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TÉCNICAS ANALÍTICAS AVANZADAS PARA LA DETERMINACIÓN DE COMPUESTOS BIOACTIVOS EN MUESTRAS VEGETALES

Presentada por:

Patricia García Salas

para optar al grado de:

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POR

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Visado en Granada a 15, de enero de 2016,

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El Prof. DR. D. ALBERTO FERNÁNDEZ GUTIÉRREZ, Profesor Emérito en el Departamento de Química Analítica "Profesor Fermín Capitán" de la Universidad de Granada y Director del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF),

CERTIFICA:

Que el trabajo que se presenta en esta tesis doctoral con el título "**TÉCNICAS ANALÍTICAS AVANZADAS PARA LA DETERMINACIÓN DE COMPUESTOS BIOACTIVOS EN MUESTRAS VEGETALES**" se ha realizado bajo mi dirección y la del Catedrático D. Antonio Segura Carretero, en los laboratorios del Departamento de Química Analítica de la Universidad de Granada, del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (Parque Tecnológico de Ciencias de la Salud), así como también, de manera parcial, en las instalaciones del Dipartimento di Scienze degli Alimenti de la Universidad de Bologna (Cesena, Italia), reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Patricia García Salas pueda optar al grado de Doctor Internacional en Química por la Universidad de Granada.

Y para que así conste, expido y firmo el presente certificado en Granada a 15 de enero de 2016:

La doctoranda Patricia García Salas y los directores de la tesis Dr. Alberto Fernández Gutiérrez y Dr. Antonio Segura Carretero, 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.

Granada a 15 de enero de 2016

Director/es de la Tesis

Fdo.: Alberto Fernández Gutiérrez Fdo.: Antonio Segura Carretero

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OBJETIVOS

El interés actual por el concepto de alimentación saludable, como herramienta para disminuir el riesgo de enfermedades, ha propiciado que numerosos estudios de investigación en ese campo se centren en la búsqueda de ingredientes bioactivos naturales para el desarrollo de productos o alimentos funcionales. Particularmente, las frutas y hortalizas contienen niveles significativos de compuestos biológicamente activos, siendo fuentes potenciales de éstos. En este contexto, el objetivo principal de la tesis doctoral que se presenta en esta memoria ha sido la búsqueda de posibles fuentes vegetales ricas en compuestos bioactivos y la caracterización de las mismas mediante técnicas analíticas avanzadas que permitan el desarrollo de métodos rápidos, robustos y fiables para la detección de este tipo de compuestos.

Para el desarrollo del objetivo principal, se definieron los siguientes objetivos parciales:

♦ Selección de diferentes matrices vegetales como fuentes de compuestos bioactivos, principalmente compuestos fenólicos y otros compuestos apolares como son, ácidos grasos, fosfolípidos, esteroles y tocoferoles.

♦ Determinación y comparación de la capacidad antioxidante de las matrices vegetales seleccionadas, utilizando tres métodos diferentes: ORAC (Oxygen Radical Absorbance Capacity), TEAC (Trolox Equivalent Antioxidant Capacity) y FRAP (Ferric Reducing Antioxidant Power).

♦ Desarrollo de metodologías analíticas a partir de técnicas separativas avanzadas como son la electroforesis capilar, la cromatografía de gases y la cromatografía líquida de alta resolución acopladas a diferentes sistemas de detección: espectrofotometría ultravioleta-visible, fluorescencia, detector evaporativo de dispersión de luz, detector de ionización de llama y espectrometría de masas con analizadores de cuadrupolo, tiempo de vuelo y cuadrupolo-tiempo de vuelo.

♦ Identificación y cuantificación de los compuestos bioactivos objeto de estudio. En el caso de los compuestos bioactivos correspondientes a la fracción lipídica, la identificación se ha llevado a cabo mediante el análisis de patrones y el uso de las bases de datos. En el caso de la determinación de compuestos fenólicos, donde se ha trabajado con detectores de espectrometría de masas con analizadores de tiempo de vuelo y cuadrupolo-tiempo de vuelo, se ha utilizado para la identificación, la gran cantidad de información que proporciona el software del instrumento, así como diferentes bases de datos de compuestos químicos.

♦ Estudio de algunos de los parámetros que pueden influir en la composición fenólica de distintas matrices vegetales, como pueden ser las condiciones climáticas, tiempo de recolección, variedad o parte del vegetal estudiada, así como otros factores presentes en el almacenamiento y procesado de las muestras, entre los que se encuentran el método de deshidratación utilizado y la temperatura y tiempo de almacenamiento.

