina[356,357].

[357] Rzepa, J.; Sajewicz, M.; Baj, T.; Gorczyca, P.; Wlodarek, M.; Kwiatkowski, S.; Kowalska, T.; Waksmundzka-Hajnos, M. The GC/MS and HPLC/DAD analysis of phenolic acids from winter savory (*Satureja montana*). *Ann. Univ. Mariae Curie-Sklodowska, Sect. DDD Pharm.* **2011**, *24* (4), 49–58.

C. PRETRATAMIENTO Y TRATAMIENTO DE LAS MUESTRAS

Los vegetales son matrices muy complejas, por lo que para llevar a cabo su análisis, es necesario realizar un pretratamiento y tratamiento de las muestras que permita: (1) mejorar la estabilidad de la muestra y sus componentes, (2) eliminar la mayor cantidad posible de impurezas y (3) aumentar la eficacia del método de extracción de los analitos[358]. Se trata, por tanto, de adecuar la matriz para optimizar la separación, detección y cuantificación de los compuestos de interés y conseguir gran calidad y reproducibilidad de los resultados analíticos.

En muestras vegetales, el primer paso, previo a su tratamiento, normalmente consiste en someterlas a un proceso de secado para su conservación por periodos de tiempo prolongados, y posteriormente, llevar a cabo su trituración, con el objetivo de aumentar la superficie de contacto con los disolventes de extracción y conseguir mejores resultados en la etapa de tratamiento siguiente.

C.1. Pretratamiento de la muestra: etapa de secado

El contenido de agua en plantas herbáceas varía entre 60-80% y en el caso de frutas y verduras es superior al 80% por lo que se consideran productos altamente perecederos [359, 360]. Para evitar el desarrollo de procesos de deterioro y el crecimiento de microorganismos patógenos, reduciendo la

[358] Watson, R. R. *Polyphenols in Plants*; Elsevier, **2014**.

[359] Sharapin, N. Materias primas vegetales para la industria de productos fitoterapéuticos. In *Fundamentos de Tecnología de Productos Fitoterapéuticos*; Pinzón S., R., Ed.; Secretaría Ejecutiva del Convenio Andrés Bello (SECAB), **2000**; pp 24–25.

[360] Orsat, V.; Changrue, V.; Raghavan, G. S. Microwave drying of fruits and vegetables. *Stewart Postharvest Rev.* **2006**, *2* (6), 1–7.

actividad de las enzimas y la velocidad a la que ocurren los cambios químicos no deseables, las muestras se deben someter a un proceso de secado o deshidratación, en el que se extrae el mayor contenido de agua posible con una alteración mínima del resto de elementos. El secado además reduce el peso y el volumen de la muestra, facilitándose el almacemaciento de la misma[361].

De manera general, los métodos de secado pueden ser clasificados en función del sistema de eliminación de agua aplicado, como: (1) secado térmico, (2) deshidratación osmótica, y (3) desaguado mecánico **(Figura 19)**. En el secado térmico se usa un gas o un medio inerte para eliminar el agua del producto, por lo que el secado térmico se puede dividir en dos tipos: (a) secado por aire, (b) secado en atmósfera con bajo contenido en aire, y (c) secado en atmósfera modificada. En la deshidratación osmótica se usan solventes o disoluciones para eliminar el agua, mientras que en el secado mecánico se aplica una fuerza física. En el secado mecánico se aplica presión o una fuerza centrífuga al producto mientras una barrera física (por ej., una membrana) mantiene separadas las fases líquida y sólida[361].

[361] Brennan, J. G. Evaporación y deshidratación. In *Manual del Procesado de los Alimentos*; Brennan, J. G., Ed.; Wiley-VCH Verlag, **2008**; pp 87–107.

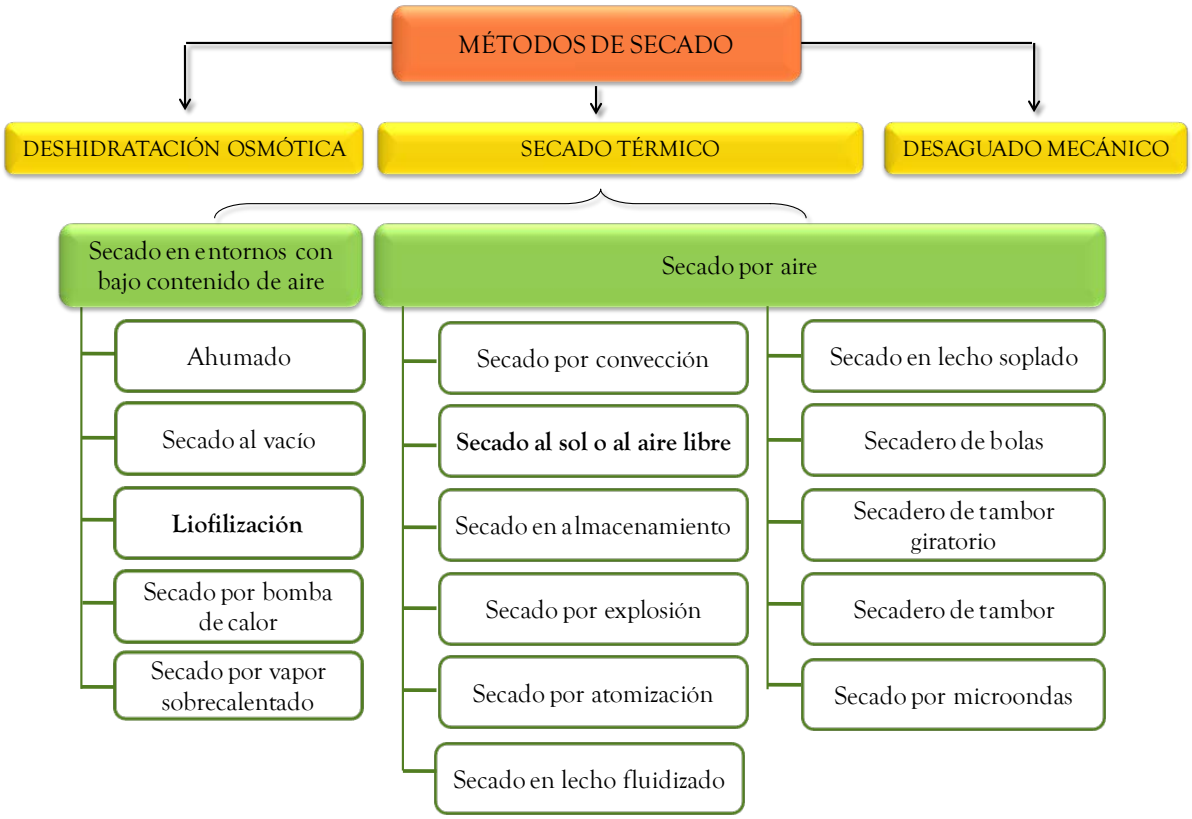

Figura 19. Principales métodos de secado empleados en muestras vegetales.

La elección del método de secado depende de varios factores como el tipo de producto, disponibilidad de secador, el coste de la deshidratación, la calidad final del producto deshidratado y la energía consumida en el proceso. Para las matrices objeto de estudio en esta tesis doctoral se emplearon el secado al aire libre para la ajedrea y la liofilización, uno de los métodos de deshidratación más utilizados en muestras vegetales, para la patata, aguacate y mango.

C.1.1. Secado al aire

El secado de plantas herbáceas reduce el contenido de agua hasta 0,5%-12%. Esto conlleva una pérdida de peso que es diferente según el órgano de la planta: hojas 20-75%, corteza 40-65%, tallo 30-70%, raíces 25-80% y flores

15-80%. La temperatura influye en el proceso de secado ya que pueden presentarse pérdidas de aceites esenciales y sustancias termolábiles. La mayoría de las plantas medicinales pueden ser secadas a temperaturas que varían entre 25 ºC y 60 ºC. Las plantas que contienen aceites esenciales o sustacias termolábiles deben ser secadas a temperaturas inferiores a 40 ºC. Además, para facilitar el secado debe facilitarse una buena circulación del aire[359].

La manera en la que va a ser realizado el secado debe determinarse experimentalmente para cada vegetal, ya que un secado lento puede causar alteraciones perjudiciales antes de que el proceso se haya terminado, debido a la acción de enzimas, hongos y/o bacterias. Sin embargo, un proceso de secado muy rápido endurece la capa superficial de las células e impide la evaporación del agua que está dentro del órgano, lo que propicia la acción de enzimas en su interior[359].

El proceso de secado puede ser realizado al aire libre al sol o a la sombra, extendiento la planta en capas finas sobre una superficie limpia. Sin embargo, este proceso no permite un control de la temperatura y debe interrumpirse cuando comienza a anochecer, guardando las plantas en un local cubierto, para impedir la absorción de la humedad durante la noche[359]. O bien, llevar a cabo todo el proceso en un lugar cubierto y bien ventilado.

C.1.2. Liofilización

La liofilización es uno de los mejores métodos para eliminar el agua de muestras biológicas. Este método de deshidratación es apropiado para células, enzimas, vacunas, virus, levaduras, sueros, derivados sanguíneos,

algas, así como frutas, vegetales, pescados y alimentos en general[362]. Consiste en la congelación de la muestra y la posterior sublimación de la fracción de hielo en la que el agua pasa de estado sólido a gas (**Figura 20**).

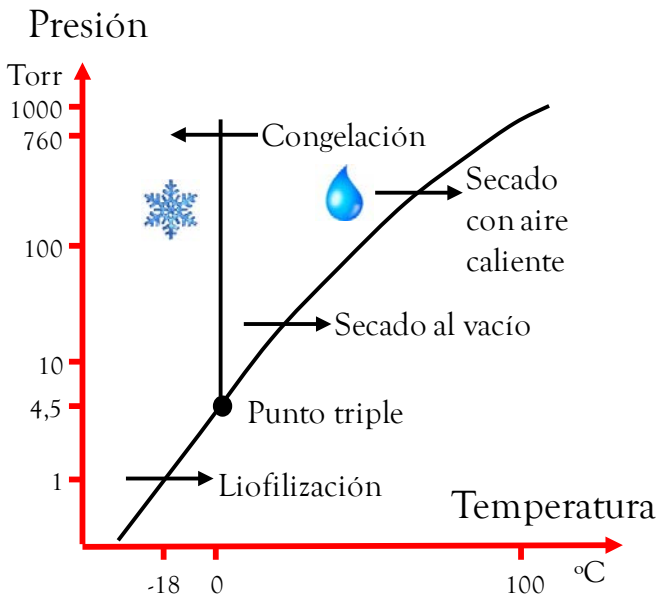

Figura 20. Diagrama de fases del agua y sistemas de secado.

El proceso se desarrolla en dos etapas, la primera consiste en la congelación de la muestra a temperaturas de -40 ºC o inferior, y posteriormente el material congelado se somete a un vacío de unos 10 mm de Hg, lo que da lugar a la sublimación del hielo, que se produce a su vez en dos fases: desecación primaria y secundaria, lo que lleva a la muestra a un estado anhidro o casi anhidro (**Figura 21**). En este proceso de secado los productos obtenidos no se ven alterados en sus propiedades. Debido a las bajas

[362] Ramírez Navas, J. S. Liofilización de alimentos. *Reciteia Rev. Revis. La Ciencia, Tecnol. E Ing. Los Aliment.* **2006**, *6* (2).

temperaturas, los procesos de degradación y la actividad microbiológica se detienen y proporciona un resultado final de mejor calidad[363].

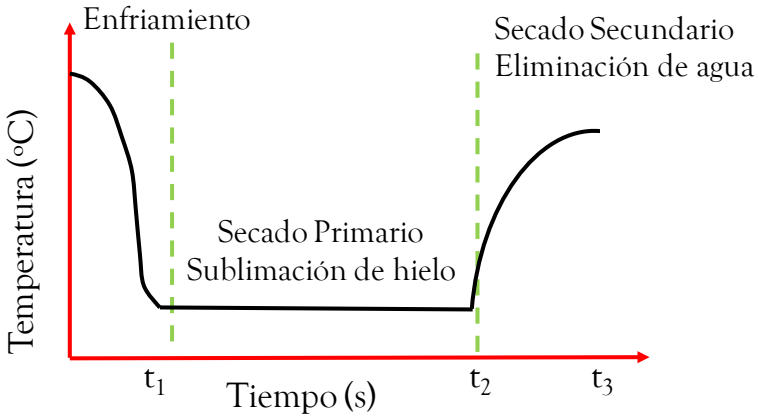

Figura 21. Etapas del proceso de liofilización.

El control y la optimización de los parámetros de liofilización son esenciales para lograr un resultado eficiente. Entre estos parámetros se encuentran (1) la naturaleza de la muestra, (2) las dimensiones del recipiente que contiene la muestra, (3) el equipo de liofilización que incluye modelo, carga en las bandejas, y tipo, número y posición de sondas, y (4) el procedimiento, que incluye la selección de temperatura, la cámara de presión y los tiempos de enfriado y ciclos de secado primario y secundario[364].

Una vez la muestra ha sido secada con el método seleccionado, es sometida a un proceso de extracción de los compuestos bioactivos de interés y que posteriormente serán analizados para su caracterización.

[363] Sagar, V. R.; Suresh Kumar, P. Recent advances in drying and dehydration of fruits and vegetables: A review. *J. Food Sci. Technol.* **2010**, *47* (1), 15–26.

[364] Franks, F. Freeze-drying of bioproducts: Putting principles into practice. *Eur. J. Pharm. Biopharm.* **1998**, *45* (3), 221–229.

C.2. Tratamiento de la muestra: extracción de compuestos bioactivos

El tratamiento de las muestras previo al análisis cromatográfico consiste en la extracción de los compuestos de interés de la matriz en la que se encuentran, y su concentración en caso de que el contenido sea muy pequeño[365]. La extracción consiste en la separación de los compuestos de interés de una matriz sólida o líquida, basándonos en las diferencias de solubilidad relativa de estos compuestros con respecto al resto de componentes de la matriz[366]. Durante esta etapa es necesario eliminar todos los elementos presentes en la matriz que puedan interferir en el análisis, así como asegurar que se obtenga un extracto compatible con la técnica analítica que se vaya a utilizar en la determinación. Este proceso juega un papel muy importante en la calidad y reproducibilidad de los resultados que se obtengan en los análisis posteriores. Existen numerosos factores que afectan a la etapa de extracción, siendo algunos de los más importantes el tipo de disolventes empleados, la temperatura, el tiempo de extracción, la relación muestra/disolvente, la composición química y las características físicas de la muestra, aunque la influencia de estos factores dependerá del tipo de técnica seleccionada.

Para el análisis de compuestos bioactivos se pueden emplear diversos sistemas de extracción recogidos en la **Figura 22**.

[365] Moldoveanu, S.; David, V. Solvent extraction. In *Modern Sample Preparation for Chromatography*; Moldoveanu, S., David, V., Eds.; Elsevier, **2015**; pp 131–189.

[366] Ignat, I.; Volf, I.; Popa, V. I. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chem.* **2011**, *126* (4), 1821–1835.

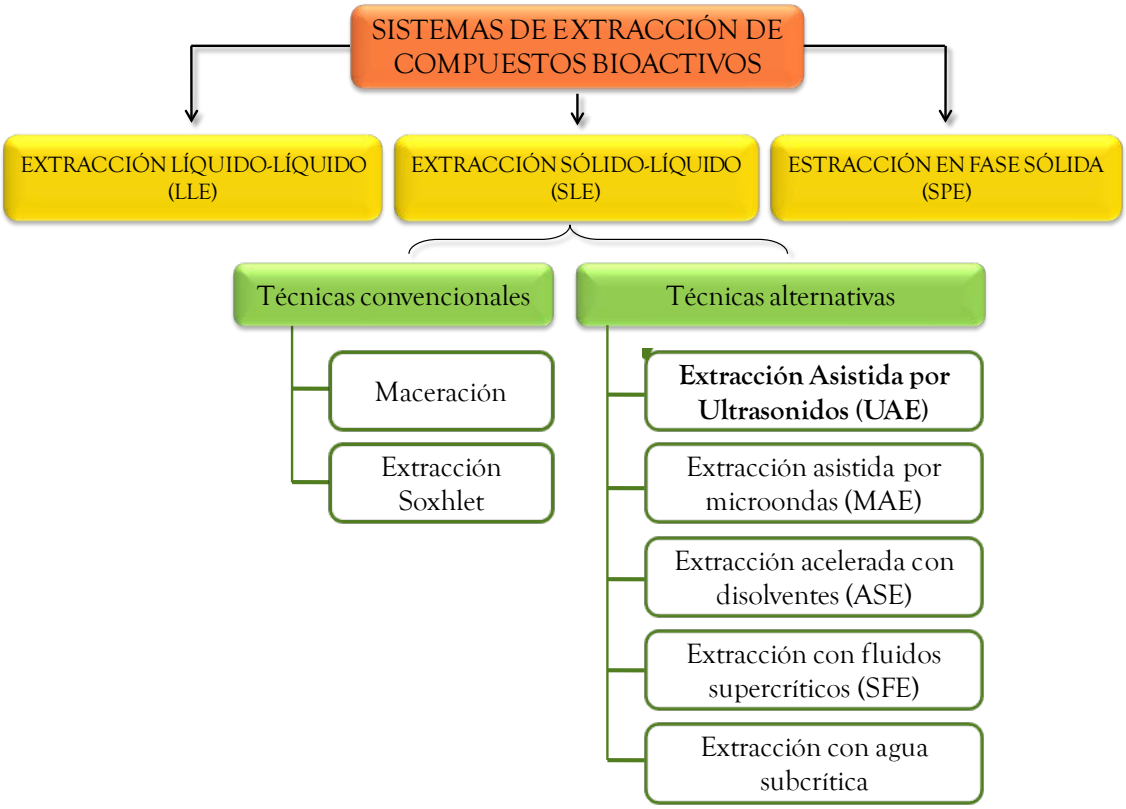

Figura 22. Principales métodos de extracción de compuestos bioactivos en vegetales.

Los vegetales son matrices generalmente complejas que contienen gran diversidad de compuestos con diferente estructura y polaridad. La solubilidad de los compuestos bioactivos depende de la naturaleza química del compuesto, de la polaridad de los disolventes que se utilicen, así como su posible interacción con otros componentes de la matriz. Por ejemplo, los compuestos fenólicos pueden asociarse a otros componentes del vegetal como carbohidratos o proteínas, y pueden dar lugar a complejos bastante insolubles.

Para la extracción de los compuestos bioactivos de las matrices vegetales objeto de esta tesis doctoral se ha utilizado la extracción sólido-líquido asistida por ultrasonidos, y además, en etapas intermedias se ha empleado la

extracción líquido-líquido. La extracción **sólido-líquido** (SLE) es una operación que permite separar los componentes presentes en una matriz sólida mediante contacto con un disolvente que disuelve selectivamente a algunos de dichos componentes. Se trata, por tanto, de una transferencia de materia en la que determinados componentes se transportan desde una fase sólida a una fase líquida que puede ser promovida por cambios en los gradientes de concentración, coeficientes de difusión o capa límite[367,368]. La eficacia de esta extracción está en función de ciertas condiciones: temperatura, índice líquido-sólido, tasa de flujo, tamaño de partícula, tiempo de contacto líquido sólido[369, 370], naturaleza del extractante (polar-polar y apolar-apolar), agitación y presión. La **extracción asistida por ultrasonido** (UAE) es la técnica más ampliamente utilizada para muestras vegetales[371]. Es un tipo de SLE que se basa en el uso de vibraciones de ultrasonido. Las ondas de ultrasonido crean una microcavitación local en el líquido que rodea las partículas de la muestra vegetal. El ultrasonido provoca un gran efecto mecánico de penetración del disolvente en las paredes celulares, facilitando la liberación del contenido celular y el calor locar del líquido favorece la difusión de la extracción[371]. A pesar de ser una técnica que

[367] Aguado, J.; Calles, J. A.; Cañizares, P.; López, B.; Santos, A.; Serrano, D. *Ingeniería de la Industria Alimentaria. Volumen II*; Rodríguez, F., Ed.; Sintesis, **2002**.

[368] Corrales, M.; García, A. F.; Butz, P.; Tauscher, B. Extraction of anthocyanins from grape skins assisted by high hydrostatic pressure. *J. Food Eng.* **2009**, *90* (4), 415–421.

[369] Hayouni, E. A.; Abedrabba, M.; Bouix, M.; Hamdi, M. The effects of solvents and extraction method on the phenolic contents and biological activities *in vitro* of Tunisian *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts. *Food Chem.* **2007**, *105* (3), 1126–1134.

[370] Pinelo, M.; Rubilar, M.; Sineiro, J.; Núñez, M. J. Extraction of antioxidant phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). *Food Chem.* **2004**, *85* (2), 267–273.

[371] Cobzac, S. C.; Gocan, S. Sample preparation for high performance liquid chromatography: Recent progress. *J. Liq. Chromatogr. Relat. Technol.* **2011**, *34* (13), 1157–1267.

depende mucho del tipo de disolvente, el tamaño de la muestra, el pH del extracto, la temperatura y presión, es rápida, simple y permite la extracción de varias muestras simultáneamente. Esta técnica ha sido aplicada satisfactoriamente para extractos de diferentes compuestos bioactivos como carotenoides, polisacáridos, proteínas, compuestos fenólicos, compuestos aromáticos o esteroles[372-374].

Conceptualmente, la extracción sólido-líquido presenta una gran similitud con la **extracción líquido-líquido**. En esta última, el objetivo que se persigue es la separación de componentes en una fase líquida por contacto con un disolvente que, aunque inmiscible en el conjunto, disuelve preferentemente a determinados componentes presentes en la misma[367]. Los dos líquidos resultantes de esta extracción son el extracto, que es el disolvente que contiene los solutos extraídos deseados, y el refinado, una solución residual baja en solutos[375].

[372] Vilkhu, K.; Mawson, R.; Simons, L.; Bates, D. Applications and opportunities for ultrasound assisted extraction in the food industry - A review. *Innov. Food Sci. Emerg. Technol.* **2008**, *9* (2), 161–169.

[373] Villares, A.; García-Lafuente, A.; Guillamón, E.; Ramos, Á. Identification and quantification of ergosterol and phenolic compounds occurring in *Tuber* spp. truffles. *J. Food Compos. Anal.* **2012**, *26* (1–2), 177–182.

[374] Wu, C.; Wang, F.; Liu, J.; Zou, Y.; Chen, X. A comparison of volatile fractions obtained from *Lonicera macranthoides* via different extraction processes: ultrasound, microwave, Soxhlet extraction, hydrodistillation, and cold maceration. *Integr. Med. Res.* **2015**, *4* (3), 171–177.

[375] Müller, E.; Berger, R.; Blass, E.; Sluyts, D.; Pfennig, A. Liquid-liquid extraction. *Ullmann´s Encyclopedia of Industrial Chemistry*; Wiley, **2008**; pp 249–307.

D. DETERMINACIÓN DE COMPUESTOS BIOACTIVOS MEDIANTE TÉCNICAS CROMATOGRÁFICAS. PLATAFORMAS ANALÍTICAS SEPARATIVAS EMPLEADAS.

Para llevar a cabo los procedimientos de separación, la química analítica dispone de numerosas técnicas que se basan en las diferencias existentes en las propiedades físico-químicas de los distintos componentes de una muestra. Las diferentes técnicas de separación, se pueden clasificar en dos grandes grupos: técnicas discontinuas y continuas **(Figura 23)**[376].

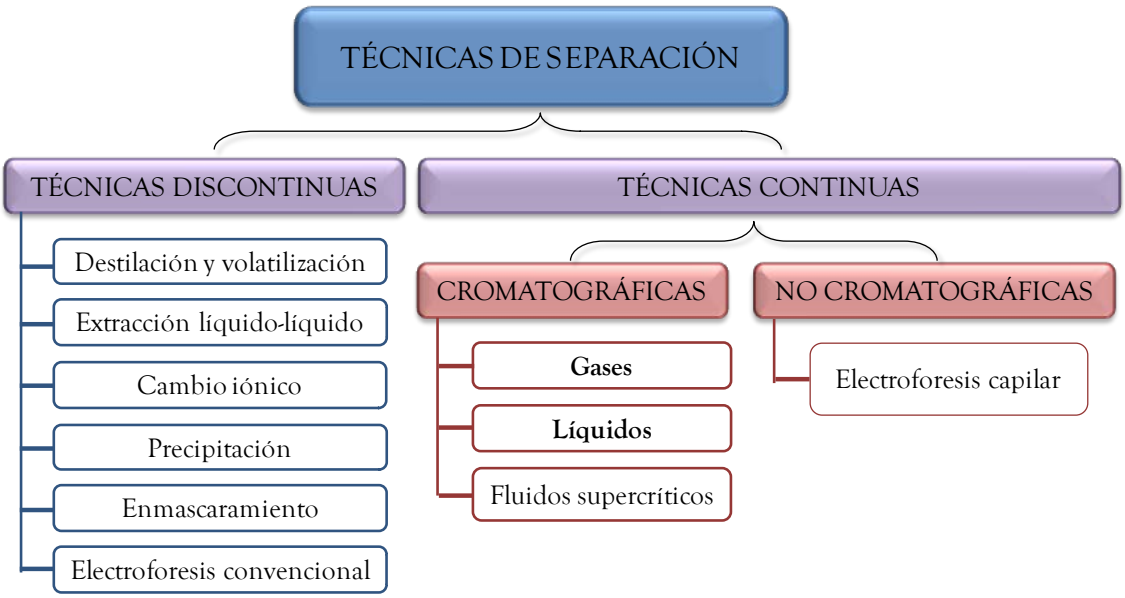

Figura 23. Clasificación de las técnicas analíticas de separación.

Las **técnicas discontinuas** son aquellas en las que no se incluye la detección de los compuestos separados, es decir, la medición de la señal analítica se efectúa de manera discontinua respecto a la separativa. Mientras que las **técnicas continuas** son aquellas que implican la detección de las especies de

[376] Luque de Castro, M. D.; Valcárcel, M.; Tena, M. T. *Extracción con Fluidos Supercríticos en el Proceso Analítico*; Reverté, S. A., **1993**; pp 19–21.

manera continúa después de ser separadas. Dentro de estas últimas técnicas se encuentran dos grandes grupos, las técnicas cromatográficas y las no cromatográficas **(Figura 23)**.

Las técnicas de mayor aplicación, hasta el momento, para el análisis de compuestos bioactivos en matrices vegetales han sido la cromatografía de gases y la cromatografía de líquidos. Estas técnicas se encuentran normalmente acopladas a sistemas de detección, por lo que el cromatógrafo actúa globalmente como instrumento suministrando información cualitativa y cuantitativa. El detector puede ser de diferentes tipos, entre los más utilizados se encuentran el detector de espectrofotometría ultravioleta-visible (UV-Vis) y el espectrómetro de masas (MS).

D.1. Técnicas cromatográficas

La cromatografía consiste en la separación de los componentes de una muestra debido a la distribución de estos dependiendo de la afinidad entre una fase móvil y una fase estacionaria. La fase estacionaria ha de ser sólida o líquida, mientras que la móvil puede ser un líquido, un gas o un fluido supercrítico. Los solutos de la muestra presentan una interacción diferencial con las fases móvil y estacionaria. La separación se da por las diferencias en las constantes de distribución de los dos componentes entre las dos fases. Los analitos que tienen más afinidad e interaccionan más fuertemente con la fase estacionaria quedarán más retenidos y mostrarán mayor tiempo de

retención que aquellos compuestos que tengan una menor afinidad e interacciones más débiles con la fase estacionaria[377].

Las técnicas cromatográficas más utilizadas para el análisis de compuestos bioactivos en matrices vegetales, y las que han sido empleadas para la realización de esta tesis doctoral, son la cromatografía líquida de alta resolución (HPLC) y la cromatografía de gases (GC)[378,314].

D.1.1. Cromatografía líquida de alta resolución (HPLC)

La cromatografía líquida de alta resolución se caracteriza por el uso de altas presiones para movilizar la fase móvil (líquido) a través de la fase estacionaria (sólido o líquido fijado a un soporte sólido). Esto permite la separación de mezclas complejas con una mayor resolución y la reducción de los tiempos de análisis. La resolución depende de la magnitud de las interacciones entre los componentes de la muestra y la fase móvil. Por tanto, la separación puede optimizarse con la selección de una correcta fase móvil y fase estacionaria. Esta técnica proporciona diferentes posibilidades para llevar a cabo la separación, lo que hace del HPLC una técnica altamente versátil y útil para un gran número de compuestos químicos[379,380].

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[377] Bélanger, J. M. R.; Bissonnette, M. C.; Paré, J. R. J. Chromatography: Principles and applications. In *Techniques and Instrumentation in Analytica Chemistry*; Paré, J. R. J., Bélanger, J. M. R., Eds.; Elsevier, **1997**; Vol. 18, pp 1–35.

[378] Talhaoui, N.; Gómez-Caravaca, A. M.; León, L.; De La Rosa, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. From olive fruits to olive Oil: Phenolic compound transfer in six different olive cultivars grown under the same agronomical conditions. *Int. J. Mol. Sci.* **2016**, *17* (3).

[379] Snyder, L. R.; Kirkland, J. J.; Dolan, J. W. *Introduction to Modern Liquid Chromatography*; Wiley, **2010**.

[380] Yashin, Y. I.; Yashin, A. Y. Liquid chromatography. In *Chemical Analysis of Food: Techniques and Applications*; Picó, Y., Ed.; Elsevier, **2012**; pp 285–310.

En este tipo de cromatografía, la fase estacionaria puede ser un sólido poroso o una fina capa líquida ligada a un soporte sólido, contenido en el interior de un tubo, generalmente de metal, y que conforma la denominada columna cromatográfica, un elemento fundamental en HPLC. Por otro lado, la fase móvil es líquida, puede estar compuesta por uno o más disolventes, y en ocasiones se modifica su pH mediante adición de ácidos, bases o sistemas tampón, dependiendo de la naturaleza de los compuestos que se deseen separar[380].

En la **Tabla 4** se muestran los diferentes tipos de HPLC que existen considerando la fase estacionaria y el tipo de fenómeno físico que provoca la separación.

Tabla 4. Clasificación de los diferentes tipos de HPLC.

Nombre	Fase estacionaria activa
Partición	Líquido retenido por un sólido soporte
Adsorción	Sólido con propiedades superficiales
Cambio iónico	Sólido con propiedades cambiadoras de iones
Afinidad	Sólido con propiedades de retención bioespecíficas
Exclusión por tamaños	Sólido con porosidad controlada
Quiral	Reactivo quiral unido a fase móvil o al soporte sólido

El tipo de cromatografía más utilizada es la cromatografía de partición o de reparto. En este tipo de cromatografía los solutos se distribuyen entre una fase móvil líquida y otra estacionaria inmiscible soportada sobre un sólido inerte. Por tanto, la causa de la separación de los solutos se encuentra, de manera genérica, en las diferencias de solubilidad. Además, según las polaridades relativas de las fases móviles y estacionarias encontramos dos tipos de cromatografía de partición: (1) cromatografía en fase normal (NP) donde la fase estacionaria es polar, mientras que la elución se lleva a cabo

con disolventes apolares, como etiléter, cloroformo o n-hexano y (2) cromatografía en fase inversa (RP) donde la fase estacionaria es apolar, tratándose normalmente de hidrocarburos tales como C_8 (n-octilo) o C_{18} (n-octadecilo) y la elución se lleva a cabo con una fase móvil de polaridad elevada, como disoluciones acuosas conteniendo ácidos, metanol, acetonitrilo o tetrahidrofurano. El modo de HPLC más empleado en la actualidad, es la cromatografía de líquidos de partición en fase inversa, ya que una gran mayoría de las muestras de interés en diversos ámbitos tienen naturaleza hidrofílica. En esta modalidad el tiempo de retención es mayor para las moléculas de naturaleza apolar, mientras que las moléculas de carácter polar eluyen más rápidamente[379].

Instrumentación HPLC

En la **Figura 24** se muestran los elementos que constituyen un sistema de HPLC, que son: (1) recipientes para el suministro de la fase móvil, (2) bombas que proporcionan presión a la fase móvil y la impulsan al resto del sistema, (3) sistema de inyección de la muestra que permite introducir una cierta cantidad de esta en el sistema, (4) columna cromatográfica que contiene la fase estacionaria y donde tiene lugar la separación de los analitos; a veces va precedida de una pre-columna que evita que lleguen a la columna componentes de la muestra que puedan dañar la fase estacionaria, (5) un horno termostatizado que mantiene la temperatura de la columna constante, asegurando una mayor reproducibilidad de la separación, (6) un detector que produce señales analíticas ante la presencia de los analitos que eluyen de la columna y (7) un sistema de adquisición de datos, hoy en día esta función la realizan ordenadores provistos de software específicos para esta tarea.

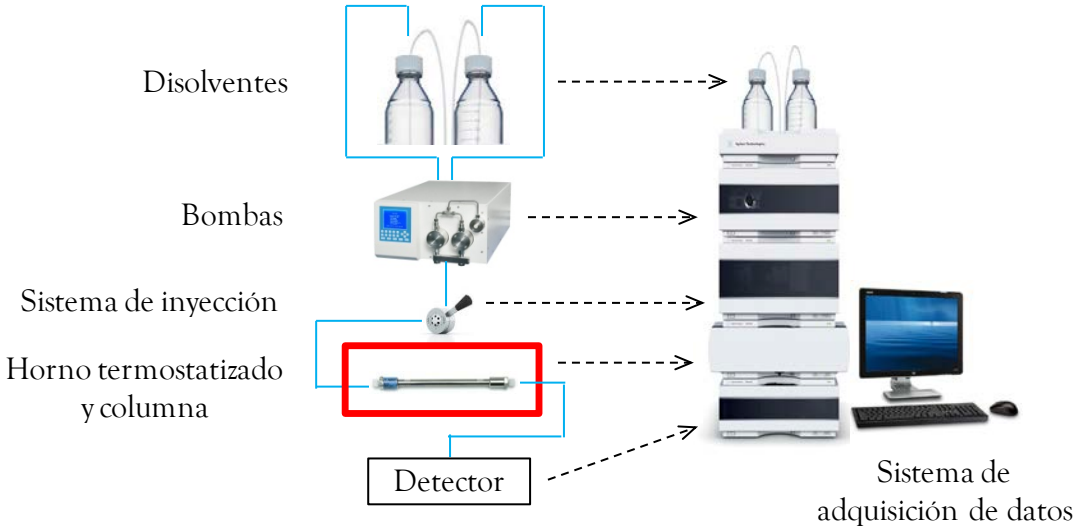


Figura 24. Esquema de un equipo HPLC.

La columna cromatográfica, como se mencionó anteriormente, es una pieza fundamental y donde tiene lugar la separación en HPLC. Actualmente se emplean columnas con tamaños de partícula cada vez más pequeños con objeto de mejorar la eficacia y la velocidad de análisis[381]. En el caso del análisis de compuestos fenólicos, las columnas más empleadas son aquellas con fase estacionaria C_{18}, con una longitud de 10-30 cm, un diámetro interno de 3.9-4.6 mm y un tamaño de partícula de 1.8-10 µm. El uso de las columnas C_{18} para el análisis de compuestos fenólicos normalmente implica el uso de un sistema binario en el que las fases móviles están constituidas por soluciones acuosas ácidas y disolventes orgánicos como metanol o acetonitrilo. La separación de los compuestos más polares depende en gran medida del pH de la fase móvil[314,378].

[381] Nguyen, D. T.-T.; Guillarme, D.; Rudaz, S.; Veuthey, J.-L. Fast analysis in liquid chromatography using small particle size and high pressure. *J. Sep. Sci.* **2006**, *29* (12), 1836–1848.

D.1.2. Cromatografía de gases

En cromatografía de gases (CG) los componentes de la muestra se separan en función de las presiones de vapor y la afinidad que presentan con la fase estacionaria [382]. La fase estacionaria puede ser (1) sólida, en la llamada **cromatografía gas-sólido (CGS),** donde el analito se adsorbe directamente sobre las partículas sólidas de la fase estacionaria o (2) líquida, como es la **cromatografía gas-líquido (CGL),** que utiliza como fase estacionaria un líquido que recubre la pared interna de una columna o un soporte sólido y donde la separación de los analitos se produce por partición. Esta última es la modalidad de CG más utilizada y la que ha sido empleada para la realización de los análisis de esteroles y alqu(en)ilresorcinoles en la presente tesis doctoral. Por otro lado, la fase móvil es un gas portador de gran pureza, generalmente He, H_2, N_2 o Ar, con un caudal conocido y controlado. La fase móvil no interacciona con el analito y su función es transportar la muestra a través de la columna. Por tanto, el único parámetro que puede alterar el equilibrio en la separación en CG es la temperatura. Los límites de temperatura normalmente oscilan entre la temperatura más baja a la cual la fase estacionaria permanece fluida y la temperatura más alta antes de que comience la degradación de la fase [383]. Actualmente, es común usar gradientes de temperatura donde esta va creciendo progresivamente, de modo que permite una mejor separación y resolución de los analitos en tiempos de análisis razonables.

[382] McNair, H. M.; Miller, J. M. *Basic Gas Chromatography*; John Wiley & Sons, Inc, **2009**.

[383] Wang, Z.; Paré, J. R. J. Gas chromatography (GC): Principles and applications. In *Instrumental Methods in Food Analysis. Techniques and Instrumentation in Analytical Chemistry*; Paré, J. R. J., Bélanger, J. M. R., Eds.; Elsevier Science, **1997**; Vol. 18, pp 61–91.

Instrumentación CG

Las partes esenciales que constituyen un cromatógrafo de gases son: (1) una fuente de suministro del gas portador, (2) reguladores de flujo y presión del gas portador, (3) un sistema de introducción de muestra, (4) una columna cromatográfica que contiene la fase estacionaria, (5) un horno en el que se encuentra la columna, (6) un detector de señales con amplificador de señal y (7) y un sistema de adquisición de datos **(Figura 25)**.

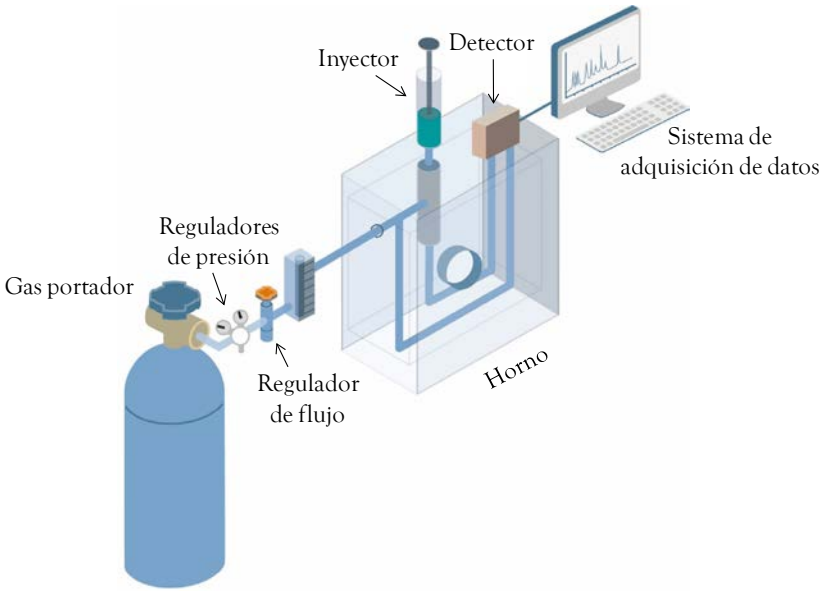


Figura 25. Diagrama de los elementos básicos de un cromatógrafo de gases.

En CG las columnas cromatográficas pueden ser de dos tipos: (1) empaquetadas, en las que la fase estacionaria líquida está retenida sobre un sólido inerte y (2) capilares, en los que la fase estacionaria se fija sobre las propias paredes del capilar **(Figuras 26 y 27)**[382].

Figura 26. Columnas empaquetadas.

Figura 27. Columna capilar.

La gran mayoría de los análisis se realizan con columnas capilares debido a la gran eficacia de separación que proporcionan, y en el caso de los esteroles y ARs, algunos autores han señalado que las columnas capilares de alta temperatura proporcionan mejores resultados en la sensibilidad y resolución de la detección[384, 385]. Este tipo de columnas son largas y estrechas, con diámetros internos típicos de 0,1-0,6 mm y longitudes típicas de 15 a 100 m. En su interior están formadas por sílice fundido (SiO_2) y recubiertas de poliimida que da soporte y previene de la humedad atmosférica. Las fases estacionarias suelen ser apolares de dimetilpolisiloxano (DB-1, DB-5 o CPSil-5) y polímeros más polares de polietilenglicol como las columnas Carbowax®, DB-Wax y HP-20M. La temperatura a la que se realiza el análisis debe ser compatible con la fase estacionaria utilizada, ya que el líquido de esta puede volatilizarse o descomponerse a altas temperaturas[386].

La ventaja que proporcionan las columnas capilares con respecto a las empaquetadas es, como ya ha sido mencionado, la elevada eficacia que

[384] Abidi, S. L. Chromatographic analysis of plant sterols in foods and vegetable oils. *J. Chromatogr. A* **2001**, *935* (1–2), 173–201.

[385] Prinsen, P.; Gutiérrez, A.; Faulds, C. B.; Del Río, J. C. Comprehensive study of valuable lipophilic phytochemicals in wheat bran. *J. Agric. Food Chem.* **2014**, *62* (7), 1664–1673.

[386] Engewald, W.; Dettmer-Wilde, K.; Rotzsche, H. Columns and stationary phases. In *Practical Gas Chromatography*; Dettmer-Wilde, K., Engewald, W., Eds.; Springer, **2014**; pp 61–69.

ofrecen en la separación, y esto permite obtener una alta resolución sin recurrir a fases estacionarias de gran selectividad, lo que simplifica la elección de la fase estacionaria[386].

En un análisis por cromatografía de gases, la muestra, bien sea un líquido volátil o un gas, se inyecta en la cámara de inyección a través de un septo, donde se volatiliza y se dirige hasta la entrada de la columna. Las técnicas de inyección más utilizadas hoy en día en cromatografía de gases capilar son las inyecciones "split/splitless" (con división o sin división de flujo)[382]. En el modo "split" la muestra se vaporiza rápidamente y sólo una fracción de esta entra en la columna. Por otro lado, el modo de inyección "splitless" es una técnica de preconcentración especialmente útil para muestras muy diluidas, tales como análisis de trazas, así como muestras con solutos térmicamente inestables. En el bloque de inyección, se introduce un volumen relativamente grande de muestra diluida en la cámara de inyección, donde se evapora y se dirige a la columna. En este punto se utiliza el llamado "efecto disolvente", que consiste en mantener la columna a una temperatura inicial de unos 10-30 ºC por debajo del punto de ebullición del disolvente, de forma que este condensa a la entrada de la columna, formando una fina película de fase estacionaria donde quedan atrapados los componentes de la muestra, produciendo un efecto de preconcentración.

El gas portador transporta los compuestos, en estado gaseoso, a través de la columna, y debido a equilibrios sucesivos entre fase móvil y estacionaria, cada componente se desplaza a una velocidad determinada. La temperatura óptima de la columna depende del punto de ebullición de la muestra y del grado de separación requerido. La temperatura tiene que ser igual o ligeramente superior al punto de ebullición promedio de los componentes

de la muestra. Para muestras con analitos que se encuentran en un amplio intervalo de ebullición, a menudo es conveniente emplear un gradiente de temperatura, aumentado la temperatura de la columna bien de forma continua o bien por etapas, al mismo tiempo que tiene lugar la separación. Para muestras con puntos de ebullición similares normalmente se emplea una temperatura constante a lo largo del análisis (isoterma)[283,387].

Una de las principales limitaciones de la CG es que los analitos deben ser volátiles y termoestables. Para poder realizar el análisis de determinados compuestos que presentan baja volatilidad, la muestra se debe someter a un proceso de derivatización previo al análisis, que transforme los analitos en otros con punto de ebullición más bajo, como sucede en el caso de los esteroles y alqu(en)ilresorcinoles estudiados en esta tesis doctoral. Su separación es posible sin derivatización pero mejora considerablemente la resolución y la sensibilidad del análisis mediante una derivatización previa que los transforme en derivados de trimetilsililo (TMS) o derivados de acetato.

Finalmente, los analitos después de ser separados llegan al detector cuya respuesta aparece en la pantalla de un ordenador o un registrador en forma de cromatograma.

D.2. Sistemas de detección

Una vez separados los compuestos mediante cromatografía, deben ser detectados. Para ello, tras la columna cromatográfica se acoplan uno o varios detectores que proporcionen una respuesta al paso de los analitos

[387] Skoog, D. A.; Holler, F. J.; Nieman, T. A. *Principios de Análisis Instrumental*; Fernández Matrid, C., Ed.; **2001**.

(absorbancia, fluorescencia, conductividad…). El procesado de esta señal produce el cromatograma, en el que se representa la respuesta obtenida por el detector frente al tiempo de retención de los analitos en la columna. La intensidad de cada pico será directamente proporcional al factor de respuesta y a la concentración del analito correspondiente en la muestra.

Para obtener buenos resultados, un detector debe cumplir unas características específicas que incluyen: elevada sensibilidad, selectividad, buena estabilidad, linealidad, tiempos de respuesta cortos, fiabilidad, no perjudicar a la eficacia, fácil de usar y bajo volumen muerto. Para la elección de un detector, además de ajustarse en la medida de lo posible a las características descritas anteriormente, hay que tener en cuenta la naturaleza y propiedades de los compuestos que se desea determinar, así como la sensibilidad requerida y el tipo de información que se desea conocer (estructural, cualitativa o cuantitativa).

Los detectores más utilizados acoplados a HPLC se pueden clasificar en tres grupos: (1) detectores ópticos, entre los que se encuentran el detector de absorción UV-Vis, de fluorescencia, espectroscopía de infrarrojos, resonancia magnética nuclear (RMN), índice de refracción, fosforescencia, quimioluminiscencia, dispersión Raman y termoóptico; (2) detectores electroquímicos como conductimetría, potenciometría, amperometría o voltámperometría; y (3) otros detectores como la espectrometría de masas (MS) o la radiometría. Por otro lado, los detectores más utilizados en CG son los detectores de conductividad térmica (TCD), de captura de electrones (ECD), de ionización de llama (FID) y la espectrometría de masas (MS).

En la presente tesis doctoral, para el análisis de compuestos bioactivos en muestras vegetales, se han utilizado los sistemas de detección mediante

espectroscopía de absorción ultravioleta-visible (UV-Vis), espectroscopía de fluorescencia (FLD) y espectrometría de masas (MS) acoplados a HPLC y detección mediante espectrometría de masas acoplada a CG.

D.2.1. Espectroscopía de absorción UV-Vis

Este método de detección se basa en la interacción de la radiación UV-Vis , que comprende la zona del espectro electromagnético entre 200-800 nm aproximadamente, y la materia, dando lugar a la absorción de radiación por parte de los analitos.

Existen detectores de absorción UV-Vis de tres tipos: (1) de longitud de onda fija, (2) de longitud de onda variable o (3) de diodos en fila (DAD). Este último (**Figura 29**) conduce la luz mediante un sistema de diodos alineados y evita la dispersión. Además, el detector DAD es especialmente útil para recoger los espectros completos de absorción UV-Vis de muestras que pasan rápidamente por una celda y permite determinar un gran número de compuestos, son fáciles de manejar y tienen un coste relativamente bajo, por lo que son los detectores más utilizados en equipos comerciales de HPLC. La detección es muy estable, por lo que da muy buenos resultados para análisis cuantitativos. Por contrapartida, presentan menor sensibilidad que otros sistemas de detección y no son selectivos, por lo que no permiten la identificación inequívoca de los compuestos sin emplear patrones.

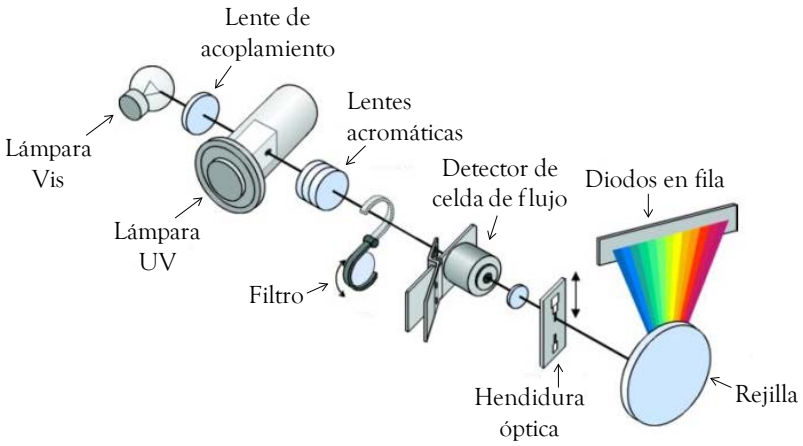


Figura 28. Diagrama de los elementos principales que componen un DAD.

Este sistema es especialmente útil para la determinación de compuestos fenólicos, ya que el gran número de enlaces conjugados que presentan hacen que se comporten como cromóforos que presentan bandas de absorción en la región UV-Vis. En general los compuestos fenólicos presentan dos bandas comunes de absorción 300-380 nm y 240-280 nm. Sin embargo, cada familia posee unas bandas de absorción diferenciales que permiten acotar la determinación de los compuestos fenólicos a la familia a la que pertenecen **(Tabla 5)**.

Tabla 5. Bandas de absorción diferenciales de las principales familias de compuestos fenólicos.

Compuestos	Bandas UV-Vis (nm)
Ácidos benzoicos	270-280
Ácidos cinámicos	305-325
Cumarinas	220-230/310-350
Chalconas	220-270/340-390
Dihidrochalconas	≈220/≈280
Auronas	240-270/340-370
Flavonas	250-270/330-350
Flavonoles	270-280
Antocianidinas	240-280/450-560
Isoflavonas	245-270/300-340
Proantocianidinas	≈280

D.2.2. Detector de fluorescencia (FLD)

Al igual que en la espectroscopia de absorción UV-Vis, los compuestos con ciertos grupos funcionales se excitan ante la absorción de una determinada energía y como resultado, emiten una radiación de mayor longitud de onda que la absorbida **(Figura 30)**. La detección de la fluorescencia va a depender de las longitudes de onda de excitación y emisión seleccionadas. Actualmente, los detectores pueden ser programados para cambiar estas longitudes de onda durante el análisis, proporcionando gran selectividad y sensibilidad en todo el cromatograma[377,388].