OBIETTIVI

L'attuale interesse per il concetto di sana alimentazione, come strumento per ridurre il rischio di malattie, ha portato numerose ricerche scientifiche in quest'ambito a concentrarsi sulla ricerca di ingredienti bioattivi naturali per sviluppare prodotti o alimenti funzionali. Particolare attenzione viene rivolta verso frutta e verdura che, essendo potenziali fonti naturali, contengono livelli significativi di composti biologicamente attivi. In questo contesto, l'obiettivo principale di questa tesi di dottorato è stata la ricerca di possibili fonti vegetali ricche di composti bioattivi, e la messa a punto di metodi rapidi, robusti ed affidabili per la loro caratterizzazione attraverso tecniche analitiche avanzate.

Per la realizzazione dell'obiettivo principale, sono stati definiti i seguenti obiettivi parziali:

♦ Selezione di diverse matrici vegetali come fonti di composti bioattivi, quali composti fenolici ed altri composti apolari come gli acidi grassi, fosfolipidi, steroli e tocoferoli.

♦ Determinazione e confronto della capacità antiossidante delle matrici vegetali selezionate, utilizzando tre diversi metodi: ORAC (OxygenRadical Absorbance Capacity), TEAC (Trolox Equivalent Antioxidant Capacity) e FRAP (Ferric Reducing Antioxidant Power).

♦ Sviluppo di metodi analitici attraverso tecniche di separazione avanzate, come elettroforesi capillare, gas cromatografia e cromatografia liquida ad elevata prestazione accoppiata a diversi sistemi di rivelazione: spettrofotometria ultravioletta-visibile, fluorescenza, rivelatore evaporativo a luce diffusa, rivelatore di ionizzazione di fiamma e spettrometria di massa con analizzatori di massa come quadrupolo, tempo di volo e quadrupolo-tempo di volo.

♦ Identificazione e quantificazione dei composti bioattivi oggetto di studio. Nel caso dei composti bioattivi corrispondenti alla frazione lipidica, l'identificazione è stata condotta utilizzando l'analisi degli standard interni e l'uso dei database. Per la determinazione dei composti fenolici, stati studiati attraverso l'uso di standard interni e l'utilizzo di rivelatori a spettrometria di massa con analizzatori a tempo di volo e quadrupolo-tempo di volo, sono state utilizzate per l'identificazione sia la grande quantità di informazioni fornite dal software dello strumento sia le varie banche dati di composti chimici.

♦ Studio di alcuni dei parametri che possono influenzare la composizione fenolica di matrici vegetali, quali le condizioni climatiche, il momento della raccolta, le varietà o parte del vegetale studiato, così come altri fattori derivanti dallo stoccaggio e trattamento dei campioni, tra i quali il metodo di disidratazione utilizzato, la temperatura ed il tempo di stoccaggio.

RESUMEN

En la presente memoria se recogen los resultados obtenidos durante la realización de la tesis doctoral titulada **"Técnicas analíticas avanzadas para la determinación de compuestos bioactivos en muestras vegetales"**. Los estudios presentados contienen la caracterización y cuantificación de varias familias de compuestos bioactivos, como son los compuestos fenólicos y los compuestos de la fracción lipídica: ácidos grasos, tocoferoles, fitoesteroles y fosfolípidos, en diferentes matrices vegetales y en subproductos derivados de las mismas. En algunos de estos casos, también se ha estudiado la influencia de las condiciones climáticas y el efecto de la temperatura sobre la composición fenólica.

La memoria consta de una primera parte denominada **"INTRODUCCIÓN"** que incluye información acerca de las bases científicas en las que se fundamenta la relación dieta-salud y su relación con alimentos de origen vegetal, los compuestos bioactivos procedentes de los vegetales, una descripción detallada sobre las distintas familias de compuestos que han sido objeto de estudio y las diferentes fuentes vegetales de los compuestos bioactivos analizados. A su vez, se exponen los diferentes procedimientos y técnicas utilizadas en la parte experimental, incluyendo el tratamiento de la muestra, la metodología para el estudio de la capacidad antioxidante, así como, las técnicas separativas y de detección empleadas en el análisis de los compuestos bioactivos de los diferentes extractos vegetales.