[388] Poole, C. F. Instrumental aspects of liquid chromatography. In *The Essence of Chromatography*; Elsevier, **2003**; pp 431–497.

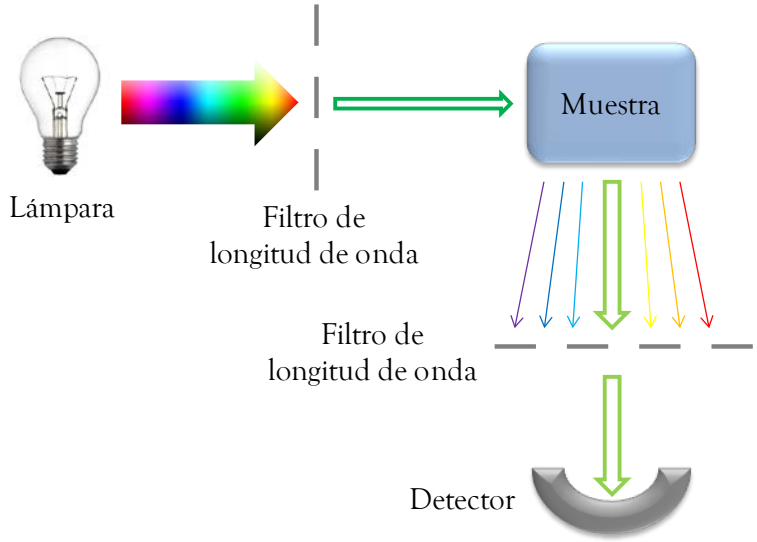


Figura 29. Representación de los elementos que constituyen un FLD.

Entre las ventajas que proporciona este sistema de detección se encuentra que la fluorescencia, al producirse a intensidades elevadas, ofrece mayor sensibilidad y por tanto posibilita la detección a nivel de trazas. Además, los detectores de fluorescencia son más selectivos que otros detectores de absorbancia, y presentan una especificidad muy alta, por lo que facilitan la detección en muestras complejas como son los vegetales.

Este sistema de detección se ha empleado en la presente tesis doctoral para el análisis de flavan-3-oles.

D.2.3. Espectrometría de masas (MS)

La espectrometría de masas (MS) se puede emplear para identificar y cuantificar cantidades de un analito de interés y se basa en la separación en condiciones de vacío de iones en estado gaseoso en función de su relación masa/carga (m/z). Actualmente es uno de los detectores más utilizados, principalmente debido a su elevada selectividad, rapidez y a que proporciona

información de la pureza y estructura del analito por lo que juega un papel fundamental en la identificación de compuestos bioactivos [389] . La espectrometría de masas puede ser utilizada directamente, sin embargo, es habitual el acoplamiento de este detector junto a una técnica separativa como HPLC o CG, ya que de esta forma se combinan la rapidez en el análisis, la alta resolución y el bajo gasto de muestra que proporciona la cromatografía con la selectividad del análisis y la información estructural que brinda la MS.

Un espectrómetro de masas se compone básicamente de tres elementos: (1) una fuente de ionización o interfase, (2) un analizador de masas, es decir, un sistema de separación de iones en base a su *m/z* y (3) un detector junto a un procesador de la señal (**Figura 31**). El proceso de detección ocurre en tres etapas principales: (1) la ionización, donde las moléculas o átomos pasan a formar iones en estado gaseoso. Esta etapa requiere de la adición o eliminación de un protón o electrón. En ocasiones, el exceso de energía de ionización puede provocar la rotura de las moléculas en fragmentos característicos[390]; (2) la separación de los iones moleculares y sus fragmentos cargados en base a su *m/z*; (3) el ión ya separado produce una señal que es transformada en una señal eléctrica que es amplificada y presentada en forma de espectro de masas. El proceso se lleva a cabo en condiciones de alto vacío, lo que permite a los iones moverse libremente en el espacio sin colisionar o interaccionar con otros radicales o moléculas neutras. La colisión podría llevar a la fragmentación del ión molecular y producir

[389] El-Aneed, A.; Cohen, A.; Banoub, J. Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. *Appl. Spectrosc. Rev.* **2009**, *44* (3), 210–230.

[390] Throck Watson, J.; David Sparkman, O. *Introduction to Mass Spectrometry*; Wiley, **2007**.

diferentes especies mediante reacciones ión-molécula, lo que reduciría la sensibilidad y la resolución, además de provocar errores en la medida[391].

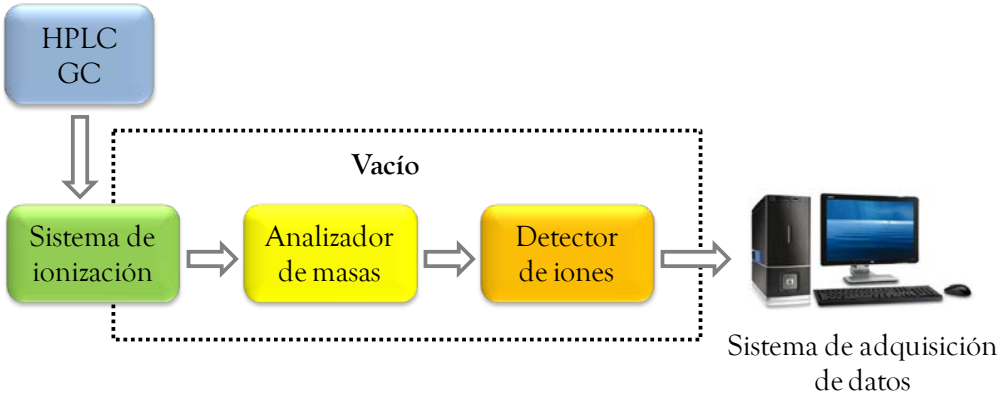


Figura 30. Principales elementos de un espectrómetro de masas.

A continuación se describen los sistemas de ionización y los analizadores utilizados en esta tesis doctoral.

D.2.3.1. Sistemas de ionización

En la fuente de ionización, dependiendo de su tipo, tiene lugar la evaporación y/o ionización de los analitos que van llegando al MS. Existen varias técnicas de ionización que pueden ser empleadas en espectrometría de masas en función de la energía interna de transferencia durante el proceso de ionización y las propiedades físico-químicas de los compuestos que se desean analizar.

Por tanto, existen sistemas de ionización fuertes como el impacto electrónico (EI) y otros más suaves como la ionización por electrospray (ESI). La **Figura**

[391] Chhabil, D. Basics of mass spectrometry. In *Fundamentals of Contemporary Mass Spectrometry*; Wiley, 2007; pp 3–14.

32 muestra los sistemas de ionización más utilizados que pueden ser acoplados a HPLC y CG, según la polaridad y tamaño de los analitos que se desea analizar (EI, ESI, ionización química a presión atmosférica (APCI), fotoionización a presión atmosférica (APPI), ionización química (CI), entre otras). Entre ellas, en esta tesis doctoral han sido empleadas la ionización por impacto electrónico (EI) y la ionización por electrospray (ESI)[392].

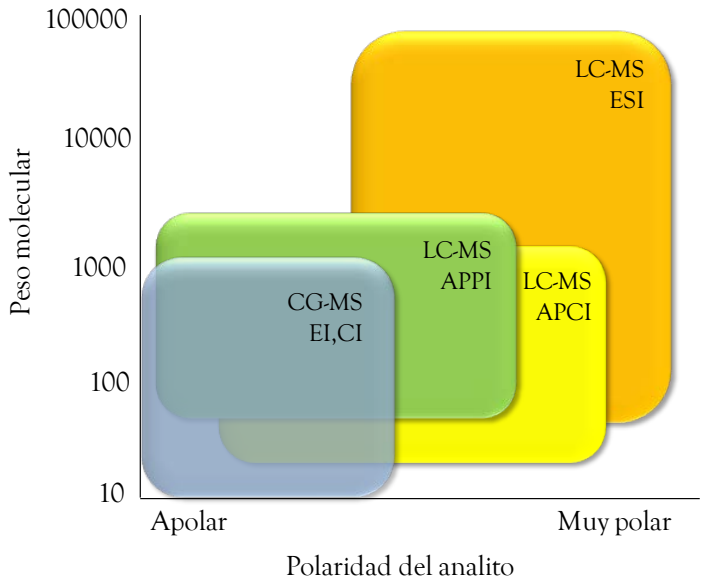


Figura 31. Tipos de ionización acoplables a HPLC y CG.

Impacto electrónico (EI)

El sistema de ionización por impacto electrónico es el sistema de ionización más ampliamente usado en acoplamientos con cromatografía de gases. Este está formado por un filamento, generalmente de tungsteno o renio, que está cargado negativamente y calentado por una corriente que circula en el

[392] Cajka, T.; Hajslova, J.; Mastovska, K. Mass spectrometry and hyphenated instruments in food analysis. In *Handbook of Food Analysis Instruments*; Ötles, S., Ed.; CRC Press, **2008**; pp 197–228.

mismo, emitiendo electrones. El proceso se inicia por la aceleración de electrones a través de un campo eléctrico con un potencial de 70 eV aplicado entre el filamento y la fuente de iones **(Figura 33)**. Las moléculas vaporizadas que van entrando en la fuente de ionización son expuestas a colisiones con los electrones emitidos por el filamento caliente[392]. La presión con la que entran las moléculas debe ser lo suficientemente baja para evitar colisiones ión-molécula. El impacto de los electrones con las moléculas causa su ionización y la fragmentación de estas de una forma característica y reproducible[393].

Esta técnica es generalmente usada en el acoplamiento GC-MS debido a la necesidad de que las moléculas estén ya previamente en estado gaseoso para ser ionizadas. Se utiliza, por tanto, para compuestos de bajo peso molecular (<800 Da) debido a su alta volatilidad, aunque también se puede utilizar para hidrocarburos fluorados y algunos complejos con metales de transición con pesos moleculares más altos pero que son suficientemente volátiles para este tipo de ionización[394].

[393] Guo, X.; Lankmayr, E. *Hyphenated Techniques in Gas Chromatography*; INTECH Open Access Publisher, **2012**.

[394] Somogyi, A. Mass spectrometry instrumentation and techniques. In *Medical Applications of Mass Spectrometry*; Vékey, K., Telekes, A., Vertes, A., Eds.; Elsevier, **2008**; pp 93–140.

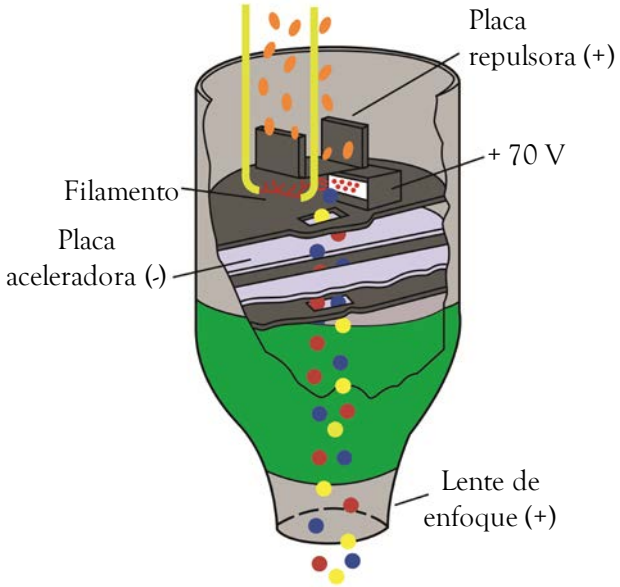


Figura 32. Diagrama de los elementos principales de un sistema de ionización EI.

Ionización por electrospray (ESI)

La ionización por electrospray (ESI) ha potenciado las aplicaciones de la espectrometría de masas, ya que facilita su acoplamiento con sistemas líquidos como en el caso de la cromatografía líquida. Principalmente es utilizada para el análisis de compuestos polares, lábiles y de elevado peso molecular.

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 solución se hace pasar a través de un capilar al que se aplica un alto potencial eléctrico, y con la ayuda de un gas nebulizador (N_2) y temperatura, se carga y dispersa simultáneamente. El disolvente de las microgotas formadas se va evaporando (desolvatación) y éstas van aumentando su densidad de carga eléctrica hasta llegar a un punto en el que la repulsión de los iones es mayor que la tensión de la superficie que mantiene unidas las gotitas, superando el denominado "límite de Rayleigh". En ese momento las

gotitas sufren un proceso conocido como "explosión de Coulomb" en el que se vuelven a formar gotas cargadas más pequeñas que seguirán sufriendo procesos de evaporación y explosión sucesivos hasta que finalmente se forman iones cargados en estado gaseoso que son atraídos a la entrada del espectrómetro de masas debido al voltaje aplicado **(Figura 34)** [395,396].

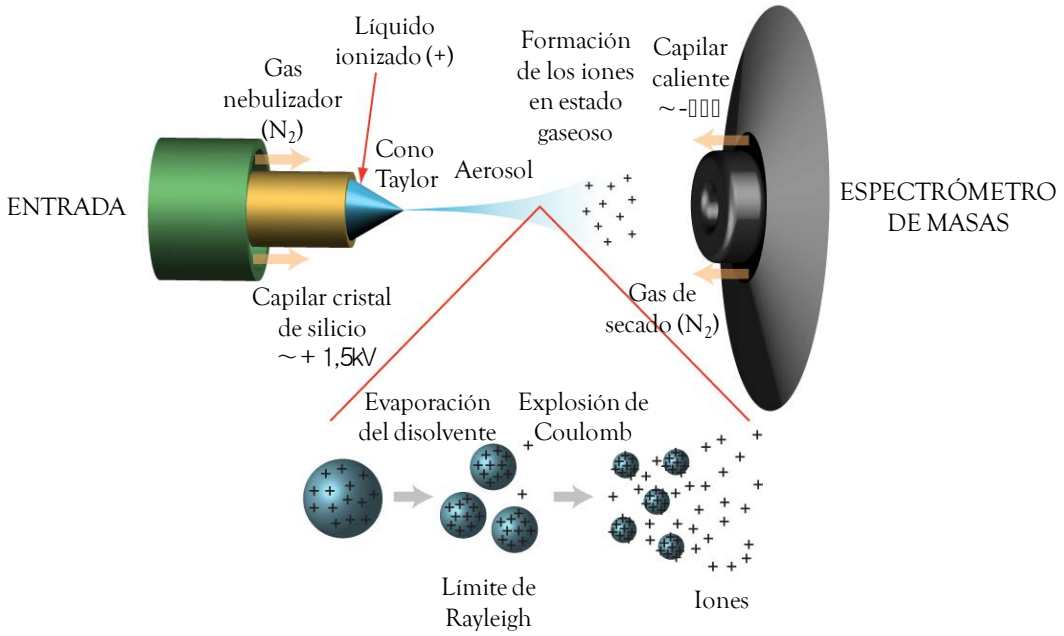


Figura 33. Proceso de formación del electrospray.

Con esta técnica de ionización se pueden formar iones mono o multicargados, por lo que al aumentar el número de cargas de una molécula, se disminuye su relación *m/z* y permite detectar compuestos de pesos moleculares elevados utilizando analizadores de masas que trabajan con un rango *m/z* limitado. Además, la ionización se puede llevar a cabo en modo

[395] Simó, C.; Cifuentes, A. *Electroforesis Capilar: Aproximación Según la Técnica de Detección*; Universidad de. Granada, **2005**; pp 409–438.

[396] Martin Smith, R. Instrumentation. In *Understanding Mass Spectra. A Basic Approach*; Busch, K. L., Ed.; Wiley, **1999**; pp 1–40.

positivo o negativo. En el modo positivo se pueden formar iones que han ganado más de un protón $(M+nH)^{n+}$. Igualmente es posible la formación de aductos con iones Na^+, Li^+, K^+, NH_4^+, etc. En el modo de ionización negativo, de forma análoga, se produce la desprotonación de las moléculas, pudiéndose originar iones por pérdida de más de un protón $(M-nH)^{n-}$.

Las ventajas de este analizador son la suavidad con la que se produce la ionización evitando la fragmentación en la fuente y la posibilidad de obtener iones cargados de forma múltiple, además de su acción en un amplio rango de masas.

Una vez las moléculas han sido transformadas en iones, estos son dirigidos al analizador de masas. Este proporciona información sobre la masa molecular de los iones con un elevado nivel de sensibilidad y selectividad.

D.2.3.2. Analizadores de masas

El analizador es donde tiene lugar la separación de los iones en estado gaseoso en función de su *m/z*, mediante un campo eléctrico y/o magnético. Actualmente existen numerosos analizadores de masas que pueden aislar iones en base a su *m/z* como: cuadrupolo (Q), trampa de iones (IT), triple cuadrupolo (QQQ), tiempo de vuelo (TOF), resonancia ion-ciclotrón con transformada de Fourier (FT-ICR) u orbitrap (OT). También existe el acoplamiento entre ellos como la combinación cuadrupolo-tiempo de vuelo (Q-TOF)[20]. Lo que marca la diferencia entre unos y otros es la resolución, el rango de masas, la capacidad para llevar a cabo experimentos de espectrometría de masas en tándem (MS/MS), el tamaño del equipo y su precio, entre otros. En el desarrollo de esta tesis doctoral han sido empleados

los analizadores Q, TOF y QTOF en los espectrómetros de masas acoplados a HPLC y QTOF en el espectrómetro de masas acoplado a CG.

Cuadrupolo (Q)

El cuadrupolo lo conforman cuatro barras metálicas (polos) dispuestas paralelamente que actúan, de alguna forma, como un filtro de masas que permite seleccionar los iones en función de su *m/z* según el voltaje aplicado. A las barras opuestas se les aplica un potencial continuo de igual signo y opuesto al de las otras dos barras. Adicionalmente, se aplica un potencial alterno asociado con una radiofrecuencia. En estas condiciones, los iones avanzarán a lo largo del analizador siguiendo trayectorias oscilantes, siendo repelidos y atraídos continuamente por las barras. Para valores definidos de los voltajes continuo y alterno sólo atraviesan el cuadrupolo los iones con determinada relación *m/z*, los demás se desestabilizan y chocan contra las barras (**Figura 35**). Al mantener constante la razón entre los potenciales continuo y alterno, pero variando su intensidad, se consigue la salida secuencial de los iones con diferente *m/z*, por el lado opuesto del cuadrupolo, generándose un espectro de masas[394].

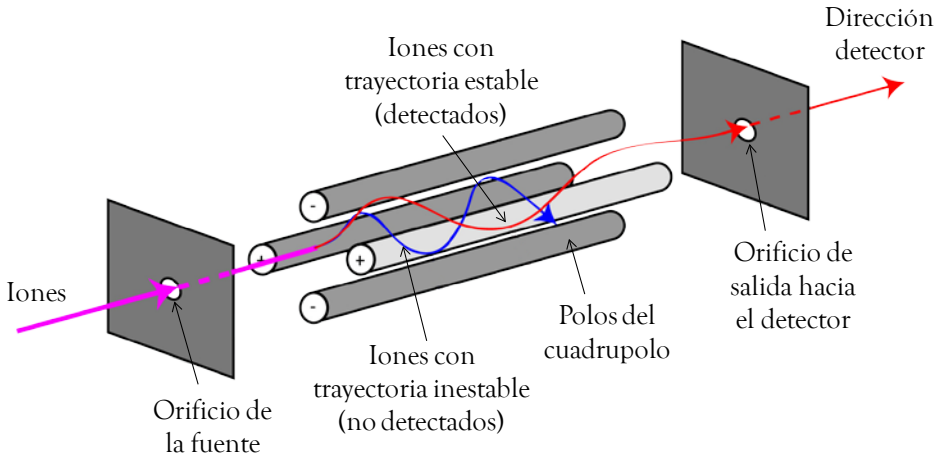


Figura 34. Diagrama de los componentes principales de un Q.

La resolución en este analizador depende del número de oscilaciones que describen los iones conforme atraviesan el cuadrupolo, siendo mayor cuanto mayor sea el número de estas. Los iones con mayor *m/z* sufren más oscilaciones por lo que se obtiene mejor resolución a expensas de un tiempo de análisis más largo[394].

Tiempo de vuelo (TOF)

El analizador de tiempo de vuelo (TOF) separa los iones según la distinta velocidad que adquieren en su interior en función de su relación *m/z*. Una vez formados los iones en la fuente de ionización, son dirigidos hacia el analizador donde se aceleran mediante la aplicación de un campo electrostático que les proporciona gran cantidad de energía cinética. Los iones, que son sometidos a un mismo potencial, adquieren velocidades distintas en el interior del tubo que son inversamente proporcionales a su *m/z*. Se consigue, por tanto, mayor resolución cuanto mayor sea la distancia que recorren los iones en el tubo de vuelo del espectrómetro de masas y cuanto menor sea la dispersión de energía cinética de los iones[396].

En la **Figura 36** se muestran los elementos que conforman un espectrómetro de masas con analizador TOF. Los iones, formados en la **cámara de nebulización**, son conducidos a través de un capilar hacia la **unidad de desolvatación**, que conecta la cámara de ionización, que se encuentra a presión atmosférica, con la zona de transmisión de iones, que se encuentra a gran vacío. La **zona de transmisión de iones o zona de transferencia óptica** está formada por tres cámaras con valores crecientes de vacío, separadas por skimmers entre sí. Los iones son transferidos por dos hexapolos hacia la zona de alto vacío y son enfocados o dirigidos hacia el analizador TOF a través de lentes. El analizador está formado por tres elementos: la zona de aceleración ortogonal, el reflector y el detector. El lugar al que llegan los iones, procedentes de la zona de transmisión de iones, es la zona de aceleración ortogonal. En esta se produce la aceleración de los iones al ser sometidos a un campo eléctrico intermitente. Los iones se desplazan por el tubo de vuelo llegando al reflector, el cual permite corregir la dispersión de la energía cinética de los iones, con lo que consigue una mayor resolución. Tras el reflector existen unas zonas de tensión, que repelen los iones y los redirigen hacia el detector. Este es un detector de impacto eléctrico, que transforma el choque de los iones que llegan a él en señales eléctricas. Está compuesto por una serie de placas que se encuentran a alto voltaje y que presentan millones de pequeños poros recubiertos internamente por una capa semiconductora, de forma que cada uno de ellos se comporta como un multiplicador de electrones **(Figura 36)**.

Las especificaciones más notables de este analizador de masas son un rango de masas entre 50-3000 *m/z*, una resolución entre 10000-15000 FWHM y una exactitud de 2 ppm cuando se realiza una calibración interna, o de 5 ppm si la calibración es externa.

La principal ventaja de este analizador es que todos los iones formados logran llegar al detector, a diferencia de lo que ocurre con otros analizadores como el cuadrupolo. Otras ventajas que proporciona este analizador son una elevada resolución y precisión de las masas, un rango de masas teóricamente ilimitado y un relativo bajo coste. Además, los valores de masas exactas junto con la distribución isotópica, permite la determinación de la fórmula molecular del compuesto, lo que facilita su identificación.

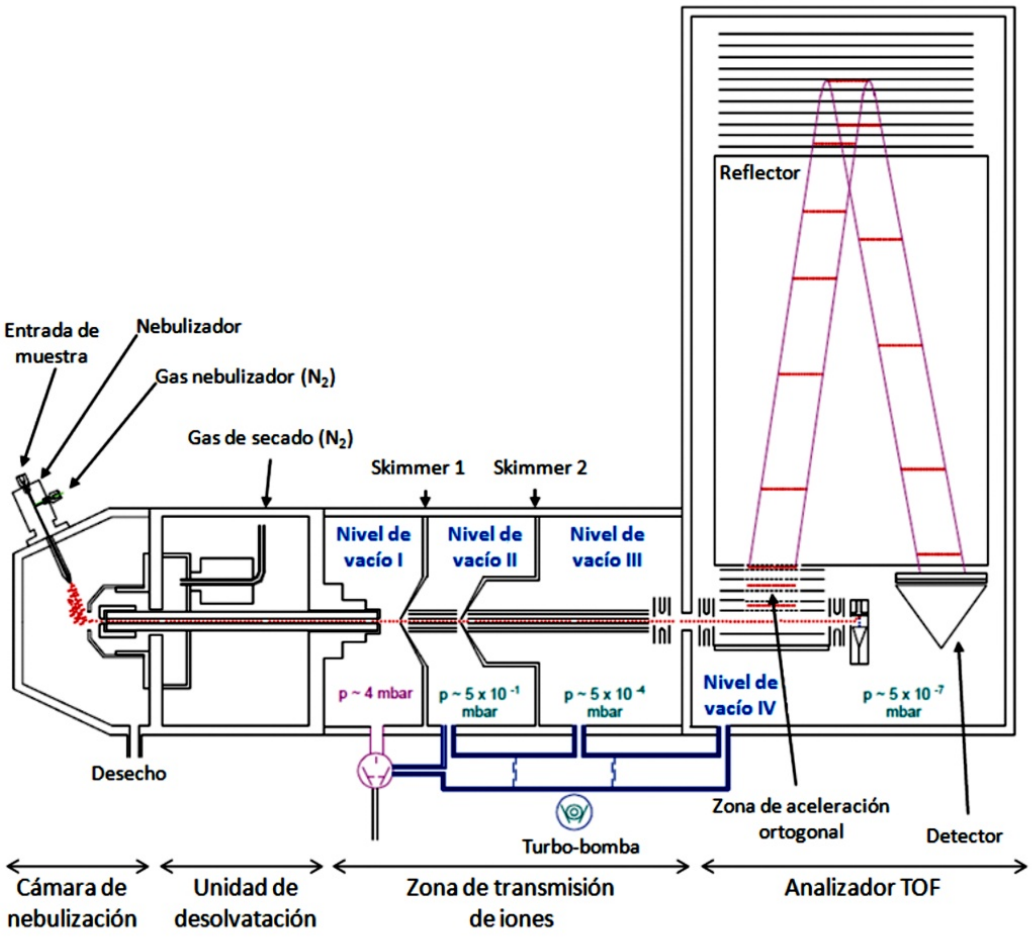


Figura 35. Diagrama de los componentes principales de un TOF-MS.

Cuadrupolo-tiempo de vuelo (QTOF)

El analizador cuadrupolo-tiempo de vuelo (QTOF) está formado por el acoplamiento de los analizadores Q y TOF, pero lo diferencia la presencia de funnels, y una celda de colisión anterior al tubo de tiempo de vuelo. Los funnels se encuentran en la zona de transmisión y sustituyen a los skimmers del TOF. Son anillos concéntricos apilados en forma de embudo, que evitan la pérdida de iones, lo que aporta una mayor sensibilidad (**Figura 37**). Los iones son seleccionados en el Q y posteriormente son fragmentados en la celda de colisión con la ayuda de un gas de colisión, generalmente N_2. La formación de iones fragmentados permite realizar análisis de MS/MS, que proporcionan información de gran utilidad para la identificación de los compuestos, lo que se suma a la masa exacta y la distribución isotópica proporcionadas por el TOF[397].

[397] Chernushevich, I. V; Loboda, A. V; Thomson, B. A. An introduction to quadrupole-time-of-flight mass spectrometry. *J. Mass Spectrom.* **2001**, *36* (8), 849–865.

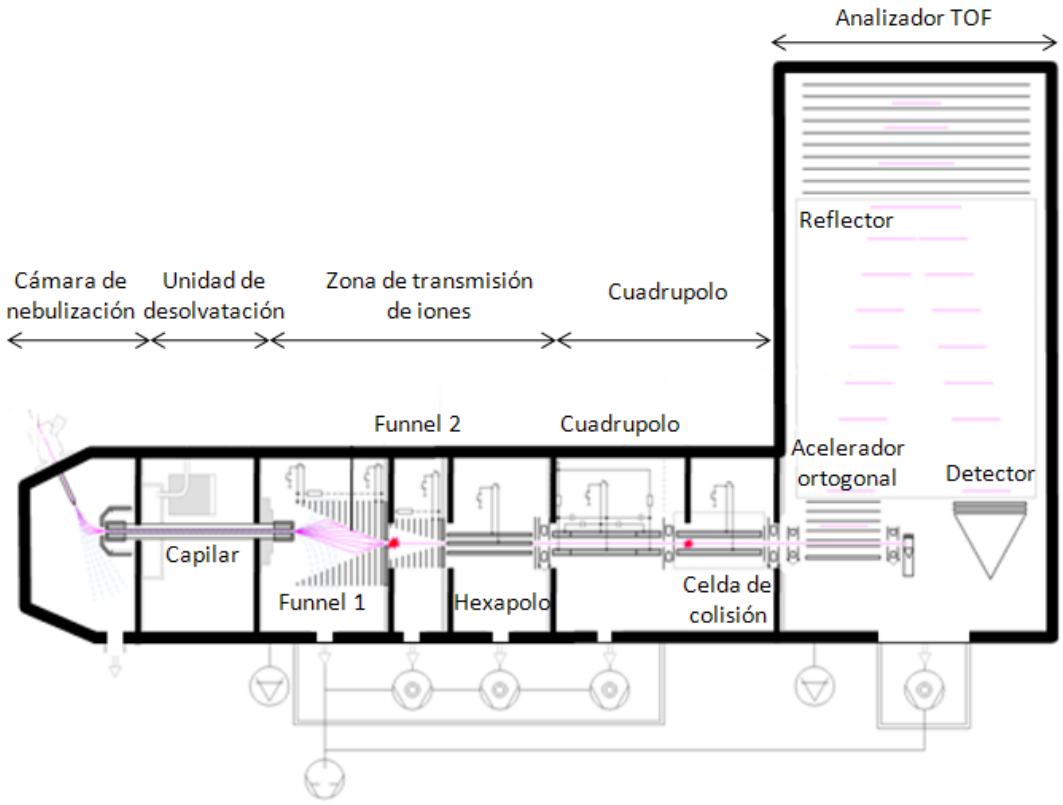
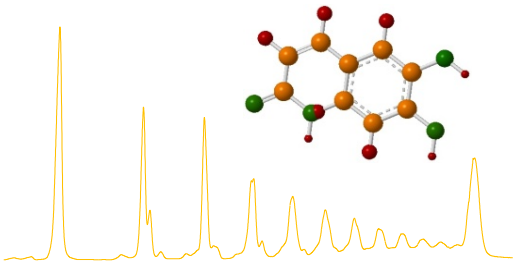


Figura 36. Diagrama de los componentes principales de un Q-TOF-MS.

Las especificaciones del analizador QTOF ofrece la posibilidad de analizar un rango de masas más amplio (50-20.000 uma) con una mayor resolución (17.500-20.000 FWHM) y una precisión de 2 ppm si se realiza una calibración interna o de 5 ppm si esta es externa, tanto de los iones moleculares como de sus fragmentos.

Parte experimental



CAPÍTULO 1

Determination of phenolic compounds and antioxidant activity of a Mediterranean plant: The case of *Satureja montana* subsp. *kitaibelii*

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Original Research Article

Distribution of phenolic compounds and other polar compounds in the tuber of *Solanum tuberosum* L. by HPLC-DAD-q-TOF and study of their antioxidant activity

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Abstract

Satureja montana subsp. *kitaibelii* extract was analyzed by HPLC-DAD-ESI-TOF-MS (high performance liquid chromatography–diode array detector-electrospray ionization-time of flight-mass spectroscopy). Forty five compounds were identified, 42 of which were identified for the first time in this plant. Quantification of the identified phenolic compounds by UV-vis (ultraviolet-visible) detection was done and chlorogenic acid was the most concentrated compound. Total phenolic content (TPC) by Folin-Ciocalteu method and antioxidant activity by DPPH and ABTS methods were also measured. TPC quantified by HPLC-DAD were 1958.44 mg/100 g dry sample. This is in agreement with TPC measured by Folin-Ciocalteu method. Antioxidant activity determined by the DPPH method expressed as EC_{50} was 116.36 ± 12.83 μg/mL and by the ABTS method was 106.59 ± 1.41 μmol trolox equivalents/g dry sample. These results reveal that *Satureja montana* subsp. *kitaibelii* could be a good functional food ingredient because

its high content of natural antioxidants as demonstrated by quantification with HPLC-DAD.

Keywords: *Satureja montana* subsp. *kitaibelii*; HPLC-DAD-ESI-TOF-MS; antioxidant activity; quantification; total phenolic compounds.

1. Introduction

The genus *Satureja* belongs to the family Lamiaceae, subfamily Nepetoideae, and the tribe Mentheae, and it is comprised of about 200 species of often aromatic herbs and shrubs. Commonly, they are annual or perennial semi-bushy aromatic plants that inhabit arid, sunny, stony and rocky habitats and are widely distributed in the Mediterranean area, Asia and boreal America[1].

Satureja montana subsp. *kitaibelii* (Wierzb. ex Heuff.) P.W. Ball is considered by some authors as a species itself. The study of Slavkovska *et al.* (2001)[2] confirmed clear differences in composition of essential oils between *S. montana* and *S. kitaibelii*, which also suggested the specificity of this bush named as *Satureja kitaibelii*. However, nowadays the most recent taxonomy classifies it as a subspecies of *Satureja montana* (*Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P. W. Ball), although the name of *Satureja kitaibelii* Wierzb. ex Heuff. is accepted as a basionym[3]. It is an endemic bush that occurs from north of Balkan Peninsula to southwest of Romania. In Serbia it is called "Rtanj tea" or "rtanjensis tea" from the name of Mount Rtanj in the east of Boljevac. It has been used as a culinary herb since antiquity in Serbia. In traditional and homeopathic medicine it has been used for various ailments, especially for digestive complaints, such as colic, and diarrhea, urinary complaints or respiratory diseases such as bronchitis or cough. Externally, it is used for skin and mucous inflammation[4]. It has also been traditionally used as stimulant, stomachic, carminative, expectorant, anticatarrhal, astringent, and aphrodisiac. These health benefits can be due to the presence of various biologically active constituents such as triterpenes[5] and phenolic compounds such as flavonoids and protocatechuic acid[6,7].

Phenolic compounds are important constituents in plants and, recently, they have received considerable attention as potential protective factors against cancer and heart diseases because of their antioxidant potency and their ubiquity in a wide range of commonly consumed foods of vegetable origin[6]. It is generally accepted that phenolic compounds behave as antioxidants as a result of the reactivity of the phenolic moiety. Many studies have reported that phenolic compounds possess other biological activities such as anti-inflammatory, antiulcer, antispasmodic, antiviral, antidiarrheal, and antitumoral properties, among others[8]. Thus, identification and quantification of phenolic compounds can provide vital information related to antioxidant function, food quality, and potential health benefits of a specific plant.

In the background, essential oil from *Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P.W. Ball, analyzed by HPLC, showed the presence of hydroxybenzoic acid derivatives (protocatechuic, syringic and vanillic acids), hydroxycinnamic acid derivatives (caffeic, *p*-coumaric and ferulic acid), and flavanols ((±)-catechin and (−)-epicatechin)[9]. Phenolic compounds in *Satureja montana* have also been studied by GC-MS and HPLC-DAD[6,10]. Other *Satureja* species such as *S. hortensis*, *S. thymbra*, *S. spinosa* have been analyzed using HPLC-DAD, GC-MS, HPLC-UV-MS, TLC-NMR or HPLC-NMR and rosmarinic acid, apigenin, naringenin, taxifolin, thymonin or thymusin were identified among others[11,12]. However, there are few studies on the profile of phenolic compounds in the methanolic extract of *Satureja montana* subsp. *kitaibelii*.

Therefore, in this work the extract of *Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P.W. Ball was analyzed for the first time by HPLC-DAD-

ESI-TOF-MS. The aims of our study were to comprehensively characterize the extract of the aerial part of *Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P.W. Ball grown in Serbia by HPLC-DAD-ESI-TOF-MS in order to identify and quantify the phenolic compounds, and also to measure the antioxidant activity by in vitro methods such as those of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl). To the best of our knowledge, the antioxidant activity of *Satureja montana* subsp. *kitaibelii* (Wierzb. ex Heuff.) P. W. Ball extract had never been analyzed before by ABTS and DPPH methods.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Labscan (Dublin, Ireland). Acetic acid, DPPH, ABTS, potassium persulphate, trolox, TPTZ (2, 6-tripyridyl-s-triazine), ferric chloride, ferrous sulphate, AAPH (2,2'-azobis-2-methylpropanimidamine dihydrochloride), fluorescein, monobasic sodium phosphate, dibasic sodium phosphate, caffeic acid, chlorogenic acid, ferulic acid, quercetin, rutin, DOPAC (3,4- dihydroxyphenilacetic acid), protocatechuic acid and Folin-Ciocalteu reagent were all purchased from Sigma Aldrich (St. Louis,MO, USA). Ethanol, sodium acetate, hydrochloric acid, sodium carbonate, and gallic acid were from Panreac (Barcelona, Spain) and acetone from Fisher Chemical (Leicestershire, UK). Double-deionized water with conductivity lower than 18.2 MΩ was purified by using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample

Aerial parts of wild population of *Satureja montana* subsp. *kitaibelii* were collected in September of 2012 at 400 m of altitude in Josanicka banja, in the south of Serbia. All the samples collected were pooled in a unique composite sample. The plant material was air-dried at room temperature in the dark and then stored at −80 °C for 2 weeks before extraction.

2.3. Extraction of phenolic compounds

A solid-liquid extraction was used to extract the phenolic compounds from *Satureja montana* subsp. *kitaibelii*. Different analyses were performed using the most common solvents to extract phenolics in plants. Three different aqueous mixtures with organic solvents at three different percentages were used to determine the optimal extraction solvent: methanol, ethanol and acetone at 80:20, 70:30, 60:40 (v of organic solvent/v of water), respectively.

All the extractions were performed as follows, 1 g of dry sample was milled and extracted via Ultra-Turrax IKA® T18 basic (Staufen, Germany) with 10 mL of solvent mixture. The system used provided milled samples of about 10 μm of particle size. Then, the sample was placed in an ultrasonic bath (10 min) and centrifuged at 1000 g for 15 min. The supernatant was removed, and the extraction was repeated twice. The supernatants were collected, evaporated, and reconstituted in 1 mL of methanol/water (50:50, v/v).The final extracts were filtered through 0.2 μm nylon syringe filters and stored at -18 °C until analyzed. All extractions were done in triplicate.

2.4. HPLC-DAD-ESI-TOF-MS analyses

The phenolic compounds were separated from the *Satureja montana* subsp. *kitaibelii* extracts on an Agilent 1200 series HPLC (Agilent Technologies, Santa Clara, CA, USA) consisting of a vacuum degasser, a binary pump, an autosampler, a column heater, and a diode array detector (DAD). This instrument was equipped with an Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm) from Agilent Technologies. A gradient elution was programmed using as a mobile phase A, acidified water (1% acetic acid), and as a mobile phase B, acetonitrile. The program was as follows: 10-15% B in 4 min, 15-16% B in 1 min, 16-18% B in 3 min, 18-20% B in 4 min, 20-22% B in 1 min, 22-25% B in 1 min, 25-28% B in 2 min, 28-30% in 1 min, 30-31% in 1 min, 31-32% B in 1 min, 32-50% B in 5 min, 50-75% B in 3 min, 75-100% B in 5 min, 2 min at 100% B and, finally, 100-10% B in 2 min. The flow rate was set at 1.00 mL/min throughout the gradient and the column temperature was maintained at 25 °C. The injection volume was 2.5 μL, and UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, and 330 nm. The effluent from the HPLC column was split using a T-type phase separator before introducing it into the mass spectrometer (split ratio 1:3).

The HPLC system was coupled to a microTOF mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with an ESI interface. Parameters of analysis were set using negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1100. The optimum values of ESI-TOF-MS parameters were: capillary voltage, 4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; and nebulizing gas pressure of 2 bar. The accurate mass data of the molecular ions were processed using the software Data

Analysis 4.0 (Bruker Daltonics GmgH, Bremen, Germany), which provided a list of possible elemental formulas by using the SmartFormula Editor. The SmartFormula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-value) for increased confidence in the suggested molecular formula. The widely accepted accuracy for confirmation of elemental compositions has been established to be 5 ppm[13].

During the use of the HPLC method, an external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, IL, USA) directly connected to the interface, passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide and 0.2% acetic acid in water/isopropanol 1:1 (v/v). With this method, an exact calibration curve was based on numerous cluster masses each differing by 68 Da ($NaCHO_2$).

2.5. Total phenolic content by Folin–Ciocalteu

The total soluble phenolic content in the extract was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method[14]. Briefly, the reaction mixture was composed by mixing 10 μL of the 1/1000 diluted methanolic extract, 600 μL of distilled water, 50 μL of the Folin-Ciocalteu's reagent, and after 10 min, 150 μL of 20% sodium carbonate and 190 μL of distilled water. After 2 h, the absorbance was read at 760 nm using a spectrophotometer (Synergy Mx, BioTek. Bad Friedrichshall, Germany). The total phenolic content has been expressed as mg GAE (gallic acid equivalents)/g dry matter.

2.6. Antioxidant activity

In order to get more information about this botanic extract, the antioxidant activity was measured by two different methods commonly used for plant extracts, ABTS and DPPH radical scavenging assays. ABTS assay, which measures the reduction of the ABTS radical cation by antioxidants, was based on a method previously described[15]. The sample was analyzed in triplicate and results were expressed as μmol trolox equivalents/g dry sample. The ability of *Satureja montana* subsp. *kitaibelii* extract to scavenge stable DPPH was also assessed by spectrophotometry. The sample was analyzed in triplicate, and the DPPH radical scavenging activity was calculated using the following formula:

$$\text{DPPH Radical Scavenging Activity (\%)} = [100 - (A_1 - A_0)] \times 100$$

Where A_1 is the absorbance of the extract, and A_0 is the absorbance of the control. Results are expressed as EC_{50} and values are referred to the lower concentration of the extracts required for the 50% of the antioxidant activity (μg/mL).

2.7. Statistical analysis

Pearson's linear correlations, at the $p<0.05$ level, was also evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussions

3.1. Evaluation of the extraction procedures and identification of phenolic compounds by HPLC–DAD–ESI–TOF–MS

To determine the phenolic profile in *Satureja montana* subsp. *kitaibelii* different solid-liquid extractions were performed as stated in **Section 2.3**. All the extracts were analyzed by HPLC coupled to DAD and ESI-TOF-MS in negative ionization mode.

Table 1. Compounds tentatively identified in *Satureja montanta* subsp. *kitaibelii* extract.

Peak	Proposed compound	Retention time (min)	*m/z* experimental $[M-H]^-$	*m/z* calculated $[M-H]^-$	Fragments	Molecular Formula	Tolerance (ppm)	Error (ppm)	mSigma value
1	Quinic acid	1.190	191.0555	191.0561	-	$C_7H_{12}O_6$	5	3.0	1.3
2	Protocatechuic acid	2.046	153.0193	153.0193	-	$C_7H_6O_4$	5	0.1	37.6
3	Dihydroxybenzoic acid glucoside isomer 1	2.127	315.0723	315.0722	153.0314	$C_{13}H_{16}O_9$	5	0.6	21.1
4	Dihydroxybenzoic acid glucoside isomer 2	2.311	315.0713	315.0722	-	$C_{13}H_{16}O_9$	5	2.7	4.1
5	Caffeoylquinic acid isomer 1	2.712	353.088	353.0878	-	$C_{16}H_{18}O_9$	5	0.6	4.1
6	Luteolin-7-*O*-glucopyranoside	3.481	429.1407	429.1402	-	$C_{19}H_{26}O_{11}$	5	1.0	5.9
7	Chlorogenic acid	3.799	353.0878	353.0878	-	$C_{16}H_{18}O_9$	5	0.0	2.2
8	Caffeoylquinic acid isomer 3	4.117	353.088	353.0878	-	$C_{16}H_{18}O_9$	5	0.6	4.1
9	Caffeic acid	4.919	179.0348	179.0350	-	$C_9H_8O_4$	5	0.9	3.9
10	Caffeoylquinic acid methyl ester	6.274	367.1039	367.1035	191.0556	$C_{17}H_{20}O_9$	5	1.1	4.7
11	Dihydroferulic acid 4-*O*-glucuronide/ 3-methoxy-4,5-dihydroxycinnamic acid glucoside	6.592	371.0999	371.0984	-	$C_{16}H_{20}O_{10}$	5	4.0	4.2
12	Quercetagetin 7-β-D-glucoside	6.926	479.0854	479.0831	-	$C_{21}H_{20}O_{13}$	5	4.7	1.5
13	Quercetin 3-β-D-glucoside	7.344	463.0898	463.0882	301.1619	$C_{21}H_{20}O_{12}$	5	3.5	2.4
14	Acacetin-rutinoside isomer 1	7.679	591.1719	591.1719	-	$C_{28}H_{32}O_{14}$	5	0.0	11
15	Acacetin-rutinoside isomer 2	8.030	591.1728	591.1719	-	$C_{28}H_{32}O_{14}$	5	1.5	7.3
16	Apigenin-6,8-di-C-β-D-glucopyranoside/ Luteolin-7-*O*β-D-rutinoside	9.050	593.151	593.1512	-	$C_{27}H_{30}O_{15}$	5	0.3	3.2
17	Luteolin-7-β-D-glucuronide	9.384	461.0739	461.0725	285.0411	$C_{21}H_{18}O_{12}$	5	2.8	21
18	Kaempferol 3-*O*-glucoside	9.618	447.0946	447.0933	285.1675	$C_{21}H_{20}O_{11}$	5	3.0	5.5
19	Patuletin 7-glucoside	10.120	493.0999	493.0988	331.0470	$C_{22}H_{21}O_{13}$	5	2.4	0.8
20	Dicaffeoylquinic acid isomer 1	10.705	515.1208	515.1195	191.0562/ 353.0889	$C_{25}H_{24}O_{12}$	5	2.5	5.2
21	Dicaffeoylquinic acid isomer 2	11.006	515.1219	515.1195	191.0875	$C_{25}H_{24}O_{12}$	5	4.7	16.3
22	Dicaffeoylquinic acid isomer 3	11.257	515.1215	515.1195	179.0353/ 191.0563/ 353.0898	$C_{25}H_{24}O_{12}$	5	3.8	3.3

23	Apigenin-7-*O*β-D-rutinoside	11.508	577.1584	577.1563	-	$C_{27}H_{30}O_{14}$	5	3.6	27.2
24	4-Succinyl-3,5-dicaffeoylquinic acid	12.344	615.1364	615.1355	179.0353/ 191.0566	$C_{29}H_{28}O_{15}$	5	1.4	4.4
25	Kaempferol-7-*O*-rhamnoside	12.478	431.1005	431.0984	-	$C_{21}H_{20}O_{10}$	5	5.0	1.4
26	Dicaffeoylquinic acid isomer 4	13.230	515.1219	515.1195	353.1117 179.0475 191.0690	$C_{25}H_{24}O_{12}$	5	4.7	3.5
27	Chrysoeriol 7-*O*β-D-glucoside	13.448	461.1108	461.1089	-	$C_{22}H_{22}O_{11}$	5	4.0	3.9
28	Kaempferol 3-*O*β-D-glucopyranoside 6"-(3-hydroxy-3-methylglutarate)	14.000	591.1371	591.1355	285.0413	$C_{27}H_{28}O_{15}$	5	2.6	2.1
29	Acacetin 7-*O*rhamnosylgalacturonide	16.993	605.1516	605.1512	-	$C_{28}H_{30}O_{15}$	5	2.4	14.6
30	3,4-Dihydroxyphenylacetic acid methyl ester	17.712	181.0513	181.0506	-	$C_9H_{10}O_4$	5	4.0	10.1
31	Kaempferol/luteoline	17.946	285.0416	285.0405	-	$C_{15}H_{10}O_6$	5	4.0	1.5
32	Quercetin 3'-methyl ether/ 6-Methoxyluteolin	18.331	315.0523	315.0510	-	$C_{16}H_{12}O_7$	5	4.2	1.3
33	Quercetin 3'-methyl ether/ 6-Methoxyluteolin	19.150	315.0508	315.0510	-	$C_{16}H_{12}O_7$	5	0.7	3.1
34	Tetrahydroxy-dimethoxyflavone	19.418	345.0615	345.0616	-	$C_{17}H_{14}O_8$	5	0.2	4.3
35	Apigenin	20.471	269.0462	269.0455	-	$C_{15}H_{10}O_5$	5	2.3	16.4
36	Luteolin methyl ether isomer 1	20.939	299.0564	299.0561	-	$C_{16}H_{12}O_6$	5	0.8	3.7
37	Luteolin methyl ether isomer 2	21.173	299.0561	299.0561	-	$C_{16}H_{12}O_6$	5	0.1	0.1
38	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	21.775	329.0681	329.0667	-	$C_{17}H_{14}O_7$	5	4.3	1.4
39	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	22.898	329.0681	329.0667	-	$C_{17}H_{14}O_7$	5	4.3	1.4
40	Trihydroxy-trimethoxyflavone isomer 1	22.929	359.0779	359.0772	-	$C_{18}H_{16}O_8$	5	2.0	9.4
41	Trihydroxy-trimethoxyflavone isomer 2	23.163	359.0781	359.0772	-	$C_{18}H_{16}O_8$	5	2.5	5.2
42	Scutellarein dimethyl ether isomer 1	24.234	313.0728	313.0718	-	$C_{17}H_{14}O_6$	5	3.3	4.9
43	Dihydroxy-trimethoxyflavone	24.652	343.083	343.0823	-	$C_{18}H_{16}O_7$	5	1.9	7.6
44	8-Methoxycirsilineol	25.555	373.0935	373.0929	-	$C_{19}H_{18}O_8$	5	1.7	4.7
45	Scutellarein dimethyl ether isomer 2	26.140	313.0729	313.0718	-	$C_{17}H_{14}O_6$	5	3.5	1.2

After the tests with the different solvent mixtures, the extractions obtained with methanol/water mixtures showed chromatogram profiles with higher number of compounds and higher intensity. Among the different methanol/water mixtures, methanol/water (70:30; v/v) gave the best chromatographic profile. This is in agreement with previous results obtained for *Satureja montana* subspecies[6,9].

Peak identification was performed on the basis of their retention times, their UV-vis and mass spectra together with the information previously reported in the literature.

Identification using UV and MS is usually called "tentative". The availability of standards makes crucial this step. In absence of some of the standards, identification is only possible based on literature, UV bands of absorption and MS spectra. Therefore, tentative or putative identification is done in most analytical studies using HPLC-UV-MS[16–18].

Figures 1a and **1b** show the base peak chromatogram (BPC) and the chromatogram at λ=280 nm of *Satureja montana* subsp. *kitaibelii* extract obtained under optimized conditions, respectively. An overview of all the compounds tentatively identified in the extract is given in **Table 1**. These compounds are summarized together with their retention time, experimental and calculated *m/z*, tolerance, error (ppm), mSigma value, molecular formula generated by the software, and the proposed compounds.