En la segunda parte se aborda la sección "**PARTE EXPERIMENTAL. RESULTADOS Y DISCUSIÓN**", que está compuesta por ocho capítulos.

El **capítulo 1**, es un trabajo de revisión bibliográfica acerca de las diferentes técnicas de extracción de compuestos fenólicos en muestras vegetales. Éste incluye una breve descripción de los distintos tipos de compuestos fenólicos y los sistemas de extracción más comunes, como son la extracción líquido-líquido (LLE), la extracción en fase sólida (SFE), la extracción con fluidos supercríticos

(SFE), la extracción con fluidos presurizados (PLE), la extracción asistida con microondas (MAE) y la extracción asistida con ultrasonidos (UAE).

El **capítulo 2** se centra, en el estudio de la capacidad antioxidante de 11 frutas y 13 verduras incluyendo las variedades más comunes cultivadas en Andalucía. Éstos fueron: espárrago (*Asparagus officinalis* L.), alcachofa (*Cynara scolymus* L.), zanahoria (*Daucus carota* L.), berenjena (*Solanum melongena* L.), haba (*Vicia faba* L.), ajo (*Allium sativum* L.), judía verde (*Phaseolus vulgaris* L.), lechuga (*Lactuca sativa* L.), cebolla (*Allium cepa* L.), pimiento (*Capsicum annuum* L.), patata (*Solanum tuberosum* L.), tomate (*Solanum lycopersicum* L.), calabacín (*Cucurbita pepo* L.), aguacate (*Persea americana* Mill.), chirimoya (*Annona cherimola* Mill.), uva (*Vitis vinifera* L.), limón (*Citrus limon* (L.) Burm. f.), níspero (*Eriobotrya japonica* (Thunb.) Lindl.), mango (*Mangifera indica* L.), melón (*Cucumis melo* L.), caqui (*Diospyros kaki*), granada (*Punica granatum* L.), membrillo (*Cydonia oblonga* Mill.), y sandía (*Citrullus lanatus* (Thunb.) Mansf.). Las técnicas de capacidad antioxidante seleccionadas fueron TEAC (Trolox Equivalent Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power) y ORAC (Oxygen Radical Absorbance Capacity).

El **capítulo 3** está dedicado a la determinación de la capacidad antioxidante, mediante los tres métodos descritos en el capítulo 2, y a la caracterización del perfil fenólico de un extracto seco de mangostán (*Garcinia mangostana*), una fruta tropical originaria del sudeste asiático. La caracterización de los compuestos fenólicos fue realizada utilizando electroforesis capilar acoplada a espectrometría de masas con analizador de tiempo de vuelo (CE-TOF-MS). Con el método puesto a punto se detectaron un total de 10 compuestos, principalmente xantonas.

En el **capítulo 4** se presenta el estudio de la influencia que los procesos tecnológicos tienen en la composición fenólica y la capacidad antioxidante de un

extracto seco de limón. Las muestras comparadas fueron un liofilizado y un deshidratado de limón, este último almacenado a una temperatura extrema de 50 ºC y a diferentes tiempos (1 y 3 meses). El estudio de la capacidad antioxidante, se realizó de nuevo usando los tres métodos comentados anteriormente. Para la identificación y cuantificación de los compuestos fenólicos se utilizó la cromatografía líquida de alta resolución acoplada a espectrofotomería Uv-Vis y a espectrometría de masas con analizador de tiempo de vuelo (HPLC-DAD-ESI-TOF-MS). La información proporcionada por ambos detectores, máximos de absorción, valores de masa exacta, y distribución isotópica ha permitido la identificación tentativa de 23 compuestos. Finalmente, se llevó a cabo un análisis de los indicadores de daño térmico, hidroxi-metilfurfural y furfural utilizando HPLC-DAD.

El **capítulo 5** se centra en la identificación y cuantificación de los compuestos fenólicos en tres variedades diferentes de berenjena (*Solanum melongena* L.) cultivadas en diferentes estaciones del año. La técnica analítica utilizada en este caso fue cromatografía líquida de alta resolución acoplada a espectrofotomería Uv-Vis y a espectrometría de masas/masas con analizador de cuadrupolo-tiempo de vuelo (HPLC-DAD-QTOF-MS) en modo de ionización positivo y negativo. El analizador de masas QTOF ofrece una elevada exactitud de los valores de masas y la distribución isotópica tanto de los espectros de masas como de los de MS/MS permitiendo la identificación de un gran número de compuestos fenólicos. Posteriormente, en función de los resultados obtenidos en la cuantificación de los compuestos identificados, se ha evaluado la influencia de las condiciones climáticas en el contenido polifenólico de las matrices.

En el **capítulo 6**, se incluye el estudio basado en la caracterización y cuantificación de los compuestos fenólicos y otros compuestos polares de dos variedades diferentes de chirimoya y sus subproductos (piel y hueso). Las variedades seleccionadas fueron ‘Fino de Jete’ y ‘Campa’, las más comunes

cultivadas en la "Costa Tropical" de Granada-Málaga. Los análisis de las diferentes muestras fueron realizados, de nuevo, mediante HPLC-DAD-QTOF-MS. Se han identificado un total de 21 compuestos en la porción comestible de la fruta (pulpa), 37 en piel y 22 en hueso, de los cuales la mayoría fueron identificados por primera vez en chirimoya. Los compuestos mayoritarios detectados en pulpa y piel fueron principalmente proantocianidinas, mientras que en hueso se encontraron ácidos orgánicos y flavonoides.

Los dos últimos capítulos de la tesis se centraron en la determinación de compuestos bioactivos apolares en diferentes matrices.

El **capítulo 7** recoge la determinación de compuestos de la fracción lipídica (ácidos grasos, tocoferoles, fitoesteroles y fosfolípidos) en las dos variedades de chirimoya estudiadas en el capítulo anterior, así como en sus subproductos. Se han cuantificado un total de 19 ácidos grasos en la pulpa, piel y hueso de la fruta mediante cromatografía de gases-fast con detector de ionización de llama (Fast-GC-FID), las pequeñas dimensiones de la columna capilar utilizada permitieron realizar el análisis de todos los ácidos grasos en un tiempo de 6 minutos, obteniendose una buena resolución. Los tocoferoles fueron determinados mediante HPLC utilizando un detector fluorimétrico, siendo cuantificados α y δ-tocoferol en piel y pulpa de las diferentes variedades de la fruta. La cromatografía de gases acoplada a espectrometría de masas con analizador de cuadrupolo (GC-Q-MS), se utilizó para la determinación de los esteroles permitiendo la detección de 8 compuestos de esta familia, β-sitosterol fue el compuesto mayoritario en todas las muestras analizadas y por primera vez se identificó γ-sitosterol en piel y pulpa. Finalmente, se realizó la determinación de los fosfolípidos mediante cromatografía líquida acoplada a un detector evaporativo de dispersión de luz (HPLC-ELSD) y de los fosfolípidos de los ácidos grasos mediante HPLC-MS.

Por último, en el **capítulo 8** se expone el estudio realizado para la caracterización de los compuestos bioactivos del aceite extraído de semillas de granada. Para la realización de este trabajo se seleccionaron un total de 17 variedades diferentes de granada de diferentes países, siendo 5 de ellas variedades de árboles no comerciales. Los compuestos objeto de estudio fueron ácidos grasos, tocoferoles, fitoesteroles y fosfolípidos, eligiéndose, para tal fin, las mismas técnicas que las descritas en el capítulo 7. Se identificaron un total de 18 ácidos grasos entre los que se encuentra el ácido punícico (CLnA). Cabe destacar, la identificación de α y β-tocotrienol, determinados por primera vez en este tipo de muestras. En cuanto a los esteroles, de los 5 cuantificados el que se encontró en mayor concentración fue el sitosterol.

RIASSUNTO

In questa relazione si riportano i risultati ottenuti durante la realizzazione della Tesi di Dottorato intitolata **"Tecniche analitiche avanzate per la determinazione di composti bioattivi in prodotti vegetali"**. Gli studi presentati trattano la caratterizzazione e quantificazione di varie famiglie di composti bioattivi, come i composti fenolici, ed i componenti della frazione lipidica come gli acidi grassi, tocoferoli, fitosteroli e fosfolipidi. Tali composti sono stati studiati in differenti matrici vegetali e su matrici ottenute dai loro sottoprodotti. In alcuni di questi casi è stata studiata l'influenza delle condizioni climatiche e come l'effetto della temperatura condizioni la composizione fenolica.