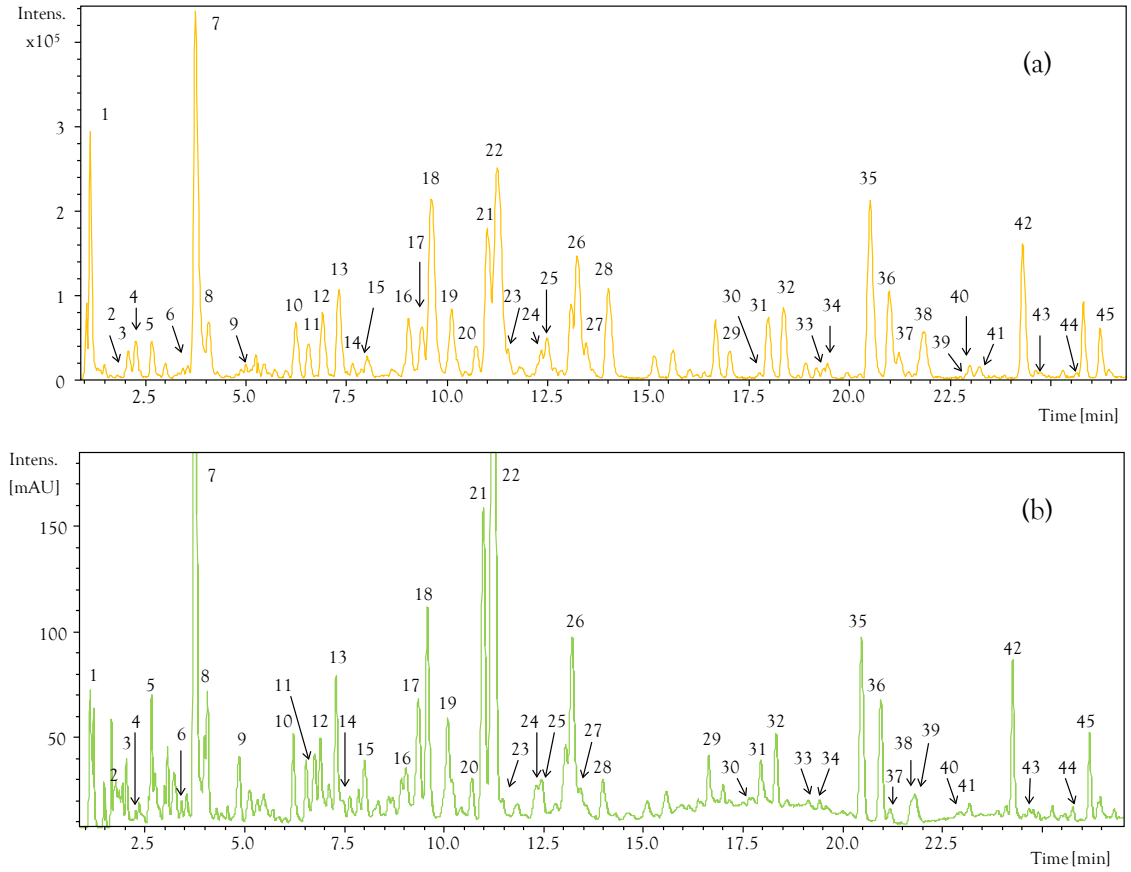

Figure 1. Chromatograms of the phenolic fraction of *Satureja montana* subsp. *kitaibelii* extract BPC obtained by mass spectrometry (a) and chromatogram obtained at λ=280 nm (b) (see **Table 1** for peak identification numbers).

In the present work, a total of 44 phenolic compounds and an organic acid were tentatively identified in *Satureja montana* subsp. *kitaibelii*, 42 of them were identified for the first time for this material. Thus, a comprehensive study of the characterized compounds is to be described later. Among the identified compounds two main families of phenolic compounds were found: phenolic acids and flavonoids, among others.

3.1.1. Phenolic acids

Among the phenolic acids identified, two main categories of compounds could be found: hydroxybenzoic and hydroxycinnamic acids.

3.1.1.1. Hydroxybenzoic acids.

Three hydroxybenzoic acids were tentatively identified in *Satureja montana* subsp. *kitaibelii*. Peak 2, identified as protocatechuic acid, was detected at 2.046 min with *m/z* 153.0193 and molecular formula $C_7H_6O_4$. To the best of our knowledge, this is one of the only three compounds previously described in *Satureja montana* subsp. *kitaibelii*[9]. Next hydroxybenzoic acids derivatives (peaks 3 and 4) were detected at retention times 2.127 and 2.311 min, with ions at the same *m/z* 315.07 and molecular formula $C_{13}H_{16}O_9$. Therefore, these compounds were proposed to be two dihydroxybenzoic acid glucoside isomers. One of these isomers has already been described in other plant such as *Castanopsis sclerophylla*[19].

3.1.1.2. Hydroxycinnamic acids.

Most hydroxycinnamic acids found in *Satureja montana* subsp. *kitaibelii* were caffeoylquinic acid derivatives. These compounds have already been reported before in other plants from the Lamiaceae family.

Three caffeoylquinic acid isomers ($C_{16}H_{18}O_9$) were found in peaks 5, 7 and 8. They were identified according to their molecular ion at *m/z* 353.0878, their retention times at 2.712, 3.799 and 4.117 min, respectively, and literature. Peak 7 was identified as 3-caffeoylquinic acid (chlorogenic acid) since it was also corroborated with its standard. This compound was previously described in *Satureja montana*[10]. Peak 5 was identified as 1-caffeoylquinic acid since it eluted before chlorogenic acid and peak 8 was identified as 4-caffeoylquinic acid since it eluted after chlorogenic acid according to Clifford *et al.* (2005)[20].

Another signal was detected in the BPC at 4.919 min, *m/z* 179.0950 and molecular formula $C_9H_8O_4$ (peak 9). According to Cetkovic *et al.* (2007)[9], this compound was tentatively considered as caffeic acid. This corresponds to the second compound described in this work that has previously been described in *Satureja montana* subsp. *kitaibelii*. Peak 10 at retention time 6.274 min and *m/z* 367.1035 was proposed as caffeoylquinic acid methyl ester ($C_{17}H_{20}O_9$) in agreement with other authors that had previously found this compound in other Lamiaceae plants[21]. Peak 11 showed a signal at retention time 6.592 min with *m/z* at 371.0984 and molecular formula $C_{16}H_{20}O_{10}$. This compound could correspond to dihydroferulic acid-4-glucuronide or 3-methoxy-4,5-dihydroxycinnamic acid glucoside[22,23]. Peaks 20, 21, 22 and 26 gave signals at *m/z* 515.12 and retention times 10.705, 11.006, 11.257 and 13.230 min, respectively. They were identified as four dicaffeoylquinic acid isomers ($C_{25}H_{24}O_{12}$). One of these isomers has been described in Lamiaceae family before[24]. At retention time 12.344 min, compound 24 gave a molecular ion at *m/z* 615.1355, which was tentatively identified as 4-succinyl-3,5-dicaffeoylquinic acid according to the molecular formula ($C_{29}H_{28}O_{15}$) provided by SmartFormula[25].

3.1.2. Phenylacetic acids

Only one phenylacetic acid was found in *Satureja montana* subsp. *kitaibelii* (peak 30). It appeared at 17.712 min, *m/z* 181.0513 and molecular formula $C_9H_{10}O_4$ and it was tentatively identified as 3, 4-dihydroxyphenylacetic acid methyl ester according to previous studies in *Satureja montana*[10].

3.1.3. Flavonoids

The second main group of phenolic compounds in *Satureja montanta* subsp. *kitaibelii* sample studied was flavonoids, specifically flavones and flavonols.

Among flavonoids, flavones were the predominant family in *Satureja montanta* subsp. *kitaibelii*. Twenty out of 26 flavonoids were flavones and only 6 were flavonols. **Table 2** summarizes single flavones and flavonols identified in *Satureja montanta* subsp. *kitaibelii*.

Table 2. Flavones and flavonols identified in *Satureja montanta* subsp. *kitaibelii*.

Peak	Compound	Previously reported	Reference
Flavones			
6	Luteolin-7-*O*glucopyranoside	Lamiaceae family	26
14	Acacetin-rutinoside isomer 1	Lamiaceae family/ *Satureja montana* subsp. *kitaibelii*	27
15	Acacetin-rutinoside isomer 2	Lamiaceae family	27
16	Apigenin-6,8-di-C-β-D-glucopyranoside/ Luteolin-7-*O*β-D-rutinoside	*Satureja montana* and *Satureja hortensis*	28,29
17	Luteolin-7-β-D-glucuronide	Genus *Satureja*	28
23	Apigenin-7-*O*β-D-rutinoside	Genus *Satureja*	28
27	Chrysoeriol 7-*O*β-D-glucoside	Lamiaceae family	30
29	Acacetin 7-*O*rhamnosylgalacturonide	*Reboulia hemisphaerica*	31
34	Tetrahydroxy-dimethoxyflavone	Lamiaceae family	32
35	Apigenin	Lamiaceae family	33
36	Luteolin methyl ether isomer 1	Genus *Satureja*	12,34
37	Luteolin methyl ether isomer 2	Genus *Satureja*	12,34
38	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	Genus *Satureja*	12
39	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	Genus *Satureja*	12
40	Trihydroxy-trimethoxyflavone isomer 1	Genus *Satureja*	12
41	Trihydroxy-trimethoxyflavone isomer 2	Genus *Satureja*	12
42	Scutellarein dimethyl ether isomer 1	Genus *Satureja*	12
43	Dihydroxy-trimethoxyflavone	Genus *Satureja*	35
44	8-Methoxycirsilineol	Genus *Satureja*	12
45	Scutellarein dimethyl ether isomer 2	Genus *Satureja*	12
Flavonols			
12	Quercetagetin 7-β-D-glucoside	Lamiaceae family	36
13	Quercetin 3-β-D-glucoside	Lamiaceae family	37

18	**Kaempferol 3-*O*glucoside**	Genus *Satureja*	38
19	**Patuletin 7-glucoside**	Asteraceae family	39
25	**Kaempferol-7-*O*rhamnoside**	Genus *Satureja*	38
28	**Kaempferol 3-*O*β-D-glucopyranoside 6"-(3-hydroxy-3-methylglutarate)**	*Pedilanthus tithymaloides*	40

Compounds 31, 32 and 33 could not be assigned to flavones or flavanols because two possible compounds could be proposed for each of them. Compound 31 at retention time 17.946 min and *m/z* 285.0405 could be identified as kaempferol and luteolin since both had the same molecular formula $C_{15}H_{10}O_6$. Both compounds had been described in genus *Satureja*[41]. Peaks 32 and 33, at retention times 18.331 and 19.150 min and *m/z* 315.0510, were proposed to be either quercetin-3'-methyl ether or 6-methoxyluteolin ($C_{16}H_{12}O_7$) as they are structural isomers. Both of them had previously been described in Lamiaceae family[24,42].

3.1.4. Organic acids

An organic acid was also detected, and this compound was assigned as quinic acid ($C_7H_{12}O_6$), at 1.190 min and *m/z* 191.0561 (peak 1). This compound had previously been identified in other Lamiaceae plant[43].

To summarize, only 3 compounds out of the 45 of the identified compounds in this work had previously been reported in *Satureja montana* subsp. *kitaibelii*, protocatechuic acid, caffeic acid and acacetin-rutinoside isomer 1. Five compounds had been reported in *Satureja montana* and 16 compounds in gene *Satureja*. The rest of the identified compounds had previously been described in Lamiaceae family and other plant families.

3.2. Quantification of phenolic compounds

Eight standard calibration curves were prepared using the following standards: caffeic acid, ferulic acid, chlorogenic acid (3-caffeoylquinic acid), quercetin, rutin, DOPAC, apigenin, and protocatechuic acid, in the range of concentrations from the limit of quantification (LOQ) to 305 µg/mL for apigenin, to 500 µg / mL for chlorogenic, and protocatechuic acids, to 515 µg /mL for caffeic acid and quercetin, to 520 µg /mL for rutin and DOPAC, and to 525 µg /mL for ferulic acid. Eight calibration points were used in each case, and the analyses were replicated three times for each calibration point (n=3). The different parameters of each one are summarized in **Table 3**: sensitivity [relative standard deviations (RSDs) (%)], linearity (r^2), calibration range and correlation coefficient.

Table 3. Analytical parameters of the method.

Analyte	RSD (%)	LOD (mg/L)	LOQ (mg/L)	Calibration range (mg/L)	Calibration equations	r^2
Caffeic acid	1.191	0.473	1.575	LOQ-515	Y=8.6244X + 2.4741	0.9998
Quercetin	0.070	1.202	4.006	LOQ-515	Y=3.3919X - 2.4995	0.9997
Rutin	1.917	2.471	8.238	LOQ-520	Y=1.6494X + 2.0719	0.9999
DOPAC	0.319	1.841	6.138	LOQ-520	Y=2.2136X + 2.8141	0.9998
Ferulic acid	1.263	0.566	1.887	LOQ-525	Y=7.2X - 3.047	0.9999
Chlorogenic acid	1.178	0.187	0.623	LOQ-500	Y=21.82X + 14.283	0.9983
Apigenin	1.325	0.625	2.148	LOQ-305	Y=6.5239X - 0.1578	0.9993
Protocatechuic acid	0.757	1.239	4.130	LOQ-500	Y=3.29X + 4.8409	0.9991

All standard phenolic compounds showed excellent linearity with correlation coefficients between peak areas and concentrations greater than 0.998 in the range studied. The LOD was determined as the signal-to-noise ratio of 10:1. The LODs ranged from 2.471 mg/L for rutin to 0.473 mg/L for caffeic acid. The LOQs were within 1.575–8.238 mg/L and they were calculated as the

concentration corresponding to 10 times the standard deviation of the background noise. Intraday and interday precisions were calculated to evaluate the repeatability of HPLC-ESI-Q-TOF-MS method. A methanol-water extract was injected on the same day (intraday precision, n=3) for 3 consecutive days (interday precision, n=9). The relative standard deviation (RSD) of analysis time and peak area were determined. The intraday repeatability (expressed as % RSDs) of the area and retention time were from 0.28 to 1.03% and 0.25 to 0.32%, respectively, whereas the interday repeatabilities were 1.45 and 1.56%, respectively.

Quantification was done using the previous calibration curves (**Table 2**). Phenolic compounds with no standard available were quantified using compounds with similar structure as usually done in literature[18,44]. The calibration curve for rutin at λ=280 nm was used to quantify quercetagetin 7-glucoside, quercetin 3-glucoside, kaempferol 3-glucoside, kaempferol 7-rhamnoside, patuletin 7-glucoside and kaempferol 3-glucopyranoside 6'-(3-hydroxy-3-methylglutarate). Likewise, the calibration curve for chlorogenic acid at λ=325 nm was used to quantify quinic acid, caffeoylquinic acid isomers, caffeoylquinic acid methyl ester, dicaffeoylquinic acid isomers and 4-succinyl-3,5-dicaffeoylquinic acid. Caffeic acid was quantified with its own standard at λ=280 nm. Quercetin 3'-methyl ether/6-methoxyluteolin was quantified with the calibration curve of quercetin at λ=280 nm. The calibration curve of apigenin at λ=280 nm was used to quantify luteolin-7-glucopyranoside, apigenin-6,8-diglucopyranoside/luteolin-7-rutinoside, acacetinrutinoside isomers, luteolin-7-glucuronide, apigenin-7-rutinoside, chrysoeriol-7-glucoside, kaempferol/luteolin, tetrahydroxydimethoxyflavone, luteolin methyl ether isomers, acacetin 7-rhamnosylgalacturonide, apigenin, 5,6,4'-trihydroxy-7,3'-dimethoxyflavone/thymusin, scutellarein dimethyl

ether isomers, 8-methoxycirsilineol, trihydroxytrimethoxyflavone isomers, and dihydroxytrimethoxyflavone. Compound 3,4-dihydroxyphenylacetic acid methyl ester was quantified with the calibration curve of DOPAC at λ=280 nm. In the same way, protocatechuic acid and dihydroxybenzoic acid glucoside isomers were quantified with the calibration curve of protocatechuic acid at λ=280 nm and the compound dihydroferulic acid 4-glucuronide/3-methoxy-4, 5-dihydroxycinnamic acid glucoside was quantified with the calibration curve of ferulic acid at λ=280 nm. Thus, compounds that were quantified with a standard were expressed as equivalents of the standard used. The methanolic extracts of *Satureja montana* subsp. *kitaibelii* were analyzed and the quantitative results are presented in **Table 4**. The sample was extracted three times and injected three times.

Table 4. Concentrations of phenolic compounds tentatively identified in *Satureja montana* subsp. *kitaibelii* extract (expressed as mg/100g dry sample + standard deviation).

Peak	Compound	Concentration
1	Quinic acid	4.88 ± 0.32
2	Protocatechuic acid	13.56 ± 0.98
3	Dihydroxybenzoic acid glucoside isomer 1	24.29 ± 1.51
4	Dihydroxybenzoic acid glucoside isomer 2	3.71 ± 0.29
5	Caffeoylquinic acid isomer 1	18.47 ± 0.88
6	Luteolin-7-*O*b-glucopyranoside	3.97 ± 0.09
7	Chlorogenic acid	471.13 ± 13.4
8	Caffeoylquinic acid isomer 3	23.29 ± 0.17
9	Caffeic acid	12.37 ± 0.73
10	Caffeoylquinic acid methyl ester	23.00 ± 1.85
11	Dihydroferulic acid 4-*O*glucuronide/ 3-Methoxy-4,5-dihydroxycinnamic acid glucoside	10.22 ± 0.63
12	Quercetagetin 7-β-D-glucoside	69.60 ± 2.79
13	Quercetin 3-β-D-glucoside	81.08 ± 3.38
14	Acacetin-rutinoside isomer 1	5.84 ± 0.14
15	Acacetin-rutinoside isomer 2	14.90 ± 1.08
16	Apigenin-6,8-di-C-β-D-glucopyranoside/ Luteolin-7-*O*β-D-rutinoside	12.34 ± 1.01

17	Luteolin-7-β-D-glucuronide	28.05 ± 1.92
18	Kaempferol 3-*O*-glucoside	153.12 ± 7.96
19	Patuletin 7-glucoside	105.07 ± 4.67
20	Dicaffeoylquinic acid isomer 1	12.85 ± 0.67
21	Dicaffeoylquinic acid isomer 2	117.27 ± 8.71
22	Dicaffeoylquinic acid isomer 3	357.45 ± 13.98
23	Apigenin-7-*O*β-D-rutinoside	4.83 ± 0.12
24	4-Succinyl-3,5-dicaffeoylquinic acid	13.77 ± 0.84
25	Kaempferol-7-*O*rhamnoside	43.07±2.06
26	Dicaffeoylquinic acid isomer 4	73.43 ± 3.85
27	Chrysoeriol 7-*O*β-D-glucoside	6.22 ± 0.44
28	Kaempferol 3-*O*β-D-glucopyranoside 6"-(3-hydroxy-3-methylglutarate)	49.08 ± 1.99
29	Acacetin 7-*O*rhamnosylgalacturonide	4.12 ± 0.21
30	3,4-Dihydroxyphenylacetic acid methyl ester	23.23 ± 1.43
31	Kaempherol/luteoline	15.28 ± 0.93
32	Quercetin 3'-methyl ether/6-Methoxyluteolin	31.07 ± 1.59
33	Quercetin 3'-methyl ether/6-Methoxyluteolin	5.20 ± 0.31
34	Tetrahydroxy-dimethoxyflavone	3.23 ± 0.11
35	Apigenin	35.35 ± 1.27
36	Luteolin methyl ether isomer 1	24.36 ± 1.18
37	Luteolin methyl ether isomer 2	3.26 ± 0.09
38	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	9.83 ± 0.75
39	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone/Thymusin	1.57 ± 0.06
40	Trihydroxy-trimethoxyflavone isomer 1	3.35 ± 0.12
41	Trihydroxy-trimethoxyflavone isomer 2	6.13 ± 0.37
42	Scutellarein dimethyl ether isomer 1	21.43 ± 1.49
43	Dihydroxy-trimethoxyflavone	1.33 ± 0.05
44	8-Methoxycirsilineol	1.08 ± 0.03
45	Scutellarein dimethyl ether isomer 2	11.79 ± 0.94
	Total	1958.44 ± 143.16

The total phenolic compound content was 1958.44 mg/ 100 g dry sample. As shown in **Table 4**, the major compounds quantified in the sample were chlorogenic acid and dicaffeoylquinic acid isomer 3 (472.2 mg/100 g and 357.5 mg/ 100 g dry sample, respectively), followed by compounds such as kaempferol 3-O-glucoside, dicaffeoylquinic acid isomer 2, patuletin 7-glucoside, quercetin 3-β-D-glucoside, dicaffeoylquinic acid isomer 4, and quercetagetin 7-β-D-glucoside.

3.3. Total phenolic content (TPC) and antioxidant activity

Phenolic compounds are a class of chemical constituents containing one or more hydroxyl residues attached to an aromatic (phenyl) ring. They are one of the most effective antioxidative constituents that contribute to the antioxidant activity of plant food[45]. Hence, it is important to quantify phenolic content and to assess its contribution to antioxidant activity. **Table 5** shows TPC, DPPH and ABTS results of *Satureja montana* subsp. *kitaibelii* extracts.

Table 5. Total phenolic content (TPC) by Folin-Ciocalteu method and antioxidant activity by DPPH and ABTS methods of *Satureja montana* subsp. *kitaibelii* extract.

	TPC (mg equivalents gallic acid/ g dry sample)	DPPH EC_{50} (µg extract/mL)	ABTS (mM equivalents Trolox/g dry sample)
Sample	25.82 ± 3.14	116.36±12.83	332.15 ± 8.4

To the best of our knowledge, TPC have been measured in *Satureja montana* subsp. *kitaibelii* for the first time in this work. The TPC result in the analyzed sample was correlated with total phenolic content obtained by HPLC analyses ($r=0.7772$; $p<0.05$); although this correlation was not very high, probably due to the nature of a spectrophotometric analysis such as Folin–Ciocalteu that is not as specific as HPLC.

Concerning antioxidant activity there are nearly no studies about *Satureja montana* L. subsp. *kitaibelii*. Only Cetkovic *et al.* (2007b) study the antioxidant activity of different *Satureja montana* L. subsp. *kitaibelii* extracts by measuring their ability to scavenge reactive hydroxyl radical during the Fenton reaction.

ABTS and DPPH are common assays to obtain antioxidant activity results in plants and food[46].Two different methods of DPPH and ABTS were used in order to evaluate the antioxidant capacity of the methanolic extracts of *Satureja montana* subsp. *kitaibelii* collected in the Southeast of Serbia. The use of different methods to measure antioxidant activity is a common practice in order to obtain complementary results[47]. As far as we are concerned, this is the first time that antioxidant activity is measured in this plant by DPPH and ABTS. DPPH expressed as EC_{50} was determined in 116.36 ± 12.83 μg/mL. This is in agreement with the high level of phenolic compounds found and it is in order with the value found in the related plant *Satureja montana* L. by Serrano *et al.* (2011)[48]. Indeed, DPPH value is highly correlated with total phenolic compounds by HPLC ($r=0.9999$; $p<0.01$). In addition, the antioxidant ability of the extracts in scavenging the blue-green colored $ABTS^{+}$ was measured relative to the radical scavenging ability of Trolox. Unlike the reactions with DPPH radical, which involves proton transfer, the reaction with ABTS radicals involves electron transfer process. The extracts showed an antioxidant activity of 332.15 ± 8.4 mM Trolox equivalents/g dry sample. This ABTS value did not correlate with total phenolics content, as DPPH is perhaps a more appropriate method to measure the antioxidant activity of *Satureja montana* subsp. *Kitaibelii*.

4. Conclusion

To the best of our knowledge, this is the first time that HPLC-DAD-ESI-TOF-MS was used to analyze the phenolic profile of *Satureja montana* subsp. *kitaibelii*. A total of 44 phenolics compounds and an organic acid were identified, 42 of which were for the first time for this material. Phenolic compounds were also quantified showing chlorogenic acid as the most

concentrated compound. Furthermore, the contents of phenolic compounds determined by HPLC were positively correlated with the DPPH antioxidant activity assay. The results of this study clearly indicate that aerial parts of *Satureja montana* subsp. *kitaibelii* are an abundant source of phenolic compounds, mainly phenolic acids and flavonoids, that serve powerful in vitro antioxidants.

HPLC-DAD-ESI-TOF-MS demonstrated to serve as a valuable tool for simultaneous and fast characterization of phenolic compounds of *Satureja montana* subsp. *kitaibelii*.

These results could explain the traditional usage of *Satureja montana* subsp. *kitaibelii* as culinary spice and folk medicine; and potential uses of this plant as a functional food ingredient.

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CAPITULO 2

Distribution of phenolic compounds and other polar compounds in the tuber of *Solanum tuberosum* L. by HPLC-DAD-QTOF and study of their antioxidant activity

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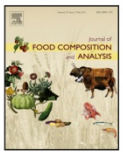

Original Research Article

Distribution of phenolic compounds and other polar compounds in the tuber of *Solanum tuberosum* L. by HPLC-DAD-q-TOF and study of their antioxidant activity

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Abstract

Potato (*Solanum tuberosum* L.) is one of the most widely grown crops worldwide. It represents a staple source of nutrients including carbohydrates, high-quality proteins, minerals, vitamins and polyphenols. This study aimed to identify mainly polyphenols in flesh, peel, and whole tuber of two potato cultivars ('Blue Bell' and 'Melody') by high performance liquid chromatography-diode array detector-electrospray ionization-quadrupole-time of flight-mass spectrometry (HPLC-DAD-ESI-QTOF-MS) analysis and to quantify the main polyphenols by ultraviolet-visible (UV-vis) in order to evaluate their distribution. The antioxidant activity of the three fractions of both cultivars by ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) was also studied. A total of 24 polar compounds were identified in less than 25 min. Three caffeoylquinic acid isomers, caffeic acid, 3,5-dicaffeoylquinic acid, and N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide were the main phenolic compounds in the three fractions of the two potato cultivars. 'Blue Bell' was the cultivar with the highest phenolic-

compound content, while the peels were the part with the highest phenolic-compound content as well as the highest antioxidant activity. The results established potato peels as an attractive by-product of the potato industry because they can be used as an alternative source of polyphenols in the food industry and used in other food products.

Keywords: *Solanum tuberosum* L.; potato peel ;potato flesh; HPLC-DAD-ESI-QTOF; phenolic compounds; antioxidant capacity; food analysis; food composition.

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most widely grown crops worldwide, and the fourth largest in terms of fresh produce, after rice, wheat, and maize[1]. Representing the staple source of nutrients and energy in many different countries, potato is an appealing crop due to its fast growth, adaptability to different environments, high yield, and response to low fertilizer input. In addition, its characteristics (up to 85% of the plant is edible as compared to ~50% in cereals) and ability to produce high yields under harsher climates and using less land than any other major crop make potato a suitable staple food in many countries[2]. Furthermore, potato is a major source of carbohydrates, high-quality proteins (proteins with a large number of essential amino acids), minerals such as potassium, sodium, iron, magnesium, and vitamins such as C, B1, B6, and B9. Furthermore, some potato cultivars are a rich source of polyphenolic compounds[3].

Phenolic compounds are secondary metabolites that are synthesized during normal plant development[4] and in response to stress conditions such as infection, wounding, and UV radiation, among others[5]. These metabolites play a decisive role in the sensory quality of fruits, vegetables, and other plants such as bitterness, astringency, color or smell[6]. They have attracted increasing attention in recent years due to their health benefits, as their consumption has been linked to lowering the risk of diseases associated with oxidative stress, such as cancer and cardiovascular diseases[7]. Potatoes contribute to the daily intake of polyphenols and therefore their consumption may be beneficial for human health.

Recent works have reported that major phenolic compounds in potato are hydroxycinnamoylquinic/hydroxycinnamoyl derivatives, mainly 5-

caffeoylquinic acid, 1-caffeoylquinic acid, 3-caffeoylquinic acid (or chlorogenic acid), caffeic acid, and caffeoyl-putrescine. In addition, Mohdaly *et al.* (2013)[8] have also reported low amounts of vanillic, sinapic, *p*-coumaric, and cinnamic acid. However, the phenolic-compound composition in potato depends on different factors such as cultivation site, climatic conditions, agricultural practices, and genotype[9,10].

Phenolic compounds are distributed mostly between the outer tissues and the skin (peel) of potato tubers[11]. Therefore, recycled potato peel, as a by-product of the food industry, could be considered as a source of polyphenol compounds and could be used to produce other food products including functional foods and nutraceuticals.

In this work, the flesh, peel, and whole tuber of two cultivars of *S. tuberosum* L. ('Blue Bell' and 'Melody') were analyzed by HPLC-DAD-ESI-QTOF-MS (high performance liquid chromatography-diode array detector-electrospray ionization-quadrupole-time of flight-mass spectrometry) to identify and quantify their phenolic and other polar compound content and were subjected to several in vitro methods based on single electron transfer mechanisms (Trolox equivalent antioxidant capacity [TEAC] and ferric reducing antioxidant power [FRAP]); and on a hydrogen-atom transfer mechanism (oxygen radical absorbance capacity [ORAC]) to measure their antioxidant activity. The distribution of phenolic compounds was also determined in the different parts of the potato tuber.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetonitrile and methanol were purchased from Labscan (Dublin, Ireland). Glacial acetic acid, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (98%), potassium persulfate (99%), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (97%), TPTZ (2,6-tripyridyl-s-triazine) (99%), ferric chloride (99%), ferrous sulfate (99%), AAPH (2,20- azobis-2-methyl-propanimidamine, dihydrochloride) (97%), fluorescein (95%), monobasic sodium phosphate (99%), dibasic sodium phosphate (99%), caffeic acid (99%), 3-caffeoylquinic acid (or chlorogenic acid) (95%), rutin (94%), and ferulic acid (99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol, sodium acetate (99%), and hydrochloric acid (37%) were from Panreac (Barcelona, Spain). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Samples

Two potato cultivars of *S. tuberosum* L. were analyzed: 'Blue Bell' and 'Melody'. 'Blue Bell' has yellow flesh and yellow peel with blue spots on it, and 'Melody' has yellow flesh and yellow peel. Potatoes of the two different varieties were grown in 2012 (from March to July) under the same agricultural conditions in open fields in Emilia-Romagna (northern Italy) in accordance with integrated pest management guidelines of the Food and Agricul-ture Organization of the United Nations[12]. Three accessions of each variety were analyzed. The peel (removed manually from the potatoes), the flesh, and whole potato were analyzed separately. Samples were frozen at -80

ºC for two days and then freeze-dried. Every accession was extracted three times and each extract was analyzed three times.

2.3. Extraction of the polar fraction

Freeze-dried samples (3 g flesh and entire potato, 2 g peel) were placed in an ultrasonic bath (15 min) with 20 mL of a solution of methanol/water (4:1, v/v) with 1% acetic acid. After centrifugation at 1000 x g for 15 min, the supernatant was removed, and the extraction was repeated three more times. The supernatants were collected, evaporated, and reconstituted with 1 mL of methanol/ water (4:1, v/v) with 1% acetic acid. The final extracts were filtered through 0.2 mm nylon syringe filters and stored at -18 ºC until analyzed.

2.4. HPLC-DAD-ESI-QTOF-MS analyses

The phenolic compounds were separated from the potato extracts using an Agilent 1200 series Rapid Resolution LC system (Agilent Technologies, CA, USA) consisting of a vacuum degasser, an autosampler, and a binary pump. This instrument was equipped with an Agilent Poroshell 120 EC-C18 column (4.6 mm x 100 mm, 2.7 mm) from Agilent Technologies. A gradient elution was programmed using as a mobile phase A, acidified water (0.5% acetic acid), and as a mobile phase B, acetonitrile. The program was as follows: 5-7% B in 2 min, 7-9% B in 2 min, 9-12% B in 3 min, 12-15% B in 1 min, 15-16% B in 1 min, 16-18% B in 3 min, 18-20% B in 2 min, 20-22% in 1 min, 22-25% in 1.50 min, 25-28% B in 1.10 min, 28-30% B in 1 min, 30-31% B in 1 min, 31-32% B in 1.50 min, 32-100% B in 3.10 min, 100-100% in 2 min. The flow rate was set at 0.80 mL min^{-1} throughout the

gradient. The injection volume was 10 mL, and UV spectra were recorded from 200 to 600 nm using the DAD detector, whereas the chromatograms were registered at 240, 280, and 330 nm. The effluent from the HPLC column was split using a T-type phase separator before introducing it into the mass spectrometer (split ratio 1:3).

The HPLC system was coupled to a microTOF-Q-MS (Bruker Daltonics, Bremen, Germany), equipped with an ESI interface. Analysis parameters were set using negative and positive ion modes with spectra acquired over a mass range from *m/z* 50 to 1100. The optimum values of ESI-QTOF-MS parameters in negative mode were: capillary voltage, -4 kV; drying gas temperature 210 °C; drying gas flow 8 L min^{-1}; and nebulizing gas pressure of 2 bar. The optimum values of the parameters in positive mode were: capillary voltage +4.5 kV; drying gas temperature 210 °C; drying gas flow 8 L min^{-1}; and nebulizing gas pressure of 2 bar.

The accurate mass data of the molecular ions were processed using the software DataAnalysis 4.0 (Bruker Daltonics GmgH, Bremen, Germany), which provided a list of possible elemental formulas by using the SmartFormula Editor. The SmartFormula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy for confirmation of elemental compositions has been established to be 5 ppm[13].

During the use of the HPLC method, an external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, IL, USA)

directly connected to the interface, passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide and 0.2% acetic acid in water/isopropanol 1:1 (v:v). With this method, an exact calibration curve was based on numerous cluster masses each differing by 68 Da ($NaCHO_2$).

2.5. Antioxidant activity

The total antioxidant activity of potato phenolic extracts was measured by three methods: Trolox equivalent antioxidant capacity (TEAC) assay, oxygen radical absorbance capacity (ORAC) assay, and ferric ion reducing power (FRAP) assay.

TEAC assay, which measures the reduction of the ABTS radical cation by antioxidants, was based on the method previously described by Laporta *et al.* (2007)[14]. The capacity of the extracts to scavenge peroxyl radicals was assayed with a validated ORAC method, which uses fluorescein (FL) as the fluorescent probe ($ORAC_{FL}$), in Laporta *et al.* (2007)[14]. The final ORAC values were calculated using a regression equation between the Trolox concentration and the net area of the FL decay curve (area under curve, AUC). The FRAP assay, which measures the capacity of antioxidant compounds to reduce the ferric ions by a single electron-transfer mechanism, was performed according to Al- Duais *et al.* (2009)[15]. Ferrous sulfate solutions (0–200 mM) were used for calibration.

2.6. Statistical analysis

One-way analysis of variance, ANOVA (Tukey's honest significant-difference multiple comparison) and Pearson's linear correlations were used with

Statistical 8.0 software (StatSoft, Tulsa, OK, USA), and p values <0.05 were considered to be statistically significant.

ANOVA was applied to the single and total phenolic compounds of each sample analyzed and also to the results of the antioxidant activity tests. Pearson's linear correlation was used to correlate the total phenolic content with TEAC, ABTS and ORAC. All chemical analyses were performed in triplicate, and the analytical data were used for statistical comparisons.

3. Results and discussion

3.1. Identification of the compounds of the polar fraction of peel, flesh, and entire tuber by HPLC-DAD-ESI-QTOF

An overview of all the compounds tentatively identified in the extracts of the two potato cultivars by HPLC-DAD-ESI-QTOF using the negative and positive modes is given in **Table 1**. These compounds are summarized together with their retention time, *m/z* experimental and calculated, tolerance, error (ppm), mSigma value, molecular formula generated by the software for the detected deprotonated molecule, classification order in the list of possibilities (sorted with respect to mSigma value), MS^2 fragments, and the proposed assignment. **Table 2** shows the distribution of these compounds in the different parts of the tuber and potato cultivars.

Table 1. Phenolic and other polar compounds tentatively identified in *Solanum tuberosum* L.

Peak	Possible compound	Retention Time(min)	UV max absorption	*m/z* experimental	*m/z* calculated	Fragment	Molecular formula	Tolerance (ppm)	Error (ppm)	mSigma
	Negative mode									
1	Citric acid	2.461	226,275	191.0204	191.0197	111	$C_6H_8O_7$	5	3.3	4.4
2	Tyrosine	2.595	226,275	180.0665	180.0666	119, 163	$C_9H_{11}NO_3$	5	0.7	2.8
3	Leucine	2.829	225,255,280	130.0812	130.0874	115	$C_6H_{13}NO_2$	5	4.4	10.7
4	Quinic acid	3.715	227,285	191.0556	191.0561	115, 129, 101, 111	$C_7H_{12}O_6$	5	2.5	14.6
5	Phenylalanine	4.083	226,257	164.0730	164.0717	147	$C_9H_{11}N0_2$	10	7.8	4.7
6	Caffeoyl putrescine	5.738	226, 292, 318	249.1238	249.1245	135, 117	$C_{13}H_{18}N_2O_3$	5	2.6	10.8
7	1-Caffeoylquinic acid	6.107	226, 295, 325	353.0881	353.0878	191	$C_{16}H_{18}O_9$	5	0.8	5.3
8	Tryptophan	6,892	226,280	203.0840	203.0826	116, 142	$C_{11}H_{12}N_2O_2$	10	7.1	28
9	Bis (dihydrocaffeoyl) spermine	7.745	226,275, 325	529.3039	529.3032	407, 365	$C_{28}H_{42}N_4O_6$	5	1.5	28.5
10	Chlorogenic acid	8.665	240,285,325	353.0905	353.0878	191	$C_{16}H_{18}O_9$	10	7.7	1.9
11	4-Caffeoylquinic acid	9.468	240,285,325	353.0887	353.0878	173, 179, 191	$C_{16}H_{18}O_9$	5	2.4	5.5
12	Caffeic acid	10.220	240,285,325	179.0352	179.0350	135	$C_9H_8O_4$	5	1.4	2.9
13	Bis(dihydrocaffeoyl) spermidine	11.023	226,280	472.2459	472.2453	308, 163	$C_{25}H_{35}N_3O_6$	5	1.2	17
14	5-O-feruloylquinic acid	12.294	240,285,325	367.1028	367.1028	191, 173, 111	$C_{17}H_{20}O_9$	5	1.7	7.4
15	Tris(dihydrocaffeoyl) spermine	14.618	230,280	693.3510	693.3505	529, 365	$C_{37}H_{50}N_4O_9$	5	0.5	0.8
16	Rutin	14.885	230,260,325	609.1454	609.1488	301	$C_{27}H_{30}O_{16}$	5	0.7	1.2
17	Ferulic acid derivative	15.354	230,285,325	309.0594	309.0616	193, 134	$C_{14}H_{14}O_8$	5	7.1	1.5
18	N-[2-hydroxy-2-(4-hydroxyfenyl) ethyl] ferulamide	16.256	230,280,325	328.1199	328.1190	310, 295	$C_{18}H_{19}NO_5$	5	2.5	13.4
19	Kaempferol-3-*O*-rutinoside	17.025	230,260,325	593.1509	593.1512	285	$C_{27}H_{30}O_{15}$	5	0.3	0.4
20	3,4-Di-caffeoylquinic	17.125	230,280,325	515.1196	515.1195	353, 179, 173	$C_{25}H_{24}O_{12}$	5	0.3	19.1
21	3,5-Di-caffeoylquinic	17.693	230,280,325	515.1227	515.1195	353, 191, 179	$C_{25}H_{24}O_{12}$	5	6.2	3.3
22	N^1,N^4,N^8-tris(dihydrocaffeoyl) spermidine	18.429	235,280,325	636.2935	636.2933	472, 514	$C_{34}H_{43}N_3O_9$	5	0.8	1.3
23	4,5-Di-caffeoylquinic	18.947	235,280,325	515.1212	515.1195	353, 179, 173	$C_{25}H_{24}O_{12}$	5	3.4	10.5
	Positive mode									
24	Methylumbelliferone	16.039	235,280,325	177.0566	177.0546	-	$C_{10}H_8O_3$	5	11.2	1.3

UV max- Ultraviolet maximum

Table 2. Distribution of polar compounds in flesh and peel of 'Blue Bell' and 'Melody' potato cultivars.

Peak	Possible compound	Flesh 'Blue Bell'	Peel 'Blue Bell'	Flesh 'Melody'	Peel 'Melody'
1	Citric acid	✓	✓	✓	✓
2	Tyrosine	✓	✓	✓	✓
3	Leucine	-	✓	✓	-
4	Quinic acid	✓	✓	-	✓
5	Phenylalanine	✓	✓	✓	✓
6	Caffeoyl putrescine	-	✓	-	✓
7	1-Caffeoylquinic acid	-	✓	✓	✓
8	Tryptophan	✓	✓	✓	✓
9	Bis (dihydrocaffeoyl) spermine	-	✓	-	✓
10	Chlorogenic acid	-	✓	✓	✓
11	4-Caffeoylquinic acid	-	✓	✓	✓
12	Caffeic acid	-	✓	-	✓
13	Bis(dihydrocaffeoyl) spermidine	-	✓	-	✓
14	5-*O*feruloylquinic acid	-	✓	-	✓
15	Tris(dihydrocaffeoyl) spermine	-	✓	-	✓
16	Rutin	-	-	✓	✓
17	Ferulic acid derivative	✓	✓	✓	✓
18	N-[2-hydroxy-2-(4-hydroxyfenyl) ethyl] ferulamide	-	✓	-	✓
19	Kaempferol-3-*O*rutinoside	-	-	✓	✓
20	3,4-Di-caffeoylquinic	-	✓	-	-
21	3,5-Di-caffeoylquinic	-	✓	-	-
22	N^1,N^4,N^8-tris(dihydrocaffeoyl) spermidine	-	✓	-	✓
23	4,5-Di-caffeoylquinic	-	✓	-	-
24	Methylumbelliferone	-	✓	-	-

✓ Presence of the compound.
- No presence of the compound.

In the present work, 24 polar compounds were tentatively identified in potato cultivars 'Blue Bell' and 'Melody' in less than 25 min by using the combination of UV, MS, and MS_2 data and the information reported in the literature. **Figures 1** and **2** show the base peak chromatogram (BPC) of the polar fraction obtained from whole potato, flesh, and peel of 'Blue Bell' and 'Melody', respectively, under the optimum conditions of the method,

respectively. In addition, **Figure 3** shows the UV chromatogram at 280 nm of the whole potato 'Blue Bell' and 'Melody' cultivars.

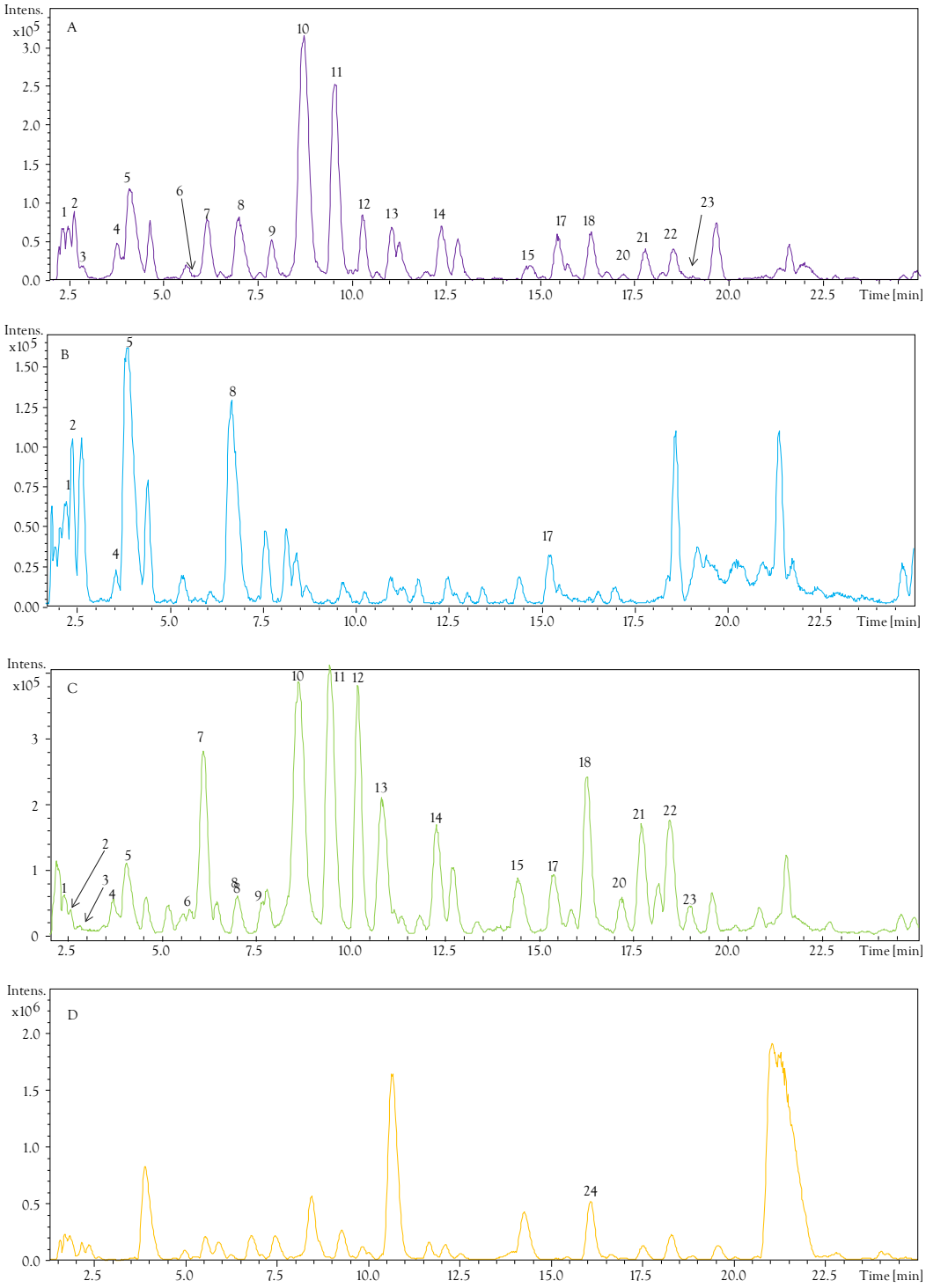

Figure 1. Base-peak chromatograms in negative mode for the polar fraction of whole potato (A), flesh (B), and peel (C), and base-peak chromatogram in positive mode for the polar fraction of peel (D) of potato cultivar 'Blue Bell'.

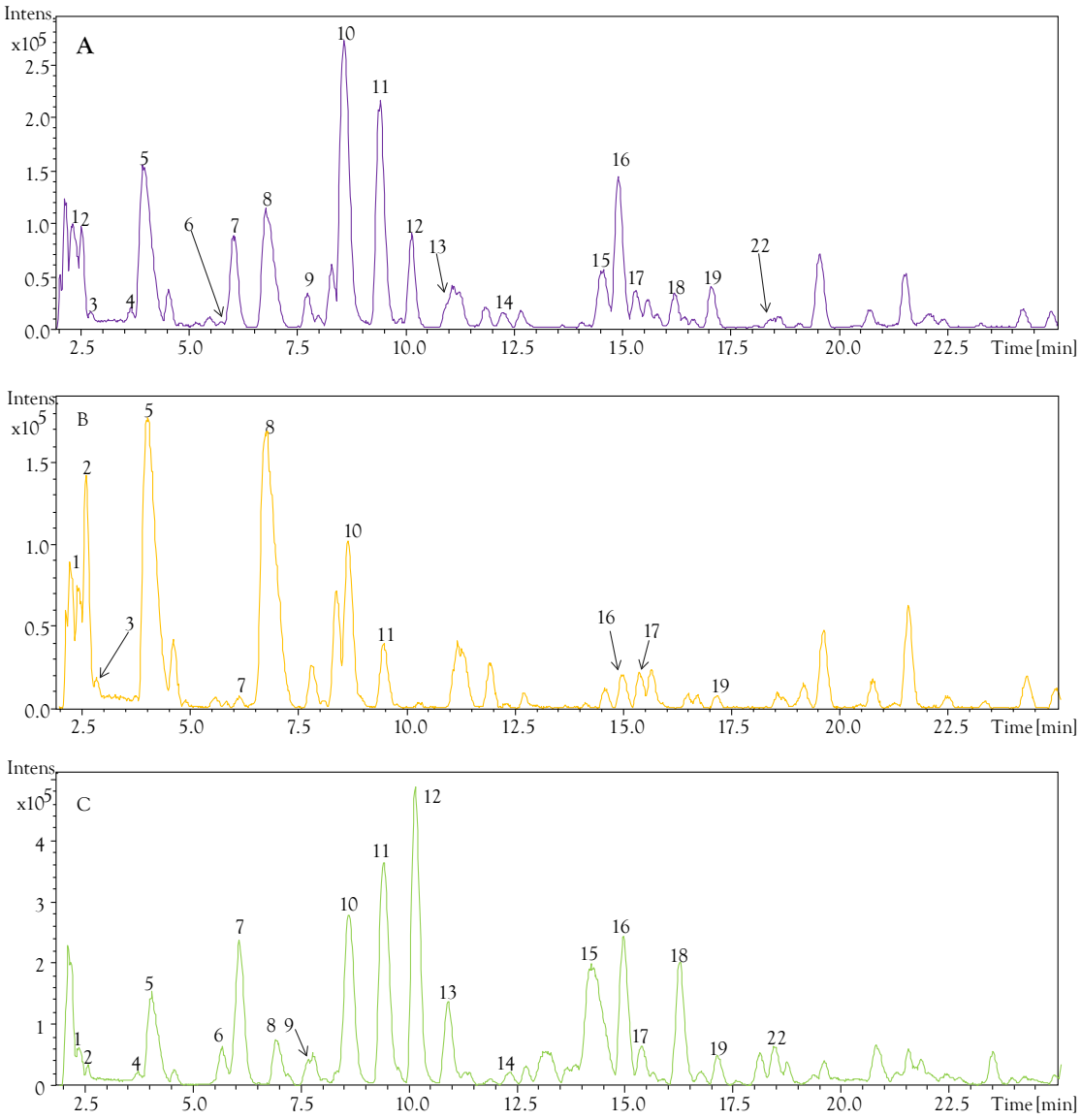

Figure 2. Base-peak chromatograms in negative mode for the polar fraction of whole potato (A), flesh (B), and peel (C) of potato cultivar 'Melody'.

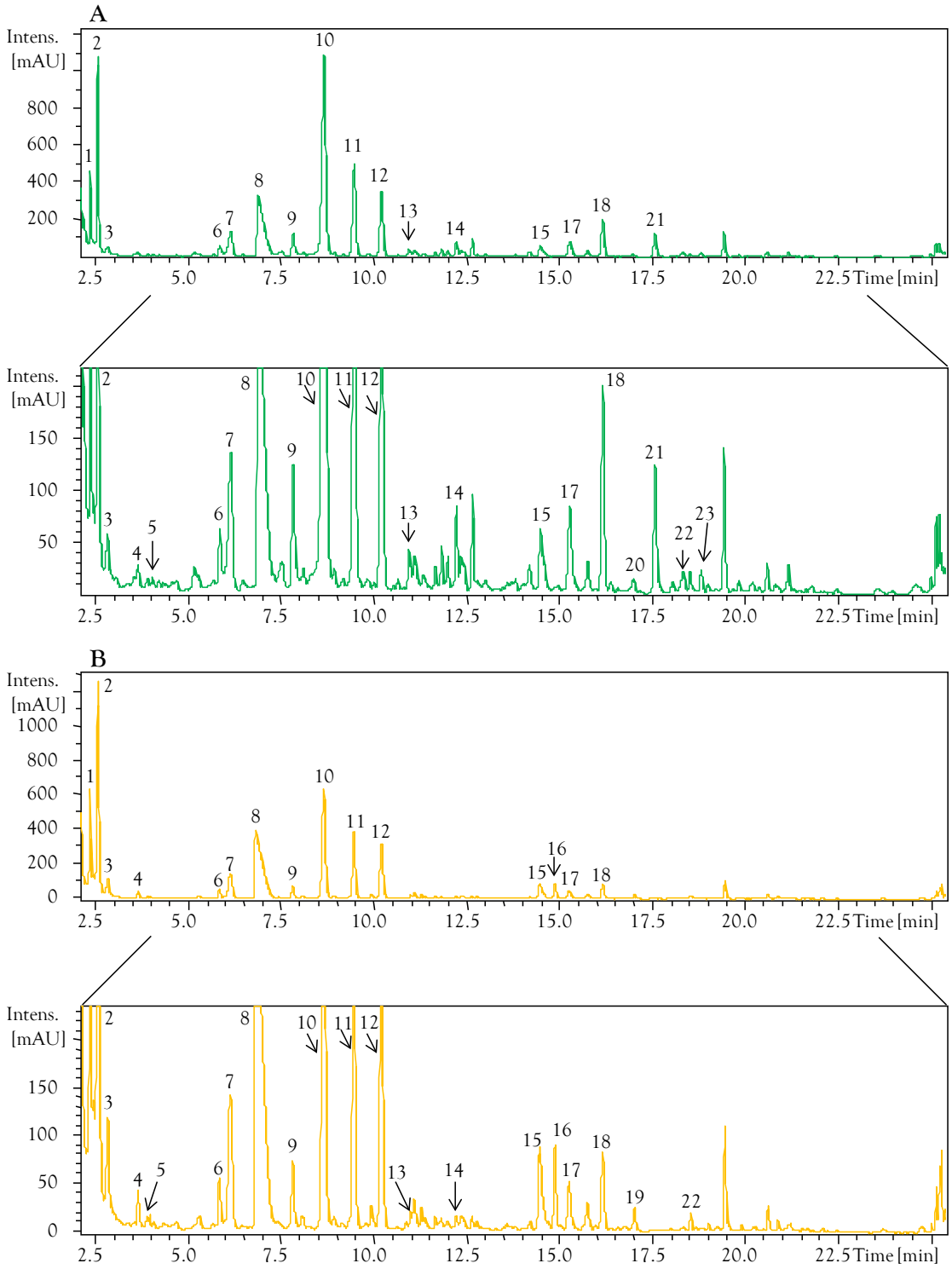

Figure 3. Chromatogram UV 280 nm of 'Blue Bell' whole potato (A) and 'Melody' whole potato (B).

A comprehensive study of the compounds characterized is described below. At the beginning of the chromatogram analysis time, two organic acids were identified corresponding to peaks 1 and 4. Peak 1 at retention time 2.461 min with *m/z* 191.0197 was tentatively identified as citric acid ($C_6H_8O_7$), according to the MS data and fragmentation pattern that show the loss of $2H_2O$ and COOH (*m/z* 111). The presence of citric acid in potato had previously been reported by Galdón *et al.* (2010)[16]. The other organic acid detected was quinic acid ($C_7H_{12}O_6$) (peak 4) with *m/z* 191.0651, which eluted at 3.715 min, and fragments at *m/z* 101, 111, 115 and 129 were shown. The presence of quinic acid in potato has been reported by Wu *et al.* (2012)[17]. These organic acids were found in all the samples analyzed except in the flesh of 'Melody', where only citric acid was detected.

Several amino acids were identified corresponding to peaks 2, 3, 5, and 8. Compound at a retention time of 2.595 min and *m/z* 180.0666 (peak 2) was tentatively identified as tyrosine ($C_9H_{11}NO_3$) due to the fragments formed by the loss of NH_2 (*m/z* 163) and the loss of CO and OH (*m/z* 119) similar to the information previously described by Shakya and Navarre (2006)[18]. Peak 3, which eluted at time 2.829 min and presented *m/z* 130.0874, gave a fragment at *m/z* 115 due to the loss of a methyl moiety. Thus, it was identified as leucine ($C_6H_{13}NO_2$); furthermore, it has been previously described in potato by Galdón *et al.* (2010)[16]. The peak at 4.083 min corresponded to phenylalanine ($C_9H_{11}NO_2$) (peak 5) because of the precursor and fragment ions at *m/z* 164.0717 and *m/z* 147, respectively, indicating the loss of a NH_2 moiety. This amino acid has been described in potato by Shakya and Navarre (2006)[18]. Peak 8 (6.892 min) had the precursor ion at *m/z* 203.0826. The ESI-MS spectra of this peak gave a MS^2

with a fragmentation pattern at *m/z* 116 and 142, in which the losses of –CH_2–CH (NH_2)–COOH, and NH_2 and COOH were shown, respectively. Thus, this compound was identified as tryptophan ($C_{11}H_{12}N_2O_2$)[18]. All amino acids were found in every sample except for leucine, which was found neither in the flesh of 'Blue Bell' nor in the peel of 'Melody'.

Phenolic compounds were detected from 5.738 min until the end of the analysis. Peak 6 corresponds to the first phenolic compound detected, assigned to caffeoyl putrescine ($C_{13}H_{18}N_2O_3$) due to *m/z* 249.1245 and a fragment at *m/z* 135. In addition, this compound has been reported by Shakya and Navarre (2006)[18]. The loss of –CO–NH–$(CH_2)_4$–NH_2 moiety resulted in a fragment of *m/z* 135. Caffeoyl putrescine was identified in the peels of the two samples studied.

Caffeoylquinic isomers ($C_{16}H_{18}O_9$) were identified in peaks 7, 10, and 11. They were identified according to their UV spectra, MS data with a base peak at *m/z* 353, their retention times at 6.107, 8.665, 9.468 min, respectively, and their MS_2 fragmentation pattern with the appearance of a fragment at *m/z* 191 (quinic acid). Caffeoylquinic acid isomer at 8.665 min was identified as chlorogenic acid because its retention time, MS and MS_2 data were corroborated with its commercial standard. This compound was previously reported in potato by Narváez-Cuenca *et al.* (2013)[19]. Peak 11 at retention time 9.468 min, was identified as the isomer 4-caffeoylquinic acid (or cryptochlorogenic acid), since it was the only one that showed a MS_2 base peak at *m/z* 173 as previously reported[20]. This isomer was also previously described in potato by Narváez-Cuenca *et al.* (2013)[19]. Peak 7 was identified as 1-caffeoylquinic acid as it showed a strong ion at *m/z* 191 (MS_2) and a weak fragment at *m/z* 179, moreover its retention time was lower than any

other caffeoylquinic acid isomer. This is in agreement with results found by Clifford *et al.* (2005)[20]. This compound was previously described in potato by Zhu *et al.* (2010)[3]. These compounds were found in all the samples except in the 'Blue Bell' flesh.

Bis (dihydrocaffeoyl) spermine ($C_{28}H_{42}N_4O_6$), *m/z* 529.3032, was identified at 7.745 min (peak 9). It was detected as a moiety at *m/z* 365 which corresponds to the loss of –CO–$(CH_2)_2$–C_6H_6–$(OH)_2$, and another moiety at *m/z* 407 which corresponded to –CH_2–C_6H_6–$(OH)_2$. This compound was previously reported by Shakya and Navarre (2006)[18]. It was found in the peel of both potato cultivars but not in the flesh.

Peak 12, at *m/z* 179.0350, eluted at 10.220 min. It was identified as caffeic acid ($C_9H_8O_4$) as reported by Wu *et al.* (2012)[17] and because the detected fragment at *m/z* 135 corresponded to the loss of a –COOH moiety. This compound was found in the peel of both cultivars.

Bis (dihydrocaffeoyl) spermidine ($C_{25}H_{35}N_3O_6$) at *m/z* 472.2453, which was found at retention time 11.023 min (peak 13), has been previously identified in potato (Shakya and Navarre, 2006)[18]. Two moieties were detected at *m/z* 163 and 308. The first one was originated by the loss of–CO–C_6H_6–$(OMe)_2$. The moiety at *m/z* 308 was due to the loss of –NH–$(CH_2)_4$–NH–$(CH_2)_3$–NH–CO–C_6H_6–$(OMe)_2$. In the present work, this compound was identified in the peel of both cultivars.

Compound 14 (12.294 min), at *m/z* 367.1028, was assigned to 5-O-feruloylquinic ($C_{17}H_{20}O_9$) since the MS_2 fragmentation pattern gave fragments at *m/z* 191, 173, and 111. The 191 moiety corresponded to quinic

acid and the 173 moiety to a quinic acid fragment. This compound has been mentioned by Shakya and Navarre (2006)[18].

Tris (dihydrocaffeoyl) spermine ($C_{37}H_{50}N_4O_9$) was identified at 14.618 min (peak 15) and *m/z* 693.3505, according to Shakya and Navarre (2006)[18]. The MS_2 fragmentation pattern showed fragments at *m/z* 529 and 365. The first one corresponded to the loss of –CO–$(CH_2)_2$–C_6H_6–$(OH)_2$. The moiety at *m/z* 365 showed the loss of –$(CH_2)_4$–NH–$(CH_2)_3$–NH–CO–$(CH_2)_2$–C_6H_6–$(OH)_2$ and the loss of 2OH. Here, this compound was found only in the peel of both cultivars.

Peak 16 (14.885 min) at *m/z* 609.1488 showed a fragment ion at *m/z* 301, which indicates quercetin, and the loss of rutinoside. Thus, this compound was proposed as rutin ($C_{27}H_{30}O_{16}$), and it appeared only in flesh and peel of 'Melody' but it was not found in 'Blue Bell'[18].

The compound at a retention time 15.354 min and *m/z* 309,0594 (peak 17) was tentatively suggested to be a ferulic acid derivative, confirmed by the appearance of the fragment ions at *m/z* 193 and 134 (ferulic acid and its fragment, respectively)[21]. It was found in all the samples analyzed from both cultivars.

Peak 18 at *m/z* 328.1190 and retention time 16.256 min, was identified as N-[2-hydroxy-2-(4-hydroxyfenyl) ethyl] ferulamide ($C_{14}H_{14}O_8$) according to the fragmentation pattern that gave *m/z* 310 and 295 moieties and the information previously reported by Wu *et al.* (2012)[17]. In the present work, this compound was found only in peels of both cultivars.

At retention time 17.025 min (peak 19) resulted in the identification of kaempferol-3-O-rutinoside ($C_{27}H_{30}O_{15}$) with *m/z* 593.1512 and a fragment at

m/z 285 which corresponded to kaempferol. The presence of this compound in potato has been reported by Shakya and Navarre (2006)[18]. Here, it was found only in the flesh and peel of 'Melody'.

Peaks 20, 21, and 23 (retention times 17.125, 17.693, 18.947 min, respectively) presented the same molecular ion at *m/z* 515 and their MS_2 fragmentation gave fragments at *m/z* 353 corresponding to caffeoylquinic acid. Thus, these peaks were assigned to dicaffeoylquinic acid isomers ($C_{25}H_{24}O_{12}$). To distinguish among these three isomers and be able to tentatively identify them, the MS_2 fragmentation of the base peak ions and the fragments of the ions present in MS were studied according to the previous report by Clifford *et al.* (2005)[20]. Thus, peaks 20 and 23 showed a moiety at *m/z* 173, which indicate the presence of a 4-acyl dicaffeoylquinic acid. In addition, MS of peak 20 showed a weak ion at *m/z* 335 and a strong ion at *m/z* 179, therefore it was identified as the isomer 3,4-dicaffeoylquinic acid. This compound was previously described in potato by Friedman (1997)[22]. Peak 23 MS showed an undetectable ion at *m/z* 335, and a strong ion m/ z 179, which indicated the isomer 4,5-dicaffeoylquinic acid. This compound was previously described in potato by Shakya and Navarre (2006)[18]. Peak 21, at 17.693 min, showed a strong moiety at *m/z* 191 which indicated a dicaffeoylquinic acid not substituted at position 4. Moreover, it showed an undetectable MS ion at *m/z* 335 and a strong ion at *m/z* 179; therefore, it was identified as the isomer 3,5-dicaffeoylquinic acid. This compound was previously described in potato by Shakya and Navarre (2006)[18]. In the present work, these compounds were found only in the peel of 'Blue Bell'.

The compound at a retention time of 18.429 min (peak 22) and *m/z* 636.2933, was tentatively suggested to be N,N,N-tris (dihy-drocaffeoyl) spermidine ($C_{34}H_{43}N_3O_9$) according to Shakya and Navarre (2006)[18]. The MS_2 fragmentation pattern showed moieties at *m/z* 472 and 514. The fragment at *m/z* 472 corresponded to the loss of $-CO-(CH_2)_2-C_6H_6-(OH)_2$. The fragment at *m/z* 514 showed the loss of $-CH_2-C_6H_6-(OH)_2$. Here, this compound was found only in the peel of both cultivars but not in the flesh.

In positive-mode ionization analysis, only one compound was identified (peak 24). This compound, at *m/z* 177.0546 and retention time 16.039 min, was proposed to be methylumbelliferone ($C_{10}H_8O_3$) according to Hasson and Laties (1976)[23]. It was found only in the peel of 'Blue Bell'.

As described above and summarized in **Table 2**, potato flesh contained a very low number of phenolic compounds. Most phenolic compounds were contained in the peel of potatoes. The main phenolic compounds of potato, such as caffeoylquinic acid isomers were found in 'Melody', as were rutin and kaempferol rutinoside. Ferulic acid derivative was identified in both 'Blue Bell' and 'Melody'.

3.2. Quantification of phenolic compounds

Quantification has been performed with UV-vis data. Although, MS data (EIC of single compounds) have been studied to check the purity of compounds. Every peak has been previously identify and confirmed by MS and, once the identity was confirmed the quantification was carried out. Peaks that could not be confirmed by this procedure were not determined.

Four calibration curves were prepared using the following standards: rutin, chlorogenic acid, caffeic acid, and ferulic acid in the range of concentrations from the limit of quantification (LOQ) to 500 mg L^{-1}, respectively. Seven calibration points were used for each and the analyses were replicated five times for each calibration point (n=5). The different parameters of each calibration curves are summarized in **Table 3**: sensitivity (RSD (%), Relative Standard Deviation), linearity (r^2), calibration range, calibration curves, and limits of detection and quantification.

Table 3. Analytical parameters of the method.

Analyte	RSD (%)	LOD (mg L^{-1})	LOQ (mg L^{-1})	Calibration range (mg L^{-1})	Calibration equations	r^2
Rutin	1.416	0.049	0.163	LOQ-500	y=4.5087x + 5.1831	0.9997
Chlorogenic acid	0.184	0.017	0.056	LOQ-500	y=47.726x – 122.78	0.9998
Caffeic acid	0.323	0.034	0.114	LOQ-500	y=23.464x + 7.2565	0.9997
Ferulic acid	0.172	0.034	0.115	LOQ-500	y=23.275x + 26.3922	0.9995

RSD - Relative standard deviation
LOD - Limit of detection
LOQ - Limit of quantification

All calibration curves showed good linearity between different concentrations depending on the analytes studied. The calibration plots indicate a good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.999 in all cases. LOQ was determined as the signal-to-noise ratio of 10:1 while the detection limit (LOD) was determined as the signal-to-noise ratio of 3:1. LOD was found to be within the range of 0.017–0.049 mg mL^{-1}, while LOQ was within 0.056–0.163 mg mL^{-1}.

Intraday and interday analyses were made to evaluate the repeatability of HPLC-ESI-QTOF method. A methanol-water extract was injected on the

same day (intraday precision n=6) for 3 consecutive days (interday precision, n=18). The relative standard deviations (RSDs) of analysis time and peak area were determined.

The intraday repeatability (expressed as % RSDs) of the retention times was from 0.37 to 1.87%, whereas the interday repeatability was from 1.21 to 2.38%. The intraday repeatability (expressed as % RSDs) of the total peak area was from 0.19 to 0.75%, whereas the interday repeatability was in the range 1.19 to 1.63%.

Quantification was made using the previous calibration curves (**Table 3**). The calibration curve of rutin was used to quantify rutin at λ=280 nm. Likewise, the calibration curve of chlorogenic acid at λ=325 nm was used to quantify caffeoylquinic acid isomers and dicaffeoylquinic acid isomers. The calibration curve of caffeic acid at λ=280 nm was used to quantify caffeoyl putrescine, caffeic acid, bis (dihydrocaffeoyl) spermine, bis (dihydrocaffeoyl) spermidine, tris (dihydrocaffeoyl) spermine, and tris (dihydrocaffeoyl) spermidine. Ferulic acid derivatives were quantified using the calibration curve of ferulic acid at λ=280 nm. Thus, compounds that were quantified with a different standard were expressed as equivalents of the standard used. The methanolic extracts of flesh, peel, and whole potato of 'Blue Bell' and 'Melody' were analyzed and the quantitative results are presented in **Table 4**. Each sample was extracted 3 times and injected 3 times.

Table 4. Quantification results of phenolic compounds expressed as mg kg^{-1} sample.

Compounds	Bluebell						'Melody'					
	Peel	RSD(%)	Flesh	RSD(%)	Entire	RSD(%)	Peel	RSD(%)	Flesh	RSD(%)	Entire	RSD(%)
Caffeic acid derivatives												
Caffeoyl putrescine	51.74b	2.1	ND	-	24.82c	1.9	64.66a	3.8	ND	-	4.65d	3.1
1-Caffeoylquinic acid	267.29a	4.5	ND	-	63.67c	1.8	184.13b	2.9	10.67e	6.7	50.82d	2.8
Bis(dihydrocaffeoyl) spermine	29.64a	3.3	ND	-	12.22b	3.5	15.71b	1.4	ND	-	8.07c	6.4
Chlorogenic acid	904.21a	9.7	ND	-	353.22b	3.7	299.51c	3.7	47.10e	1.6	168.89d	1.9
4-Caffeoylquinic acid	461.74a	5.2	ND	-	154.04c	2.3	351.32b	4.0	16.03e	2.8	90.19d	5.8
Caffeic acid	249.96b	3.6	ND	-	59.16c	2.1	324.01a	4.2	ND	-	29.40d	4.6
Bis(dihydrocaffeoyl) spermidine	29.30a	1.5	ND	-	17.74c	1.6	21.37b	4.3	ND	-	3.65d	2.7
Tris(dihydrocaffeoyl) spermine	39.24b	2.3	ND	-	17.80c	1.1	42.72a	1.3	ND	-	12.54d	2.6
Rutin	ND	-	ND	-	ND	-	198.97a	2.3	10.76c	5.7	36.00b	7.1
Kaempferol-3-*O*rutinoside	ND	-	ND	-	ND	-	87.98a	2.0	5.85c	4.9	17.29b	2.3
3,4-Di-caffeoyl quinic acid	46.84a	1.4	ND	-	18.75b	1.6	ND	-	ND	-	ND	-
3,5-Di-caffeoyl quinic acid	205.55a	2.9	ND	-	52.66b	2.3	ND	-	ND	-	ND	-
N^1,N^4,N^8-tris(dihydrocaffeoyl) spermidine	25.15b	0.9	ND	-	21.76b	1.6	59.69a	1.7	ND	-	1.70c	6.0
4,5-Di-caffeoyl quinic acid	38.35a	1.1	ND	-	15.87b	2.5	ND	-	ND	-	ND	-
Total	2349.01a	6.8	-	-	799.51c	6.8	1650.06b	6.9	90.41e	2.6	423.20d	7.3
Of which caffeoylquinic acid isomers	1923.97a	6.1	-	-	658.22c	7.9	834.96b	7.1	73.80e	1.8	309.90d	6.9
Ferulic acid derivatives												
5-*O*feruloyl quinic acid	25.18a	1.3	ND	-	17.90b	1.5	26.22a	9.4	ND	-	1.93c	1.7
Ferulic acid derivative	35.36a,b	2.2	10.05	3.6	41.37a	2.7	30.38b	1.4	3.94d	4.8	6.66c	3.4
N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide	167.76a	6.4	ND	-	41.00c	2.4	126.96b	3.5	ND	-	8.10d	5.6
Total	228.30a	3.3	10.05e	2.9	100.27c	1.3	183.56b	2.9	3.94f	6.1	16.69d	4.1
TOTAL	2577.31a	4.0	10.05f	3.1	899.78c	7.6	1833.63b	8.9	94.36e	7.6	439.90d	6.8

ND - Not detected
RSD(%)- relative standard deviation among samples
Different letters in the same row indicate significant differences ($p<0.05$)
Three accessions of each variety were analyzed. Each accession was extracted three times and injected three times (n=27).
Rutin LOD 0.049 mg L^{-1} equivalent to 0.02 mg kg^{-1} sample
Chlorogenic acid LOD 0.017 mg L^{-1} equivalent to 0.009 mg kg^{-1} sample
Caffeic acid LOD 0.034 mg L^{-1} equivalent to 0.017 mg kg^{-1} sample
Ferulic acid LOD 0.034 mg L^{-1} equivalent to 0.017 mg kg^{-1} sample.

As shown in **Table 4**, levels of phenolic compounds were found to be higher in the potato peels than in the flesh, in agreement with previous data reported by Ieri *et al.* (2011)[21]. The three caffeoylquinic acid isomers, caffeic acid, rutin, 3,5-dicaffeoylquinic acid, and N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide were the major compounds identified. Caffeoylquinic isomers were the major phenolic compounds in the two potato cultivars; furthermore, the peel was the richest part in these compounds (Wu *et al.*, 2012)[17]. The three caffeoylquinic acid isomers showed higher concentrations in 'Blue Bell' peel than in 'Melody' peel with the following percentages: 31.13% for 1-caffeoylquinic acid, 66.88% for the chlorogenic acid and 23.91% for 4-caffeoylquinic acid.

Likewise, the concentrations of the three caffeoyl acid isomers were higher in whole 'Blue Bell' potato than in whole 'Melody' potato with the following percentages: 20.25% 1-caffeoylquinic acid, 52.18% chlorogenic acid, and 41.43% 4-caffeoylquinic acid.

Regarding potato flesh, caffeoylquinic acid isomers were identified and quantified only in the flesh of 'Melody'. Comparing the same weight of samples, the concentration of 1-caffeoylquinic acid was 76.17% higher in 'Blue Bell' peel sample than in whole potato sample, whereas the same compound was 72.41% higher in 'Melody' peel sample than in whole potato sample. Likewise, chlorogenic acid content was 60.94% higher in peel sample than in entire potato sample in 'Blue Bell' cultivar and 43.61% higher in 'Melody'. 4-Caffeoylquinic acid presented 66.65% less concentration in whole potato than in peel of 'Blue Bell'; similarly, 'Melody' presented 74.32% less concentration in whole potato than in peel.

Caffeic acid has been reported as another important compound in potato composition with high levels of antioxidant activity[17]. This compound was detected in higher concentrations in the peel fractions. 'Melody' peel showed 22.84% more caffeic acid concentration than did 'Blue Bell'. Nevertheless, 'Blue Bell' whole potato showed a higher level of caffeic acid than did 'Melody' whole potato: 50.34% higher concentration of caffeic acid in whole 'Blue Bell' than in whole 'Melody'. Whole 'Blue Bell' presented 76.32% less concentration of caffeic acid than did the peel, while in 'Melody' the difference was 90.93% less in the whole potato than in the peel. Caffeic acid was not detected in flesh of either potato cultivar.

Rutin was detected in peel, flesh, and whole potato of 'Melody' but was not detected at all in 'Blue Bell'. Peel presented 81.91% higher level of rutin than did whole potato.

3,5-Dicaffeoylquinic acid was detected only in 'Blue Bell' peel and whole potato, while this acid was not detected in the flesh. Whole potato presented 74.36% less concentration of 3,5-dicaffeoylquinic acid than did the peel.

N-[2-Hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide was 24.31% higher in 'Blue Bell' peel than in 'Melody' peel. 'Blue Bell' whole potato also had higher levels of N-[2-hydroxy-2-(4- hydroxyphenyl) ethyl] ferulamide than did 'Melody' whole potato, registering 80.24% higher concentration in 'Blue Bell' than in 'Melody'. Regarding the 'Blue Bell' whole potato, the N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide concentration was 75.57% lower than in 'Blue Bell' peel, while in 'Melody' whole potato the concentration was 93.62% lower than in 'Melody' peel. This compound was not found in the flesh of either cultivar. The presence of N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl] ferulamide in potato peel represents around the 7% of

total phenolic compounds; therefore, potato peel could have an extra-add value, since this compound has demonstrated to selectively induce apoptosis in cancerous white blood cells[24].

In the flesh of both cultivars, levels of a ferulic acid derivative were low. 'Blue Bell' flesh had 61.00% more ferulic acid derivative than in 'Melody' flesh. This compound was detected also in the peel, showing 14.12% more concentration of ferulic acid derivative in 'Blue Bell' peel than in 'Melody' peel. Regarding whole potato, 'Blue Bell' showed 83.82% higher concentration of this compound than 'Melody' whole potato.

The potato flesh presented very low concentration of phenolic compounds, and also whole potato samples showed significantly lower phenolic contents compared to potato peel samples considering the same weight of sample. The difference was 63.54% and 76.01% for 'Blue Bell' and 'Melody' whole potatoes, respectively, and 99.61% and 94.85% for 'Blue Bell' and 'Melody' flesh, respectively. Finally, the phenolic content of 'Blue Bell' peel was the highest of all the potato fractions. This agrees with results found by other authors affirming that blue and purple potato cultivars have a higher phenolic content than do yellow cultivars[25–27].

3.3. Antioxidant activity of the extracts

Three different methods were used in order to evaluate the antioxidant capacity of the methanolic extracts of flesh, peel and whole potatoes of the two potato cultivars 'Blue Bell' and 'Melody'. The TEAC and FRAP methods are based on single electron transfer mechanisms (SET). Both have been used in a large variety of food (TEAC) and biological samples (FRAP)[28]. On the other hand, the ORAC method is based on hydrogen-atom transfer

(HAT) reactions, which scavenge the generation of peroxyl radicals through the breakdown of azo compounds[28]. **Table 5** shows the antioxidant values found for each fraction and cultivar extract.

Table 5. In vitro antioxidant capacity by ABTS, FRAP, and ORAC methods of flesh, peel, and whole tuber of cultivars 'Blue Bell' and 'Melody'.

		TEAC (µmol Trolox equivalents g^{-1} extract (dw))	CV(%)	FRAP (µmol Fe^{2+} equivalents g^{-1} extract (dw))	CV(%)	ORAC (µmol Trolox equivalents g^{-1} extract (dw))	CV(%)
'Blue Bell'	Flesh	1.133 d	2.4	3.529 e	6.1	0.189 c	4.3
	Peel	12.990 a	3.3	39.940 a	4.9	0.524 a	2.2
	Entire	3.326 b	2.7	14.586 c	5.8	0.233 b	3.1
'Melody'	Flesh	0.132 e	3.1	2.728 e	4.3	0.240 b	4.7
	Peel	2.160 c	4.3	25.767 b	3.5	0.578 a	3.1
	Entire	0.324 e	5.1	10.317 d	3.7	0.222 b	8.4

Different letters in the same column indicate significant differences ($p<0.05$)
TEAC - Trolox Equivalent Antioxidant Capacity
FRAP - Ferric Reducing Antioxidant Power
ORAC - Oxygen Radical Absorbance Capacity
CV(%) - coefficient of variation among samples
Three accessions of each variety were analyzed. Each accession was extracted three times and injected three times (n=27).

As it can be seen, samples that presented a higher phenolic content also showed the most effectiveness as free-radical scavengers, which is in agreement with Albishi *et al.* (2013)[26]. In fact, phenolic compound content correlated positively with all the antioxidant activity assays made: TEAC ($r=0.8289$, $p<0.05$), FRAP ($r=0.9679$, $p<0.05$), and ORAC ($r=0.8222$, $p<0.05$).

'Blue Bell' peel, a slightly blue partially-colored potato peel, was the fraction with the highest antioxidant activity in TEAC, FRAP, and ORAC assays. Blue and purple potatoes have been demonstrated to have a higher content

of antioxidant activity than in yellow cultivars[25], in agreement with the present results.

TEAC was higher in the peel of both cultivars, showing sharply lower values of 74.40 and 91.28%, respectively, in the whole potato and potato flesh of 'Blue Bell'; 'Melody' values were 85.01 and 93.90% lower, respectively. TEAC values of flesh and whole potato did not significantly differ in 'Melody' or in 'Blue Bell'. However, TEAC results were much lower in the yellow 'Melody' potato than in 'Blue Bell' potato that presents blue spots. TEAC in the peel, flesh, and whole potato were 83.4%, 88.4%, and 90.3% lower, respectively.

Regarding FRAP assay, 'Blue Bell' peel had the highest value, followed by the 'Melody' peel, which was 35.5% lower. FRAP values were significantly lower in the whole potato than in the peel, i.e. 63.5% and 59.9% for 'Blue Bell' and 'Melody', respectively. In addition, FRAP values in the flesh were also lower than in the peel, by 91.2% and 89.4% for 'Blue Bell' and 'Melody', respectively. 'Melody' FRAP results were 22.7 to 35.5% lower than in 'Blue Bell'.

ORAC values in peel were similar for both potato cultivars, and the same occurred for the whole potato. However, ORAC values in the whole potato were lower than in the peel. Finally, ORAC values in 'Melody' flesh was similar to the values reported for the whole potatoes, but values were significantly lower for 'Blue Bell' flesh.

The higher levels of flesh and peel antioxidant activity of 'Blue Bell' may be due to the higher content of caffeoylquinic acid isomers of this potato cultivar[29,30].

4. Conclusions

A total of 24 phenolic compounds and other polar compounds were simultaneously identified by HPLC-DAD-QTOF in flesh, skin and whole tuber of two varieties of potatoes that had never been studied before for this purpose. As far as we are concerned, this is the first time that a quadrupole-time of flight mass spectrometer coupled to HPLC-DAD has been used for the simultaneous determination of the polar fraction of potato in a reduced analysis time.

Phenolic compounds were also quantified and were observed to be positively correlated with all the antioxidant activity assays carried out (TEAC, FRAP and ORAC). The hyphenation of HPLC to MS, which combines the advantages of traditional HPLC with a fused-core C18 column, has demonstrated to represent a valuable tool and a good alternative for simultaneous and faster characterization of the polar fraction of potato. In fact, it has been possible to evaluate the distribution of single phenolic compounds in potato tuber for the first time by this analytical technique. Furthermore, it has been seen that potato peels, which are discarded in the potato industry (i.e. in the production of potato chips and fries) merit more attention, as they could be recycled in the food industry in other products.

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CAPÍTULO 3

HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado

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HPLC-DAD-ESI-QTOF-MS and HPLC-FLD-MS as valuable tools for the determination of phenolic and other polar compounds in the edible part and by-products of avocado

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Abstract

Avocado is a tropical fruit increasingly cultivated around the world due to global interest and rising consumption. Thus, there is also a surge in avocado by-products that needs assessment. The aim of this work is to compare the phenolic profile of avocado pulp, peel and seed when the fruit is at optimal ripeness for consumption and when overripe. Two analytical techniques were used: (1) HPLC-DAD-ESI-QTOF-MS was used for the first time to determine phenolic and other polar compounds in avocado peel and seed. Phenolic compounds quantified with these methods were in higher concentration in overripe than in pulp and seed of optimally ripe fruit. (2) HPLC-FLD-MS was used to specifically determine flavan-3-ols. Procyanidins to degree of polymerization 13 have been quantified singularly here for the first time. In addition, A- and B-type procyanidins from the degree of polymerization 2 to 6 were differentiated and quantified. The procyanidin concentration increased after ripening probably due to the release of tannins linked to cell-wall structures. Because of this situation and the presence of A-type procyanidins, avocado peel and seed from overripe fruit, the main by-

products of avocado processing, hold interest for developing functional foods, nutraceuticals and cosmetics.

Keywords: Avocado, HPLC-DAD-ESI-QTOF-MS, phenolic compounds, flavan-3-ols, by-product.

1. Introduction

Avocado, *Persea americana* Miller, of family Lauraceae, is a large drupe with the highest oil content of all fruits, except perhaps the olive. It is an evergreen tree with the feature that avocado fruits mature on the trees and ripen after harvest. Indigenous to Tropical America, today the avocado tree is cultivated in various subtropical countries[1]. Among the numerous varieties of avocado fruit around the world, the cultivar 'Hass' dominates the international market[2]. Avocado production has considerably expanded in recent years due to rising consumption[3].

Avocado is generally recognized as a popular and healthy food source supplying proteins and lipids to the human diet. The pulp contains several bioactive phytochemicals including carotenoids, B vitamins, vitamins C and E, D-mannoheptulose, β-sitosterol, and persenone A and B. These constituents have demonstrated in vitro antifungal, antitumor and antioxidant activities[4]. Avocado pulp also has in vitro growth-inhibitory effects against cancer and is also well known for its beneficial skin properties. The unsaponifiable fraction of avocado pulp in combination with unsaponifiable soybean oil is used to treat osteoarthritis, and some research shows its anti-carcinogenic and anti-inflammatory effects[4–6]. Although not as thoroughly studied as the pulp, avocado peel and seed reportedly contain great amounts of phenolic compounds and display a higher antioxidant activity than the pulp[2,7]. The phenolic content and antioxidant capacity of avocado seeds and peels are several-fold greater than that reported for raw blueberry, which is known for its high antioxidant capacity[8].

Industrially, avocados are processed into oil and paste, which leaves 21-30% of the fruit weight to be discarded[2,7]. These waste products represent a

potential source of molecules with applications in the food, pharmaceutical, and cosmetic industries.

Thus, the aim of this work was to comprehensively determine the distribution of the phenolic compounds in extracts of seed, peel and pulp of the 'Hass' avocado cultivar by HPLC-DAD-ESI-QTOF-MS. Flavan-3-ols were also determined by HPLC-FLD-MS to quantify all polymerization grades of procyanidins. To the best of our knowledge, the phenolic compounds of peel and seed of avocado have never been analyzed before by HPLC-DAD-ESI-QTOF-MS.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetic acid and HPLC-MS-grade acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and methanol from Panreac (Barcelona, Spain). Double-deionized water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Ferulic acid, catechin, procyanidin B2, chlorogenic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid quinic acid and citric acid were acquired from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples

About 10 kg of 'Hass' avocado from the subtropical coast of Granada were provided by Miguel García Sánchez e Hijos, S.A. (Motril, Spain) in July 2014. Half of the fruits were left in the laboratory until the optimal consumption ripeness (CRA), whereas half of avocados were left until

overripeness (ORA). Then, pulp, peel and seed of the samples were manually separated in an ice bath, frozen at -80 ºC and freeze-dried in a lyophilizer (Advantage Plus EL-85 freeze dryer, SP Scientific, Ipswich, Suffolk, UK). Afterwards, dried samples were milled (IKA M20-IKAWERKE GmbH & Co. KG, Staufen, Germany) and kept at -18 ºC until used.

2.3. Extraction of phenolic compounds

A solid-liquid extraction was used to obtain the polar fraction of avocado pulp, peel and seed following a protocol from the literature with slight modifications[9]. Briefly, 2 g of freeze-dried sample powder were extracted with 20 mL hexane to eliminate lipids and then dissolved in 15 mL of a solution of methanol/water (80:20, v/v). The mixture was placed in an ultrasonic bath for 15 min at room temperature and then centrifuged for 15 min at 1000 g. The supernatant was removed, and the extraction was repeated twice more. The supernatants were collected, evaporated, and reconstituted in 2 mL of methanol/water (80:20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 mm (Millipore, Bedford, MA, USA) and stored at -18 ºC until analyzed.

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

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, autosampler, binary pump, and DAD was used for the chromatographic determination.

A Poroshell 120 EC-C18 (4.6mm x 100 mm, particle size 2.7 mm) (Agilent Technologies) was used to separate the compounds. Due to the different

nature of the three matrices analyzed (especially avocado pulp, which has a high number of phenolic acid derivatives compared to avocado peel and seed, which contain mainly procyanidins, some flavonoids and a lower quantity of phenolic acid derivatives), two analytical methods were used. Avocado pulp was analyzed using water containing 1% acetic acid as the solvent system A and acetonitrile as solvent system B and the following gradient elution: 0 min, 1% B; 15 min, 40% B; 16 min, 40% B; 19 min, 100% B; 21 min, 1% B; and 23 min, 1% B. The sample volume injected was 3 mL and the flow rate used was 1.6 mL min^{-1}. Avocado peel and seed were analyzed using the same gradient elution. Mobile phase A was water containing 1% acetic acid and mobile phase B was acetonitrile and they were used as follows: 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; and 18 min, 5% B. The sample volume injected was 3 mL and the flow rate used was 1.2 mL min^{-1}. The column was maintained at 25 °C. All samples were analyzed in triplicate.

UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, and 330 nm. The effluent from the HPLC column was split using a T-type phase separator before introducing it into the mass spectrometer (split ratio 1:3). MS analyses were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass Q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization interface in negative ionization mode under the following conditions: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage and scan range, 3500 V and *m/z* 50-1500, respectively. Automatic MS/MS experiments were conducted using the following collision-energy values: *m/z* 100, 30 eV; *m/z*

500, 35 eV; *m/z* 1000, 40 eV; and *m/z* 1500, 45 eV. Reference mass correction of each sample was performed with a continuous infusion of Agilent TOF mixture containing two mass references. The detection window was set to 100 ppm. Data acquisition (2.5 Hz) in the centroid mode was governed via the Agilent MassHunter Workstation. Integration and data elaboration were carried out using Mass-Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

The calibration curves using ferulic acid, chlorogenic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid, quinic acid and citric acid were prepared for the quantification of phenolic compounds and organic acids. Quantification was performed using DAD chromatograms of ferulic acid, tyrosol, rutin, gallic acid, hydroxybenzoic acid at λ=280 nm and chlorogenic acid at λ=330 nm. The calibration curves of citric acid and quinic acid were performed using the MS chromatogram. The calibration curves were prepared from the limit of quantification (LOQ) to 125 mg/mL. All calibration curves showed good linearity among different concentrations (>0.999). The analytical parameters of the method such as limit of detection (LOD), LOQ, intraday and interday precision (expressed as % RSDs) of the retention times and the total peak area are summarized in **Table 1**.

Table 1. Precision, limit of detection and limit of quantification of the methods proposed.

	Pulp					
Analyte	**Intraday RSD(%) Rt**	**Interday RSD(%) Rt**	**Intraday RSD(%) Area**	**Interday RSD(%) Area**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Citric acid	0.57	1.54	0.66	1.27	0.130	0.768
Gallic acid	0.13	1.31	0.56	1.07	0.037	0.128
Quinic acid	0.78	1.04	0.31	0.83	0.097	0.301
Chlorogenic acid	2.63	2.97	0.42	0.94	0.045	0.194
Tyrosol	0.85	1.83	0.59	1.14	0.064	0.232
Ferulic acid	0.84	1.74	0.43	1.02	0.080	0.255
Rutin	1.67	2.31	0.52	0.99	0.015	0.046
Hydroxybenzoic acid	0.53	1.36	0.38	0.88	0.019	0.067
	Peel and Seed					
Analyte	**Intraday RSD(%) Rt**	**Interday (%) Rt**	**Intraday RSD(%) Area**	**Interday RSD(%) Area**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Citric acid	0.62	1.38	0.67	1.31	0.170	0.683
Gallic acid	0.11	1.02	0.52	0.98	0.096	0.291
Quinic acid	0.65	0.96	0.28	0.69	0.102	0.316
Chlorogenic acid	2.34	2.85	0.45	0.87	0.035	0.112
Tyrosol	0.74	1.43	0.49	1.04	0.051	0.166
Ferulic acid	0.71	1.44	0.40	0.79	0.083	0.263
Rutin	1.39	2.17	0.37	0.84	0.012	0.041
Hydroxybenzoic acid	0.77	1.21	0.35	0.91	0.049	0.157

The calibration curve of citric acid was used to quantify citric and succinic acid; the calibration curve of ferulic acid was used to quantify hydroxycinnamic acid derivatives; the calibration curve of quinic acid was used to quantify quinic acid, the calibration curve of chlorogenic acid was used to quantify quinic acid derivatives; the calibration curve of tyrosol was used to quantify hydroxytyrosol glucoside, tyrosol glucoside and tyrosol hexoside-pentoside; the calibration curve of rutin was used to quantify rutin and other quercetin derivatives; the calibration curve of gallic acid was used to quantify octyl gallate; and the calibration curve of hydroxybenzoic acid was used to quantify protocatechuic acid -4-glucoside and vanillic acid

glucoside. It has to be taken into account that the response of the standards can differ from the response of the derivatives present in avocado samples, and consequently the quantification of these compounds is only an estimation of their actual concentrations.

2.5. Determination of flavan-3-ols by HPLC-FLD-MS

An Agilent 1200 Series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system, a degasser, an autosampler, a fluorimetric detector (FLD) and a massspectrometer detector (MSD) was used for the analyses. The methodology was the same reported by Verardo *et al.* (2015)[10].

Avocado samples were extracted using a solution of acetone/water (4:1, v/v). A Develosil Diol 100 Å column 5 μm, 250 x 4.6mm ID (Phenomenex, Torrance, CA, USA) was used. Mobile phase A and B consisted of an acidic acetonitrile ((A), CH_3CN:CH_3COOH, 98:2; v/v) and acidic aqueous methanol ((B), CH_3OH:H_2O:CH_3COOH, 95:3:2; v/v/v). The gradient elution was the following: 7% B for 3 min, 37.6% B for 57 min, and 100% B for 7 min. Then the initial conditions were set, 7% B during 5 min. Fluorescence detection was performed with an excitation wavelength of 230 nm and an emission wavelength of 321 nm. The injection volume was 5 mL. All the analyses were carried out at 35 °C.

Mass spectrometer analyses were carried out using an electrospray ionization (ESI) interface under the following conditions: drying gas flow (N_2), 9.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 3500 V; fragmentor voltage and scan range variables. The

fragmentor and *m/z* ranges used for HPLC-ESI/MS analyses were: 120 V and *m/z* 50-1000, 140 V and *m/z* 1000-2000.

2.6. Statistical analysis

One-way analysis of variance, ANOVA (Tukey's honest significant difference multiple comparison), was evaluated using Statistica 8.0 software (StatSoft, Tulsa, OK, USA), and p values <0.05 were considered to be statistically significant.

3. Results and discussion

3.1. Identification of phenolic and other polar compounds by HPLC-DAD-ESI-QTOF-MS

Avocado pulp, peel and seed extracts obtained by solid-liquid extraction were analyzed using an HPLC coupled to DAD and ESI-QTOF-MS in the negative ionization mode in order to identify phenolic and other polar compounds. Peak identification was performed on basis of their relative retention time values, their UV-Vis spectra and mass spectra obtained using ESI-QTOF-MS together with information previously reported in the literature. **Table 2** summarizes the tentatively identified compounds in the three fractions of avocado. Compounds which were not identified in pulp were subsequently numbered in peel and seed.

Flavan-3-ols have not been included in this identification table because specific analyses for these compounds have been performed by HPLC-FLD-MS.

3.1.1. Avocado pulp

A total of 23 compounds were tentatively identified in avocado pulp (**Table 2**). These belonged to various metabolite types that included organic acids, amino acids, sugars, nucleosides, phenolic acids (hydroxybenzoic and hydroxycinnamic), phenolic alcohols and iridoids.

Table 2. Identification of phenolic and other polar compounds in pulp, peel and seed of avocado by HPLC-DAD-QTOF-MS.

Peak	Proposed compound	Retention time (min)	UV λmax (nm)	*m/z* experimental $[M-H]^-$	*m/z* calculated $[M-H]^-$	Fragments MS/MS	Molecular formula	Error (ppm)	Score
PULP									
1	Perseitol	0.58	242/265	211.0829	211.0823	101	$C_7H_{16}O_7$	2.7	97.51
2	Quinic acid*	0.74	230/262	191.0571	191.0561	111	$C_7H_{12}O_6$	5.7	93.18
3	Citric acid*	1.16	230	191.0200	191.0197	111, 101, 113	$C_6H_8O_7$	1.4	99.04
4	Succinic acid	1.58	230/265	117.0194	117.0193	100	$C_4H_6O_4$	0.93	86.35
5	Uridine	1.59	228/262	243.0625	243.0631	227	$C_9H_{12}N_2O_6$	3.48	96.54
6	Protocatechuic acid-4-glucoside	3.17	240/311	315.0722	315.0722	108, 153	$C_{13}H_{16}O_9$	0.08	99.63
7	Penstemide	3.81	230/272	443.1934	443.1923	101,113	$C_{21}H_{32}O_{10}$	1.72	98.11
8	Sinapic acid-C-hexoside	4.50	234/306	385.1143	385.114	223	$C_{17}H_{22}O_{10}$	0.64	99.37
9	Caffeic acid glucoside	4.71	237/308	341.0886	341.0878	161, 133, 179	$C_{15}H_{18}O_9$	2.36	96.88
10	Penstemide isomer I	5.12	230/272	443.1929	443.1923	101, 113, 161	$C_{21}H_{32}O_{10}$	1.13	98.82
11	Tyrosol-hexoside-pentoside	5.19	228/278	431.1562	431.1559	299,119, 113, 137, 131, 149,179, 161	$C_{19}H_{28}O_{11}$	0.85	98.51
12	*p*-coumaric acid glucoside	5.68	233/283/314	325.0935	325.0929	163,145	$C_{15}H_{18}O_8$	1.88	97.47
13	*p*-coumaric acid hexoside pentoside	5.96	284/315	457.1360	457.1351	145,163,307,323	$C_{20}H_{26}O_{12}$	1.75	98.27
14	*p*-coumaric acid glucoside isomer I	6.05	233/283/314	325.0934	325.0929	145, 117, 119	$C_{15}H_{18}O_8$	1.52	98.59
15	Ferulic acid glucoside	6.22	240/295/329	355.1039	355.1035	193, 175	$C_{16}H_{20}O_9$	1.3	97.83
16	*p*-coumaric acid rutinoside	6.30	240/279/315	471.1516	471.1508	163, 145	$C_{21}H_{28}O_{12}$	1.72	97.92
17	Ferulic acid glucoside isomer I	6.54	240/295/329	355.1039	355.1035	193, 175	$C_{16}H_{20}O_9$	1.17	99.25
18	Octyl gallate	6.55	272/322	281.1393	281.1394	121, 139	$C_{15}H_{22}O_5$	0.57	98.83
19	3-feruloylquinic acid	7.18	282/315	367.1035	367.1035	193, 191	$C_{17}H_{20}O_9$	0.01	98.97
20	Coumaric acid	7.49	236/279/308	163.0396	163.0401	119	$C_9H_8O_3$	2.26	98.64
21	5-feruloylquinic acid	7.58	235/288/316	367.1039	367.1035	173, 191	$C_{17}H_{20}O_9$	0.97	97.56
22	4-feruloylquinic acid	7.95	281/312	367.1039	367.1035	173, 191	$C_{17}H_{20}O_9$	1.06	99.42
23	Agoniadin	8.20	240/290/317	469.1354	469.1351	163, 145	$C_{21}H_{26}O_{12}$	0.52	98.26

PEEL									
1	Perseitol	0.868	242/265	211.0825	211.0823	101	$C_7H_{16}O_7$	1.0	99.77
2	Quinic acid	0.914	230/262	191.0566	191.0561	111	$C_7H_{12}O_6$	2.38	98.29
10	Penstemide	4.344	230/272	443.1934	443.1923	101, 113	$C_{21}H_{32}O_{10}$	2.4	97.01
24	Chlorogenic acid*	4.496	234/295/326	353.0889	353.0878	191	$C_{16}H_{18}O_9$	2.92	96.31
25	Quercetin-diglucoside	6.456	238/280/352	625.1418	625.141	301	$C_{27}H_{30}O_{17}$	1.02	98.86
26	Quercetin-3-*O*arabinosyl-glucoside	7.059	236/279/354	595.1308	595.1305	301	$C_{26}H_{28}O_{16}$	0.36	99.42
27	Rutin*	9.855	234/282/350	609.1474	609.1461	301	$C_{27}H_{30}O_{16}$	1.98	97.34
SEED									
1	Perseitol	0.872	242/265	211.0827	211.0823	101	$C_7H_{16}O_7$	1.88	99.01
2	Quinic acid	0.917	230/262	191.0567	191.0561	111	$C_7H_{12}O_6$	4.05	93.95
3	Citric acid*	1.183	230	191.0197	191.0197	111, 101, 113	$C_6H_8O_7$	0.04	99.88
28	Hydroxytyrosol glucoside	3.126	234/280	315.1094	315.1085	135, 153	$C_{14}H_{20}O_8$	2.48	96.57
29	1-caffeoylquinic acid	3.585	239/293/324	353.088	353.0878	191, 179, 135	$C_{16}H_{18}O_9$	0.55	99.84
30	Tyrosol glucoside	4.012	229/276	299.1138	299.1136	119, 137	$C_{14}H_{20}O_7$	0.43	99.42
10	Penstemide	4.344	234/295/326	443.1924	443.1923	101,113	$C_{21}H_{32}O_{10}$	0.18	99.74
31	3-*O*-*p*-coumaroylquinic acid	4.538	238/285/311	337.093	337.0929	163	$C_{16}H_{18}O_8$	0.27	99.83
32	4-caffeoylquinic acid	5.075	241/284/326	353.0887	353.0878	135, 173, 191	$C_{16}H_{18}O_9$	2.54	97.08
33	Vanillic acid glucoside	6.366	239/279	329.0878	330.0951	123, 167	$C_{14}H_{18}O_9$	0.14	99.06
34	(1'S, 6'R)-8'-hydroxyabscisic acid beta-D-glucoside	8.546	242/274	441.177	441.1766	330,139	$C_{21}H_{30}O_{10}$	0.67	99.24

*Compounds checked against an authentic standard.