La relazione si compone di una parte iniziale, **"INTRODUCCIÓN"**, che include informazioni sulle basi scientifiche per le quali si fonda la relazione dieta-salute ed i legami con gli alimenti di origine vegetale, una descrizione dettagliata sulle famiglie di composti bioattivi oggetto di studio e le differenti fonti vegetali analizzate. Successivamente vengono descritti i diversi procedimenti e tecniche utilizzate nella parte sperimentale, includendo: trattamento dei campioni, metodo di studio della capacità antiossidante, tecniche separative e di determinazione utilizzate per l'analisi dei composti bioattivi dei differenti estratti vegetali.

La seconda parte è costituita dalla sezione **"PARTE EXPERIMENTAL. RESULTADOS Y DISCUSIÓN"**, divisa in otto capitoli.

Nel **capítulo 1** si riporta il lavoro di revisione bibliografica sulle differenti tecniche di estrazione dei composti fenolici in campioni vegetali. Si include una breve descrizione dei distinti tipi di composti fenolici ed i sistemi di estrazione più comuni tra cui, estrazione liquido-liquido (LLE), estrazione in fase solida (SFE), estrazione con fluidi supercritici (SFE), estrazione con fluidi pressurizzati (PLE), estrazione assistita con microonde (MAE) ed estrazione assistita con ultrasuoni (UAE).

Nel **capítulo 2** viene trattato lo studio della capacità antiossidante di undici varietà di frutta e tredici varietà di verdura, includendo le varietà più coltivate in Andalusia: asparago (*Asparagus officinalis* L.), carciofo (*Cynara scolymus* L.), carota (*Daucus carota* L.), melanzana (*Solanum melongena* L.), fava (*Vicia faba* L.), aglio (*Allium sativum* L.), fagiolino (*Phaseolus vulgaris* L.), lattuga (*Lactuca sativa* L.), cipolla (*Allium cepa* L.), peperone (*Capsicum annuum* L.), patata (*Solanum tuberosum* L.), pomodoro (*Solanum lycopersicum* L.), zucchina (*Cucurbita pepo* L.), avocado (*Persea americana* Mill.), chirimoya (*Annona cherimola* Mill.), uva (*Vitis vinifera* L.), limone (*Citrus limon* (L.) Burm. f.), nespolo (*Eriobotrya japonica* (Thunb.) Lindl.), mango (*Mangifera indica* L.), melone (*Cucumis melo* L.), cachi (*Diospyros kaki*), melograno (*Punica granatum* L.), mela cotogna (*Cydonia oblonga* Mill.) e cocomero (*Citrullus lanatus* (Thunb.) Mansf.). Le tecniche di capacità antiossidante selezionate sono state: TEAC (Trolox Equivalent Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power) ed ORAC (Oxygen Radical Absorbance Capacity).

Il **capítulo 3** è dedicato alla determinazione della capacità antiossidante attraverso i tre metodi descritti nel capítulo 2, e la caratterizzazione del profilo fenolico di un estratto secco di mangostano (*Garcinia mangostana*), un frutto tropicale originario del sudest asiatico. La caratterizzazione dei composti fenolici è stata realizzata utilizzando elettroforesi capillare accoppiata a spettrometria di massa con analizzatore a tempo di volo (CE-TOF-MS). Con il metodo messo a punto sono stati determinati un totale di dieci composti principalmente xantoni.

Nel **capítulo 4** viene presentato lo studio sull'influenza che esercitano i processi tecnologici sulla composizione fenolica e la capacità antiossidante di un estratto secco di limone. Sono stati messi a confronto due campioni di limone: un liofilizzato ed un disidratato. Quest'ultimo conservato ad una temperatura estrema di 50 ºC, ed a tempi differenti (1 e 3 mesi). Lo studio della capacità antiossidante è stato realizzato utilizzando i tre metodi commentati

precedentemente. Per la identificazione e quantificazione dei composti fenolici è stata utilizzata la cromatografia liquida ad elevata prestazione accoppiata a detectors UV-Visibile ed a spettrometria di massa con analizzatore a tempo di volo (HPLC-DAD-ESI-TOF-MS). L'informazione ottenuta mezzo di entrambi i rivelatori, della assorbanza massima, del valore di massa esatta, e della distribuzione isotopica, a permesso una identificazione di ventitré composti. Infine è stata condotta l'analisi degli indicatori del danno termico, l'hidroxi-metilfurfurale e furfurale, utilizzando la tecnica HPLC-UV.