Table 3. Phenolic and other polar compounds in avocado pulp expressed as mg/100 g dry matter[a].

Pulp compounds	CRA		ORA	
	Mean	SD	Mean	SD
Quinic acid	0.12a	0.01	0.10a	0.00
Citric acid	3.61b	0.19	3.89a	0.18
Succinic acid	1.10b	0.09	1.96a	0.02
Protocatechuic acid-4-glucoside	0.24a	0.01	0.27a	0.03
Sinapic acid-C-hexoside	0.74a	0.002	0.74a	0.04
Caffeic acid glucoside	0.98a	0.03	0.78b	0.00
Tyrosol-hexoside-pentoside	2.28a	0.10	2.04b	0.06
p-coumaric acid glucoside	8.44b	0.15	9.35a	0.07
p-coumaric acid hexoside pentoside	1.06b	0.02	1.57a	0.00
p-coumaric acid glucoside isomer I	1.03a	0.02	0.56b	0.04
Ferulic acid glucoside	1.95b	0.07	2.44a	0.10
p-coumaric acid rutinoside	1.64b	0.02	3.58a	0.08
Ferulic acid glucoside isomer I	0.74a	0.02	0.66b	0.01
Octyl gallate	0.95b	0.02	2.18a	0.17
3-feruloylquinic acid	0.77b	0.03	1.23a	0.09
Coumaric acid	4.69a	0.05	2.76b	0.02
5-feruloylquinic acid	7.62b	0.09	11.55a	0.60
4-feruloylquinic acid	0.81a	0.03	0.67b	0.09
Total	38.75b	0.86	44.99a	1.01

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Citric and succinic acid were quantified in mg equivalent of citric acid /100 g dry matter.
Quinic acid was quantified in mg equivalent of quinic acid /100 g dry matter.
Caffeic acid glucoside were quantified in mg equivalent of chlorogenic acid /100g dry matter.
Sinapic acid-C-hexoside, *p*-coumaric acid glucoside, *p*-coumaric acid hexoside pentoside, *p*-coumaric acid glucoside isomer I, ferulic acid glucoside, *p*-coumaric acid rutinoside, ferulic acid glucoside isomer I and 3-feruloylquinic acid, coumaric acid, 5-feruloylquinic acid and 4-feruloylquinic acid were quantified in mg equivalent of ferulic acid /100g dry matter.
Tyrosol-hexoside-pentoside was quantified in mg equivalent of tyrosol/100g dry matter.
Octyl gallate was quantified in mg equivalent of gallic acid/100g dry matter.
Protocatechuic acid-4-glucoside was quantified in mg equivalent of hydroxybenzoic acid/100g dry matter.
[a] Different letters in the same row indicate significant differences ($p<0.05$).

A sugar (perseitol), three organic acids (quinic acid, citric acid and succinic acid), and an amino acid (uridine) were identified, in agreement with previous studies on avocado pulp[11–13].

In addition, seven hydroxycinnamic acid derivatives (sinapic acid-*C*-hexoside, *p*-coumaric acid glucoside and one of its isomers, ferulic acid glucoside and its isomer, *p*-coumaric acid rutinoside and coumaric acid) and one hydroxybenzoic acid derivative (octyl gallate) were also identified, as previously described[12,13].

Furthermore, another six hydroxycinnamic acid derivatives that, to our knowledge, had not been identified in avocado pulp before were found using the methodology previously described in **Section 2.4** by HPLC-DAD-ESI-QTOF-MS. Protocatechuic acid linked to a hexose (peak 6) was identified at *m/z* 315.0722 and a retention time 3.17 min in avocado pulp. The fragment at *m/z* 153 corresponded to a protocatechuic acid moiety. This compound could be tentatively identified as protocatechuic acid -4-glucoside, as previously described in literature for other fruits and plants[14,15]. Peak 9, *m/z* 341.0886 at 4.71 min, presented fragments at *m/z* 161 and 179. This finding could indicate the loss of a hexoside moiety (probably glucoside) and the presence of caffeic acid. Thus, this compound was identified as caffeic acid glucoside[16]. Peak 13 was identified as p-coumaric acid hexoside pentoside. Its fragmentation pattern showed fragments at *m/z* 323 due to the loss of a pentoside moiety, at *m/z* 307 because of the loss of a hexoside moiety. Characteristic fragments of coumaric acid moieties were also present (*m/z* 163 and 145).

Three isomers of feruloylquinic acid (peaks 19, 21 and 22) were found at *m/z* 367.103 and retention times of 7.18, 7.58 and 7.95 min, respectively.

They were identified as 3, 5 and 4-feruloylquinic acids, respectively based on their fragmentation pattern and the elution order found in literature for similar separation conditions (Clifford *et al.*, 2003) [17]. Finally, at *m/z* 469.1354 and a retention time of 8.20 min, agoniadin was identified. Fragments at *m/z* 163 and 145 corresponding to the coumaric acid moiety of this compound were generated after the MS/MS analysis.

One phenolic alcohol was found at 5.19 min and *m/z* 431.1562. It generated different fragments, at *m/z* 137 that corresponded to a tyrosol moiety, at *m/z* 299 that matched the loss of a pentose moiety tyrosol hexoside and further fragmentation of *m/z* 299 resulted in a pattern matching that of tyrosol hexoside[18]. Therefore, this compound was identified as tyrosol hexoside pentoside. Moreover, two iridoids (peaks 7 and 10) were found at *m/z* 443.193 and presented fragments at *m/z* 101. Thus, these compounds were identified as penstemide and its isomer as previously described by other authors[19]. These compounds have also been identified for the first time in avocado in this study.

3.1.2. Avocado peel

Seven compounds were identified in this fraction of avocado. Three of them, belonging to the flavonol family (peaks 25, 26 and 27), were quercetin derivatives: quercetin-diglucoside, quercetin-3-*O*-arabinosyl-glucoside and rutin. All showed a fragment at *m/z* 301 due to a quercetin moiety[20].

A derivative of caffeic acid such as chlorogenic acid was identified in avocado peel. It showed *m/z* 353.0889 and a fragment at *m/z* 191 due to the quinic acid moiety. This compound was also confirmed by the comparison with its commercial standard.

Perseitol, quinic acid and penstemide were also found in avocado peel. To the best of our knowledge, this is the first time that these compounds have been identified in avocado peel.

Table 4. Phenolic and other polar compounds in avocado peel expressed as mg/100 g dry matter[a].

Peel compounds	CRA		ORA	
	Mean	SD	Mean	SD
Quinic acid	0.43a	0.01	0.12b	0.02
Chlorogenic acid	189.89a	1.07	159.24b	0.82
Quercetin-3,4'-diglucoside	270.05a	2.19	29.50b	3.73
Quercetin-3-*O*arabinosyl-glucoside	19.76b	0.22	60.78a	0.30
Rutin	35.31b	0.61	46.96a	2.09
Total	515.45a	0.00	296.61b	2.22

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Quinic acid was quantified in mg equivalent of quinic acid /100 g dry matter.
Chlorogenic acid was quantified in mg equivalent of chlorogenic acid /100g dry matter.
Quercetin-3,4'-diglucoside, quercetin-3-*O*-arabinosyl-glucoside, rutin were quantified in mg equivalent of rutin /100g dry matter.
[a] Different letters in the same row indicate significant differences ($p<0.05$).

3.1.3. Avocado seed

A total of 11 compounds were identified in avocado seed. Perseitol, quinic acid, citric acid and penstemide were also found in the seed and they presented the same fragmentation pattern as in avocado peel. These compounds have not previously been described in avocado seed.

Two phenolic alcohol derivatives were identified in seed. Hydroxytyrosol 1-glucoside (peak 28) showed an *m/z* at 315.1094 and two fragments, one at *m/z* 153 corresponding to the presence of hydroxytyrosol and another one at *m/z* 135 due to the loss of a glucose moiety. Tyrosol hexoside was also found in seed. The presence of tyrosol derivatives in avocado seed has previously

been described by other authors[21,22]. (1'S, 6'R)-8'-hydroxyabscisic acid β-D-glucoside was also identified in avocado seed[22].

Finally, four hydroxycinnamic acid derivatives were also found in avocado seed: 3-*O*-*p*-coumaroylquinic acid was previously described by other authors[20]. Meanwhile, vanillic acid glucoside (peak 33) at *m/z* 329.0878 and fragments at *m/z* 123 and 167 indicating the presence of a vanillic acid moiety, was identified for the first time in avocado seed.

Two caffeoylquinic acid isomers were found in seed. The presence of the isomer corresponding to chlorogenic acid was ruled out because they did not match with the commercial standard of this compound. According to Clifford *et al.*[23], peak 29 was identified as 1-caffeoylquinic acid because it showed a strong fragment at *m/z* 191 and a weak fragment at *m/z* 179, and peak 32 was identified as 4-caffeoylquinic acid because it was the only isomer that showed a MS/MS base peak at *m/z* 173. These specific chlorogenic derivative compounds were identified for the first time in avocado seed.

3.2. Quantification of phenolic compounds and other compounds

The pulp, peel and seed of CRA and ORA samples were quantified for phenolic compounds and organic acids. Each compound and also the total compound contents were compared between CRA and ORA. The phenolic content of fruits is known to be influenced by the degree of ripeness. However, few studies investigated phenolic and other polar metabolites in avocado[13,24]. Only avocado pulp has been studied in this sense, and to our knowledge, there are no studies about the evolution of these compounds during fruit ripening in avocado by-products such as peel and seed.

Regarding avocado pulp **(Table 3)**, quinic acid showed no significant differences between ORA and CRA pulp, whereas succinic acid proved to be significantly higher in ORA pulp than in CRA pulp. This agrees with previously reported results[13]. Citric acid was also significantly higher in ORA pulp than in CRA pulp. Most phenolic compound derivatives of avocado pulp were significantly higher in ORA than in CRA pulp. Sinapic acid-*C*-hexoside did not change with ripening, whereas only caffeic acid glucoside, tyrosol-hexoside-pentoside, *p*-coumaric acid glucoside, ferulic acid glucoside isomer I, coumaric acid and 4-feruloylquinic acid were less concentrated in ORA than in CRA pulp. Furthermore, the total content of these compounds in avocado pulp was 45.03 mg/100 g dry matter in ORA pulp and 38.79 mg/100 g in CRA pulp. A similar trend in avocado pulp has been detected by other authors[13]. This is probably due to the effect of phenylalanine ammonia lyase (PAL), which has been demonstrated to increase in activity with the ripening of avocado[25].

On the contrary, phenolic and other polar compounds in avocado peel were much lower in ORA than in CRA peel, 296.65 and 515.52 mg/100 g, respectively **(Table 4)**. However, quercetin-3-*O*-arabinosyl-glucoside and rutin were significantly higher in ORA than in CRA peel. No qualitative differences were found between the two samples. It is possible that external conditions affect mainly the peel deteriorating these kinds of compounds.

Finally, avocado seed **(Table 5)** showed a significantly higher content of all single compounds in ORA seed than in CRA seed, except for hydroxytyrosol 1-glucoside, which decreases with ripening. Quinic acid content did not change with ripening. The total content of phenolic and other polar

compounds in avocado seed was also higher in ORA seed (676.45 mg/100 g) than in CRA seed (400.07 mg/100 g).

Table 5. Phenolic and other polar compounds in avocado seed expressed as mg/100 g dry matter[a].

Seed compounds	CRA		ORA	
	Mean	SD	Mean	SD
Quinic acid	0.08a	0.01	0.08a	0.01
Citric acid	4.63b	0.14	12.39a	0.30
Hydroxytyrosol glucoside	38.95a	0.61	25.22b	1.32
1-caffeoylquinic acid	112.29b	0.41	243.78a	9.52
Tyrosol glucoside	223.66b	1.33	339.14a	11.85
3-*O*-*p*-coumaroylquinic acid	7.01b	0.05	37.56a	1.73
4-caffeoylquinic acid	6.69b	0.05	10.39a	0.62
Vanillic acid glucoside	6.74b	0.27	7.86a	0.01
Total	400.05b	0.00	676.43a	24.74

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
Citric acid was quantified in mg equivalent of citric acid /100 g dry matter.
Quinic acid was quantified in mg equivalent of quinic acid /100 g dry matter.
1-caffeoylquinic acid, 3-O-p-coumaroylquinic acid and 4-caffeoylquinic acid were quantified in mg equivalent of chlorogenic acid /100g dry matter.
Hydroxytyrosol glucoside and tyrosol glucoside were quantified in mg equivalent of tyrosol/100g dry matter.
Vanillic acid glucoside was quantified in mg equivalent of hydroxybenzoic acid/100g dry matter.
[a] Different letters in the same row indicate significant differences ($p<0.05$).

3.3. Identification of flavan-3-ols by HPLC-FLD-MS

To identify flavan-3-ols, fluorimetric detection and mass spectrometry were used to confirm the peak identity. As previously described in literature, flavan-3-ols eluted according to their degree of polymerization (DP), firstly eluting the monomers and then the different oligomers[26]. A-type and B-type procyanidins (PCs) were identified in avocado peel and seed until the hexamer DP. This is the first time that avocado procyanidins have been

differentiated in A-type and B-type from DP from 3 to 6. As reported by other authors, A-type procyanidins eluted before B-type procyanidins[27,28]. **Figure 1** lists the flavan-3-ols identified in avocado pulp, peel and seed.

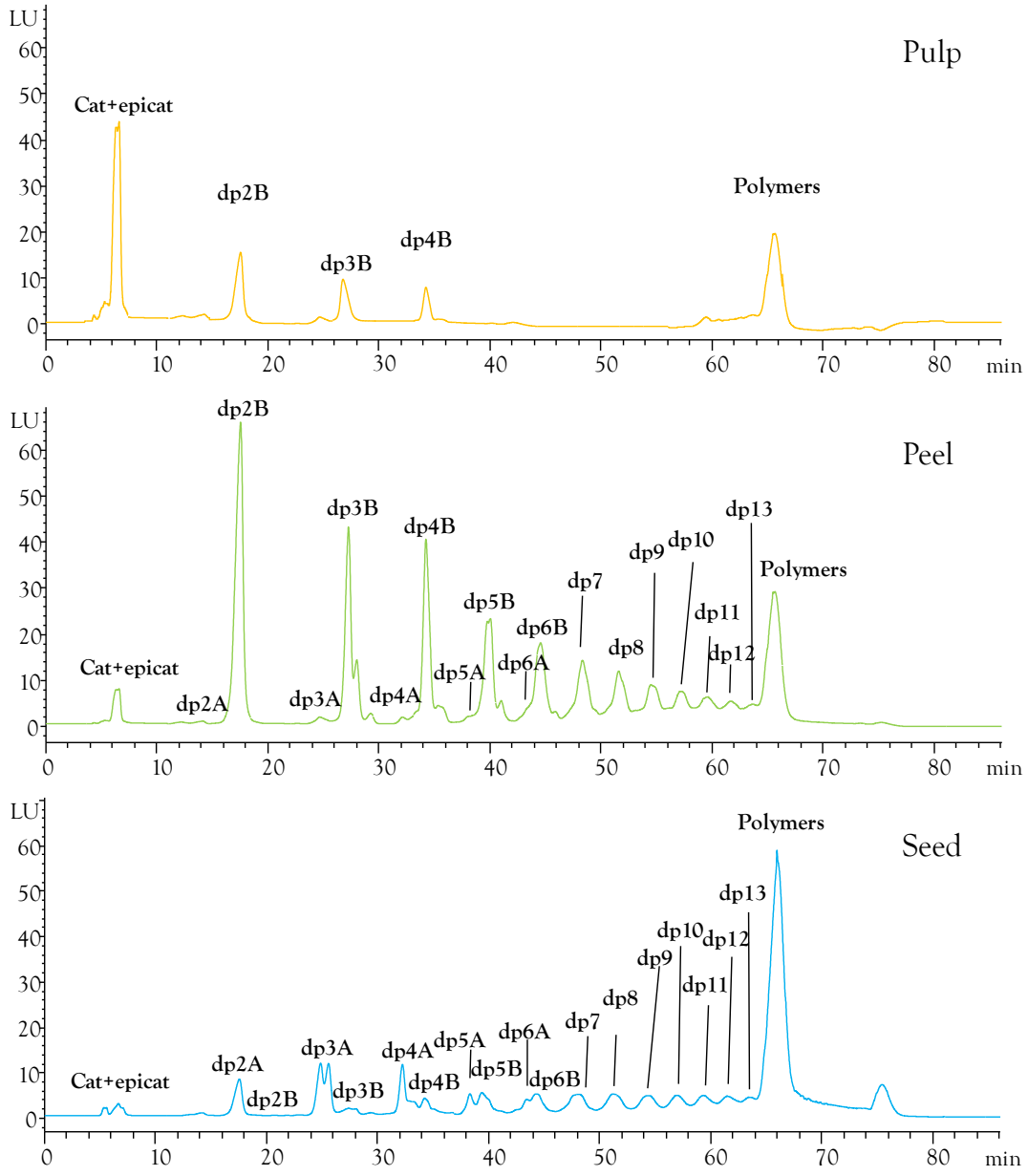

Figure 1. Fluorescence chromatograms of the identified flavan-3-ols in avocado pulp, peel and seed. (DP: degree of polymerization). Excitation wavelength of 230 nm and emission wavelength of 321 nm.

For avocado pulp, the first peak corresponded to the monomers catechin and epicatechin, and showed a molecular ion at *m/z* 289 and fragments ion at *m/z* 245 ([M-H-CO_2]-) and *m/z* 139 (Retro Diels Alder fragmentation)[10]. B-type to DP 4 oligomeric procyanidins were identified in avocado pulp. A B-type PC dimer was found at *m/z* 577 and showed two principal fragments at *m/z* 425 and 289[29]. The B-type PC trimer registered a molecular ion at *m/z* 865 and several fragments at *m/z* 739, 713 and 289. A B-type PC tetramer at *m/z* 1153 and fragments at *m/z* 865 and 577 was also found[10,29]. Finally the polymers were detected.

Avocado seed and peel flavan-3-ols profiles were qualitatively similar. The monomer catechin/epicatechin was found in these fractions of avocado. PCs A and B-type from DP 2 to 13 were identified. Major PCs were identified by MS and FLD (DP from 2 to 5), whereas for PCs from DP up to 6, only FLD identification was possible. This was due to the limited ionization of big polymers and their lower sensibility in MS compared to FLD. However, identification was possible because peak pattern in all cases was the same.

The B-type PC dimer, trimer and tetramer had the same mass spectra as previously described for avocado pulp. A B-type PC pentamer was found at *m/z* 1441 and showed fragments at *m/z* 865 and 577.

Concerning A-type PCs, the A-type PC dimer showed a molecular ion at *m/z* 575 and a fragment at *m/z* 289; the A-type PC trimer presented a molecular ion at *m/z* 863 and fragments at *m/z* 577 and 289; the A-type PC tetramer had a molecular ion at *m/z* 1151 and fragments at *m/z* 577 and 289 and the A-type PC pentamer showed a molecular ion at *m/z* 1439 and fragments at *m/z* 577 and 289.

3.4. Quantification of flavan-3-ols by HPLC-FLD-MS

Fluorimetric detection was used for quantification because it is known to be more selective and provides a stronger signal than MS detection for procyanidins. Calibration curves of (+)-catechin and procyanidin B2 were prepared from 5 to 500 and 1-500 mg/mL, respectively, at 6 concentration levels. Correction factors suggested by Robbins *et al.* (2009)[26] were used for PCs.

Table 6 shows the quantification results of flavan-3-ols in avocado pulp, peel and seed. Total flavan-3-ols content were 0.152, 19.69 and 16.49 mg/g dry matter in CRA pulp, peel and seed, respectively. These results agree with those reported by Wang *et al.* (2010)[7]. However, total flavan-3-ols content in ORA samples was 19.14%, 16.84% and 12.00% higher than CRA, respectively, for pulp, peel and seed. The increase of PCs during avocado ripening are probably be due to the release of tannins linked to cell-wall structures after softening. The total PCs content in avocado peel and seed were in the range of food commonly known for its high PCs content, such as chocolate and cocoa powder[30].

Table 6. Flavan-3-ols in avocado pulp, peel and seed expressed as mg/g dry matter[a].

Compounds	pulp CRA		pulp ORA		peel CRA		peel ORA		seed CRA		seed ORA	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
cat+epic	0.071e	0.002	0.130d	0.001	3.58b	0.19	4.16a	0.29	1.77c	0.18	1.70c	0.03
dp2 A					0.96b	0.01	1.01a	0.01	0.94c	0.01	0.93c	0.01
dp2 B	0.016d	0.001	0.015d	0.002	5.94b	0.16	7.46a	0.27	0.23c	0.04	0.23c	0.01
dp3 A					0.12b	0.01	0.12b	0.01	0.65a	0.02	0.68a	0.03
dp3 B	0.018d	0.001	0.019d	0.001	1.41b	0.01	1.67a	0.08	0.19c	0.02	0.17c	0.02
dp4 A					0.121b	0.003	0.15b	0.03	0.34a	0.02	0.31a	0.03
dp4 B	0.0067e	0.0003	0.0079d	0.0001	0.86b	0.02	1.09a	0.06	0.16c	0.01	0.17c	0.01
dp5 A					0.071c	0.003	0.08c	0.02	0.130a	0.001	0.113b	0.001
dp5 B					0.59b	0.03	0.80a	0.03	0.189d	0.004	0.205c	0.003
dp6 A					0.09a	0.01	0.10a	0.01	0.108a	0.005	0.101a	0.008
dp6 B					0.42b	0.02	0.61a	0.04	0.18c	0.01	0.178c	0.001
dp7					0.26b	0.01	0.37a	0.04	0.168d	0.004	0.175c	0.001
dp8					0.178b	0.005	0.25a	0.03	0.138c	0.005	0.139c	0.003
dp9					0.110b	0.001	0.15a	0.02	0.087d	0.001	0.093c	0.001
dp10					0.055b	0.001	0.07a	0.01	0.050c	0.005	0.054b	0.002
dp11					0.043b	0.001	0.06a	0.01	0.044b	0.001	0.045b	0.001
dp12					0.034b	0.002	0.04a	0.01	0.036b	0.002	0.037b	0.001
dp13					0.024b	0.002	0.031a	0.004	0.027a	0.001	0.026a	0.001
polymer	0.0394e	0.0004	0.016f	0.001	4.83d	0.15	5.43c	0.25	11.05b	0.01	13.378a	0.001
Total	0.152f	0.003	0.188e	0.001	19.69b	0.57	23.68a	1.20	16.49d	0.22	18.74c	0.06

CRA: optimal consumption ripening avocado; ORA over-ripped avocado.
From DP 3 to polymers were quantified using the corrections factors suggested by Robbins *et al.* (2009)[26].
[a] Different letters in the same row indicate significant differences ($p<0.05$).

The compound present in the highest concentration in avocado pulp was catechin/epicatechin with a 46.9% in CRA and 69.1% in ORA pulp. This increase could be due to the breakup of the polymer into smaller moieties. In fact, polymer in CRA represents 25.9% and decrease to 8.5% in ORA pulp.

In avocado peel, there were no significant differences in terms of the percentages of each individual PC between CRA and ORA. It bears highlighting that B-type PCs were predominant in avocado peel. The major PC was the B-type PC dimer with 30.2% and 31.5% in CRA and ORA peel, respectively. It was followed by polymer (24.5% and 23.0% in CRA and ORA peel, respectively) and catechin/epicatechin (18.2% and 17.6% in CRA and ORA peel, respectively).

CRA and ORA seed also presented a similar composition in terms of the percentages of each individual PC. In this avocado by-product, A-type PC dimer, trimer and tetramer together with Btype PC pentamer and hexamer B-type predominated. The major component was the polymer that represented 67.0% and 71.4% in CRA and ORA seed, respectively. The second compound in terms of concentration was catechin/epicatechin, which represented 10.8% and 9.1% in CRA and ORA seed, respectively.

The presence of A-type PCs in avocado peel and seed could provide additional health benefits to these avocado by-products. In fact, A-type PCs have proven to be more resistant to microbial catabolism[31] and this could explain why A-type PCs from cranberries have been suggested to prevent urinary-tract infections[32].

As far as we are concerned, this is the first time that a quantification of single PCs until DP 13 has been performed in avocado samples. Besides, A and B-type PCs have been quantified separately from DP 2 to 6.

4. Conclusions

Two different methods were set up with HPLC-DAD-ESI-QTOF-MS to analyze phenolics in avocado fruit and its by-products. For the first time, ESI-QTOF-MS has been applied to the study of these compounds in avocado peel and seed proving to be a valuable tool. Nine compounds in avocado pulp, three in avocado peel and three in avocado seed were identified for the first time using HPLC-DAD-ESI-QTOF-MS. Phenolic compounds quantified with these methods were in higher concentration in ORA pulp and seed than in CRA pulp and seed, probably due to the effect of PAL, which could increase its activity with fruit ripening.

PCs were also determined by HPLC-FLD-MS and catechin/epicatechin, PCs up to DP 13, and the polymers were also identified. It bears emphasizing that this is the first time that PCs up to DP 13 have been quantified singularly, and that A and B-type PCs were differentiated and quantified from DP 2 to 6. The PC content was higher in the three fractions of ORA analyzed than in CRA, possibly because of the hydrolysis of complex tannins.

Finally, the peel and seed from overripe avocado fruit, the main by-products of avocado processing, have demonstrated to possess high concentrations of PCs and they also present A-type PCs. These characteristics make avocado by-products useful matrices to develop functional food, nutraceutical or cosmetics.

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CAPÍTULO 4

HPLC-DAD-q-TOF-MS as a powerful platform for the determination of phenolic and other polar compounds in the edible part of mango and its by-products (peel, seed, and seed husk)

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Research Article

HPLC-DAD-q-TOF-MS as a powerful platform for the determination of phenolic and other polar compounds in the edible part of mango and its by-products (peel, seed, and seed husk)

Free and bound phenolic and other polar compounds in mango edible fraction and its by-products (peel, seed, and seed husk) have been determined by HPLC-DAD-ESI-qTOF-MS. This analytical technique has demonstrated to be a valuable platform for the identification and quantification of these compounds in mango. In fact, UV-Vis and mass spectra data allowed the determination of 91 free compounds and 13 bound (cell wall linked) compounds taking into account the four fractions of mango. To our knowledge, this is the first time that mango seed husk has been studied regarding its phenolic compounds. The method proposed showed LODs between 0.006 and 0.85 μg/mL and accuracy ranged from 94.8 and 100.7%. Mango peel presented the highest concentration of free polar compounds followed by seed, pulp, and seed husk. It is also important to highlight that bound phenolic compounds had never been determined in mango pulp, seed, and seed husk before. Furthermore, ellagic acid was the most abundant bound compound in the four mango fractions analyzed. These results show that mango pulp and its by-products are a good source of phenolic and other polar compounds. In particular, mango seed contains a high total concentration of ellagic acid (650 mg/100 g dry weight).

Keywords:
By-products / Free and bound phenolic compounds / HPLC-DAD-ESI-qTOF-MS / Mango
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Abstract

Free and bound phenolic and other polar compounds in mango edible fraction and its by-products (peel, seed, and seed husk) have been determined by HPLC-DAD-ESI-QTOF-MS. This analytical technique has demonstrated to be a valuable platform for the identification and quantification of these compounds in mango. In fact, UV-Vis and mass spectra data allowed the determination of 91 free compounds and 13 bound (cell wall linked) compounds taking into account the four fractions of mango. To our knowledge, this is the first time that mango seed husk has been studied regarding its phenolic compounds. The method proposed showed LODs between 0.006 and 0.85 μg/mL and accuracy ranged from 94.8 and 100.7%. Mango peel presented the highest concentration of free polar compounds followed by seed, pulp, and seed husk. It is also important

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Keywords: By-products, free and bound phenolic compounds, HPLC-DAD-ESI-QTOF-MS, mango.

1. Introduction

Mango (*Mangifera indica* Linn.) is a tropical fruit originated in the Indo-Burmese region. It is a wide consumed fruit and its production was 1 567 439 tons of mango pulp in 2013. The main producers of mango are India, Egypt, Thailand, Cuba, and Philippines[1]. However, nowadays, mangoes are widely available not only in tropics and subtropics, but also in North America, Japan, and Europe[2].

The high consumption and popularity of mango is due to its excellent sensorial properties such as color, taste, and flavor[3]. Furthermore, mango is a great source of bioactive compounds such as ascorbic acid[4], carotenoids[5], and phenolic compounds[6,7]. These compounds have demonstrated different health-promoting properties such as antioxidative, anticarcinogenic, antiatherosclerotic, antimutagenic, and angiogenesis inhibitory properties[8–10].

Tropical and subtropical fruits processing arise high quantities of by-products. In fact, mango processing produces around 35-60% of peel and seed taking into account the total fruit[11]. These mango by-products have also reported great antioxidant activities attributed to phenolic compounds, carotenoids, tocopherols, and sterols[12,13].

Regarding phenolic compounds, different families of compounds have been described in mango and all of them present different health benefits: phenolic acid derivatives, flavones, gallotannins, benzophenone derivatives (maclurin derivatives), and xanthone glycosides (mangiferin derivatives)[8,14–16]. The distribution of the phenolic compounds depends on the part of mango

(pulp, peel, and seed), being flavonols, xanthone glycosides, gallotannins, and benzophenone derivatives predominant in mango peel and seed[6,12,17].

Despite the economic importance of mango and the interest about the bioactive compounds of this fruit, there are few studies that determine the phenolic profiles of mango pulp and its by-products and most of them are focused on the study of free phenolic compounds. They have mainly been analyzed by HPLC coupled to UV-Vis and/or MS detectors and using C18 as stationary phase[7,12,14,16,18]. This kind of polar low molecular weight compounds has mostly been analyzed by LC using C18 as stationary phase in different matrices[19,20]. There are nearly no studies about the determination of individual bound phenolic compounds in mango pulp and its by-products[21]. Besides, phenolic compounds have never been studied in mango seed husk. Thus, the aim of this study was the determination of individual free and bound phenolic compounds in mango pulp, peel, seed, and seed husk by HPLC-DAD-ESI-QTOF-MS. The expansion of knowledge about phenolic compounds in mango by-products will lead to provide add-value to these food by-products that could help to their revalorization in the food industry as natural ingredients for the development of new functional foods.

2. Materials and methods

2.1. Samples

About 10 kg of 'Keitt' mango from the Tropical Coast of Granada were provided by Miguel García Sánchez e Hijos, S.A. (Motril, Spain) in July 2014. Pulp, peel seed and seed husk of samples were manually separated and freeze dried in a lyophilizer (Advantage Plus EL-85 freeze dryer, SP Scientific,

Ipswich, Suffolk, UK). Finally, dried samples were milled and kept at -18°C until use.

2.2. Chemicals and reagents

HPLC grade acetic acid and HPLC-MS grade ACN were purchased from Fisher Scientific (Leicestershire, UK), and methanol was purchased from Panreac (Barcelona, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Double-DI water with conductivity lower than 18.2 MΩ was obtained with a Milli-system from Millipore (Bedford, MA, USA). The following phenolic standards were supplied by Sigma-Aldrich (St. Louis, MO, USA): gallic acid, coumaric acid, ferulic acid, vanillic acid, catechin, quercetin, quinic acid, citric acid, mangiferin, and ellagic acid.

2.3. Extraction of the free polar fraction of mango

The polar fractions of mango pulp, peel, seed, and seed husk were extracted using a solid-liquid extraction. Briefly, 0.5 g of freeze-dried sample powder were dissolved in 10 mL of a solution of methanol/water (80:20, v/v). The mixture was placed in an ultrasonic bath for 15min at room temperature and then it was centrifuged for 15 min at 1000 × g; the supernatant was removed, and the extraction was repeated twice more.

The supernatants were collected, evaporated, and reconstituted in 3 mL of methanol/water (80:20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 μm (Millipore) and stored at -18°C until the analyses.

2.4. Extraction of the bound polar fraction of mango

After the extraction with aqueous methanol was performed to discharge free phenolic compounds, bound phenols were collected by alkaline hydrolysis following the method proposed by Gomez-Caravaca *et al.*[22]. Mango samples were digested with 100 mL of 2 M NaOH at room temperature for 20 h by shaking under nitrogen gas. The mixture was then brought to pH 2-3 by adding 10 M hydrochloric acid in a cooling ice bath and extracted with 500 mL of hexane to remove the lipids. The final solution was extracted three times with 100 mL of 1/1 diethyl ether/ethyl acetate (v/v). The organic fractions were pooled and evaporated to dryness. The phenolic compounds were reconstituted in 2 mL of methanol/water (1:1, v/v). The final extracts were filtered through 0.22 μm PTFE syringe filters and stored at -18°C until the analyses.

2.5. HPLC-DAD-ESI-QTOF-MS analysis of the polar fraction of mango

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, autosampler, binary pump, and DAD was used for the chromatographic determination. The column was a Poroshell 120 EC-C18 (4.6 mm × 100 mm, particle size 2.7 μm) (Agilent Technologies). As reported by manufacturer, this stationary phase is a superficially porous microparticulate column packing. Superficially porous silica particles have a solid silica core and a porous silica outer layer. The EC-C18 bonded phase is made by first chemically bonding a dense monolayer of dimethyl-n-octadecyl silane stationary phase to the porous shell of the Poroshell 120 silica support. The bonded phase packing is then endcapped for using proprietary reagent and procedures to obtain maximum

deactivation of the silica surface. The temperature was established at 25°C. Mango pulp, peel, seed, and seed husk were analyzed using a mobile phase consisting of 1% acetic acid as solvent system A and ACN as solvent system B and the following gradient elution: 0 min, 0.8% B; 5.5 min, 6.8% B; 16 min, 20% B; 20 min, 25% B; 25 min, 35% B; 29 min, 100% B; 32 min, 100% B; 34 min, 0.8% B; 36 min, 0.8% B. The column was equilibrated for 3 min prior to every analysis. The sample volume injected was 3 mL and the flow rate used was 0.8 mL/min. UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, and 330 nm.

MS analysis were carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass Q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in negative ionization mode at the following conditions: drying gas flow (N_2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370°C; capillary voltage, 3500 V; fragmentor voltage, and scan range were 3500 V and *m/z* 50-1500, respectively. Automatic MS/MS experiments were carried out using the followings collision energy values: *m/z* 100, 30 eV; *m/z* 500, 35 eV; *m/z* 1000, 40 eV; and *m/z* 1500, 45 eV. Integration and data elaboration were performed using MassHunter Workstation software (Agilent Technologies).

3. Results and discussion

3.1. Analytical parameters of the method

An analytical validation of the method proposed was carried out considering linearity, sensitivity, repeatability, and accuracy. **Table** 1 summarizes the analytical parameters of the ten standards used including linear range,

calibration curves, determination coefficients, RSD, LOD, LOQ, and accuracy.

Table 1. Analytical parameters of the method proposed.

Analyte	Calibration range (µg/mL)	Calibration equations	r^2	RSD (%)	LOD (µg/mL)	LOQ (µg/mL)	Accuracy (% RSD)		
							0.1 µg/mL	50 µg/mL	125 µg/mL
Quinic acid	LOD-125	y=538080x + 481653	0.993	0.31	0.04	0.13	99.3	97.5	99.7
Citric acid	LOD-125	y=203401x - 191543	0.998	0.57	0.11	0.35	100.1	99.4	99.6
Gallic acid	LOD-125	y=357804x + 254028	0.994	0.41	0.06	0.20	98.2	96.1	100.4
Coumaric acid	LOD-125	y=370029x + 102682	0.996	0.92	0.06	0.19	98.7	99.2	97.6
Vanillic acid	LOD-125	y=108999x + 103196	0.998	0.85	0.20	0.66	99.1	100.7	98.9
Ferulic acid	LOD-125	y=287194x - 29240	0.992	0.84	0.08	0.25	98.1	100.3	99.2
Catechin	LOD-125	y=509740x + 155190	0.997	0.67	0.02	0.08	97.8	98.1	96.4
Quercetin	LOD-125	y=931681x + 412330	0.999	0.53	0.04	0.14	96.9	94.8	97.9
Mangiferin	LOD-125	y=999318x + 478555	0.992	0.36	0.01	0.02	98.5	97.8	100.3
Ellagic acid	LOD-125	y=9525,5x + 99751	0.992	0.95	0.85	2.83	99.4	96.5	98.7

The external calibration curves were prepared with the standards (gallic acid, coumaric acid, ferulic acid, vanillic acid, catechin, quercetin, quinic acid, citric acid, mangiferin, and ellagic acid) at eight different concentration levels from the LOQ to 125 µg/mL. All calibration curves showed good linearity among different concentrations, and the determination coefficients (r^2) were higher than 0.992 in all cases.

The LOQ was determined as the S/N of 10:1, and the LOD was determined as a S/N of 3:1. The method used for showed LODs within the range 0.01-0.85 µg/mL, whereas the LOQs were within 0.02-2.83 µg/mL.

The precision of the analytical procedure was measured as repeatability. The intraday and interday repeatability (expressed as % RSDs) were obtained for six consecutive injections of each analyte at an intermediate concentration value of the calibration curve, carried out within the same day and on three different days. The intraday repeatability of the retention times was in the

range from 0.11 to 2.49%, whereas the interday repeatability was from 1.09 to 3.02%. The intraday repeatability of the total peak area was 0.25-0.71%, whereas the interday repeatability was 0.85-1.13%.

Accuracy was evaluated with separately prepared individual primary stock solutions, mixtures, and working solutions of all standards. It was calculated over the linear dynamic range at three different concentration levels (low (0.1 μg/mL), medium (50 μg/mL), high (125 μg/mL)) via three assays per concentration on different days. The analyte concentrations were calculated and the accuracy was obtained by the ratio of this calculated concentration versus the theoretical (spiked) concentration (**Table 1**).

3.2. Method application to the analysis of mango extracts

3.2.1. Identification of free polar compounds in mango extracts

In this study, a total of 91 compounds were tentatively identified in the four parts of mango analyzed (pulp, peel, seed, and seed husk). Peak identification was performed on basis of their relative retention time values, their UV-Vis spectra, and mass spectra obtained using QTOF-MS together with information previously reported in the literature. The identified compounds of the four fractions of mango are summarized in **Table 2** including the retention time (min), molecular formula, experimental and calculated *m/z*, fragments, error (ppm), score, and the presence of the compounds in the different parts of mango. They can be classified in different families such as organic acids, gallates and gallotannins, flavonoids, xanthones, ellagic acid and derivatives, benzophenone derivatives, and other phenolic acid derivatives. **Figure 1** shows the base peak chromatograms with the

identification of free phenolic and polar compounds in mango pulp, peel, seed, and seed husk.

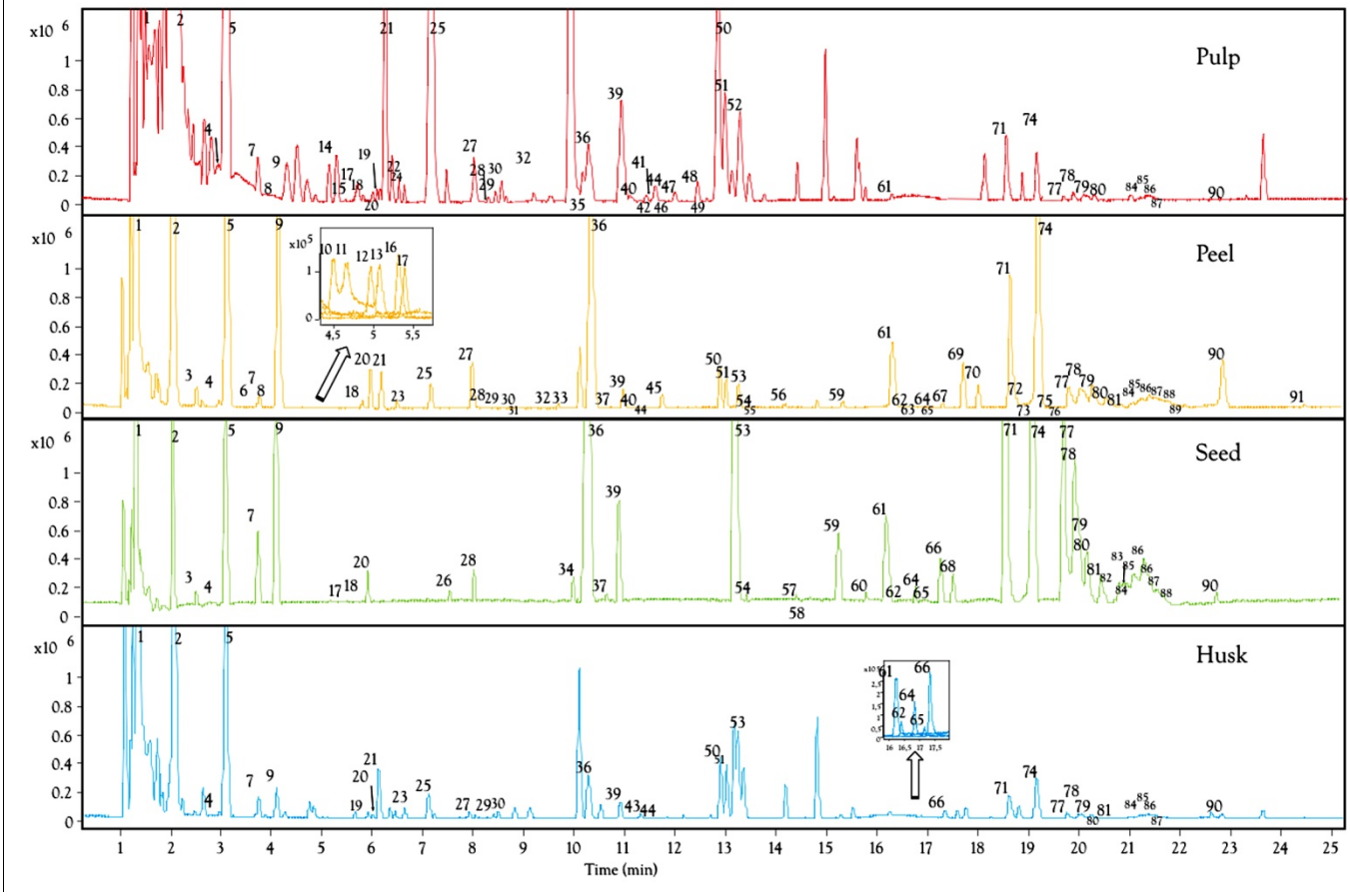

Figure 1. Base peak chromatograms with the identification of phenolic and polar compounds in mango pulp, seed, and seed husk. See **Table 2** for identification numbers.

Table 2. Free polar compounds identified in mango pulp, peel, seed and husk by HPLC-DAD-QTOF-MS.

Peak	Proposed compound	Retention time (min)	*m/z* experimental $(M-H)^-$	*m/z* calculated $(M-H)^-$	Fragments	Molecular Formula	Error (ppm)	Score	Pulp	Peel	Seed	Husk
1	Quinic acid	1.315	191.057	191.056	-	$C_7H_{12}O_6$	-5.57	93.89	✓	✓	✓	✓
2	Citric acid	2.067	191.020	191.020	-	$C_6H_8O_7$	-1.70	99.42	✓	✓	✓	✓
3	Galloyl diglucoside	2.532	493.121	493.120	169	$C_{19}H_{26}O_{15}$	-1.56	98.62	-	✓	✓	-
4	Galloylglucose	2.975	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-1.98	98.52	✓	✓	✓	✓
5	Galloylglucose isomer I	3.113	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-2.60	97.30	-	✓	✓	-
6	Galloylglucose isomer II	3.722	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-2.02	98.04	-	✓	-	-
7	Gallic acid	3.767	169.0147	169.0142	125	$C_7H_6O_5$	-2.63	98.73	✓	✓	✓	✓
8	Vanillic acid glucoside	3.942	329.088	329.088	108/152/167	$C_{14}H_{18}O_9$	-1.07	96.73	✓	✓	✓	✓
9	5-galloylquinic acid	4.126	343.068	343.067	127/169/191	$C_{14}H_{16}O_{10}$	-2.11	97.72	✓	✓	-	-
10	Galloylglucose isomer III	4.459	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-1.35	99.22	-	✓	-	-
11	Galloylglucose isomer IV	4.619	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-1.12	99.26	-	✓	-	-
12	*p*-hydroxybenzoic acid glucoside	4.923	299.078	299.077	137	$C_{13}H_{16}O_8$	-1.54	99.06	-	✓	-	-
13	Galloylglucose isomer V	5.029	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-1.44	99.11	✓	-	-	-
14	*p*-hydroxybenzoic acid glucoside dimer	5.034	599.163	599.162	137/299	$C_{26}H_{32}O_{16}$	-2.40	96.22	-	✓	-	-
15	Galloyl diglucoside isomer I	5.057	493.120	493.120	169	$C_{19}H_{26}O_{15}$	-1.00	98.86	✓	-	-	-
16	3-galloylquinic acid	5.267	343.068	343.067	169/191	$C_{14}H_{16}O_{10}$	-1.32	83.93	-	✓	-	-
17	Galloyl diglucoside isomer II	5.344	493.120	493.120	169	$C_{19}H_{26}O_{15}$	-0.93	99.51	✓	✓	✓	-
18	Galloyl diglucoside isomer III	5.792	493.120	493.120	169	$C_{19}H_{26}O_{15}$	-0.79	99.51	✓	✓	✓	-
19	Dihydroxybenzoic acid glucoside	5.959	315.073	315.072	108/152/199	$C_{13}H_{16}O_9$	-1.82	98.45	✓	✓	✓	✓
20	Galloyl diglucoside isomer IV	6.033	493.121	493.120	169	$C_{19}H_{26}O_{15}$	-1.73	98.35	✓	-	-	✓

21	Galloylglucose isomer VI	6.169	331.068	331.068	125/169	$C_{13}H_{16}O_{10}$	-1.85	98.48	✓	✓	-	✓
22	Galloyl diglucoside isomer V	6.496	493.120	493.120	169	$C_{19}H_{26}O_{15}$	-0.48	99.32	✓	-	-	-
23	Vanillic acid glucoside isomer I	6.723	329.088	329.088	108/152/167	$C_{14}H_{18}O_{9}$	-1.07	96.73		✓	-	✓
24	Dihydroxybenzoic acid glucoside isomer I	6.996	315.073	315.072	108/152/199	$C_{13}H_{16}O_{9}$	-1.47	99.20	✓	-	-	-
25	*p*-hydroxybenzoic acid glucoside	7.149	299.078	299.077	137	$C_{13}H_{16}O_{8}$	-1.64	98.96	✓	✓	-	✓
26	Maclurin C-glucoside	7.578	423.094	423.093	193/303	$C_{19}H_{20}O_{11}$	-1.67	98.48	-	-	✓	-
27	Digalloylglucose	7.951	483.0789	483.078	125/169	$C_{20}H_{20}O_{14}$	-1.61	98.15	✓	✓	-	✓
28	Maclurin C-glucoside isomer I	8.073	423.094	423.093	193/303	$C_{19}H_{20}O_{11}$	-0.62	99.40	✓	✓	✓	-
29	Vanillic acid glucoside isomer II	8.489	329.088	329.088	108/152/167	$C_{14}H_{18}O_{9}$	-1.01	99.56	✓	✓	-	✓
30	Digalloylglucose isomer I	8.588	483.0787	483.078	125/169	$C_{20}H_{20}O_{14}$	-1.26	99.13	✓	✓	-	✓
31	3,5-digalloylquinic acid	9.042	495.079	495.078	343/191/169	$C_{21}H_{20}O_{14}$	-0.57	99.65	-	✓	-	-
32	Vanillic acid glucoside isomer III	9.360	329.088	329.088	108/152/167	$C_{14}H_{18}O_{9}$	-1.01	99.56	✓	✓	-	-
33	5-(digalloyl)quinic acid	9.656	495.079	495.078	169/191/343	$C_{21}H_{20}O_{14}$	-0.98	99.37	-	✓	-	-
34	Iriflophenone glucoside	10.013	407.099	407.098	151/245/285	$C_{19}H_{20}O_{10}$	-1.08	98.60	-	-	✓	-
35	Ferulic acid hexoside	10.266	355.104	355.104	134/193	$C_{16}H_{20}O_{9}$	-1.30	98.95	✓	-	-	-
36	Methylgallate	10.304	183.030	183.030	169	$C_{8}H_{8}O_{5}$	-1.20	99.32	✓	✓	✓	✓
37	Maclurin galloyl glucoside	10.702	575.105	575.104	193/303/333	$C_{26}H_{24}O_{15}$	-0.81	99.49	-	✓	✓	-
38	Digallic acid	10.874	321.026	321.025	125/169	$C_{14}H_{10}O_{9}$	-0.72	99.24	-	✓	-	-
39	Catechin	10.929	289.073	289.072	109/123	$C_{15}H_{14}O_{6}$	-2.71	97.20	✓	✓	✓	✓
40	Hydroxybenzoyl galloyl glucoside	11.134	451.089	451.088	137/169	$C_{20}H_{20}O_{12}$	-0.47	97.45	✓	✓	-	-
41	Sinapic acid hexoside	11.230	385.114	385.114	208/223	$C_{17}H_{22}O_{10}$	-0.48	99.77	✓	-	-	-
42	Coumaric acid hexoside pentoside	11.269	457.163	457.135	145/163/325	$C_{20}H_{26}O_{12}$	-1.73	98.17	✓	-	-	-
43	Sinapic acid hexoside isomer I	11.326	385.114	385.114	208/223	$C_{17}H_{22}O_{10}$	-0.38	99.14	-	-	-	✓
44	Coumaric acid glucoside	11.394	325.0933	325.0929	117/145/163	$C_{15}H_{18}O_{8}$	-1.11	98.78	✓	✓	-	✓

45	Trigalloyl glucose	11.715	635.091	635.089	169/465	$C_{27}H_{24}O_{18}$	-2.07	96.76	-	✓	-	-
46	Coumaric acid hexoside pentoside isomer I	12.050	457.136	457.135	145/163/325	$C_{20}H_{26}O_{12}$	-1.38	98.21	✓	-	-	-
47	Coumaric acid glucoside isomer I	12.249	325.093	325.093	117/145/163	$C_{15}H_{18}O_8$	-0.83	99.64	✓	-	-	-
48	Ferulic acid hexoside isomer I	12.564	355.104	355.104	134/193	$C_{16}H_{20}O_9$	-1.39	98.90	✓	-	-	-
49	Ferulic acid hexoside isomer II	12.714	355.104	355.104	134/193	$C_{16}H_{20}O_9$	-1.23	99.28	✓	-	-	-
50	Sinapic acid hexoside-pentoside	12.922	517.23	517.2291	153/205/223/385	$C_{24}H_{38}O_{12}$	-1.88	97.58	✓	✓	-	✓
51	Dihydro sinapic acid hexoside-pentoside	13.044	519.2459	519.2447	225/387	$C_{24}H_{40}O_{12}$	-1.99	97.39	✓	✓	-	✓
52	Ferulic acid hexoside isomer II	13.190	355.104	355.104	134/193	$C_{16}H_{20}O_9$	-1.66	96.43	✓	-	-	-
53	Mangiferin	13.265	421.0785	421.0776	272/301/331	$C_{19}H_{18}O_{11}$	-1.92	98.02	-	✓	✓	✓
54	Maclurin digalloyl glucoside	13.498	727.1157	727.1152	169/303/575	$C_{33}H_{28}O_{19}$	-0.58	99.61	-	✓	✓	-
55	Trigalloyl glucose isomer I	13.514	635.089	635.089	169/465	$C_{27}H_{24}O_{18}$	-0.54	99.63	-	✓	-	-
56	Coumaroyl galloyl glucoside	14.151	477.1045	477.1038	119/163/169/313	$C_{22}H_{22}O_{12}$	-3.35	68.68	-	✓	-	-
57	Methoxylmangiferin	14.441	435.0938	435.0933	272/285/315/345	$C_{20}H_{20}O_{11}$	-1.14	99.16	-	-	✓	-
58	Tetragalloyl glucose	14.558	787.1006	787.100	169/393	$C_{34}H_{28}O_{22}$	-1.36	97.09	-	-	✓	-
59	Syringic acid	15.313	197.046	197.046	124	$C_9H_{10}O_5$	-1.75	99.31	-	✓	✓	-
60	Eriodictyol	15.825	287.0562	287.0561	109/121/135/151	$C_{15}H_{12}O_6$	-0.32	99.75	-	-	✓	-
61	Methyl-digallate ester	16.276	335.041	335.041	124/183	$C_{15}H_{12}O_9$	-0.67	99.66	✓	✓	✓	✓
62	Tetragalloyl glucose isomer I	16.370	787.101	787.100	169	$C_{34}H_{28}O_{22}$	-1.09	98.01	-	✓	✓	✓
63	Coumaroyl galloyl glucoside isomer I	16.559	477.1043	477.1038	119/163/169/313	$C_{22}H_{22}O_{12}$	-1.52	96.04	-	✓	-	-
64	Tetragalloyl glucose isomer II	16.863	787.101	787.100	169	$C_{34}H_{28}O_{22}$	-1.39	98.02	-	✓	✓	✓
65	Tetragalloyl glucose isomer III	17.223	787.101	787.100	169/393	$C_{34}H_{28}O_{22}$	-0.67	99.54	-	✓	✓	✓
66	Ellagic acid	17.314	300.999	300.999	145/257/300	$C_{14}H_6O_8$	-1.16	99.05	-	-	✓	✓
67	Coumaroyl galloyl glucoside pentoside	17.494	629.116	629.115	169/313	$C_{29}H_{26}O_{16}$	-1.11	97.96	-	✓	-	-
68	7-*O*galloyltricetiflavan	17.558	441.083	441.083	125/169/271	$C_{22}H_{18}O_{10}$	-1.29	98.28	-	-	✓	-

69	Quercetin glucoside	17.715	463.089	463.088	300	$C_{21}H_{20}O_{12}$	-1.62	98.32	-	✓	-	-
70	Quercetin galactoside	18.014	463.089	463.088	300	$C_{21}H_{20}O_{12}$	-1.25	98.88	-	✓	-	-
71	Pentagalloyl glucose	18.645	939.112	939.111	169/617/769	$C_{41}H_{32}O_{26}$	-0.91	98.71	✓	✓	✓	✓
72	Quercetin xyloside	18.678	433.078	433.078	300	$C_{20}H_{18}O_{11}$	0.47	99.79	-	✓	-	-
73	Quercetin arabinopyranoside	19.038	433.078	433.078	300	$C_{20}H_{18}O_{11}$	-0.59	99.76	-	✓	-	-
74	Methyl-digallate ester isomer	19.188	335.042	335.041	124/183	$C_{15}H_{12}O_9$	-2.30	97.60	✓	✓	✓	✓
75	Coumaroyl galloyl glucoside pentoside isomer I	19.348	629.115	629.115	169/313	$C_{29}H_{26}O_{16}$	-0.35	99.61	-	✓	-	-
76	Quercetin arabinofuranoside	19.548	433.078	433.078	300	$C_{20}H_{18}O_{11}$	-0.64	99.22	-	✓	-	-
77	Hexagalloylglucose	19.786	1091.124	1091.122	169/545	$C_{48}H_{36}O_{30}$	-1.79	96.89	✓	✓	✓	✓
78	Hexagalloylglucose isomer I	20.029	1091.124	1091.122	169/545	$C_{48}H_{36}O_{30}$	-1.49	97.65	✓	✓	✓	✓
79	Hexagalloylglucose isomer II	20.262	1091.124	1091.122	169/545	$C_{48}H_{36}O_{30}$	-1.73	96.98	✓	✓	✓	✓
80	Hexagalloylglucose isomer III	20.549	1091.123	1092.122	169/546	$C_{48}H_{36}O_{30}$	-0.86	99.13	✓	✓	✓	✓
81	Hexagalloylglucose isomer IV	20.826	1091.123	1092.122	169/546	$C_{48}H_{36}O_{30}$	-0.72	99.15	-	✓	✓	✓
82	Heptagalloylglucose isomer I	20.857	1243,135	1243,133	545/621/939	$C_{55}H_{40}O_{34}$	-1,08	98,69	-	-	✓	-
83	Heptagalloylglucose isomer II	20.929	1243,134	1243,133	545/621/939	$C_{55}H_{40}O_{34}$	-1,05	98,72	-	-	✓	-
84	Heptagalloylglucose isomer III	21.042	1243.134	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-0.69	99.42	✓	✓	✓	✓
85	Heptagalloylglucose isomer IV	21.241	1243.134	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-1.08	98.48	✓	✓	✓	✓
86	Heptagalloylglucose isomer V	21.402	1243.135	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-1.46	97.47	✓	✓	✓	✓
87	Heptagalloylglucose isomer VI	21.457	1243.135	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-1.21	98.12	✓	✓	✓	✓
88	Heptagalloylglucose isomer VII	21.651	1243.134	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-0.60	99.45	-	✓	✓	-
89	Heptagalloylglucose isomer VIII	22.038	1243.133	1243.133	545/621/939	$C_{55}H_{40}O_{34}$	-0.02	99.88	-	✓	-	-
90	Ethyl 2,4-dihydroxy-3-(3,4,5-trihydroxybenzoyl)oxybenzoate	22.799	349.054	349.057	124/189/197	$C_{16}H_{14}O_9$	-0.62	99.73	✓	✓	✓	✓
91	Rhamnetin hexoside	24.513	477.105	477.104	299/314	$C_{22}H_{22}O_{12}$	-1.73	97.39	-	✓	-	-

3.2.1.1. Mango seed husk

Among the mango fractions studied, to our knowledge, the mango seed husk has never been studied before. Therefore, this fraction merits special attention because, as mango by-product, mango seed husk could be used as food ingredient for the development of functional food or nutraceuticals.

Two organic acids were identified: peak 1, at 1.31 min and *m/z* 191.057 was identified as quinic acid and peak 2, at 2.07 and *m/z* 191.020 was assigned to citric acid. Both compounds were corroborated with their standards and their presence was in agreement with literature for mango pulp[23].

Different compounds belonging to the family of gallates and gallotannins were also found in this fraction. Peaks 4 and 21, at 2.975 and 6.169 min, respectively, presented *m/z* 331.068 and fragments at *m/z* 169 due to a gallic acid moiety after the loss of glucose. Thus, these compounds were identified as two isomers of galloylglucose as previously described in mango pulp, peel, and seed[12]. Peak 7, at 3.767 min, *m/z* 169.0147 and molecular formula $C_7H_6O_5$, was identified as gallic acid. Indeed, it showed a characteristic fragment at *m/z* 125. This compound is in agreement with previous findings in mango pulp, peel, and seed[12,14,24]. Peak 9 was identified as 5-galloylquinic acid according to Clifford *et al.*[25] because it presented *m/z* at 343.068 and the MS/MS spectrum showed a major fragment at *m/z* 191 followed by another fragment at *m/z* 169. This compound was previously described in mango peel[26]. At 5.959 min and *m/z* 493.121, it was identified a galloyl diglucoside isomer (peak 20). The fragments at *m/z* 169.0147 and 125 corroborated the presence of a gallic acid moiety. This compound has previously been reported by Krenek *et al.* for mango pulp[24]. Two isomers of digalloylglucose (peaks 27 and 30) were also found at 7.951 and 8.588 min

and *m/z* 483.0789. The fragments at *m/z* 169 and 125 confirmed the fragmentation pattern described by Dorta *et al.* for this compound in mango peel[14]. Peak 36 showed a molecular ion at *m/z* 183.030 and 10.034 min with a molecular formula $C_8H_8O_5$. The fragmentation gave cause to an ion at *m/z* 169 due to a gallic acid moiety. Thus, the compound was identified as methylgallate as previously described in mango pulp[23]. Two isomers of methyldigallate ester were also identified at 16.276 and 19.188 min (peaks 61 and 74). The molecular formula $C_{15}H_{12}O_9$, molecular ion at *m/z* 335.041, and the fragments at *m/z* 183 and 124 due to a methylgallate moiety confirmed the identification[23]. Three isomers of tetragalloyl glucoside, a pentagalloyl glucose, five hexagalloyl glucose isomers, and four heptagalloyl glucose isomers were also identified in mango seed husk (peaks 62, 64, 65, 71, 77-81, 84-87). The fragmentation pattern of these compounds (**Table 2**) corresponded with the one reported previously by other authors for mango pulp, peel, and seed[6].

One flavonoid could also be identified: peak 39, at 10.929 min and *m/z* 289.073, presented $C_{15}H_{14}O_6$ as molecular formula and it showed fragments at *m/z* 109 and 123. Therefore, it was identified as catechin. This compound was previously described in mango pulp fiber waste[27].

Concerning xanthones, at *m/z* 421.0785 and 13.265 (peak 53) it was found mangiferin. It presented a molecular formula $C_{19}H_{18}O_{11}$ and typical fragments at *m/z* 272, 301, and 331. This was in agreement with other studies in mango pulp, peel, and seed[12,23].

Peak 66 showed a molecular ion at *m/z* 300.999 and 17.314 min. The MS/MS spectrum showed fragments at *m/z* 145 corresponding to the loss of two-CO2 moieties and 257 due to the loss of four-OH moieties. This was

identified as ellagic acid in agreement with previous results in mango pulp, peel, and seed[12]. Its identity was confirmed by coelution with its commercial standard.

Finally, eight phenolic acid derivatives were also observed in mango seed husk. Peaks 23 and 29 showed a molecular ion at *m/z* 329.088 and fragments at *m/z* 167 due to a vanillic acid moiety after the loss of a glucose, at *m/z* 152 [167-CH_3] and *m/z* 108 [152-CO_2]. According to the fragmentation pattern, these compounds were assigned as isomers of vanillic acid glucoside. Vanillic acid has been previously described in mango pulp[17,28]; however, to our knowledge, this is the first time that vanillic acid glucoside has been identified in mango. Peak 25 was found at 7.149 min and *m/z* 299.078 and presented a fragment at *m/z* 137 after the loss of a glucose moiety. According to Krenek *et al.* it was identified as *p*-hydroxybenzoic acid glucoside[24]. Peaks 43, 44, and 50 were assigned to sinapic acid hexoside, coumaric acid glucoside, and sinapic acid hexoside pentoside, respectively. Their mass spectra and fragmentation patterns (**Table 2**) were in agreement with data reported for mango pulp before[21]. Peak 51, at 13.044 min and *m/z* 519.2459, showed as molecular formula $C_{24}H_{40}O_{12}$ (only 2 H more than sinapic acid hexoside pentoside) and fragments at *m/z* 387 and 225, both fragments with two more H than the fragments for sinapic acid hexoside pentoside and corresponding to the loss of a pentose moiety and, then, a successive loss of a glucose. Thus, it was identified as dihydrosinapic acid hexosidepentoside. Dihydroxybenzoic acid glucoside was identified in mango seed husk (peak 19) at *m/z* 315.073 and fragments at *m/z* 169 [M-H-glucose]$^-$ and 125. Ethyl 2,4-dihydroxy-3-(3,4,5-trihydroxybenzoyl) oxybenzoate (peak 90) was identified according to data reported by Dorta *et al.* for mango pulp,

peel, and seed[14]. As far as we are concerned, this is the first time that vanillic acid glucoside, dihydroxybenzoic acid glucoside, and dihydro sinapic acid hexoside pentoside have been identified in mango and particularly in mango seed husk.

3.2.1.2. Mango pulp

Forty-seven free polar compounds were identified in mango pulp. This is the first time that a HPLC-DAD-ESI-QTOF-MS methodology has allowed identifying such number of compounds in the same analysis for mango pulp.

Peaks 1, 2, 4, 7, 9, 19, 20, 21, 25, 27, 29, 30, 36, 39, 44, 50, 51, 61, 71, 74, 77-80, 84-87, and 90 were identified following the same procedure explained for mango seed husk. Concerning the rest of the compounds, some new gallates and gallotannins were found. Four new galloyl diglucose isomers (peaks 15, 17, 18, and 22) were identified; thus, mango pulp presented a total of five galloyl diglucose isomers. Besides, concerning isomers of methyldigallate ester (peaks 61 and 74), only one isomer of the two found in mango had previously been described in mango peel[23]. Peak 40 at 11.134 min and *m/z* 451.089 showed fragments at *m/z* 169 due to a gallic acid moiety and at *m/z* 137 due to a hydroxybenzoic acid moiety. Therefore, this compound that apparently has not been described before in mango was identified as hydroxybenzoyl galloyl glucoside.

Regarding benzophenones, maclurin C-glucoside (peak 28) was identified at 8.073 min and *m/z* 423.094. The fragmentation pattern showed ions at *m/z* 303 because of the cleavage of the C-glycoside moiety and at *m/z* 193 by the loss of 110 Da after the fragmentation of maclurin moiety. This pattern was in agreement with data reported previously in other fractions of mango[14].

Two new vanillic acid glucoside isomers (peaks 8 and 32) were found in mango pulp. A total of four isomers of this compound could be identified in this fraction. As stated before, these compounds had never been identified before in mango. Peak 14 ($C_{26}H_{32}O_{16}$) was identified as *p*-hydroxybenzoic acid glucoside dimer. It showed a molecular ion at *m/z* 599.163 and fragments at *m/z* 299 (due to the monomer) and 137 (loss of a glucoside moiety). The fragmentation pattern agrees with the one reported for the monomer[21]. Another isomer of dihydroxybenzoic acid glucoside was identified in mango pulp (peak 24) at *m/z* 315.073 and 6.996 min. Three isomers of ferulic acid hexoside (peaks 35, 48, and 49) were found in mango pulp. The molecular formula $C_{16}H_{20}O_9$, molecular ion at *m/z* 355.104, and fragments at *m/z* 193 and 134 allowed identifying them according to Krenek *et al.*[24]. Nevertheless, it has been the first time that three isomers of this compound have been identified in mango pulp. At 11.230 min it was identified a sinapic acid hexoside (peak 41) as described before for another isomer in mango seed husk. Two isomers (peaks 42 and 46) were found at 11.269 and 12.050 min with *m/z* 457 and fragments at *m/z* 325.093 due to the loss of a pentoside moiety at *m/z* 163 [coumaric acid-H]$^-$ and *m/z* 145 [coumaric acid-H-H_2O]$^-$. These compounds were identified as coumaric acid hexoside pentoside. The fragmentation pattern is similar to coumaric acid hexoside as reported earlier[29]. Finally, another isomer of coumaric acid glucoside was found at 12.249 min (peak 47). Thus, two isomers of this compound were present in mango pulp, one more than the earlier found in literature[21].

It is important to highlight the absence of flavonoids, ellagic acid and derivatives, and xanthones as mangiferin in mango pulp. Indeed, that this is

the first time that five isomers of galloyl diglucose have been identified in mango pulp, previous literature only reported one of these isomers in this fraction of mango[21]. Besides, methyldigallate ester isomers, hydroxybenzoyl galloyl glucoside, maclurin C-glucoside, vanillic acid glucoside isomers, *p*-hydroxybenzoic acid glucoside dimer, three isomers of ferulic acid hexoside, and coumaric acid hexoside pentoside have been reported in mango pulp for the first time.

3.2.1.3. Mango peel

Sixty-eight free polar compounds were tentatively identified in mango peel. This is also the first time that so many compounds have been identified in the same analysis by HPLC-DAD-ESI-QTOF-MS. Mango peel showed compounds belonging to the seven families mentioned before: organic acids, gallates and gallotannins, flavonoids, xanthones, ellagic acid and derivatives, benzophenone derivatives, and other phenolic acid derivatives.

Many of these compounds coincide with the compounds reported in mango pulp and seed husk above. In fact, compounds 1, 2, 4, 7–9, 17, 18, 20, 21, 23, 25, 27-30, 32, 36, 39, 40, 44, 50, 51, 53, 61, 62, 64, 65, 71, 74, 77-81, 84–87, and 90 were present in mango peel and their mass data were the same as previously described for mango pulp and/or seed husk.

As far as gallates and gallotannins concerns, mango peel presented five more isomers of galloylglucose than pulp and seed husk (peaks 5, 6, 10, 11, 13). The fragmentation pattern of the isomers was the same as the described above and in literature[12]. This mango fraction also showed four isomers of galloyl diglucose, and one of them (peak 3) was different from the reported ones in mango pulp and seed husk. Peak 16 at 5.267 min was another

isomer of galloylquinic acid. According to Clifford *et al.*[22] this compound was identified as 3-galloylquinic acid because the main fragment was *m/z* 169 and the second fragment was *m/z* 191 with an abundance of 50%. Peaks 31 and 33 at 9.042 and 9.656 min presented a molecular formula $C_{21}H_{20}O_{14}$, molecular ion at *m/z* 495.079, and fragments at *m/z* 343 [M-H-gallic acid]$^-$, *m/z* 191 (quinic acid), and 169 (gallic acid). In agreement with Clifford *et al.*[22] they were assigned to 3,5-digalloylquinic acid and 5-(digalloyl) quinic acid, respectively. Peak 38, at 10.874 min and *m/z* 321.026, presented fragments at *m/z* 169 (gallic acid) and 125. Thus it was identified as digallic acid as previously described in mango before[30]. Peaks 45 and 55 were identified as two isomers of trigalloylglucose, they presented as molecular formula $C_{27}H_{24}O_{18}$, a molecular ion at *m/z* 635.091, and fragments at *m/z* 465 [M-H-glucose]$^-$ and *m/z* 169 due to a gallic acid moiety. This compound has been reported in mango leaves earlier[31]. Concerning heptagalloylglucose isomers, mango peel showed two more isomers (peaks 88 and 89).

Other type of gallate derivatives could also be seen in mango peel: peaks 37 and 54 were identified as maclurin galloyl glucoside (*m/z* 575.105) and maclurin digalloyl glucoside (*m/z* 727.1157), respectively. The fragmentation pattern (**Table 2**) was in agreement with the one proposed previously by Berardini *et al.* for mango peel[6]. Peaks 56 and 63 were assigned to coumaroyl galloyl glucoside. Their mass spectra showed a molecular ion at *m/z* 477.1045 and the MS/MS spectra presented fragments at *m/z* 313 due to the loss of a glucose moiety, at *m/z* 169 because of a gallic acid moiety and at *m/z* 163 that indicates the presence of a coumaric acid moiety. The fragmentation pattern and literature about this family of compounds[32] confirmed their identity. Peaks 67 and 75, at *m/z* 629.116 were identified as

coumaroyl galloyl glucoside pentoside. They presented fragments at *m/z* 477 confirming the presence of coumaroyl galloyl glucoside after the loss of a pentose moiety and the other fragments were the same as the ones described before for coumaroyl galloyl glucoside.

Regarding flavonoids, mango peel was the fraction richer in these compounds. Five quercetin derivatives were identified (peaks 69, 70, 72, 73, and 76). All of them showed a fragment at *m/z* 300 due to the presence of a quercetin moiety. According to Dorta *et al.*[14] they were assigned to quercetin glucoside, quercetin galactoside, quercetin xyloside, quercetin arabinopyranoside, and quercetin arabinofuranoside. Peak 91, at 24.513 min and *m/z* 477.105, showed fragments at *m/z* 299 and 314 [M-H-glucose]$^{\cdot}$. This pattern was assigned to rhamnetin hexoside as described by other authors before[12].

Finally, two more phenolic acids were identified. Peak 12, at 4.293 min and *m/z* 299.078, with molecular formula $C_{13}H_{16}O_8$ and a fragment at *m/z* 137 (*p*-hydroxybenzoic acid) was assigned to *p*-hydroxybenzoic acid glucoside in agreement with Krenek *et al.* for mango pulp[24]. Syringic acid (peak 59) was identified in mango peel as previously described by Ajila *et al.*[33].

As far as we are concerned, this is the first time that seven of galloylglucose have been identified in mango peel. Furthermore, no reports have previously described the two isomers of digalloylquinic acid, the two isomers of trigalloylglucose, the two isomers coumaroyl galloyl glucoside, the two isomers of *p*-hydroxybenzoic acid glucoside, the four isomers of vanillic acid glucoside, dihydro sinapic acid hexoside pentoside, and hydroxybenzoyl galloyl glucoside in mango pulp.

Most of the compounds (1-5, 7, 9, 17, 18, 20, 28, 36, 37, 39, 53, 54, 59, 61, 62, 64-66, 71, 74, 77-81, 84-88, and 90) have already been described in the other fractions studied. However, there are some compounds that were exclusively present in mango seed. Two isomers of heptagalloylglucose (peaks 82 and 83) that were not present in the other fractions of mango were identified in mango seed. Maclurin C-glucoside (peak 26) and iriflophenone glucoside (peak 34) were identified as previously described by Barreto et al[15]. A new compound was identified at 14.441 min and *m/z* 435.0938 (peak 57). This compound showed fragments at 272/285/315/345 and this pattern was in agreement with previous literature[34]; thus, this compound was identified as methoxylmangiferin. Peak 58 corresponded to an isomer of tetragalloylglucose that was only present in mango seed. Peak 60, apparently, was another new compound. Its molecular ion at *m/z* 287.0562 and fragments at *m/z* 109/121/135/151 were in agreement with the fragmentation pattern of eriodictyol[35]. Peak 68 showed a molecular ion at *m/z* 441.083 and a molecular formula $C_{22}H_{18}O_{10}$. Besides, the MS/MS spectrum reported fragments at *m/z* 271 [M-H-gallic acid]$^-$, *m/z* 169 due to a gallic acid moiety, and *m/z* 125. These data were in agreement with results previously reported for *Pithecellobium clypearia*[36] and, therefore this compound was identified as 7-*O*-galloyltricetiflavan. To our knowledge, vanillic acid glucoside, methoxylmangiferin, eriodictyol, and 7-*O*-galloyltricetiflavan were identified for the first time in mango seed.

3.2.2. Identification of bound phenolic compounds in mango extracts

Some phenolic compounds appear linked to structural compounds of plants such as proteins, dietary fiber, etc.[37]. Therefore, these phenolic compounds

cannot be seen if a conventional extraction is performed. However, there are only few studies that analyze bound phenolic compounds in mango[18,30] and, as far as we are concerned, they have only been studied in mango peel. Because of that, a basic hydrolysis has been carried out in order to release and analyze phenolic compounds in mango pulp, peel, seed, and seed husk. A total of 13 bound phenolic compounds were identified taking into account the four mango parts analyzed. Almost all of them were phenolic acids or derivatives and also ellagic acid was found in all the fractions. **Table 3** summarizes the tentatively identified compounds including the retention time (min), molecular formula, experimental and calculated *m/z*, fragments, error (ppm), score, and the presence of the compounds in the different parts of mango. **Figure 2** shows the base peak chromatograms with the identification of bound phenolic compounds in mango pulp, peel, seed, and seed husk.

Table 3. Bound phenolic compounds identified in mango pulp, peel, seed and husk by HPLC-DAD-QTOF-MS.

Peak	Proposed compound	Retention time (min)	*m/z* experimental $(M-H)^-$	*m/z* calculated $(M-H)^-$	Fragments	Molecular Formula	Error (ppm)	Score	Pulp	Peel	Seed	Husk
1	Gallic acid	3.85	169.0145	169.0142	125	$C_7H_6O_5$	-1.64	99.13	✓	✓	✓	✓
2	Galloylglucose	6.246	331.068	331.067	125/169	$C_{13}H_{16}O_{10}$	-0.96	99.05	✓	✓	-	-
3	*p*-hydroxybenzoic acid	9.213	137.0243	137.0247	108/119	$C_7H_6O_3$	0.11	98.92	✓	✓	✓	✓
4	*p*-hydroxybenzoic acid	9.855	137.0247	137.0247	108/119	$C_7H_6O_3$	-1.71	99.48	✓	✓	✓	✓
5	Vanillic acid	9.984	167.0348	167.035	108/152	$C_8H_8O_4$	1.04	99.58	-	✓	-	✓
6	Acetosyringenin	12.142	195.0667	195.0663	108/122/150	$C_{10}H_{12}O_4$	-2.01	98.84	-	-	-	✓
7	Syringic acid	12.569	197.046	197.0455	123	$C_9H_{10}O_5$	-1.93	99.09	-	-	-	✓
8	Protocatechuic acid	14.715	153.0192	153.0192	107	$C_7H_6O_4$	0.67	99.88	-	✓	✓	-
9	*p*-hydroxybenzoic acid	15.159	137.0245	137.0244	107/119	$C_7H_6O_3$	-0.65	99.68	-	-	✓	-
10	trans p-coumaric acid	15.595	163.0402	163.0401	119	$C_9H_8O_3$	-1.69	97.24	✓	✓	✓	✓
11	cis-*p*-coumaric acid	16.735	163.0403	163.0401	119	$C_9H_8O_3$	-2.41	98.28	✓	✓	-	-
12	Ferulic acid	17.062	193.0507	193.0506	133	$C_{10}H_{10}O_4$	-1.45	95.30	✓	✓	✓	✓
13	Ellagic acid	17.505	300.9992	300.999	145	$C_{14}H_6O_8$	-0.77	99.65	✓	✓	✓	✓

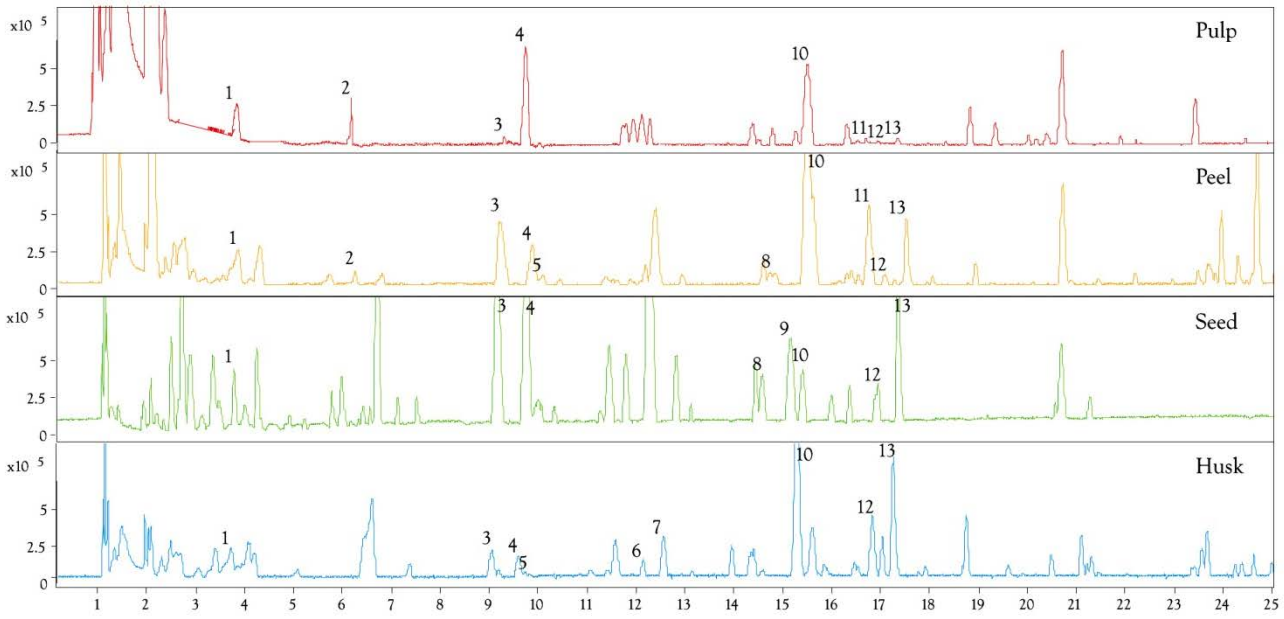

Figure 2. Base peak chromatograms with the identification of bound phenolic and polar compounds in mango pulp, peel, seed, and seed husk. See **Table 3** for identification numbers.

Peaks 1, 7, 8, and 12 at *m/z* 169.0145, 197.046, 153.0192, and 193.0507 were identified as gallic acid, syringic acid, protocatechuic acid, and ferulic acid, respectively, according to results about mango peel found in literature[18,30]. Gallic acid and ferulic acid were detected in the four mango parts, syringic acid was present only in seed husk, and protocatechuic acid was identified in peel and seed.

As far as we are concerned, the remaining compounds have been detected for the first time as bound phenolic compounds in mango. Several compounds were phenolic compounds derivatives from compounds that have previously been identified in the free polar fractions of mango. Thus, they were identified as galloyl glucose (*m/z* 331.068, peak 2),three p-hydroxybenzoic acid isomers (*m/z* 137.024, peaks 3, 4 and 9), vanillic acid

(*m/z* 167.0348, peak 5), acetosyringenin (*m/z* 195.0667, peak 6), two isomers of *p*-coumaric acid (*m/z* 163.04, peaks 10 and 11), and ellagic acid (*m/z* 300.999, peak 13) and they were detected in the different parts of mango.

3.2.3. Quantification of free and bound polar compounds in mango extracts

The method performed was used to quantify free and bound polar compounds in mango pulp, peel, seed, and seed husk. The quantification was performed using the previous calibration curves obtained by MS. Catechin, citric acid, quinic acid, and ellagic acid were quantified with the calibration curves of their own standards. The calibration curve of gallic acid was used to quantify gallates, gallotannins, and maclurin derivatives, coumaric acid for coumaric acid derivatives, ferulic acid for ferulic acid derivatives and other hydroxycinnamic acid derivatives, vanillic acid for vanillic acid derivatives, quercetin for quercetin derivatives, and mangiferin for mangiferin derivatives. It has to be taken into account that the response of the standards can be different from the response of the derivatives present in mango samples, and consequently, the quantification of these compounds is only an estimation of their actual concentrations.

The results of the quantification of free polar compounds in mango are showed in **Table 4**. The comparison between the obtained data and literature of every single compound was not easy due to the high number of phenolic compounds identified in this work.

Table 4. Free polar compounds in mango pulp, peel, seed and husk by HPLC-DAD-QTOF-MS expressed as mg/100 g dry matter.

Peak	Proposed compound	Retention time (min)	Pulp	Peel	Seed	Husk
1	Quinic acid	1.315	11.52±0.81	74.47±3.60	61.94±2.09	29.54±0.57
2	Citric acid	2.067	295.71±6.76	396.51±6.13	60.27±1.99	122.75±2.11
3	Galloyl diglucoside	2.532	n.d.	23.16±0.95	3.77±0.14	n.d.
4	Galloylglucose	2.975	60.05±1.16	9.85±0.39	0.38±0.03	34.87±1.11
5	Galloylglucose isomer I	3.113	n.d.	280.99±6.99	56.98±3.18	n.d.
6	Galloylglucose isomer II	3.722	n.d.	8.33±0.71	n.d.	n.d.
7	Gallic acid	3.767	2.08±0.02	12.18±0.39	17.55±0.74	2.48±0.05
8	Vanillic acid glucoside	3.942	2.66±0.16	n.d.	n.d.	n.d.
9	5-galloylquinic acid	4.126	0.90±0.03	200.20±5.45	59.94±2.75	3.55±0.08
10	Galloylglucose isomer III	4.459	n.d.	3.25±0.21	n.d.	n.d.
11	Galloylglucose isomer IV	4.619	n.d.	4.37±0.55	n.d.	n.d.
12	*p*-hydroxybenzoic acid glucoside	4.923	n.d.	1.61±0.05	n.d.	n.d.
13	*p*-hydroxybenzoic acid glucoside dimer	5.024	3.43±0.32	n.d.	n.d.	n.d.
14	Galloylglucose isomer V	5.029	n.d.	2.31±0.13	n.d.	n.d.
15	Galloyl diglucoside isomer I	5.057	0.065±0.007	n.d.	n.d.	n.d.
16	3-galloylquinic acid	5.267	n.d.	2.91±0.16	n.d.	n.d.
17	Galloyl diglucoside isomer II	5.344	0.168±0.005	1.71±0.12	n.q.	n.d.
18	Galloyl diglucoside isomer III	5.792	0.60±0.02	7.85±0.20	1.45±0.09	n.d.
19	Galloyl diglucoside isomer IV	5.959	1.56±0.10	29.76±0.90	6.33±0.31	0.49±0.03
20	Dihydroxybenzoic acid glucoside	6.033	0.33±0.01	n.d.	n.d.	0.67±0.02
21	Galloylglucose isomer VI	6.169	30.25±0.99	30.23±0.51	n.d.	5.01±0.23
22	Galloyl diglucoside isomer V	6.496	0.191±0.006	n.d.	n.d.	n.d.
23	Vanillic acid glucoside isomer I	6.723	n.d	n.d.	n.d.	3.78±0.14
24	Dihydroxybenzoic acid glucoside isomer I	6.996	0.81±0.07	n.d.	n.d.	n.d.
25	*p*-hydroxybenzoic acid glucoside	7.149	30.81±0.40	17.24±0.45	n.d.	2.70±0.20
26	Maclurin C-glucoside	7.578	n.d.	n.d.	4.38±0.32	n.d.
27	Digalloylglucose	7.951	2.03±0.04	47.28±1.54	n.d.	0.84±0.03
28	Maclurin C-glucoside isomer I	8.073	0.81±0.03	3.28±0.45	8.37±0.33	n.d.
29	Vanillic acid glucoside isomer II	8.489	2.89±0.17	n.d.	n.d.	2.20±0.16
30	Digalloylglucose isomer I	8.588	n.q	2.97±0.25	n.d.	n.q.
31	3,5-digalloylquinic acid	9.042	n.d.	2.73±0.27	n.d.	n.d.
32	Vanillic acid glucoside isomer III	9.360	2.66±0.16	n.d.	n.d.	n.d.
33	5-(digalloyl)quinic acid	9.656	n.d.	10.33±0.42	n.d.	n.d.
34	Iriflophenone glucoside	10.013	n.d.	n.d.	7.31±0.54	n.d.
35	Ferulic acid hexoside	10.266	0.61±0.03	n.d.	n.d.	n.d.
36	Methylgallate	10.304	3.22±0.10	120.33±3.35	197.31±9.85	7.13±0.69
37	Maclurin galloyl glucoside	10.702	n.d.	3.57±0.32	3.28±0.15	n.d.
38	Digallic acid	10.874	n.d.	2.30±0.01	n.d.	n.d.

39	Catechin	10.929	3.43±0.10	11.56±0.42	18.25±1.04	1.68±0.08
40	Hydroxybenzoyl galloyl glucoside	11.134	0.374±0.005	2.32±0.07	n.d.	n.d.
41	Sinapic acid hexoside	11.230	0.41±0.02	n.d.	n.d.	n.d.
42	Coumaric acid hexoside pentoside	11.269	0.202±0.002	n.d.	n.d.	n.d.
43	Sinapic acid hexoside isomer I	11.326	n.d	n.d.	n.d.	0.23±0.02
44	Coumaric acid glucoside	11.394	0.23±0.02	1.93±0.16	n.d.	0.15±0.01
45	Trigalloyl glucose	11.715	n.d	17.70±0.54	n.d.	n.d.
46	Coumaric acid hexoside pentoside isomer I	12.050	0.168±0.006	n.d.	n.d.	n.d.
47	Coumaric acid glucoside isomer I	12.249	0.86±0.03	n.d.	n.d.	n.d.
48	Ferulic acid hexoside isomer I	12.564	0.38±0.01	n.d.	n.d.	n.d.
49	Ferulic acid hexoside isomer II	12.714	0.272±0.006	n.d.	n.d.	n.d.
50	Sinapic acid hexoside-pentoside	12.922	12.55±0.65	32.49±1.12	n.d.	8.97±0.16
51	Dihydro sinapic acid hexoside-pentoside	13.044	5.69±0.16	22.77±0.95	n.d.	7.59±0.23
52	Ferulic acid hexoside isomer II	13.190	0.215±0.002	n.d.	n.d.	n.d.
53	Mangiferin	13.265	n.d	6.23±0.49	71.39±1.61	3.64±0.08
54	Maclurin digalloyl glucoside	13.498	n.d	1.82±0.08	3.54±0.17	n.d.
55	Trigalloyl glucose isomer I	13.514	n.d	1.32±0.10	n.d.	n.d.
56	Coumaroyl galloyl glucoside	14.151	n.d	5.16±0.13	n.d.	n.d.
57	Methoxylmangiferin	14.441	n.d	n.d.	2.59±0.24	n.d.
58	Tetragalloyl glucose	14.558	n.d	n.d.	n.q.	n.d.
59	Syringic acid	15.313	n.d	7.63±0.18	26.82±1.86	n.d.
60	Eriodictyol	15.825	n.d	n.d.	4.92±0.46	n.d.
61	Methyl-digallate ester	16.276	0.547±0.003	48.81±2.93	35.21±1.36	1.56±0.14
62	Tetragalloyl glucose isomer I	16.370	n.d	1.94±0.10	1.86±0.12	n.q.
63	Coumaroyl galloyl glucoside isomer I	16.559	n.d	4.61±0.14	n.d.	n.d.
64	Tetragalloyl glucose isomer II	16.863	n.d	6.42±0.35	5.69±0.14	0.11±0.01
65	Tetragalloyl glucose isomer III	17.223	n.d	1.23±0.07	0.42±0.04	n.q.
66	Ellagic acid	17.314	n.d	n.d	614.89±20.44	56.31±3.78
67	Coumaroyl galloyl glucoside pentoside	17.494	n.d	1.32±0.07	n.d.	n.d.
68	7-*O*galloyltricetiflavan	17.558	n.d	n.d.	9.65±0.44	n.d.
69	Quercetin glucoside	17.715	n.d	42.16±0.80	n.d.	n.d.
70	Quercetin galactoside	18.014	n.d	20.24±0.30	n.d.	n.d.
71	Pentagalloyl glucose	18.645	1.64±0.05	22.47±2.03	159.76±5.04	0.80±0.02
72	Quercetin xyloside	18.678	n.d	8.36±0.74	n.d.	n.d.
73	Quercetin arabinopyranoside	19.038	n.d	4.27±0.18	n.d.	n.d.
74	Methyl-digallate ester isomer	19.188	2.15±0.35	179.68±6.78	119.08±4.91	6.83±0.60
75	Coumaroyl galloyl glucoside pentoside isomer I	19.348	n.d	0.72±0.04	n.d.	n.d.
76	Quercetin arabinofuranoside	19.548	n.d	3.88±0.05	n.d.	n.d.
77	Hexagalloylglucose	19.786	n.q.	21.03±1.07	61.67±4.56	n.q
78	Hexagalloylglucose isomer I	20.029	n.q	18.21±0.89	34.82±2.27	n.q
79	Hexagalloylglucose isomer II	20.262	n.q	12.32±0.67	18.52±1.77	n.q
80	Hexagalloylglucose isomer III	20.549	n.q	3.37±0.12	7.78±0.52	n.q

81	Hexagalloylglucose isomer IV	20.826	n.d	0.50±0.04	2.66±0.19	n.q
82	Heptagalloylglucose isomer I	20.029	n.d	n.d	n.q	n.d
83	Heptagalloylglucose isomer II	20.262	n.d	n.d	n.q	n.d
84	Heptagalloylglucose isomer III	21.042	n.q	4.34±0.41	n.q.	n.q
85	Heptagalloylglucose isomer IV	21.241	n.q	7.13±0.55	2.18±0.16	n.q
86	Heptagalloylglucose isomer V	21.402	n.q	8.31±0.71	3.71±0.29	n.q
87	Heptagalloylglucose isomer VI	21.457	n.q	5.37±0.40	n.q	n.q.
88	Heptagalloylglucose isomer VII	21.651	n.d	4.72±0.40	n.q.	n.q
89	Heptagalloylglucose isomer VIII	22.038	n.d	1.57±0.12	n.d.	n.q
90	Ethyl 2,4-dihydroxy-3-(3,4,5-trihydroxybenzoyl)oxybenzoate	22.799	0.17±0.04	7.17±0.52	6.29±0.58	0.14±0.01
91	Rhamnetin hexoside	24.513	n.d	6.16±0.20	n.d.	n.d.
	Total		483.45±11.30	1822.80±99.44	1700.54±24.61	304.21±6.99

Regarding free polar compounds, mango peel presented the highest concentration (1822.80 mg/100 g of dry weight (d.w.)) followed by seed that also showed a high concentration (1700.54 mg/100 g d.w.), pulp had less of a third of free polar compounds compared with peel and seed (483.45 mg/100 g d.w.), and, finally, seed husk contained 304.21 mg/100 g d.w. These concentrations of phenolic compounds are in the range of the quantities found in other tropical fruits with demonstrated high antioxidant activity and health benefits such as avocado[38,39].

The compound present in the highest concentration in mango pulp was citric acid and represented the 61.2%. The concentration of citric acid was within the range (200–327 mg/100 g) described by the Souci Fachmann Kraut Online Database[40]. The citric acid content in mango pulp is enormously higher than the content in other tropical fruit pulps (avocado, pineapple, banana, papaya, passion fruit, watermelon, and melon)[41].

As noticed for other tropical fruits[42], most compounds were phenolic acid derivatives, being especially abundant the gallic acid derivatives. Two of the isomers of galloyl glucose were the major compounds after citric acid with

concentrations of 60.05 and 30.25 mg/100 g d.w., respectively. Gallic acid derivatives have demonstrated high antioxidant activities and health benefits such as neuroprotective effect[43], citoprotective effect[44], potential utility for treatment of hypertension, diarrhea, and cystic fibrosis[45].

Mango peel presented free polar compounds of all the families. Citric acid was also the major compound, it represented the 21.7%. As reported for the pulp, citric acid content in peel was also higher than in other tropical fruits[41].

The concentration of gallic acid derivatives was also very high; the most concentrated ones were galloylglucose isomer I, 5-galloylquinic acid, and methyl-digallate ester isomer. This fraction was also the most abundant in gallotannins and flavonoids.

Mango seed was characterized by the presence of ellagic acid. It was the most concentrated compound corresponding to 36.2% of total free polar compounds. This compound has demonstrated antioxidant, estrogenic/antiestrogenic, antimicrobial, anti-inflammatory, and prebiotic effects[46]. Its concentration as free ellagic acid is much higher than that found in different berries, nuts, and walnuts[47] and, although the quantity is lower than the hydrolyzed ellagic acid in pomegranate peel[48], known as source of ellagic acid, it is important to highlight that mango seed is a significant source of free ellagic acid. Besides, methylgallate, pentagalloyl glucose, and methyl digallate ester isomer were also present in high concentrations. Mango seed also showed the highest concentration of mangiferin (71.39 mg/100 g d.w.) compared to the other fractions. Mangiferin has demonstrated to improve dyslipidemia[49] and may alter transcription contributing to positive health benefits[50].

Mango seed husk showed as major compound the citric acid (40.3%), followed by ellagic acid (18.5%) and galloylglucose (11.5%). Its total concentration of free polar compounds was only slightly lower than the concentration of mango pulp. Therefore, this fraction, which has not been paid any attention until date, represents a good by-product as source of bioactive compounds.

Table 5 reports the quantification results of bound phenolic compounds in mango pulp, peel, seed, and seed husk. On the one hand, seed and seed husk showed similar and the highest concentrations of bound phenolic compounds, 41.71 and 39.59 mg/100 g d.w, respectively. On the other hand, pulp had the lowest concentration of bound phenolic compounds with 2.51 mg/100 g d.w. The four fractions of mango analyzed presented ellagic acid as the highest concentrated compound. It represented the 62.78%, 88.65%, 87.41%, 89.20% in mango pulp, peel, seed, and seed husk, respectively. Besides, mango seed husk was the only fraction where syringic acid and its derivative acetosyringenin were determined.

Table 5. Bound phenolic compounds in mango pulp, peel, seed and husk by HPLC-DAD-QTOF-MS expressed as mg/100 g dry matter.

Peak	Proposed compound	Retention time (min)	Pulp	Peel	Seed	Husk
1	Gallic acid	3.85	0.019±0.001	0.718±0.062	0.310±0.015	0.487±0.017
2	Galloylglucose	6.246	0.368±0.002	0.186±0.016	n.d.	n.d.
3	*p*-hydroxybenzoic acid	9.213	0.079±0.001	0.686±0.078	2.186±0.065	0.326±0.005
4	*p*-hydroxybenzoic acid	9.855	0.426±0.006	0.322±0.006	0.982±0.072	0.186±0.005
5	Vanillic acid	9.984	n.d.	0.089±0.005	n.d.	1.266±0.008
6	Acetosyringenin	12.142	n.d.	n.d.	n.d.	0.164±0.004
7	Syringic acid	12.569	n.d.	n.d.	n.d.	0.396±0.012
8	Protocatechuic acid	14.715	n.d.	0.156±0.006	0.434±0.014	n.d.

9	***p*-hydroxybenzoic acid**	15.159	n.d.	n.d.	0.846±0.027	n.d.
10	**trans *p*-coumaric acid**	15.595	0.013±0.001	0.838±0.007	0.107±0.005	0.723±0.009
11	**cis-*p*-coumaric acid**	16.735	0.011±0.001	0.688±0.006	n.d.	n.d.
12	**Ferulic acid**	17.062	0.018±0.001	0.165±0.003	0.382±0.020	0.721±0.014
13	**Ellagic acid**	17.505	1.579±0.228	29.869±0.015	36.456±2.852	35.315±0.231
	Total		2.515±0.217	33.692±0.022	41.706±2.931	39.586±0.231

4. Concluding remarks

HPLC-DAD-ESI-QTOF-MS has been used to analyze free and bound phenolic and other polar compounds of mango edible part and its by-products (peel, seed, and seed husk). To our knowledge, this is the first time that mango seed husk has been studied to determine its phenolic compounds. This fraction has shown a concentration of free polar compounds that is in the same order than mango pulp, whereas bound phenolic compounds concentration of mango seed husk is similar to mango peel and seed. Besides, the method proposed by HPLC-DAD-ESI-QTOF-MS has demonstrated to be an excellent platform to determine polar compounds in mango. A total of 91 different free polar compounds have been determined among the different mango fractions analyzed. Sixteen of them have been determined for the first time in mango. Seventeen were present for the first time in mango pulp, twenty one in mango peel, and four in mango seed.

It is also important to highlight that this is the first time that mango pulp, seed, and seed husk have been studied to determine bound phenolic compounds. They stand out because ellagic acid is the major compound in the four mango fractions analyzed.

The results obtained in this work encourage the use of mango by-products for the production of functional ingredients. In fact, mango by-products could be used as a source of ellagic acid, especially mango seed that contains around 650 mg/100 g d.w. of ellagic acid in form of free and bound ellagic acid.

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CAPÍTULO 5

Use of HPLC- and GC-QTOF to determine hydrophilic and lipophilic phenols in mango fruit (Mangifera indica L.) and its by-products

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Use of HPLC- and GC-QTOF to determine hydrophilic and lipophilic phenols in mango fruit (*Mangifera indica* L.) and its by-products

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Abstract

Mango industry processing generates high quantities of mango by-products such as peels and seeds (35%-60% of the fruit). Indeed, it is known that mango and its by-products contain different families of bioactive compounds that possess several health benefits. Thus, the aimof this study has been the determination of different families of phenolic derivatives (free and bound phenolic compounds and alk(en)ylresorcinols (ARs)) in mango edible part and its by-products (peel, seed and seed husk) fromthree different cultivars. This is the first study that evaluates the phenolic compounds and ARs in the four fractions of mango of three different cultivars. Special attention has been paid to the determination of anthocyanins and ARs, because these families of compounds had not been studied in depth in mango. In fact, petunidin rutinoside-(*p*-coumaric acid) gallate was found in mango pulp, peel, seed and seed husk of the three cultivars and, it had never been described in mango before. It is also important to highlight that this is the first time that the identification and quantification of ARs have been performed in mango seed and seed husk; besides, four and five out of eleven

alk(en)ylresorcinols detected in peel and pulp, respectively, were identified for the first time in these mango fractions. Furthermore, antioxidant activity was measured by ABTS and FRAP assays. Seed free and bound phenolic extracts showed the highest antioxidant capacity.

Keywords: mango, *Mangifera indica* L., HPLC-DAD-ESI-QTOF-MS, GC-QTOF-MS, phenolic compounds, alk(en)ylresorcinols, by-products.

1. Introduction

Mango (*Mangifera indica* L.), which belongs to the family Anacardiaceae, is grown naturally or mainly cultivated in tropical and subtropical regions. It is one of themost popular edible fruit and its production, at present, ranks seventh in world fruit production, just after watermelons, bananas, apples, grapes, oranges and coconuts[1].