Il **capítulo 5** tratta dell'identificazione e quantificazione dei composti fenolici in tre differenti varietà di melanzana *(Solanum melongena L.)*, coltivate in differenti periodi dell'anno. In questo caso, è stata utilizzata la cromatografia liquida ad elevata prestazione accoppiata a detectors UV-Visibile ed a spettrometria di massa con fonte di ionizzazione elettrospray (in modalitá positiva e negativa) e analizzatore quadrupolo-tempo di volo (HPLC-DAD-QTOF-MS). L'analizzatore di massa QTOF offre una elevata fedeltà dei valori di massa e la distribuzione isotopica sia degli spettri di massa che gli MS/MS, permettendo l'identificazione di un grande numero di composti fenolici. Successivamente, in funzione dei risultati ottenuti durante la quantificazione dei composti, è stata valutata l'influenza delle condizioni climatiche sul contenuto polifenolico delle matrici.

Nel **capítulo 6** è stato incluso lo studio sulla caratterizzazione e quantificazione dei composti fenolici ed altri composti polari di due differenti varietà di chirimoya ed relativi sottoprodotti (pelle ed osso). Le varietà selezionate sono state 'Fino de Jete' e 'Campa', le più comunemente coltivate sulla "Costa Tropical" di Granada-Málaga. Le analisi dei campioni sono state realizzate attraverso HPLC-DAD-QTOF-MS: sono stati identificati un totale di ventuno composti nella parte commestibile del frutto (polpa), trentasette nella pelle, e ventidue nell'osso, la maggior parte dei quali sono stati identificati per la prima volta nella chirimoya. I composti maggioritari determinati nella polpa e nella

pelle sono principalmente proantocianidine, mentre acidi orgánici y flavonoidi nell'osso.
Gli ultimi due capitoli della tesi si focalizzano sulla determinazione dei composti bioattivi apolari in differenti matrici.

Il **capítulo 7** tratta della determinazione della frazione lipidica (acidi grassi, tocoferoli, fitosteroli e fosfolipidi) delle due varietà di Chirimoya studiate nel capitolo precedente, così come nei suoi sottoprodotti. Attraverso l'utilizzo della fast-gascromatografia con rivelatore a ionizzazione di fiamma (Fast-GC-FID) sono stati quantificati un totale di diciannove acidi grassi nella polpa, buccia ed nocciolo del frutto. Le ridotte dimensioni della colonna capillare utilizzata hanno permesso di realizzare con una buona risoluzione l'analisi di tutti gli acidi grassi in un tempo di sei minuti. I tocoferoli sono stati determinati mediante HPLC utilizzando un rivelatore fluorimetrico e sono stati quantificati il α e δ-tocoferol nella pelle e polpa delle differenti varietà di frutta. La tecnica gascromatografia accoppiata a spettrometria di massa con analizzatore a singolo quadrupolo (GC-Q-MS) è stata utilizzata per la determinazione degli steroli permettendo la determinazione di otto composti di questa famiglia. Il β-sitosterolo è risultato essere il composto maggioritario in tutti i campioni analizzati e, per la prima volta, è stato identificato il γ-sitosterolo nella buccia e polpa. Inoltre è stata effettuata la determinazione dei fosfolipidi mediante cromatografia liquida accoppiata a rivelatore evaporativo a luce diffusa e gli acidi grassi dei fosfolipidi attraverso HPLC-MS.

Nel **capítulo 8** viene esposto lo studio realizzato per la caratterizzazione dei composti bioattivi nella frazione lipidica nell'olio estratto dai semi di melograno. Per la realizzazione di questo lavoro sono stati selezionati un totale di diciassette diverse varietà di melograno provenienti da diversi paesi, eccetto cinque, che appartengono a varietà non commerciali. I composti oggetto di studio sono stati: acidi grassi, tocoferoli, fitosteroli e fosfolipidi. Le tecniche utilizzate sono state le

stesse descritte nel capítulo 7. Sono stati identificati un totale di diciotto acidi grassi tra i quali i l'acido punico (ClnA). Inoltre si evidenzia l'identificazione, per la prima volta in questo tipo di matrice, dell' α e β-tocotrienolo. Per quanto riguarda gli steroli, dei cinque quantificati, quello più abbondante è risultato essere il sitosterolo.