Mango is generally consumed as a dessert fruit, but there has been an increase in consumption of mango products such as juice, nectar, powder, canned mango slices in syrup, and chutneys. Processing of mango fruits generates a significant amount of by-products such as peels and seeds[2], which represent from 35% to 60% of the fruit, approximately. There are many previous research works that have studied the bioactive compounds content such as phenolic compounds, carotenoids, tocopherols, or sterols[3–6]. The daily intake in the diet of these bioactive compounds has been shown to have possible health benefits due to their antiviral, antibacterial, analgesic, anti-inflammatory, and immunomodulatory activities[7]. Indeed, they have demonstrated in vitro antiamoebic activity[8], interesting R-amylase and R-glucosidase inhibitory activities[9] and cardiotonic and diuretic properties[10].

Phenolic compounds are secondary metabolites that are synthesized during normal plant development in response to stress conditions[11,12]. They play a protective role and because of that, they are mainly distributed in the outer layers of the fruit. The main phenolic and other polar compounds identified in mango fruits have been flavonol glycosides (quercetin and kaempferol derivatives and rhamnetin hexoside), xanthone glycosides (mangiferin derivatives), and gallotannin and benzophenone derivatives (maclurin and iriflophenone glycosides). Anthocyanins and alk(en)ylresorcinols (ARs) are

two other important families of phenolic compounds contained in mango that have not been studied in depth yet.

Anthocyanins are widely distributed in most vascular plants where they serve as inducible sunshields[13]. Cyanidin-3-*O*-galactoside and 7-*O*-methylcyanidin 3-*O*-β-D-galactopyranoside are the main anthocyanin compounds found in the red color of mango fruit peel[14,15].

Alk(en)ylresorcinols represent a class of amphiphilic phenolic lipids which are predominantly produced by higher plants such as cereals as well as in mango peels and pulp, peas (*Pisum sativum* L.), and ginkgo (*Ginkgo biloba* L.)[16–20]. Several potential health effects related to the intake of ARs, such as anti-microbial, anti-inflammatory, anti-mutagenic, and anti-carcinogenic activities, have been demonstrated in animal and model systems[19,21–23]. Due to their amphiphilic nature, ARs interact with membranes and DNA and, thus, they have been suggested as potential inhibitors of bacterial, fungal, and protozoal growth[24]. Mango peel and pulp alk(en)ylresorcinols have previously been studied by Geerkens *et al.* (2015)[24] and Kienzle *et al.* (2014)[16]; however, to the best of our knowledge, the present study is the first evidence for their presence in mango seed and husk.

Some studies have shown that the qualitative and quantitative phenolic composition of the extracts studied depends mainly on the variety, the maturity stage and the part of the mango analyzed (peel, pulp or seed)[5,18,25–27].

Taking into account the fewstudies on the determination and quantification of anthocyanins and alk(en)ylresorcinols in mango, and the important role of phenolic compounds in health, the aim of this study was to determine the

free and bound phenolic compounds profile, paying special attention to anthocyanins profile, and alk(en)ylresorcinols profile of pulp, peel, seed and seed husk of three cultivars of mango fruit ('Keitt', 'Osteen' and 'Sensación') by HPLC-DAD-ESI-QTOF-MS and GC-QTOF-MS. Furthermore, the antioxidant activity of the phenolic extracts of the four parts of the three mango cultivars was measured by ABTS and FRAP methods.

2. Materials and methods

2.1. Samples

Three mango cultivars ('Keitt', 'Osteen' and 'Sensación') cultivated under the same agronomical and environmental conditions were provided by Miguel García Sánchez e Hijos, S.A. (Motril, Spain) in July 2015. About 10 kg of fruit from each cultivar at optimal consumption ripeness established by the company (13-16°Brix) were manually separated in pulp, peel, seed and seed husk, and then, freeze-dried in a lyophilizer (Advantage Plus EL-85 freeze dryer, SP Scientific, Ipswich, Suffolk, UK). The samples were milled (IKA M20-IKAWERKE GmbH & Co. KG, Staufen, Germany) and kept at -18 °C until use.

2.2. Chemicals and reagents

HPLC-grade acetic acid, HPLC-MS-grade acetonitrile, hexane, diethyl ether, ethyl acetate and dichloromethane were purchased from Fisher Scientific (Leicestershire, UK), and methanol, ethanol, sodium acetate, hydrochloric acidwere purchased from Panreac (Barcelona, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA).

Double-deionized water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). The following standards and reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA): gallic acid, coumaric acid, ferulic acid, vanillic acid, catechin, quercetin-glucoside, quinic acid, citric acid, mangiferin, ellagic acid, cyanidin 3-*O*-β-D-galactopyranoside, methylbehenate, nonadecylresorcinol, acetic acid, hydroxide sodium, ABTS, potassium persulfate, trolox, TPTZ, ferric chloride and ferrous sulfate. Pyridine was purchased from VWR (Chemicals Prolabo, Fontenaysous-Bois, France). Trimethylchlorosilane and anhydrous sodium sulfate were supplied by Merck KGaA (Darmstadt, Germany), and hexamethyldisilazanewas supplied by Alfa AesarGmbH& Co KG (Karlsruhe, Germany).

2.3. Extraction of the free polar fraction of mango

The extraction of the free polar fraction was carried out according to Gómez-Caravaca *et al.* (2016)[28]. Briefly, 0.5 g of sample powder were dissolved in 10 mL of a solution of methanol/water (80:20, v/v). The mixture was placed in an ultrasonic bath for 15 min and then it was centrifuged for 15 min at 1000 g; the supernatant was removed, and the extraction was repeated twice more.

The supernatants were collected, evaporated, and reconstituted in 3 mL of methanol/water (80:20, v/v). The final extracts were filtered with regenerated cellulose filters 0.2 μm (Millipore, Bedford, MA, USA) and stored at -18 °C until the analyses. Three extraction replicates were done for each sample.

2.4. Extraction of the bound polar fraction of mango

Bound compounds are linked to the cellular walls and they are not taken into account only with a conventional extraction. Thus, a second extraction was performed following the method previously proposed by Verardo *et al.* (2011)[29]. Once the extraction with aqueous methanol was performed to discharge free polar compounds, mango samples were digested with 100 mL of 2 M NaOH. The mixture was then brought to pH 2-3 and extracted with 500 mL of hexane. The final solution was extracted three times with 100 mL of diethyl ether/ethyl acetate (1:1, v/v). The organic fractionswere pooled and evaporated to dryness. The phenolic compounds were reconstituted in 2 mL of methanol/water (1:1 v/v). The final extracts were filtered and stored at -18 °C until the analyses. Three extraction replicates were done for each sample.

2.5. Extraction of alk(en)ylresorcinols in mango

0.5 g of sample powder were extracted using 10 mL of dichloromethane in an ultrasonic bath during 10 min. Afterwards, it was centrifuged for 10 min at 1000 g; the supernatant was removed, and the extraction was repeated twice more. The supernatants were collected, evaporated, reconstituted in 1 mL of dichloromethane and stored at -18 °C until the analyses. Three extraction replicates were done for each sample.

2.6. HPLC-DAD-ESI-QTOF-MS analysis of the polar fraction

The free and bound polar extracts obtained from pulp, peel, seed and seed husk of the three mango cultivars were analyzed by HPLC-DADESI-QTOF-MS. The chromatographic determination was performed by an Agilent 1200

series HPLC (Agilent Technologies, Santa Clara, CA, USA) consisting of a vacuum degasser, a binary pump, an autosampler, a column heater, and a diode array detector (DAD). This instrument was equipped with an Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm) from Agilent Technologies. Separation was performed using different gradient elution programmes depending on the phenolic classes. Previously optimised and validated methodologies were used to perform the analyses.

Anthocyanins were determined using the method proposed by Gómez-Caravaca *et al.* (2013)[30]. Briefly, the mobile phases consisted of 5% formic acid inwater (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 6 min, 12% B; 8 min, 15% B; 9 min, 16% B; 10 min, 17% B; 11 min, 17.5% B; 12 min, 18% B; 13 min, 100% B; 17 min, 100% B; 18 min, 5% B. The initial conditions were maintained for 5 min. The flow rate was set at 0.80 mL/min throughout the gradient. The other phenolic families were determined as described previously[28], briefly, a gradient elution was programmed using as a mobile phase A, acidified water (1% acetic acid), and as a mobile phase B, acetonitrile. The program was as follows: 0 min, 0.8% B; 5.5 min, 6.8% B; 16 min, 20% B; 20 min, 25% B; 25 min, 35% B; 29 min, 100% B; 32 min, 100% B; 34 min, 0.8% B; 36 min, 0.8% B. The column was equilibrated for 3 min before analyses. The sample volume injected was 3 μL and the flow rate used was 0.8 mL/min. UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, 330 nm and 520 nm (for anthocyanins). The HPLC system was coupled to a 6540 Agilent Ultra-High-Definition Accurate-Mass QTOF- MS, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface operating in negative ionmode for the analysis of phenolic compounds, with the

exception of anthocyanins, which were analyzed in the positive mode, at the following conditions: drying gas flow (N2), 12.0 L/min; nebulizer pressure, 50 psi; gas drying temperature, 370 °C; capillary voltage, 3500 V; fragmentor voltage and scan range were 3500 V and *m/z* 50-1500, respectively. Automatic MS/MS experiments were carried out using the following collision energy values: *m/z* 100, 30 eV; *m/z* 500, 35 eV; *m/z* 1000, 40 eV; and *m/z* 1500, 45 eV. Integration and data elaboration were performed using MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA).

Eleven external calibration curves were prepared with the following standards (gallic acid, coumaric acid, ferulic acid, vanillic acid, catechin, quercetin, quinic acid, citric acid, mangiferin, ellagic acid and cyanidin 3-*O*-β-D-galactopyranoside) at eight different concentration levels from the LOQ to 125 μg/mL.

2.7. Antioxidant activity

ABTS and FRAP methods were used to measure the antioxidant activity of the pulp, peel, seed and seed husk of the three mango cultivars. ABTS assay, which measures the reduction of the ABTS radical cation by antioxidants, was based on a method previously described[31]. The samples were analyzed in triplicate and results expressed as μmol Trolox equivalents/g dry sample. The FRAP assay, which measures the capacity of antioxidant compounds to reduce the ferric ions by a single electron-transfer mechanism, was performed according to Al-Duais *et al.* (2009)[32]. The samples were analyzed in triplicate and results expressed as μmol $FeSO_4$ equivalents/g dry sample.

2.8. GC-QTOF-MS analysis

The alk(en)ylresorcinols extracts were silylated according to Sweeley, Bentley, Makita, and Wells (1963)[33] and they were analyzed using a GC-QTOF-MS (Agilent Technologies, Santa Clara, CA, USA) consisting of a gas chromatograph (7890B Agilent Technologies), a QTOF mass spectrometer (7200 Agilent Technologies) and an autosampler (GC Sampler 120 Agilent Technologies). The capillary column was an HP-5ms (30 m × 0.25 mm inner diameter, 0.25 μm film thickness) from Agilent Technologies. The chromatographic conditions of the method were as follows: initial temperature 200 °C; temperature gradient 8 °C min^{-1} until 320 °C was reached and hold temperature during 13 min. The injector temperature was 320 °C and the transfer line temperature was 300 °C. The injection volume was 1 μL in split mode with 1:10 split ratio.

Instrumental conditions used for the MS detector were as follows: acquisition mode, total ion current; ion source temperature, 230 °C; quadrupole temperature, 150 °C; detector voltage, 70 eV; scan range, from 40 to 600 *m/z*; acquisition rate, 4 scans/s; solvent delay time, 6 min. Mass spectrometry grade PFTBA (perfluorotri-n-butylamine) was used for the daily mass calibration. Data were filed and processed by the software MassHunter Workstation (Agilent Technologies, Santa Clara, CA, USA).

Methylbehenate was used as internal standard and a calibration curve was built using nonadecylresorcinol. The calibration curve was built using the analyte/internal standard peak area ratio versus analyte concentration for the GC analysis. ARs were quantified as equivalents of nonadecylresorcinol.

2.9. Statistical analysis

Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson's linear correlation, at the $p < 0.05$ level, were evaluated using Statistica 8.0 software (2007, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Determination of free polar compounds in mango

3.1.1. Identification

Mango pulp, peel, seed and seed husk extracts of 'Keitt', 'Osteen' and 'Sensación' mango cultivars were analyzed using HPLC coupled to DAD and ESI-QTOF-MS in negative and positive ionization modes. Compound identification was performed on basis of their relative retention time, their UV-Vis spectra and mass spectra obtained using Q-TOF-MS together with information previously reported in the literature.

Ninety one compounds were determined in negative mode in the four parts of mango as previously reported by Gómez-Caravaca *et al.* (2016)[28]. Regarding anthocyanins, three compounds were determined in positive mode analysis. The first anthocyanin eluted at retention time 8.74 min and was identified as cyanidin 3-*O*-β-D-galactopyranoside. It was found in the peel of the three cultivars and showed *m/z* 449.1080 and molecular formula $C_{21}H_{20}O_{11}$. It was identified with an error of -0.22 ppm and score 99.66. This compound had already been described in mango by Berardini *et al.* (2005)[14] and corroborated in the present work by comparison with its standard. At retention time 11.152 min and *m/z* 463.1245 it was identified

7-*O*-methylcyanidin-3- *O*-β-D-galactopyranoside with molecular formula $C_{22}H_{22}O_{11}$. It was found in the peel of the three cultivars with an error of -2.42 ppm and score of 96.65. This compound had previously been described in mango by Berardini *et al.* (2005)[15]. A third anthocyanin was tentatively identified at retention time 14.717 min, *m/z* 923.1110 and molecular formula $C_{41}H_{30}O_{25}$ as petunidin rutinoside-(*p*-coumaric acid) gallate. It was found in pulp, peel, seed and seed husk of the three cultivars and, to the best of our knowledge, this is the first time that this compound has been described in mango. The identification of this compound was based on a previous work and the fragments detected[34]. A fragment was observed at *m/z* 317.0640 corresponding to petunidin ($C_{16}H_{13}O_7$), and another fragment was shown at *m/z* 479.1150 corresponding to petunidin glucoside ($C_{22}H_{23}O_{12}$). A third fragment at *m/z* 771.0981 was detected corresponding to the loss of gallate.

3.1.2. Tentative quantification

Quantification of free polar compounds was performed according to the method used by Gómez-Caravaca *et al.* (2016)[28]. Catechin, citric acid, quinic acid, and ellagic acid were quantified with the calibration curves of their own standards. The calibration curve of gallic acid was used to quantify gallates, gallotannins, and maclurin derivatives, coumaric acid for coumaric acid derivatives, ferulic acid for ferulic acid derivatives and other hydroxycinnamic acid derivatives, vanillic acid for vanillic acid derivatives, quercetin for quercetin derivatives, mangiferin for mangiferin derivatives and cyanidin 3-*O*-β-D-galactopyranoside for anthocyanins. **Table 1** shows the quantification of phenolic compounds previously identified in negative and

positive mode. In relation with negative mode, the highest total amount of compounds was found in the peel of the three cultivars (from 1811.43 to 2476.08 mg/100 g dw in 'Keitt' and 'Sensación', respectively). The second fraction with the highest amount of phenolic compounds was seed (1715.27 mg/100 g dw, 1497.34 mg/100 g dw and 1548.67mg/100 g dwin 'Keitt', 'Osteen' and 'Sensación', respectively). However, 'Keitt' cultivar did not show statistical differences between peel and seed fractions. And finally pulp (474.78 mg/100 g dw, 323.50 mg/100 g dw and 502.02 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) and seed husk (301.75 mg/100 g dw, 196.55 mg/100 g dw and 477.54 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) had the lowest content of free polar compounds and according to the statistical analysis they both did not show significant differences.

Table 1. Free polar compounds in mango pulp, peel, seed and husk of 'Keitt', 'Osteen' and 'Sensación' cultivars determined by HPLC-DAD-QTOF-MS expressed as mg/100 g dry matter.

Peak	Proposed compound	Pulp			Peel			Seed			Husk		
		Keitt	Osteen	Sensación	Keitt	Osteen	Sensación	Keitt	Osteen	Sensación	Keitt	Osteen	Sensación
Negative mode													
1	Quinic acid	10.72± 0.81 ab	8.57± 0.3a	11.45± 0.37 ab	70.31± 3.60 g	68.11± 0.58 d	85.74± 1.49 h	63.40± 2.09 c	67.51± 0.62 d	62.53± 1.19 c	28.08± 1.0 e	15.19± 0.52 b	35.55± 1.01 f
2	Citric acid	291.65± 6.76 g	205.43± 7.2 e	242.33± 2.64 f	400.52± 6.14 h	458.55± 5.77 j	452.77± 41.91 i	63.04± 2.0 a	75.15± 3.45 c	59.77± 1.36 a	121.99± 2.11 b	89.93± 3.13 d	110.16± 10.61 b
3	Galloyl diglucoside	n.d.	n.d.	n.d.	22.60± 0.96 c	33.39± 0.62 d	51.81± 5.37 e	3.80± 0.14 a	9.51± 0.19 b	2.54± 0.04 a	n.d.	n.d.	n.d.
4	Galloylglucose	58.97± 1.17 d	48.34± 1.18 h	81.19± 0.41 i	10.35± 0.39 c	7.87± 0.17 bc	14.63± 1.22 e	0.44± 0.06 a	2.71± 0.02 ab	n.q.	34.67± 1.12 g	30.08± 1.33 f	55.87± 5.30 d
5	Galloylglucose isomer I	n.d.	n.d.	n.d.	273.43± 6.99 d	252.03± 1.64 c	372.97± 23.25 e	59.88± 3.18 a	101.36± 2.67 b	63.10± 3.48 a	n.d.	n.d.	n.d.
6	Galloylglucose isomer II	n.d.	n.d.	n.d.	8.33± 0.82 b	7.56± 0.43 a	14.13± 0.009 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	Gallic acid	2.48± 0.03 ab	0.93± 0.59 a	3.58± 0.09 b	12.51± 0.39 b	12.98± 0.003 b	25.62± 2.62 d	18.27± 0.94 e	13.24± 0.71 b	26.08± 1.11 d	2.52± 0.05 ab	1.24± 0.03 ab	3.12± 0.01 ab
8	Vanillic acid glucoside	1.59± 0.08 b	0.59± 0.03 c	2.67± 0.05 d	n.d.	n.d.	n.d.	62.69± 2.76 a	16.86± 0.64 a	n.d.	n.d.	n.d.	n.d.
9	5-galloylquinic acid	0.89± 0.03 a	n.q.	1.14± 0.04 a	194.03± 5.46 d	0.29± 0.02 a	16.66± 0.49 b	n.d.	n.d.	21.89± 0.76 c	3.57± 0.08 a	n.q.	1.13± 0.08 a
10	Galloylglucose isomer III	n.d.	n.d.	n.d.	3.54± 0.41 a	3.09± 0.76 a	5.03± 0.44 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
11	Galloylglucose isomer IV	n.d.	n.d.	n.d.	4.39± 0.55 b	2.53± 0.31 a	4.09± 0.33 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	*p*-hydroxybenzoic acid glucoside	n.d.	n.d.	n.d.	1.53± 0.05 b	0.84± 0.03 a	0.64± 0.05 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

13	***p*-hydroxybenzoic acid glucoside dimer**	3.17± 0.32 c	1.78± 0.13 a	2.67± 0.12 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	**Galloylglucose isomer V**	n.d.	n.d.	n.d.	2.62± 0.34 b	1.80± 0.28 a	3.78± 0.32 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	**Galloyl diglucoside isomer I**	0.062± 0.007 a	n.q.	0.09± 0.003 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	**3-galloylquinic acid**	n.d.	n.d.	n.d.	2.74± 0.16 a	4.33± 0.08 b	4.05± 0.12 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	**Galloyl diglucoside isomer II**	0.163± 0.005 a	0.06± 0.003 a	0.92± 0.04 c	1.90± 0.22 b	2.70± 0.06 b	3.90± 0.40 d	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
18	**Galloyl diglucoside isomer III**	0.585± 0.02 a	0.6± 0.06 a	3.51± 0.10 b	8.00± 0.21 d	12.46± 0.04 e	15.70± 1.0 f	1.43± 0.09 a	5.19± 0.11 c	1.53± 0.07 a	n.d.	n.d.	n.d.
19	**Galloyl diglucoside isomer IV**	1.48± 0.10 a	2.17± 0.12 ab	9.25± 0.36 cd	28.67± 0.91 e	39.33± 0.38 f	63.26± 1.93 g	6.56± 0.32 bc	13.44± 0.33 d	7.46± 0.32 c	0.53± 0.04 a	1.76± 0.06 a	3.13± 0.14 ab
20	**Dihydroxybenzoic acid glucoside**	0.31± 0.01 c	0.17± 0.02 b	1.68± 0.02 e	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.69± 0.03 a	1.21± 0.07 d	0.73± 0.08 a
21	**Galloylglucose isomer VI**	29.50± 1 d	17.7± 0.5 c	38.58± 0.34 f	29.64± 0.52 d	28.35± 0.04 e	18.91± 0.56 c	n.d.	n.d.	n.d.	5.20± 0.23 b	2.76± 0.12 ab	5.77± 0.57 b
22	**Galloyl diglucoside isomer V**	0.18± 0.01 b	0.10± 0.013 a	0.25± 0.01 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
23	**Vanillic acid glucoside isomer I**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.86± 0.14 b	4.47± 0.19 c	2.58± 0.13 a
24	**Dihydroxybenzoic acid glucoside isomer I**	0.74± 0.07 a	1.0± 0.02 b	0.96± 0.05 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	**p-hydroxybenzoic acid glucoside isomer I**	30.45± 0.40 h	10.35± 0.40 d	27.57± 0.44 g	16.66± 0.45 f	6.34± 0.09 c	14.43± 0.78 e	n.d.	n.d.	n.d.	2.86± 0.21 a	1.86± 0.08 a	3.69± 0.32 b
26	**Maclurin C-glucoside**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.64± 0.32	n.q.	n.q.	n.d.	n.d.	n.d.
27	**Digalloylglucose**	2.07± 0.04 a	0.15± 0.01 a	n.q.	45.35± 1.54 c	37.75± 0.20 b	0.53± 0.007 a	n.d.	n.d.	n.d.	0.87± 0.03 a	0.21± 0.02 a	n.q.
28	**Maclurin C-glucoside isomer I**	0.79± 0.03 a	n.q.	n.q.	3.64± 0.45 c	1.05± 0.0001 a	1.70± 0.11 b	8.68± 0.33 e	4.17± 0.17 d	11.77± 0.58 f	n.d.	n.d.	n.d.

29	Vanillic acid glucoside isomer II	2.73± 0.18 d	0.32± 0.003 b	1.34± 0.03 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.34± 0.17 c	4.02± 0.22 e	4.53± 0.18 a
30	Digalloylglucose isomer I	n.q	n.q.	n.q.	2.37± 0.55 a	0.63± 0.09 b	3.21± 0.32 a	n.d.	n.d.	n.d.	n.q.	n.q.	n.q.
31	3,5-digalloylquinic acid	n.d.	n.d.	n.d.	2.69± 0.27	n.q.	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
32	Vanillic acid glucoside isomer III	2.53± 0.16 a	n.q.	0.86± 0.04 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
33	5-(digalloyl)quinic acid	n.d.	n.d.	n.d.	10.12± 0.43 a	n.q.	0.34± 0.04 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	Iriflophenone glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.84± 0.54 c	0.61± 0.05 a	2.13± 0.07 b	n.d.	n.d.	n.d.
35	Ferulic acid hexoside	0.57± 0.04 a	1.50± 0.10 b	1.76± 0.15 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
36	Methylgallate	3.19± 0.10 a	2.92± 1.91 a	10.63± 0.20 a	117.85± 2.74 bc	167.60± 2.65 c	121.77± 0.94 bc	183.98± 9.9 d	271.19± 16.13 e	94.43± 27.21 b	6.01± 0.99 a	7.36± 0.75 a	n.d.
37	Maclurin galloyl glucoside	n.d.	n.d.	n.d.	4.01± 0.62 b	2.36± 0.03 c	4.73± 0.49 d	3.31± 0.15 b	0.86± 0.08 a	0.93± 0.07 a	n.d.	n.d.	n.d.
38	Digallic acid	n.d.	n.d.	n.d.	2.29± 0.01 a	2.84± 0.05 a	10.83± 0.23 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
39	Catechin	3.53± 0.10 cd	4.68± 0.34 bd	1.83± 0.04 a	11.09± 0.42 e	6.37± 0.03 b	27.87± 1.04 h	19.63± 1.05 f	10.91± 0.26 e	25.37± 0.67 g	1.60± 0.08 a	3.00± 0.10 ac	6.08± 0.40 b
40	Hydroxybenzoyl galloyl glucoside	0.37± 0.01 a	n.q.	n.q.	2.22± 0.07 b	0.58± 0.03 a	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
41	Sinapic acid hexoside	0.40± 0.03 b	0.26± 0.01 a	0.34± 0.02 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
42	Coumaric acid hexoside pentoside	0.20± 0.002 b	0.02± 0.00003 a	2.04± 1.18 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
43	Sinapic acid hexoside isomer I	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.24± 0.02 c	0.19± 0.006 a	0.20± 0.03 b
44	Coumaric acid glucoside	1.24± 0.02 d	0.34± 0.03 ab	7.72± 0.59 e	1.54± 0.26 c	2.26± 0.02 c	11.58± 0.26 f	n.d.	n.d.	n.d.	0.16± 0.01 ab	n.q.	0.64± 0.01 b

45	**Trigalloyl glucose**	n.d	n.d.	n.d.	17.04± 0.54 a	25.68± 0.30 b	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
46	**Coumaric acid hexoside pentoside isomer I**	0.17± 0.01 b	0.02± 0.00003 a	1.11± 0.003 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
47	**Coumaric acid glucoside isomer I**	0.89± 0.03 b	0.27± 0.02 a	4.10± 0.02 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
48	**Ferulic acid hexoside isomer I**	0.38± 0.01 b	0.03± 0.005 a	1.18± 0.08 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
49	**Ferulic acid hexoside isomer II**	0.28± 0.01 a	0.63± 0.05 c	0.42± 0.006 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
50	**Sinapic acid hexoside-pentoside**	12.00± 0.65 d	5.33± 0.15 bc	5.14± 0.12 bc	31.26± 1.12 f	50.99± 0.45 g	24.15± 0.81 e	n.d.	n.d.	n.d.	8.88± 0.17 cd	3.07± 0.23 ab	2.83± 0.02 ab
51	**Dihydro sinapic acid hexoside-pentoside**	5.48± 0.17 a	7.99± 0.23 ab	16.40± 0.52 d	21.76± 0.95 e	51.15± 0.76 f	62.59± 0.45 g	n.d.	n.d.	n.d.	7.64± 0.24 ab	10.33± 0.72 ab	11.73± 0.53 bd
52	**Ferulic acid hexoside isomer II**	0.21± 0.002 b	0.01± 0.00001 a	0.67± 0.04 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
53	**Mangiferin**	n.d	n.d.	n.d.	6.60± 0.49 b	4.14± 0.01 a	29.78± 0.61 e	72.75± 1.62 g	22.48± 0.32 d	50.58± 0.93 f	3.56± 0.09 a	1.98± 0.02 a	16.75± 1.13 c
54	**Maclurin digalloyl glucoside**	n.d	n.d.	n.d.	1.89± 0.08 c	0.53± 0.03 a	1.30± 0.05 b	3.76± 0.17 d	0.47± 0.04 a	1.26± 0.07 b	n.d.	n.d.	n.d.
55	**Trigalloyl glucose isomer I**	n.d	n.d.	n.d.	1.22± 0.11 a	1.53± 0.06 a	1.45± 0.03 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
56	**Coumaroyl galloyl glucoside**	n.d	n.d.	n.d.	5.05± 0.14 a	2.34± 0.01 b	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
57	**Methoxylmangiferin**	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	3.03± 0.35 b	2.24± 0.008 a	4.43± 0.23 c	n.d.	n.d.	n.d.
58	**Tetragalloyl glucose**	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
59	**Syringic acid**	n.d	n.d.	n.d.	7.47± 0.18 ac	24.09± 0.27 b	13.76± 0.73 c	25.44± 1.86 b	54.47± 0.89 d	29.36± 0.74 b	n.d.	n.d.	n.d.
60	**Eriodictyol**	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	5.35± 0.47 b	n.q.	2.98± 0.10 a	n.d.	n.d.	n.d.

61	Methyl-digallate ester	0.55± 0.004 a	0.26± 0.003 a	2.17± 0.03 a	46.73± 2.40 b	66.55± 1.52 b	50.70± 1.25 b	33.75± 1.37 d	47.65± 0.81 b	19.61± 0.82 c	1.26± 0.23 a	1.11± 0.03 a	1.54± 0.13 a
62	Tetragalloyl glucose isomer I	n.d	n.d.	n.d.	1.87± 0.11 b	4.71± 0.14 a	4.03± 0.39 a	2.00± 0.12 b	1.04± 0.05 c	3.52± 0.09 a	n.q.	n.q.	0.12± 0.01 d
63	Coumaroyl galloyl glucoside isomer I	n.d	n.d.	n.d.	4.43± 0.14 a	4.35± 0.04 b	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
64	Tetragalloyl glucose isomer II	n.d	n.d.	n.d.	6.37± 0.36 de	5.76± 0.23 bc	4.54± 0.08 b	5.63± 0.15 cd	6.78± 0.52 e	2.14± 0.10 f	0.13± 0.02 a	0.13± 0.01 a	0.07± 0.005 a
65	Tetragalloyl glucose isomer III	n.d	n.d.	n.d.	1.12± 0.07 b	1.41± 0.08 b	2.37± 0.29 c	0.46± 0.06 a	0.67± 0.03 a	n.q.	n.q.	n.q.	n.q.
66	Ellagic acid	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	637.78± 20.44 d	224.28± 11.58 b	835.65± 29.01 e	58.39± 3.79 c	20.73± 0.17 a	202.47± 14.28 b
67	Coumaroyl galloyl glucoside pentoside	n.d	n.d.	n.d.	2.24± 0.45 a	2.0± 0.003 a	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
68	7-*O*galloyltricetiflavan	n.d	n.d.	n.d.	n.d.	n.d.	n.d.	9.85± 0.48 b	5.09± 0.12 a	12.26± 0.25 c	n.d.	n.d.	n.d.
69	Quercetin glucoside	n.d	n.d.	n.d.	32.62± 4.91 a	120.78± 1.52 b	188.69± 1.62 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
70	Quercetin galactoside	n.d	n.d.	n.d.	16.25± 2.06 a	77.31± 1.25 b	120.17± 1.13 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
71	Pentagalloyl glucose	1.63± 0.05 a	1.03± 0.01 a	3.72± 0.25 a	24.02± 2.78 b	36.63± 0.16 b	33.44± 0.13 b	153.83± 5.04 d	207.41± 13.38 e	92.60± 7.95 c	0.78± 0.02 a	0.22± 0.02 a	0.83± 0.09 a
72	Quercetin xyloside	n.d	n.d.	n.d.	6.93± 1.17 a	24.24± 0.36 b	39.18± 4.13 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
73	Quercetin arabinopyranoside	n.d	n.d.	n.d.	4.35± 0.12 b	16.52± 0.34 a	14.97± 0.39 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
74	Methyl-digallate ester isomer	2.42± 0.35 a	1.27± 0.01 a	11.98± 0.39 a	174.89± 5.54 bc	222.06± 1.57 b	192.43± 1.68 b	114.46± 4.92 e	150.88± 3.59 c	70.09± 3.70 d	5.61± 0.96 a	4.70± 0.11 a	6.94± 0.69 a
75	Coumaroyl galloyl glucoside pentoside isomer I	n.d	n.d.	n.d.	1.85± 0.57 a	1.99± 0.11 a	n.q.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
76	Quercetin arabinofuranoside	n.d	n.d.	n.d.	4.19± 0.12 b	19.41± 0.43 a	17.59± 0.56 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

77	**Hexagalloylglucose**	n.q.	n.q.	0.22± 0.00002 a	20.28± 0.88 c	56.45± 0.90 e	77.90± 0.81 b	63.47± 4.56 f	82.79± 1.95 b	33.02± 2.03 d	n.q.	n.q.	0.02± 0.002 a
78	**Hexagalloylglucose isomer I**	n.q.	n.q.	0.37± 0.007 a	17.44± 0.89 b	51.56± 0.61 cd	73.81± 0.76 e	33.20± 2.28 c	46.56± 2.94 d	10.0± 0.04 ab	n.q.	n.q.	n.q.
79	**Hexagalloylglucose isomer II**	n.q.	n.q.	0.15± 0.007 a	11.74± 0.67 bc	29.05± 0.45 d	36.26± 0.50 e	20.95± 1.89 cd	29.91± 2.38 e	7.98± 0.34 b	n.q.	n.q.	n.q.
80	**Hexagalloylglucose isomer III**	n.q.	n.q.	n.q.	3.33± 0.12 ab	27.70± 0.52 e	10.97± 0.26 cd	7.69± 0.53 bc	15.54± 0.26 de	0.98± 0.13 a	n.q.	n.q.	n.q.
81	**Hexagalloylglucose isomer IV**	n.d.	n.d.	n.d.	0.46± 0.05 a	4.60± 0.10 b	3.02± 0.24 b	2.79± 0.20 b	10.25± 0.23 c	0.54± 0.07 a	n.q.	n.q.	n.q.
82	**Heptagalloylglucose isomer I**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
83	**Heptagalloylglucose isomer II**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
84	**Heptagalloylglucose isomer III**	n.q.	n.q.	n.d.	3.71± 0.44 a	10.39± 0.70 b	35.04± 2.72 c	n.q.	n.q.	n.q.	n.d.	n.d.	n.d.
85	**Heptagalloylglucose isomer IV**	n.q.	n.q.	n.d.	7.35± 0.55 b	17.54± 0.15 c	48.18± 5.98 d	2.32± 0.16 ab	0.59± 0.27 a	2.19± 0.20 ab	n.d.	n.d.	n.d.
86	**Heptagalloylglucose isomer V**	n.q.	n.q.	n.d.	7.80± 0.72 b	29.02± 0.22 c	44.86± 4.69 d	3.31± 0.46 ab	2.03± 0.19 a	5.24± 0.94 ab	0.18± 0.02 a	n.q.	0.44± 0.02 a
87	**Heptagalloylglucose isomer VI**	n.q.	n.q.	n.d.	5.27± 0.40 b	15.94± 1.67 c	28.43± 3.20 d	n.q.	0.05± 0.02 a	1.03± 0.0006 a	n.d.	n.d.	n.d.
88	**Heptagalloylglucose isomer VII**	n.d	n.d	n.d	4.56± 0.40 a	23.33± 0.07 b	20.32± 1.56 b	n.q.	0.022± 0 a	0.51± 0.04 a	n.d.	n.d.	n.d.
89	**Heptagalloylglucose isomer VIII**	n.d	n.d	n.d	1.38± 0.22 a	6.54± 0.34 b	7.38± 2.82 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
90	**Ethyl 2,4-dihydroxy-3-(3,4,5-trihydroxybenzoyl) oxybenzoate**	0.21± 0.04 a	n.q.	n.q.	7.17± 0 d	n.q.	n.q.	5.85± 0.73 c	10.85± 0.35 b	11.30± 0.15 b	0.14± 0.004 a	n.q.	n.q.
91	**Rhamnetin hexoside**	n.d.	n.d.	n.d.	5.82± 0.27 a	5.22± 0.07 b	28.03± 0.19 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	TOTAL	474.78± 11.32 a	323.50± 14.31 a	502.02± 1.45 a	1811.43± 99.53 bc	2281.30± 4.42 c	2476.08± 330.15 d	1715.27± 24.62 b	1497.34± 44.22 b	1548.67± 39.21 b	301.75± 6.99 a	196.55± 11.15 a	477.54± 20.63 a

Positive mode													
1	**Cyanidin 3-*O*β-D-Galactopyranoside**	n.d.	n.d.	n.d.	0.138± 0.002 a	0.753± 0.002 b	1.108± 0.024 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	**7-*O*Methylcyanidin 3-*O*β-D-galactopyranoside**	n.d.	n.d.	n.d.	0.582± 0.022 a	0.692± 0.001 b	3.627± 0.092 c	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	**Petunidin rutinoside (*p*-coumaric acid) gallate**	0.545± 0.027 ce	0.505± 0.028 ce	0.699± 0.005 f	0.424± 0.003 abc	0.375± 0.011 ab	0.588± 0.082 ef	0.463± 0.041 bc	0.373± 0.001 ab	0.437± 0.003 abc	0.320± 0.023 ad	0.245± 0.013 d	0.329± 0.011 ad
	TOTAL	0.545± 0.027 d	0.505± 0.028 cd	0.699± 0.005 f	1.144± 0.017 g	1.820± 0.008 h	5.323± 0.034 i	0.463± 0.041 cd	0.373± 0.001 be	0.437± 0.003 ce	0.320± 0.023 ab	0.245± 0.013 a	0.329± 0.011 ab

* Different letters in the same raw indicate significant differences ($p<0.05$)

Regarding cultivars and according to the statistical analysis no significant differences were found between 'Keitt', 'Osteen' and 'Sensación' in pulp, seed and seed husk. However, there were statistical differences among cultivars in peel fraction. 'Sensación' showed the highest quantity of total free polar compounds (2476.08 mg/100 g dw), followed by 'Osteen' (2281.30 mg/100 g dw) and 'Keitt' (1811.43 mg/100 g dw). These concentrations are in the order of the compounds quantified in mango and other tropical fruit by-products such as pineapple and passion fruit[35].

The compound present in the highest content in peel of the three cultivars was citric acid which represented a similar percentage in the three cultivars, 22.11%, 20.1%, and 18.29% in 'Keitt', 'Osteen' and 'Sensación', respectively. Some gallic acid derivatives were also present in a high concentration such as galloylglucose isomer I (15.09%, 11.05% and 15.06% in 'Keitt', 'Osteen' and 'Sensación' respectively), methyldigallate ester isomer (9.65%, 9.73% and 7.77%, in the three cultivars respectively) or methylgallate (6.51%, 7.35% and 4.92%, in the three cultivars respectively). Besides, 5-galloylquinic acid was only found in high concentration in 'Keitt' cultivar (11%), whereas it was quite low in 'Osteen' (0.01%) and 'Sensación' (0.67%). The most abundant families found in peel fraction were gallotannins and flavonoids.

The main mango seed compound was ellagic acid, which represented 37.50% in 'Keitt', 14.98% in 'Osteen' and "53.96% in 'Sensación' cultivar. Ellagic acid has been reported to have some health benefit suchas antioxidant, estrogenic/anti-estrogenic, antimicrobial, anti-inflammatory, anti-cancer and prebiotic effects[36–38] and due to the high amount found, mango seed could be a significant source of this compound. Some gallic acid

derivatives were also found in high quantity in the three cultivars such as methylgallate (10.73%, 18.11% and 6.10%, respectively), pentagalloyl glucose (8.97%, 13.85% and 5.98%, respectively) and methyl-digallate ester isomer (6.67%, 10.08% and 4.53%, respectively). Besides, mangiferin quantified in seed (72.75 mg/100 g dw, 22.48 mg/100 g dw and 50.58 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) was higher than mangiferin in peel (6.60 mg/100 g dw, 4.14 mg/100 g dw and 29.78 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) or seed husk (3.56 mg/100 g dw, 1.98 mg/100 g dw and 16.75 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively), except in 'Osteen' peel and 'Keitt' and 'Osteen' seed husk which did not show significant differences.

Citric acid was the major polar compound found in pulp of the three mango cultivars. It represented 60.38% in 'Keitt', 63.50% in 'Osteen' and 48.27% in 'Sensación'. In addition to citric acid, galloylglucose (58.97 mg/100 g dw, 48.34 mg/100 g dw and 81.19 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) and galloylglucose isomer VI (29.50 mg/100 g dw, 17.73 mg/100 g dw and 8.58 mg/100 g dw in 'Keitt', 'Osteen' and 'Sensación', respectively) were the following compounds in terms of concentration in the pulp of the three cultivars.

Related to seed husk the main compounds depended on the cultivar. Citric acid was the highest compound in 'Keitt' and 'Osteen', whereas ellagic acid was the main compound in 'Sensación'. The percentages of citric acid were 40.10%, 45.75% and 23.07% in 'Keitt', 'Osteen' and 'Sensación' cultivars, respectively, and ellagic acid percentages were 19.19%, 10.55% and 42.40% in 'Keitt', 'Osteen' and 'Sensación' cultivar, respectively. Quinic acid and

galloylglucose were also two major compounds in mango seed husk of the three cultivars.

Regarding compounds identified in positive mode, the concentration of anthocyanins in peel was higher than in the other mango fractions, followed by pulp, seed and seed husk. 'Sensación' showed the highest anthocyanins content in peel (5.323 mg/100 g dw), followed by 'Osteen' (1.820 mg/100 g dw) and 'Keitt' (1.144 mg/100 g dw). Cyanidin 3-*O*-β-D-galactopyranoside and 7-*O*-methylcyanidin 3-*O*-β-D-galactopyranoside were only detected in mango peel. Cyanidin 3-*O*-β-D-galactopyranoside concentration ranged 0.138-1.108 mg/100 g dw and it was in the order of the quantification reported in mango peel by Berardini *et al.* (2005)[14]. The only anthocyanin identified in pulp, seed and husk was petunidin rutinoside-(*p*-coumaric acid)-gallate. 'Sensación' had the highest concentration followed by 'Keitt' and 'Osteen' in mango pulp and peel, whereas 'Keitt' showed the highest concentration in seed, although there were not significant differences with 'Sensación'. Its content in pulp was in the range 310.11-698.58 μg/100 g dw, whereas the concentration in peel was between 375.05 and 588.03 μg/100 g dw, in seed ranged from 373.17 to 463.10 μg/100 g dw and in husk 244.60-329.11 μg/100 g dw. Cultivars did not show significant differences in seed husk fraction.

3.2. Determination of bound phenolic compounds in mango

3.2.1. Identification

Mango extracts were analyzed with the method proposed in **Section 2.6** in negative ionization mode in order to identify bound phenolic compounds.

Compound identification was performed according to their relative retention time values, their UV–Vis spectra andmass spectra obtained using QTOF-MS together with information previously reported in the literature. Thirteen bound phenolic compounds were identified and reported as previously described by Gómez-Caravaca *et al.* (2016)[28].

3.2.2. Tentative quantification

Table 2 shows the quantification results of bound phenolic compounds. The seed of 'Keitt' and 'Sensación' presented the highest concentration of total compounds (42,340.03 μg/100 g dw and 51,118.04 μg/100 g dw, respectively), followed by 'Keitt' and 'Sensación' seed husk, which also had a high amount of bound phenolic compounds (39,586.72 μg/100 g dw and 29,389.95 μg/100 g dw, respectively). However, there were no statistical differences between seed and seed husk in 'Keitt'. Next fractions in terms of concentration were 'Keitt' and 'Sensación' peel (33,828.19 μg/100 g dw and 15,559.52 μg/100 g dw, respectively) and, finally, 'Keitt' and 'Sensación' pulp had the lowest content of bound polar compounds (2121.82 μg/100 g dw and 3057.52 μg/100 g dw, respectively). Regarding 'Osteen' peel was the fraction with the highest content of bound polar compounds (35,431.94 μg/100 g dw), followed by seed husk (19,794.45 μg/100 g dw), then seed (11,325.20 μg/100 g dw) and finally pulp (3826.76 μg/100 g dw).

Table 2. Bound polar compounds in mango pulp, peel, seed and husk of 'Keitt', 'Osteen' and 'Sensación' cultivars determined by HPLC-DAD-QTOF-MS expressed as μg/100 g dry matter.

Peak	Proposed compound	Pulp			Peel			Seed			Husk		
		Keitt	Osteen	Sensación	Keitt	Osteen	Sensación	Keitt	Osteen	Sensación	Keitt	Osteen	Sensación
1	Gallic acid	19.23± 1.29 b	78.41± 0.61 d	16.46± 1.60 b	772.30± 9.01 g	940.58± 9.01 h	261.75± 0.31 c	310.42± 15.23 a	317.57± 0.50 a	333.04± 9.61 a	487.78± 17.46 e	263.43± 1.23 c	556.37± 34.59 f
2	Galloylglucose	367.85± 2.57 e	382.68± 1.18 f	35.99± 2.96 b	200.90± 2.31 c	208.86± 3.21 d	13.20± 1.63 a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	*p*-hydroxybenzoic acid isomer I	79.41± 1.19 b	412.32± 1.36 a	96.21± 0.99 b	754.49± 8.20 c	968.36± 2.78 e	307.40± 2.86 a	2186.12± 65.22 h	1379.60± 114.54 f	835.62± 5.57 cd	326.48± 5.92 a	1979.40± 2.47 g	913.25± 3.09 de
4	*p*-hydroxybenzoic acid isomer II	426.78± 6.73 c	195.48± 14.16 a	51.56± 1.40 e	322.08± 6.59 b	173.97± 4.31 a	541.69± 3.97 f	982.15± 72.18 g	729.36± 42.06 d	760.07± 22.68 d	186.31± 5.78 a	245.32± 3.96 a	383.56± 32.34 bc
5	Vanillic acid	n.d.	n.d.	n.d.	89.20± 5.48 b	30.47± 3.00 a	48.21± 4.00 ab	n.d.	n.d.	n.d.	1266.11± 8.75 d	1508.96± 2.99 e	1128.39± 83.72 c
6	Acetosyringenin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	164.37± 4.28 b	243.29± 0.44 c	152.24± 1.46 a
7	Syringic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	396.03± 12.40 b	351.86± 3.78 a	461.44± 3.65 c
8	Protocatechuic acid	n.d.	n.d.	n.d.	156.98± 5.99 a	158.44± 11.63 a	254.52± 4.33 b	434.24± 14.41 d	479.05± 0.66 e	295.82± 17.90 c	n.d.	n.d.	n.d.
9	*p*-hydroxybenzoic acid isomer III	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	846.12± 27.51 c	259.42± 10.22 a	720.43± 27.30 b	n.d.	n.d.	n.d.
10	Trans *p*-coumaric acid isomer	13.51± 0.24 a	15.31± 0.27 a	5.40± 0.03 a	838.90± 6.79 d	764.95± 63.80 cd	1081.61± 8.30 e	107.88± 5.27 b	129.33± 1.89 b	155.55± 3.75 b	723.07± 9.17 c	1367.13± 6.54 f	791.56± 73.22 cd
11	Cis *p*-coumaric acid isomer	11.15± 0.36 b	8.42± 0.03 ab	4.51± 0.26 ab	688.88± 6.11 c	752.87± 11.01 d	861.14± 5.64 e	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	Ferulic acid	9.72± 0.71 a	16.68± 0.77 a	9.91± 0.13 a	135.05± 3.30 c	80.58± 2.09 b	106.98± 0.40 bc	382.81± 20.03 e	368.37± 35.59 de	341.11± 5.91 d	721.31± 14.57 g	980.67± 0.12 h	588.78± 3.77 f
13	Ellagic acid	1194.17± 1.62 a	2717.46± 23.32 a	2837.47± 287.83 a	29869.40± 15.53 c	31368.11± 2178.66 c	12083.01± 180.20 b	36456.61± 2852.53 d	7521.86± 704.67 e	47182.66± 536.48 g	35315.26± 231.62 d	12854.40± 458.43 b	24414.36± 309.36 f
	TOTAL	2121.82± 14.71 a	3826.76± 41.70 a	3057.52± 295.21 a	33828.19± 15.80 b	35431.94± 2107.03 b	15559.52± 138.70 e	42340.03± 2983.67 c	11325.20± 870.56 d	51118.04± 572.04 h	39586.72± 231.98 c	19794.45± 453.86 f	29389.95± 366.58 g

* Different letters in the same raw indicate significant differences ($p<0.05$)

Ellagic acid was the main compound in the four fractions in all the cultivars. 'Sensación' seed presented the highest percentage (92.3%) which was quite similar to pulp (90.44%). 'Osteen' had the highest percentage in peel (88.53%) and 'Keitt' presented percentages very similar in seed husk (89.21%), peel (88.30%) and seed (86.10%). Besides, syringic acid and acetosyringenin were only determined in seed husk in the three cultivars.

3.3. Antioxidant activity

ABTS and FRAP methods were used in order to evaluate the antioxidant capacity of the methanolic extracts of the free and bound polar fractions: ABTS based on a hydrogen transfer mechanisms (HAT) and FRAP based on single electron transfer (SET).

Table 3 shows the antioxidant activity results for each mango fraction and cultivar extract. Regarding free phenolic compounds extracts, seed showed the highest antioxidant capacity, followed by peel, pulp and finally seed husk showed the lowest value of antioxidant activity. Pearson correlation showed correlation between total free polar compounds and antioxidant activity by FRAP in the four mango fractions ($r=0.7847$, $p=0.003$; $r=0.6331$, $p=0.027$; $r=0.9850$, $p=0.0001$; $r=0.8321$, $p=0.003$; in pulp, seed, seed husk and peel, respectively). However, statistical analysis did not find a correlation between total free phenolic compounds and antioxidant activity by ABTS method in seed and peel ($r=0.6627$, $p=0.019$; $r=-0.3076$, $p=0.331$; $r=0.9521$, $p=0.0001$; $r=0.4013$, $p=0.250$, in pulp, seed, seed husk and peel, respectively).

Table 3. Antioxidant activity of pulp, peel, seed and husk of 'Keitt', 'Osteen' and 'Sensación' cultivars determined by FRAP ($FeSO_4$ (µM)/mg dw) and ABTS (µM eq Trolox/mg dw) methods.

		FREE		BOUND	
		FRAP [FeSO4 (µM)/mg dw]	ABTS [µM eq Trolox /mg]	FRAP [FeSO4 (µM)/mg dw]	ABTS [µM eq Trolox /mg]
PULP	Keitt	116.9±8.0	61.4±6.4	0.85±0.04	0.79±0.01
	Osteen	94.1±4.8	59.2±2.9	1.07±0.17	0.99±0.04
	Sensación	174.9±4.4	102.3±9.0	1.11±0	1.08±0.05
PEEL	Keitt	620.4±14.6	475.4±8.7	3.25±0.50	1.56±0.13
	Osteen	570.9±26.0	468.2±4.8	3.81±0.52	2.04±0.19
	Sensación	996.4±30.6	486.5±1.0	2.84±0.82	1.53±0.15
SEED	Keitt	2062.6±213.2	484.3±1.0	19.41±2.20	29.08±1.88
	Osteen	1795.8±46.9	482.8±6.0	22.83±1.56	20.96±0.43
	Sensación	1822.6±58.7	485.6±2.7	20.35±1.80	20.09±3.17
HUSK	Keitt	59.8±4.7	48.6±3.1	12.17±1.73	10.15±1.42
	Osteen	35.0±1.5	26.7±1.3	17.37±0.5	11.59±0.60
	Sensación	83.1±1.7	60.7±1.0	10.78±0.37	10.74±0.73

Regarding to bound phenolic compound extracts, seed was the fraction with the highest antioxidant activity, followed by seed husk, peel and pulp. Statistical analysis correlated total bound phenolic compounds and antioxidant activity in husk (r=-0.7345, p=0.007; r=-0.8442, p=0.001, in FRAP and ABTS, respectively).With respect to seed, there was correlation between total bound phenolic compounds and antioxidant activity measured by FRAP (r=-0.6491, p=0.022) but not by ABTS (r=0.2225, p=0.487). Phenolic compounds in pulp (r=0.1988, p=0.536; r=-0.1080, p=0.738, in FRAP and ABTS, respectively) and peel (r=-0.0253, p=0.945; r=0.3203, p=0.367, in FRAP and ABTS, respectively) did not show any correlation with antioxidant activity. These results could be justified by the different

mechanism of the two methods or by the presence, in the extracts, of other compounds that react with the radicals.