INTRODUCCIÓN

1. ASPECTOS CIENTÍFICOS DE LA RELACIÓN DIETA-SALUD: ALIMENTOS DE ORIGEN VEGETAL.

La vinculación entre la alimentación y su efecto beneficioso sobre la salud se conoce desde la antigüedad, de hecho, en el año 500 a. C. Hipócrates, considerado como el padre de la medicina moderna, ya hizo pública su conocida frase en la que recomendaba "que vuestra medicina sea vuestro alimento, y que vuestro alimento sea vuestro medicamento"[1]. Actualmente este principio sigue siendo la base de la ciencia que estudia la causa-efecto entre alimentación y salud.

Desde el desarrollo de la bioquímica a comienzos del siglo XX, hasta hace unos años, una gran parte de los estudios científicos sobre alimentación se han centrado en los aspectos nutritivos de los alimentos. Así, la mayoría de las recomendaciones para la ingesta siguen estando limitadas al aporte de nutrientes y las cantidades recomendadas normalmente están destinadas a la prevención de enfermedades ocasionadas por déficit de los mismos. Si bien es cierto que el papel principal de los alimentos es suministrar energía para el mantenimiento del organismo y sus funciones, también se sabe que, además, la alimentación produce una serie de efectos fisiológicos beneficiosos que influyen en la reducción de riesgo de enfermedad. En base a esto, en el binomio alimentación y salud no sólo entran en juego aspectos puramente nutricionales sino que se deberían reconocer otros beneficios[2,3].

Desde finales del siglo XX, diversas áreas de la ciencia atienden a esta nueva perspectiva de la relación alimentación y salud propiciada por las nuevas

[1] Panagiotakos, D. B.; Georgousopoulou, E. N.; Pitsavos, C.; Chrysohoou, C.; Skoumas, I.; Pitaraki, E.; Georgiopoulos, G. A.; Ntertimani, M.; Christou, A.; Stefanadis, C. Exploring the path of Mediterranean diet on 10-year incidence of cardiovascular disease: The ATTICA study (2002–2012). *Nutr. Metab. Cardiovasc. Dis*. **2015**, 25 (3), 327–335.

[2] Clydesdale, F. M. A proposal for the establishment of scientific criteria for health claims for fuctional foods. *Nutr. Rewiews* **1997**, *55* (12), 413–422.

[3] Juárez, M.; Olano, A.; Morais, F. *Alimentos funcionales*. Fundación Española para la Ciencia y la Tecnología (FECYT), 2005.

demandas de la sociedad, la innovación tecnológica en la industria alimentaria y la biotecnología moderna. Esto ha dando lugar a una nueva concepción de los alimentos y a la aparición de nuevos productos alimentarios o suplementos como ingredientes funcionales, alimentos con alto valor añadido y nutracéuticos, lo que incrementa notablemente el potencial de creación de riqueza del sector agroalimentario y abre la puerta a nuevas oportunidades de innovación[3,4]. De esta forma, se podrán establecer futuras recomendaciones sobre alimentos con un efecto beneficioso para problemas de salud concretos y con una base científica sólida.

En este contexto, juegan un papel fundamental los alimentos de origen vegetal, en general, y las frutas y hortalizas en particular. Diversos estudios científicos *in vitro* e *in vivo*, epidemiológicos y/o clínicos, indican que el consumo de productos naturales, tales como frutas y hortalizas, está íntimamente relacionado con una reducción de numerosos factores de riesgo en el desarrollo de enfermedades crónicas[5]. La mayoría de estas enfermedades están vinculadas al proceso de envejecimiento y relacionadas con el daño oxidativo, esto explica el interés sobre los productos vegetales, los cuales tienen la característica de poseer una elevada capacidad antioxidante y podrían ejercer un papel importante en la prevención o reducción de dichas enfermedades[6,7].

Estas propiedades se atribuyen al gran número de antioxidantes naturales que forman parte de la composición de los alimentos vegetales. Además de macro y micronutrientes, éstos contienen una serie de sustancias que, a pesar de no tener una función nutricional clásicamente definida, o no ser consideradas esenciales para la salud, pueden tener, como se ha explicado anteriormente, un impacto