3.4. Determination of alk(en)ylresorcinols in mango

3.4.1. Identification

As **Table 4** shows, eleven ARs were identified in mango fruit. The mass spectra of all the trimethylsilyl (TMS) derivatives of ARs identified showed a base fragment at *m/z* 268, due toMcLafferty rearrangement of the phenolic ring, and another fragment at *m/z* 281[39]. Peaks 1 and 2 were identified as pentadecenylresorcinols (C15:1). Their *m/z* were 462.3358 and 462.3342, respectively, and they were detected at retention times 9.141 and 9.172 min, respectively. Peak 1 was detected in all the fractions and in all the cultivars, except in seed of 'Sensación'; whereas peak 2 was found only in 'Sensación' peel. Peak 3 at 9.293 min and *m/z* 464.3540, was identified as pentadecylresorcinol (C15:0). It was found in all the parts of mango and in all the cultivars, except in 'Sensación' seed and seed husk. At retention time 10.690 min and *m/z* 488.3524, heptadecadienylresorcinol (C17:2) was detected (peak 4). Three heptadecenylresorcinols (C17:1) belonging to peaks 5, 6 and 8 were detected at retention times 10.780, 10.825 and 10.873 and their *m/z* was 490.3662. Compounds 5 and 6 were found in all the fractions of the three cultivars, whereas compound 8 was only found in 'Keitt' pulp and seed husk. Heptadecylresorcinol (C17:0) (peak 7) was found at 10.859 min and m/z 492.3819. It was only detected in peel of 'Keitt'.

Table 4. Alk(en)ylresorcinols identified in mango pulp, peel, seed and husk determined by GC-QTOF-MS.

Peak	Proposed compound	Alkyl chain	Retention Time (min)	Silylated molecular formula	m/z experimental	m/z calculated	Fragments
1	Pentadecenylresorcinol	C15:1	9.14	$C_{27}H_{50}O_2Si_2$	462.3358	462.3349	268; 281
2	Pentadecenylresorcinol	C15:1	9.17	$C_{27}H_{50}O_2Si_2$	462.3342	462.3349	268; 281
3	Pentadecylresorcinol	C15:0	9.29	$C_{27}H_{52}O_2Si_2$	464.3540	464.3506	268; 281
4	Heptadecadienylresorcinol	C17:2	10.69	$C_{29}H_{52}O_2Si_2$	488.3524	488.3506	268; 281
5	Heptadecenylresorcinol	C17:1	10.78	$C_{29}H_{54}O_2Si_2$	490.3696	490.3662	268; 281
6	Heptadecenylresorcinol	C17:1	10.82	$C_{29}H_{54}O_2Si_2$	490.3694	490.3662	268; 281
7	Heptadecylresorcinol	C17:0	10.86	$C_{29}H_{56}O_2Si_2$	492.3813	492.3819	268; 281
8	Heptadecenylresorcinol	C17:1	10.87	$C_{29}H_{54}O_2Si_2$	490.3659	490.3662	268; 281
9	Nonadecenylresorcinol	C19:1	12.28	$C_{31}H_{58}O_2Si_2$	518.3950	518.3975	268; 281
10	Nonadecenylresorcinol	C19:1	12.39	$C_{31}H_{58}O_2Si_2$	518.3986	518.3975	268; 281
11	Nonadecenylresorcinol	C19:1	12.44	$C_{31}H_{58}O_2Si_2$	518.3989	518.3975	268; 281

Three nonadecenylresorcinols (C19:1) corresponding to peaks 9, 10 and 11 were identified at retention times 12.276, 12.389, 12.439 min, and *m/z* 518.3975. Peak 9 was found in all the fractions of the three cultivars, except in seed husk of 'Osteen' cultivar. Peak 10 was found in all the parts of all the cultivars except in pulp and seed husk of 'Sensación', whereas compound 11 was found in every part of the three mango cultivars.

Four out of eleven ARs detected in mango peel and five out of eleven ARs detected in pulp have been identified for the first time in this work. Seven of them were previously reported in peel by other authors[24,40] and six of them were identified previously in mango pulp by Geerkens *et al.*, (2015)[41].

3.4.2. Tentative quantification

The method performed was used to quantify ARs in mango pulp, peel, seed and seed husk of the three cultivars.

The results of the quantification of ARs in mango are showed in **Table 5**. Mango peel was the fraction that presented the highest amounts of ARs. 'Osteen' peel had the highest amount of ARs (1897.65 μg/g dw), followed by 'Sensación' peel (1172.85 μg/g dw) and 'Keitt' peel (1059.92 μg/g dw), although the two last did not show significant differences between them. Next fraction in content of ARs was 'Keitt' seed husk (694.23 μg/g dw). Concerning 'Osteen' and 'Sensación' cultivars, next fraction in content of ARs was pulp (292.19 μg/g dw and 165.11 μg/g dw, respectively), then seed husk (111.82 μg/g dw and 152.12 μg/g dw, respectively) and finally seed had the lowest concentration of ARs (34.40 μg/g dw and 10.85 μg/g dw, respectively). However, in 'Keitt' cultivar, after mango peel the next fraction with the highest amount of ARs were pulp and seed husk (203.90 μg/g dw) and finally seed presented the lowest content of ARs (30.07 μg/g dw). From a statistical point of view there were no significant differences between pulp and seed in the three cultivars and seed husk in 'Osteen' and 'Sensación'.

The main compounds found in peel in the three cultivars were heptadecadienylresorcinol (C17:2) (peak 4) and two heptadecenylresorcinols (C17:1) (peaks 5 and 6). 'Sensación' peel had the highest amount of compound 4, 602.26 μg/g dw which represent 51.35% of the total ARs. 'Osteen' and 'Keitt' cultivars also presented a high quantity of compound 4, 284.73 μg/g dw (15%) and 224.77 μg/g dw (21.21%), respectively. Regarding compound 5, 'Osteen'was the cultivar with the highest quantity and it had 596.07 μg/g dw (31.41%). 'Keitt' also presented a high concentration of compound 5 with 330.23 μg/g dw (31.16%). Peak 6 was also an important compound in the three cultivars. 'Osteen' had the highest percentage, 46.06% (873.99 μg/g dw). 'Sensación' presented 35.44% (415.69 μg/g dw) of compound 6 and 'Keitt' 25.61% (271.40 μg/g dw).

Besides, compound 7, another heptadecylresorcinol (C17:1) was only found in 'Keitt' peel which represented 10.97% (116.29 μg/g dw), a significant percentage of the total.

With respect to pulp, 'Osteen' had the highest amount of total ARs (292.19 μg/g dw), followed by 'Keitt' (203.90 μg/g dw) and 'Sensación' (165.11 μg/g dw) without significant differences. Compounds 4, 5 and 6 were also the most important in pulp. 'Osteen' had the highest concentrations of compounds 5 and 6 (102.82 μg/g dw and 154.62 μg/g dw, respectively), whereas 'Sensación' had the highest concentration of compound 4 (94.41 μg/g dw).

Regarding seed husk, 'Keitt' was the cultivar with the highest content in AR (694.23 μg/g dw), followed by 'Sensación' (152.12 μg/g dw) and 'Osteen' (111.82 μg/g dw). 'Keitt' had significant quantity of compounds 4, 5, 6, 8 and 11. On the one hand, compound 5 was the majority (180.42 μg/g dw), followed by compounds 4 and 8 (131.63 μg/g dw and 128.07 μg/g dw). On the other hand, compound 8 was only detected in 'Keitt' pulp and seed husk. Compounds 5 and 6 were also the main compounds in 'Osteen' and 'Sensación' cultivars.

Table 4. Alk(en)ylresolcinols in pulp, peel, seed and husk of 'Keitt', 'Osteen' and 'Sensación' mango cultivars determined by GC-QTOF-MS expressed as μg/g dry matter.

		1 (C15:1)	2 (C15:1)	3 (C15:0)	4 (C17:2)	5 (C17:1)	6 (C17:1)	7 (C17:0)	8 (C17:1)	9 (C19:1)	10 (C19:1)	11 (C19:1)	TOTAL
Peel	**Keitt**	12.35± 0.52 bc	n.d.	56.68± 1.34 c	224.77± 12.27 c	330.23± 25.94 f	271.40± 12.50 c	116.29± 8.54 a	n.d.	1.71± 0.15 e	1.89± 0.16 d	44.61± 3.86 b	1059.92± 65.28 c
	Osteen	16.50± 0.09 c	n.d.	110.76± 4.56 e	284.73± 14.48 c	596.07± 65.56 g	873.99± 89.69 e	n.d.	n.d.	4.95± 0.04 f	5.73± 0.09 e	4.92± 0.27 a	1897.65± 174.77 e
	Sensación	3.87± 0.30 a	1.77± 0.15 a	61.13± 5.74 d	602.26± 46.68 d	75.88± 2.93 bcd	415.69± 32.05 d	n.d.	n.d.	5.38± 0.31 g	1.20± 0.10 cd	5.66± 0.39 a	1172.85± 88.66 c
Pulp	**Keitt**	26.56± 0.36 c	n.d.	6.71± 0.11 a	36.98± 0.33 a	55.70± 1.69 abc	50.03± 1.92 a	n.d.	17.02± 0.43 a	0.30± 0.01 ab	0.41± 0.01 ab	10.19± 0.14 a	203.90± 21.58 ab
	Osteen	1.74± 0.18 a	n.d.	6.29± 0.57 a	25.48± 1.59 a	102.82± 7.75 de	154.62± 12.07 b	n.d.	n.d.	0.17± 0.01 ab	0.57± 0.06 abc	0.50± 0.05 a	292.19± 14.53 b
	Sensación	0.54± 0.04 a	n.d.	7.33± 0.34 a	94.41± 4.02 b	7.82± 0.27 a	53.70± 2.39 a	n.d.	n.d.	0.71± 0.04 bc	n.d.	0.60± 0.06 a	165.11± 7.16 ab
Seed	**Keitt**	0.53± 0.02 a	n.d.	0.36± 0.01 a	2.13± 0.13 a	8.12± 0.67 a	4.53± 0.36 a	n.d.	n.d.	7.74± 0.27 h	5.13± 0.06 e	2.06± 0.04 a	30.07± 1.90 ab
	Osteen	0.96± 0.02 a	n.d.	0.88± 2.07 a	2.96± 0.22 a	14.32± 0.46 ab	12.54± 0.72 a	n.d.	n.d.	1.14± 0.02 cde	1.27± 0.10 abcd	0.35± 0.02 a	34.40± 1.42 ab
	Sensación	n.d.	n.d.	n.d.	0.84± 0.08 a	3.29± 0.14 a	3.41± 0.33 a	n.d.	n.d.	1.59± 0.10 de	1.45± 0.14 bcd	0.26± 0.01 a	10.85± 0.22 a
Husk	**Keitt**	8.26± 0.87 ab	n.d.	33.76± 2.46 b	131.63± 1.91 b	180.42± 19.65 e	118.83± 1.32 b	n.d.	128.07± 6.99 b	1.37± 0.14 cd	2.05± 0.07cd	89.85± 3.85 b	694.23± 8.60 d
	Osteen	0.59± 0.05 a	n.d.	2.83± 0.28 a	5.57± 0.53 a	37.83± 2.28 ab	64.70± 4.80 a	n.d.	n.d.	n.d.	0.18± 0.01 ab	0.14± 0.01 a	111.82± 7.96 a
	Sensación	5.67± 0.27 ab	n.d.	n.d.	n.d.	86.23± 8.34 cd	59.47± 4.12 a	n.d.	n.d.	0.51± 0.01 abc	n.d.	0.24± 0.01 a	152.12± 12.74 ab

* Different letters in the same column indicate significant differences ($p<0.05$)

4. Conclusions

In summary, HPLC-DAD-ESI-QTOF-MS and GC-QTOF-MS have demonstrated to be adequate tools to identify and quantify free and bound phenolic compounds and ARs in pulp, peel, seed and seed husk of three mango cultivars named 'Keitt', 'Osteen' and 'Sensación'. This is the first study that evaluates the phenolic compounds and ARs in the four fractions of mango of three different cultivars.

Concerning free phenolic compounds, to our knowledge, the anthocyanin petunidin rutinoside-(*p*-coumaric acid) gallate, has been described in peel, pulp, seed and seed husk of mango for the first time. Concentrations of free phenolic compounds were higher in peel followed by seed, seed husk and pulp. 'Sensación' was the cultivar with the highest total free phenolic compounds in peel, pulp and seed husk, whereas 'Keitt' was the one with the highest amount in seed. Regarding bound phenolic compounds, seed, peel and seed husk were the fractions with the highest concentrations. 'Keitt' showed the highest quantity of total bound phenolic compounds in seed and seed husk, whereas 'Osteen' presented the highest quantity in peel and pulp.

It is important to highlight that this is the first time that the identification and quantification of ARs have been performed in mango seed and seed husk. As far as we know, four out of eleven ARs detected in mango peel and five out of eleven ARs detected in pulp have been identified for the first time. Peel, seed and seed husk were the fractions with the highest concentration of ARs. 'Osteen' cultivar showed the highest concentration of ARs in peel, seed and pulp, but 'Keitt' presented the highest quantity in seed husk.

Therefore, results obtained in this work confirm mango by-products (seed, peel and seed husk) as rich sources of bioactive compounds and encourage their use in the production of functional ingredients.

5. Appendix A. Supplementary data

Structure and names of alk(en)ylresorcinols identified in mango fruit

R	Retention time (min)	Proposed compound
C15:1	9.14	Pentadecenylresorcinol
C15:1	9.17	Pentadecenylresorcinol
C15:0	9.29	Pentadecylresorcinol
C17:2	10.69	Heptadecadienylresorcinol
C17:1	10.78	Heptadecenylresorcinol
C17:1	10.82	Heptadecenylresorcinol
C17:0	10.86	Heptadecylresorcinol
C17:1	10.87	Heptadecenylresorcinol
C19:1	12.28	Nonadecenylresorcinol
C19:1	12.39	Nonadecenylresorcinol
C19:1	12.44	Nonadecenylresorcinol

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CAPÍTULO 6

Comparison of two stationary phases for the determination of sterols and tocopherols in mango edible part and its by-products by GC-QTOF-MS

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Abstract

Two different GC-QTOF-MS methodologies were carried out for the analysis of phytosterols and tocopherols in the edible part of three mango cultivars and their by-products (pulp, peel and seed). To that end, a non-polar column (HP-5ms) and a mid-polar column (Rtx-200MS) were used. The analysis time for Rtx-200MS was much lower than the one obtained with HP-5ms. Furthermore, the optimized method for Rtx-200MS column had a higher sensibility and precision of peak area than HP-5ms methodology. However, Rtx-200MS produced an overlapping between β-sitosterol and Δ^5-avenasterol. Four phytosterols and two tocopherols were identified in mango samples. As far as we are concerned, this is the first time that phytosterols have been studied in mango peel and that Δ^5-avenasterol has been reported in mango pulp. α- and ϒ-tocopherol were determined in peel, and α-tocopherol was the major tocopherol in this fraction (up to 81.2%); however only α-tocopherol was determined in pulp. Peel was the fraction with the highest total concentration of sterols followed by seed and pulp, and 'Sensación' was the cultivar with the highest concentration of total phytosterols in most of the cases. There were no significant differences between quantification of tocopherols with both columns. However, in most cases quantification of phytosterols was higher with Rtx-200MS than with HP-5ms column.

Keywords: mango, GC-QTOF-MS, phytosterols, tocopherols, by-product, unsaponifiable.

1. Introduction

Mango (*Mangifera indica* L.), a member of Anacardiaceae family in the order of Sapindales is native from Southeast of Asia and is widely cultivated at both tropical and subtropical latitudes[1]. There are more than 70 genera, and 1000 varieties, and it is considered one of the most important tropical fruit[2]. Presently, mango is cultivated on an area of approximately 3.7 million ha worldwide. Besides, mango fruit is in the 2nd position as a tropical crop, only behind bananas in terms of production and acreage used[3].

Mango fruits provide energy, dietary fibre, carbohydrates, proteins, fats and phenolic compounds, which are vital to normal human growth, development and health. Moreover, it has been well documented that mango fruits are an important source of micronutrients, vitamins such as tocopherols and other phytochemicals such as phytosterols[4–6]. It is well known that phytosterols produce a wide spectrum of biological activities in human. Particularly, phytosterols are considered an efficacious cholesterol-lowering agent[7,8].

Mango puree, slices in syrup, nectar, leather, pickles, canned slices and chutney are the main industrial products obtained from mango fruits. The main by-products produced in the processing of mango fruits are peels and seeds. Peels represent between 7% and 24% of the total weight of mango fruit[9,10] and depending on the cultivar, kernel represents between 45% and 85% of the seed and approximately 20% of the whole fruit[11–13]. These by-products have been recognized as natural source of bioactive phenolic compounds and their use in a commercial purpose could be an important and sustainable opportunity for reducing pollution from bio-wastes[14].

In the past decade, fats from mango, in particular mango seeds, have been extracted, fractionated, and evaluated. In fact, the interest about mango fat has widely increased due to its unique physicochemical characteristics, which are similar to those of cocoa butter[3,13,15,16]. However, there is no information about the distribution of sterols and tocopherols in mango edible part and its by-products.

Phytosterols and tocopherols have previously been studied in mango seed[5,17,18] and tocopherols have also been studied in mango pulp and peel[19,20]. However, as far as we are concerned, there is only one previous work of phytosterols in mango pulp[6].

Thus, the aim of this study has been the optimization and comparison of two different GC-QTOF-MS methodologies for the analysis of phytosterols and tocopherols in pulp, peel and seed of three different mango cultivars: 'Keitt', 'Osteen' and 'Sensación'. To our knowledge, this is the first time that phytosterols have been studied in mango peel.

2. Materials and methods

2.1. Samples

Mangoes were provided by Miguel García Sánchez e Hijos, S.A. (Motril, Spain) from October 2015 harvest. About 10 kg of mango fruits of the cultivars 'Keitt', 'Osteen' and 'Sensación' were manually separated in peel, pulp and seed. The samples were freeze-dried in a lyophilizer (Advantage Plus EL-85 freeze dryer, SP Scientific, Ipswich, Suffolk, UK) and, then, milled and kept at -18 ºC until use.

2.2. Chemicals and reagents

Ethanol, potassium hydroxide, diethyl ether, *n*-hexane and isopropanol were purchased from Fisher Scientific (Leicestershire, UK). Double-deionized water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). The following phenolic standards and reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA): α-tocopherol, β-sitosterol, stigmasterol, campesterol, dihydrocholesterol. Pyridine was purchased from VWR Chemicals Prolabo (France). Trimethylchlorosilane and anhydrous sodium sulphate were supplied by Merk KGaA (64271 Darmstadt, Germany), and hexamethyldisilazane was supplied by Alfa Aesar GmbH & Co KG (Germany).

2.3. Isolation of unsaponifiable fraction by hot saponification

Hot saponification was performed according to Caligiani *et al.*[21] with some modifications. Briefly, 1.0 mL of dihydrocholesterol (c=1.0 mg/mL) was added to 15 g of sample powder and saponification was carried out by boiling and stirring for 1 h with 100 mL of 1.0 N potassium hydroxide in ethanol-water solution (4/1 v/v). The sample was transferred to a separating funnel and 100 mL of distilled water was added. Then, a extraction was carried out with 50 mL of diethyl ether and repeated four times. The ether extracts were pooled into a separating funnel and washed four times with 50 mL of distilled water. The organic phase was dried with anhydrous sodium sulphate, filtered, dried in a rotary evaporator and the residue was weighed and kept at -18 ºC until use.

2.4. Determination of phytosterols and tocopherols by GC-QTOF-MS

Phytosterols and tocopherols were analysed in a gas chromatograph coupled to a quadrupole-time of flight mass spectrometer (GC-QTOF-MS) (Agilent Technologies, Santa Clara, CA, USA) consisting of a gas chromatograph (7890B Agilent Technologies) a QTOF mass spectrometer (7200 Agilent Technologies) and an autosampler (GC Sampler 120 Agilent Technologies).

Two methodologies were developed to separate the phytosterols and tocopherols from the different parts of mango by GC-QTOF-MS. To that end, two GC columns were used: HP-5MS (Agilent Technologies, Santa Clara, CA, USA) and Rtx-200MS (Restek Corporation, Bellefonte, PA, USA). HP-5MS is a non-polar column with very low bleed characteristics; this column has been widely used for the determination of sterols and tocopherols. Rtx-200MS is a mid-polar column that offers exceptional thermal stability, low bleed, and superior inertness, even for active compounds. This column has also been used for the analysis of sterols in the last years[22].

Before the analysis, the samples were silylated according to Sweeley *et al.*[23]. Separations were carried out with different methods depending on the capillary column used.

2.4.1. HP-5ms column

The first column used was an HP-5ms (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) from Agilent Technologies. The chromatographic conditions of the method were as follows: initial temperature 250 °C; temperature gradient 10 °C min^{-1} until reach 270 °C and hold temperature

during 25 min; temperature gradient 10 °C min^{-1} until reach 310 °C and hold temperature during 5 min;. The injector temperature was 280 °C and the transfer line temperature was 300 °C. The injection volume was 1µL in split mode with 1:10 split ratio. The carrier gas was helium at a flow rate 1.2 mL/min.

2.4.2. Rtx-200MS column

The second column was an Rtx-200MS (30m × 0.25 mm inner diameter, 0.25 µm film thickness) from Restek Corporation. The chromatographic conditions of the method were as follows: initial temperature 210 °C; temperature gradient 15 °C min^{-1} until reach 260 °C and hold temperature during 10 min; temperature gradient 15 °C min^{-1} until reach 310 °C and hold temperature during 2 min. The injector temperature was 280 °C and the transfer line temperature was 300 °C. The injection volume was 1µL in split mode with 1:50 split ratio. The carrier gas was helium at a flow rate 1.2 mL/min.

Instrumental conditions used for the MS detector were as follows: acquisition mode, total ion current; ion source temperature, 230 °C; detector voltage, 70 eV; scan range, from 50 to 700 *m/z*; scan speed, 1250 u/s; solvent delay time, 6 min. Data were filed and processed by the software MassHunter Workstation (Agilent Technologies, Santa Clara, CA, USA).

3. Results and discussion

3.1. Optimization of GC-QTOF-MS methods

The GC methods were optimized using a real sample extract of mango peel in full scan mode.

Temperature of the injection port was checked to establish the considered optimal one. It was ranged between 200 and 300 ºC and 280 ºC was selected as optimal for both columns. Furthermore, the gradient of temperatures of the oven and the gas flow were tested to choose the best compromise between resolution and analysis time. The initial column temperature was checked for both columns (150-250 ºC) and 210 ºC was selected for Rtx-200MS, whereas 250 ºC was chosen for HP-5ms. The higher polarity of Rtx-200MS column provided a lower retention of compounds in the stationary phase and a lower resolution among them. Thus, a lower initial temperature was needed to obtain a good separation. After that, an increase of temperature was required until reach an isothermal step: 260 ºC for Rtx-200MS and 270 ºC for HP-5ms. The elution of the compounds of interest was produced in this step; the analysis time for Rtx-200MS was much lower than the one obtained with HP-5ms; however, a proper resolution between peaks was achieved with HP-5ms whereas Rtx-200MS produced an overlapping between β-sitosterol and Δ^5-avenasterol. An isothermal step below 260 ºC produced an excessive retention of the peaks increasing their width and the resolution was not significantly improved. Then, the final column temperature was optimized and 310 ºC was selected for both columns.

The parameters related to the mass detection method were optimized taking into account the area of the MS signal of the standards compounds available (α-tocopherol, β-sitosterol, stigmasterol, campesterol and dihydrocholesterol). These parameters were chosen to get a compromise solution to obtain the maximum signal for most of the peaks detected in the samples under study.

3.1. Methods validation

Four calibration curves were prepared using the following standards: tocopherol, campesterol, stigmasterol and β-sitosterol. Dihydrocholesterol was used as internal standard (IS). Calibration curves were built using the standard/IS peak area ratio versus standard concentration. Standards were injected under the optimum conditions of the method found for both HP-5ms and Rtx-200MS columns. **Table 1** summarizes the different parameters of each standard compound for both columns. All calibration curves showed good linearity between different concentrations depending on the analytes in question. The calibration plots reveal good correlation between peak areas and analyte concentrations, and the regression coefficients were always higher than 0.99.

Table 1. Analytical parameters of the method proposed.

Analyte	Column	Calibration Range (µg/mL)	Calibration equations	R^2	LOD (µg/mL)	LOQ (µg/mL)	Accuracy (% RSD)		
							0.1 µg/mL	50 µg/mL	100 µg/mL
Campesterol	HP	LOD-100	y = 175991x - 692519	0.9962	0.883	2.942	99.3	97.5	99.7
	Rtx	LOD-100	y = 245482x - 89617	0.9913	0.578	1.925	98.9	98.8	99.1
Stigmasterol	HP	LOD-100	y = 172642x - 699942	0.9961	0.900	2.999	100.1	99.4	99.6
	Rtx	LOD-100	y = 325223x - 200715	0.9900	0.589	1.963	99.7	100.2	99.9
β-sitosterol	HP	LOD-100	y = 119251x + 444578	0.9944	1.304	4.342	98.2	96.1	100.4
	Rtx	LOD-100	y = 180878x - 101182	0.9952	0.852	2.841	99.0	98.3	99.5
β-tocopherol	HP	LOD-100	y = 124615x - 807670	0.9958	1.247	4.155	98.7	99.2	97.6
	Rtx	LOD-100	y = 186088x - 178465	0.9989	0.816	2.719	97.6	99.3	98.7

LOD and LOQ were calculated based on *S/N* ratio. They were established injecting the standard solutions by the injection of the smallest amounts which provide *S/N*=3 for LOD and *S/N*=10 for LOQ. LOD was found to be within the range 0.883-1.304 µg/mL whereas LOQ was within 2.942-4.342 µg/mL for HP-5ms column, whilst for Rtx-200MS column LOD was within 0.578-0.852 µg/mL and LOQ was within 1.925-2.841 µg/mL. As it can be seen, LOD and LOQ of the method optimized using the Rtx-200MS column were lower than those obtained for HP-5ms column. Therefore, it can be said that the optimized method for the analysis of tocopherols and sterols using Rtx-200MS column has a higher sensibility than the HP-5ms methodology.

Method accuracy was determined by the closeness of the test value to the nominal value and was evaluated with separately prepared individual primary stock solutions, mixtures and working solutions of all standards. It was calculated over the linear dynamic range at three concentration levels: low (0.1 µg/mL), medium (50 µg/mL), high (100 µg/mL) via three assays per

concentration on different days. The analyte concentrations were calculated from calibration curves and, accuracy was calculated by the ratio of this calculated concentration versus the theoretical one (**Table 1**). The recoveries were very close to 100% (96.1% to 100.4%) for both columns used.

Moreover, intraday and interday repeatability were developed to assess the performances of the method, taken into account the standards at the three levels of concentration. Because of that, one mango extract was injected (n=3) during the same day (intraday precision) for 7 consecutive days (interday precision, n=21). The relative standard deviations (%RSD) of analysis time and peak area were determined (**Table 2**) for Rtx-200MS and HP-5ms columns. Intra- and interday precision of analysis time were very similar for both columns, whereas the intra- and interday precision of peak area were lower for HP-5ms compared to Rtx-200MS.

Table 2. Intraday and interday repeatability (expressed as % RSDs) of the area and retention time of the compounds, in the HP-5ms and Rtx-200MS columns.

Compound	HP				Rtx			
	Intraday RT	Intraday peak area	Interday RT	Interday peak area	Intraday RT	Intraday peak area	Interday RT	Interday peak area
α-tocopherol	0.06-0.10	0.84-1,97	0.17-0.23	2.53-3.14	0.01-0.03	0.99-2.41	0.015-0.023	1.27-2.14
Campesterol	0.04-0.09	0.05-0.08	0.019-0.22	1.35-1.76	0.005-0.02	0.98-1.49	0.009-0.017	1.51-1.99
Stigmasterol	0.02-0.13	0.47-0.68	0.20-022	1.29-1.54	0.02-0.03	0.85-1.73	0.013-0.027	1.67-1.96
β-sitosterol	0.02-0.07	0.52-0.71	0.19-0.23	1.31-1.50	0.01-0.03	0.97-1.4	0.014-0.025	1.87-2.61

3.2 Identification of phytosterols and tocopherols of mango fractions

Both optimized methodologies were applied to mango samples and peak identification was done (**Table 3**).

Six compounds: two tocopherols and four phytosterols were detected in mango samples. The identification of every compound was corroborated with its standard as exception of ϒ-tocopherol and Δ^5-avenasterol. ϒ and α–tocopherols eluted in the first place. ϒ-tocopherol (peak 1) at *m/z* 488, presented a fragment at *m/z* 223 [M-255] due to the loss of the side chain ($C_{16}H_{33}$) and another fragment at *m/z* 263 [M-(225+40)], because of the cleavage of the side chain accomplished by the breakdown of chroman structure with hydrogen rearrangement and loss of a methyl acetylene fragment[24]. This compound was only detected in mango peel. α-Tocopherol (peak 2), at *m/z* 502, showed fragments at *m/z* 237 and 277 due to a similar fragmentation pattern previously indicated for ϒ-tocopherol. α-Tocopherol was detected in mango pulp and peel. Previous research reports the presence of α -tocopherol in pulp[6].

Table 3. Phytosterols and tocopherols identification in mango pulp, peel and seed by GC-QTOF-MS.

Peak	Proposed compound	Retention time (min)		m/z experimental (M^+)		m/z calculated (M^+)	Fragments	Molecular Formula	Pulp	Peel	Seed
		HP	Rtx	HP	Rtx						
1	**ϒ-tocopherol**	8.47	9.368	488.4010	488.4037	488.4050	263/223	$C_{31}H5_6O_2Si$	-	✓	-
2	**α-tocopherol**	11.282	10.518	502.4170	502.4170	502.4206	277/237	$C_{32}H_{58}O_2Si$	✓	✓	-
3	**Campesterol**	14.201	11.860	472.4074	472.4095	472.4100	382/367/343	$C_{31}H5_6OSi$	✓	✓	✓
4	**Stigmasterol**	15.110	12.040	484.4070	484.4073	484.4100	394/379/355	$C_{32}H5_6OSi$	✓	✓	✓
5	**β-sitosterol**	17.029	12.882	486.4220	486.4243	486.4257	396/381/357	$C_{32}H_{58}OSi$	✓	✓	✓
6	**Δ^5-avenasterol**	17.523	17.584	484.4070	484.4075	484.4100	386	$C_{32}H5_6OSi$	✓	✓	✓

All of the identified phytosterol were detected in pulp, peel and seed of mango. The first phytosterol (peak 3) at *m/z* 472, was identified as campesterol. It showed three typical fragments: at *m/z* 213, due to the the

loss of the side chain (SD) and the D ring; *m/z* 129 and its complement *m/z* 343 [M-129]$^+$. Other fragments found were *m/z* 315 [M-SC-2]$^+$, *m/z* 255 [M-SC-90]$^+$, *m/z* 253 [M-SC-90-2]$^+$, 457 [M-CH3]$^+$, *m/z* 382 [M-TMSOH]$^+$ and *m/z* 367 [M-CH3-TMSOH]+. This fragmentation pattern is in agreement with previous literature and also with the spectra of its standard[25,26].

Peak 4 at *m/z* 484 and fragments at *m/z* 469, 394, 379, 355, 343, 255, 253, 213 and 129, that coincide with the same fragmentation pattern as campesterol, was identified as stigmasterol. This identification was corroborated by the injection of its standard and it is in agreement with Pelillo *et al.*[25]

β-sitosterol was detected at *m/z* 486 (peak 5) and differents fragments at *m/z* 471, 396, 381, 343, 357, 255, 253, 213 and 129 were detected according to Pelillo *et al.* [25]. The identification was also confirmed by its standard.

Campesterol, stigmasterol and β-sitosterol were previously reported in mango pulp and seed[5,6].

Last phytosterol determined in mango was Δ^5-avenasterol (peak 6) at *m/z* 484 and with the typical fragments at 469, 394, 379, 355, 343, 255, 253, 213 and 129. Besides, it showed an important fragment at *m/z* 386 corresponding to [M-98]$^+$. This compound was previously reported in seed[5] and, as far as we are concerned, this is the first time that Δ^5-avenasterol has been identified in mango peel and pulp.

On the one hand, it is interesting to highlight that Rtx-200MS column was not able to clearly separate Δ^5-avenasterol in any fraction of mango. On the other hand, total time of analysis was almost 5 min shorter using Rtx-200MS than HP-5ms.

To our knowledge, this is the first time phytosterols and tocopherols have been analyzed in mango peel; therefore, this is the first time that these compounds have been identified and quantified in mango peel. Furthermore, as far as we are concerned, Δ^5-avenasterol has been reported in mango pulp for the first time in this work.

3.3. Quantification of phytosterols and tocopherols of mango fractions

The methods performed were used to quantified phytosterols and tocopherols in mango pulp, peel and seed. The results of the phytosterols and tocopherols quantification in different mango fractions and cultivars are showed in **Table 4**.

In relation to tocopherols, they only were present in peel and pulp; however, peel exhibited higher total concentration of tocopherols than pulp. The concentration of tocopherols was 74.4-79.8% higher in peel for the different cultivars when HP-5MS was used and 75.5-79.4% when Rtx-200MS was used. Regarding cultivars, 'Sensación' presented the highest content in total tocopherols in all the fractions studied, followed by 'Keitt' and 'Osteen'. α and ϒ-tocopherol were determined in peel, whereas α-tocopherol was only identified in pulp. α-tocopherol was the major tocopherol in peel. Its percentage with HP-5ms column was 77.39 %, 72.28 % and 75.10 %, in 'Keitt', 'Osteen' and 'Sensación', respectively, and with Rtx-200MS column was 81.19 %, 72.75 % and 73.77 %, in 'Keitt', 'Osteen' and 'Sensación', respectively.

Table 4. Phytosterols and tocopherols quantification in mango pulp, peel and seed of 'Keitt', 'Osteen' and 'Sensación' cultivars measured with HP and Rtx columns by GC-QTOF-MS expressed as μg/g dry matter.

		Peel			Pulp			Seed		
		Keitt	Osteen	Sensación	Keitt	Osteen	Sensación	Keitt	Osteen	Sensación
ϒ-tocopherol	**HP**	6.13±0.41b	7.09±0.17c	14.41±0.48d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Rtx	5.19±0.20b	6.22±0.07c	17.73±0.60d	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
α-tocopherol	**HP**	20.99±0.74f	18.47±0.39e	43.45±1.38g	6.72±0.17c	5.16±0.14b	14.81±0.26d	n.d.	n.d.	n.d.
	Rtx	22.39±1.06e	16.61±0.31b	49.86±1.20f	6.62±0.69d	4.71±0.17c	16.56±0.40b	n.d.	n.d.	n.d.
TOTAL TOCOPHEROLS	**HP**	27.12±1.15c	25.56±0.56c	57.86±1.86e	6.72±0.17b	5.16±0.14b	14.81±0.26d	n.d.	n.d.	n.d.
	Rtx	27.58±1.26f	22.83±0.38e	67.59±1.80g	6.62±0.69c	4.71±0.17b	16.56±0.40d	n.d.	n.d.	n.d.
Campesterol	**HP**	62.89±0.76c	58.80±1.43b	57.16±1.57b	51.84±0.43f	45.06±1.11e	62.62±0.73c	39.09±1.36d	25.53±0.32a	27.79±1.56a
	Rtx	58.25±2.38c	54.01±2.51ac	53.06±2.30a	52.82±1.71a	45.10±0.78e	63.04±1.78f	39.23±1.44d	25.50±0.89b	28.97±2.18b
Stigmasterol	**HP**	47.84±4.54c	42.40±0.75b	61.62±0.94f	8.48±0.42a	7.21±0.40a	14.23±0.41f	47.73±1.87c	42.02±0.55b	35.37±1.31e
	Rtx	57.38±1.96b	42.54±1.96f	86.09±3.31g	14.61±1.09d	8.15±0.32c	20.77±1.51e	57.33±2.14b	51.58±1.04a	49.82±3.67a
β-sitosterol	**HP**	442.92±18.11e	473.89±9.07d	472.89±9.07d	180.49±3.59a	172.24±1.15a	239.88±1.83b	299.56±8.47c	244.61±3.21b	293.19±5.83c
	Rtx	504.65±12.77c	565.28±15.34f	497.59±11.01c	217.66±3.02a	218.29±2.58a	290.25±11.43b	382.73±6.23d	307.68±6.00b	421.81±11.03e
Δ^5-avenasterol	**HP**	12.23±0.91a	18.91±0.44b	11.55±0.93a	13.15±0.36a	20.52±1.44b	26.79±0.82c	27.31±0.68c	5.36±0.09d	34.30±2.39e
	Rtx	22.98±1.76ad	30.73±2.38ab	16.16±0.96c	18.13±1.28cd	25.94±2.82ab	27.76±2.29ab	30.08±2.57b	7.87±0.78e	43.27±4.55f
TOTAL STEROLS	**HP**	565.88±24.32e	594.00±11.69f	603.41±12.37g	253.96±4.80a	245.03±4.10a	343.52±3.79d	413.69±12.38b	317.52±4.17c	390.65±11.09b
	Rtx	643.26±18.87f	692.56±22.19c	652.90±21.98c	311.44±7.10a	297.48±6.50a	401.82±17.01b	509.37±12.38d	392.63±8.71b	543.87±21.43e

Different letters in the same raw indicate significant differences ($p<0.05$)

Regarding phytosterols, peel was also the fraction with the highest total concentration of sterols followed by seed and pulp. 'Sensación' was the cultivar with the highest concentration of total phytosterols in most of the cases; although, the analyses performed with Rtx-200MS column in peel fraction showed the highest amount in 'Osteen' cultivar. Furthermore, analyses with HP-5ms column in seed showed the highest total phytosterols concentration in 'Keitt' cultivar. The most abundant phytosterol in mango was β-sitosterol. It reached with HP-5ms column in peel 78.27 %, 79.77 % and 78.40 % in 'Keitt', 'Osteen' and 'Sensación', respectively, and with Rtx-200MS column 78.45 %, 81.62 % and 76.21 % in 'Keitt', 'Osteen' and 'Sensación', respectively. Its percentages in seed with HP-5ms column were 72.41 %, 77.04 % and 75.05 % in 'Keitt', 'Osteen' and 'Sensación', respectively and 75.14 %, 78.37 % and 77.56 % in 'Keitt', 'Osteen' and 'Sensación', respectively, with Rtx-200MS column. Regarding pulp, β-sitosterol percentages with HP-5ms column were 71.07 %, 70.29 % and 69.83 % in 'Keitt', 'Osteen' and 'Sensación', respectively, and with Rtx-200MS column were 71.78 %, 73.38 % and 72.23 % in 'Keitt', 'Osteen' and 'Sensación', respectively.

Concentrations of tocopherols and sterols in mango are in the same range of concentrations that results found by Vilela et al.[6] in pulp and Jin et al.[27] in seed. However, differences observed can mainly be attributed to agronomical and environmental factors.

It is important to notice that there was no difference between quantification of tocopherols with both columns. However, in most cases quantification of phytosterols was higher with Rtx-200MS column than with HP-5ms column.

Statistical analyses were used to determine the correlation between quantifications carried out with HP-5ms and Rtx-200MS columns. The results showed that there was a high correlation between total tocopherols and total sterols concentration found with HP-5ms and Rtx-200MS columns, respectively ($r=0.9931$; $p<0.01$ and $r=0.9707$; $p<0.01$, respectively).

4. Conclusions

Two methodologies have been developed for the determination of tocopherols and sterols in pulp, peel and seed of three mango cultivars ('Keitt', 'Osteen' and 'Sensación') by GC-QTOF-MS using two columns with different stationary phases. The analysis time was shorter using Rtx-200MS column than with HP-5ms. Moreover, the optimized Rtx-200MS method allowed obtaining a higher sensibility and precision of peak area than the HP-5ms methodology. However, Rtx-200MS column gave cause to an overlapping between β-sitosterol and Δ^5-avenasterol.

Four phytosterols and two tocopherols were identified in the different mango fractions studied and no significant differences were found for the quantification results of tocopherols obtained with the two columns. Nevertheless, in most cases quantification of phytosterols was higher with Rtx-200MS column than with HP-5ms column. Indeed, the results showed that there was a high correlation between total tocopherols and total sterols concentration found with HP-5ms and Rtx-200MS columns

Mango fat has demonstrated similar characteristics to cacao butter; however, there is a lack of information about the distribution of some of the families of mango fat in mango fruit. Thus, this work provides evidences about the distribution of sterols and tocopherols in mango pulp, peel and seed.

Besides, this is the first time that sterols have been studied in mango peel and that Δ^5-avenasterol has been reported in mango pulp.

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Conclusiones

1. Se han determinado, por primera vez, los compuestos fenólicos de la parte aérea de *Satureja montana* subsp. *kitaibelii* mediante HPLC-DAD-ESI-TOF-MS. Esta técnica ha demostrado ser una potente herramienta para la identificación y cuantificación de los compuestos fenólicos y otros compuestos polares en esta matriz. Se determinaron un total de 45 compuestos fenólicos y ácidos orgánicos, de los cuales 42 no habían sido descritos anteriormente en esta matriz. Los principales compuestos cuantificados fueron ácidos fenólicos y flavonoides, siendo el más abundante el ácido clorogénico (471.13 mg/100 g m.s.). Además se determinó la actividad antioxidante mediante DPPH y ABTS y se comprobó la existencia de una correlación positiva entre los resultados de DPPH con los compuestos fenólicos totales analizados mediante HPLC. Estos resultados han demostrado que la parte aérea de *Satureja montana* subsp. *kitaibelii* podría ser un buen ingrediente funcional debido a su elevado contenido natural en compuestos antioxidantes.

2. Se ha llevado a cabo la identificación y cuantificación de 24 compuestos fenólicos y otros compuestos polares mediante HPLC-DAD-Q-TOF en pulpa, piel y tubérculo completo de 2 variedades de patata: 'Blue Bell' y 'Melody'. Esta ha sido la primera vez que estas variedades se han estudiado con el fin de conocer la distribución de estos compuestos en el tubérculo de patata. Además, se ha utilizado por primera vez el analizador QTOF-MS acoplado a HPLC-DAD con una columna core-shell para la determinación de la fracción polar de patata, obteniéndose tiempos reducidos de análisis. Por ello, esta técnica ha demostrado ser una herramienta valiosa y una buena alternativa para la determinación de los compuestos fenólicos de la patata. La fracción con mayor contenido de compuestos fenólicos fue la piel. Por último, se realizaron 3 ensayos de medida de la actividad antioxidante

(ABTS, FRAP y ORAC) y se observó una correlación positiva del contenido en compuestos fenólicos de todas las fracciones de patata con los 3 ensayos. Por todo ello, ya que la piel de patata se considera un desecho en la industria de la patata (ej. patatas fritas, chips, etc), esta merecería mayor atención, ya que podría ser reutilizada con el fin de aprovechar sus componentes bioactivos.

3. Se han puesto a punto tres métodos analíticos para el estudio del perfil de compuestos fenólicos mediante HPLC en la pulpa, piel y hueso de aguacate (*Persea americana* Mill.) en dos estados de maduración (óptima y con exceso de maduración). Las dos primeras metodologías se optimizaron mediante HPLC-DAD-ESI-Q-TOF-MS para el análisis global de compuestos fenólicos en pulpa, piel y hueso de aguacate, demostrándose por primera vez la efectividad de esta técnica para esta aplicación en piel y hueso. Se identificaron 34 compuestos fenólicos, de los cuales 9 de la pulpa, 3 de la piel y 3 del hueso no habían sido descritos anteriormente en estas matrices. Se observó un mayor contenido de compuestos fenólicos en pulpa y hueso de aguacates en un estado de maduración ligeramente pasado con respecto a aguacates en un punto óptimo de maduración, lo que puede ser debido a la mayor actividad que presenta la fenilalanina amonio liasa (PAL) en estados más avanzados de maduración. La segunda metodología se puso a punto mediante HPLC-FLD-MS para la determinación de flavan-3-oles en las 3 fracciones de aguacate. Este método permitió la identificación y, por primera vez, la cuantificación de procianidinas tipo A y B hasta un grado de polimerización 13. Además, se diferenciaron y cuantificaron procianidinas tipo A y B con grado de polimerización entre 2-6. El contenido de procianidinas resultó ser mayor en las tres fracciones de aguacate en estado de maduración óptimo, y esto podría ser debido a la hidrólisis de los taninos

complejos durante el proceso de maduración. Por último, cabe destacar que los principales subproductos del procesado del aguacate, piel y hueso, contienen altas concentraciones de procianidinas y, además, presentan procianidinas tipo A. Estas características confieren a estos subproductos de aguacate utilidad para la producción de ingredientes funcionales.

4. Se ha realizado el análisis de los compuestos fenólicos libres y enlazados y otros compuestos polares en la pulpa, piel, hueso y cáscara del hueso de mango (*Mangifera indica* L.) mediante HPLC-DAD-ESI-Q-TOF-MS. El método propuesto demostró ser una excelente herramienta para determinar los compuestos polares en mango. Fueron identificados 13 compuestos fenólicos enlazados y 91 libres. Los compuestos fenólicos enlazados no se habían descrito previamente en pulpa, hueso ni cáscara del hueso y, en cuanto a los compuestos fenólicos libres, 16 de ellos se describieron por primera vez en mango. Esta parece ser la primera evidencia del estudio de compuestos fenólicos en la cáscara del hueso de mango. El mayor contenido de compuestos fenólicos libres lo presentó la piel, seguida del hueso, pulpa y cáscara del hueso. Esta última fracción presentó una concentración de compuestos fenólicos libre similar a la de la pulpa de mango, mientras que la concentración de compuestos fenólicos enlazados se asemejaba a las de la piel y hueso del mango. Por otro lado, ha sido la primera vez que los compuestos enlazados han sido estudiados en pulpa, hueso y cáscara del hueso del mango, y los resultados en la concentración fueron muy similares en cáscara de hueso, piel y hueso, siendo el ácido elágico el compuesto mayoritario en las cuatro fracciones. De hecho, los subproductos del mango, especialmente el hueso, podrían emplearse como fuente de ácido elágico.

5. HPLC-DAD-ESI-Q-TOF-MS y GC-Q-TOF-MS han demostrado ser herramientas adecuadas para la identificación y cuantificación de los compuestos fenólicos libres y enlazados y alqu(en)ilresorcinoles (ARs) en pulpa, piel, hueso y cáscara del hueso de diferentes variedades de mango (*Mangifera indica* L.): 'Keitt', 'Osteen' y 'Sensación'. Este trabajo representa la primera evidencia acerca de la determinación simultánea de compuestos fenólicos y ARs en mango en las cuatro fracciones de mango de 3 variedades diferentes. En cuanto al análisis de los compuestos fenólicos libres, se identificó por primera vez la antocianina petunidina rutinósido-(-*p*-cumaroil) galato en mango. La piel fue la fracción con mayor contenido en compuestos fenólicos libres seguida del hueso, cáscara del hueso y pulpa, y 'Sensación' fue la variedad con mayor concentración de estos compuestos en piel, pulpa y cáscara del hueso, sin embargo, fue 'Keitt' la variedad con mayor contenido en hueso. En cuanto a los compuestos fenólicos enlazados, la fracción con mayor concentración fue el hueso, seguido de la piel y la cáscara del hueso. La variedad 'Keitt' presentó la mayor concentración de estos compuestos en hueso y cáscara del hueso, mientras que 'Osteen' mostró mayor cantidad en piel y pulpa. Cabe destacar que esta ha sido la primera vez que se han estudiado los ARs en el hueso y cáscara del hueso de mango, además, de los 11 ARs identificados, 4 se identificaron por primera vez en piel y 5 en pulpa. La piel, el hueso y la cáscara del hueso fueron las fracciones que presentaron mayor concentración de ARs. 'Osteen' fue la variedad con mayor concentración de ARs en piel, hueso y pulpa, sin embargo 'Keitt' fue la que presentó mayor concentración de ARs en la cáscara del hueso.

6. Se han desarrollado y comparado dos metodologías de GC-Q-TOF-MS para el análisis de esteroles y tocoferoles en la pulpa y subproductos (piel, hueso y cáscara del hueso) de tres variedades de mango (*Mangifera indica* L.): 'Keitt',

'Osteen' and 'Sensación'. Para ello se emplearon dos columnas con distinta fase estacionaria: Rtx-200MS y HP-5ms. La metodología llevada a cabo con Rtx-200MS mostró un tiempo de análisis significativamente menor y mayor sensibilidad y precisión en las áreas de picos, que en la llevada a cabo con HP-5ms. Sin embargo Rtx-200MS producía un solapamiento entre β-sitosterol and Δ^5-avenasterol. Con ambos métodos se identificaron 4 esteroles y 2 tocoferoles en las fracciones de mango estudiadas y no se encontraron diferencias significativas en la cuantificación de los tocoferoles con ambas columnas. Sin embargo, la concentración de esteroles obtenida con la columna Rtx-200MS fue superior en la mayoría de los casos. Además, los resultaron demostraron que existía una fuerte correlación positiva entre la concentración de esteroles y tocoferoles totales obtenidos con ambas columnas. Este trabajo ha estudiado por primera vez los esteroles en la piel del mango, y además, ha sido descrito por primera vez el Δ^5-avenasterol en pulpa.

Anexo 1. Indice de tablas

INDICE DE TABLAS

ANEXO 2. *Indice de figuras*

INDICE FIGURAS