[4] Barrrera, A. Nuevas realidades, nuevos paradigmas: la nueva revolución agrícola. *Comuniica, Innovación tecnológica* **2011**, 10–21.
[5] Henriquez C.; Zúñiga E.; Luth, M. Polifenoles. Salud desde el reino vegetal. *Agron. y For.* **2009**, *39*, 30–33.
[6] Blasa, M.; Gennari, L.; Angelino, D.; Ninfali, P. *Bioactive Foods in Promoting Health*. Elsevier, 2010.
[7] Shashirakha M. N.; Mallikarjuna S. E.; Rajarathnam, S. Status of Bioactive Compounds in Foods, with Focus on Fruits and Vegetables. *Crit. Rev. Food Sci. Nutr. Publ.* **2015**, *55*, 1324–1339.

decisivo en el desarrollo de alguna enfermedad. Estas sustancias bioactivas, que normalmente son metabolitos secundarios de las plantas se denominan también fitoquímicos o fitonutrientes. Sin embargo, a pesar de que actualmente, se han aislado e identificado un gran número de compuestos bioactivos en productos vegetales, un gran porcentaje de los mismos sigue siendo desconocido[8].

Por todo ello, gracias a las importantes propiedades biosaludables de estos fitoquímicos, los alimentos de origen vegetal ocupan en la actualidad un área de investigación emergente y multidisciplinar centrada en el estudio químico de la composición de los mismos y en el análisis fisiológico de los mecanismos de acción de estas sustancias activas[7,9]. No obstante, los efectos protectores de los compuestos bioactivos están subordinados a diversos factores como accesibilidad y capacidad de extracción de los alimentos, absorción intestinal, metabolismo, acción biológica final en el cuerpo humano e interacción potencial con los tejidos diana[10].

En base a todo esto, los estudios de composición química son fundamentales como primer paso para estudiar la relación entre los alimentos de origen vegetal y la salud, así como para el desarrollo de nuevos productos funcionales con propiedades preventivas para ciertas enfermedades y/o trastornos patológicos. Para tal finalidad, la química analítica ofrece multitud de herramientas que permiten la detección e identificación de estos compuestos saludables.

[8] Liu, R. H. Dietary bioactive compounds and their health implications. *J. Food Sci.* **2013**, *78* (S1), A18–A25.

[9] Martínez-Navarrete, N.; Camacho Vidal, M.; Martínez Lahuerta, J. Los compuestos bioactivos de las frutas y sus efectos en la salud. *Act. Dietética* **2008**, *2*, 64–68.

[10] Lamuela-Raventós, R. M.; Vallverdú-Queralt, A.; Jáuregui, O.; Martínez-Huélamo, M.; Quifer-Rada, P. Improved Characterization of Polyphenols Using Liquid Chromatography. In *Polyphenols in Plants: Isolation, Purification and Extract Preparation*; Watson, R. R.; Elsevier, 2014, pp 261–292.

2. COMPUESTOS BIOACTIVOS DE LOS ALIMENTOS VEGETALES.

Como se comentó en el apartado anterior, numerosos estudios demuestran que los beneficios de ciertos tipos de dietas, basadas en el consumo de frutas, verduras, legumbres, aceite de oliva y cereales, no se limitan a su contenido en nutrientes sino que existen otro tipo de componentes "no nutrientes", denominados compuestos bioactivos[11–13].

Los compuestos bioactivos se definen como aquellos que, a pesar de no tener una función nutricional básica, poseen cierta actividad biológica dentro del organismo ya que están implicados en diferentes procesos metabólicos. Esto se traduce en una reducción del riesgo de padecer distintas enfermedades y por tanto, en la mejora del estado de bienestar del individuo[14].

Dentro del término de actividad biológica se deben tener en cuenta tres aspectos importantes[13]:

- Función: papel esencial que desempeñan los componentes bioactivos en el organismo.
- Efecto: respuestas, beneficiosas o adversas, fisiológicas o farmacológicas de los compuestos bioactivos.
- Asociación: correlaciones de los componentes funcionales de los alimentos con alguna finalidad fisiológica o clínica que puede o no mostrar una relación causal.

Los diferentes compuestos bioactivos poseen estructuras y actividades biológicas muy diferentes, en base a esto pueden clasificarse en diferentes categorías como

[11] Martin, C.; Zhang, Y.; Tonelli, C.; Petroni, K. Plants, Diet, and Health. *Annu. Rev. Plant Biol.* **2013**, *64*, 19–46.

[12] Poole, S.; Blades, M. The Mediterranean diet – a review of evidence relevant to the food and drink industry. *Nutr. Food Sci.* **2013**, *43* (1), 7–16.

[13] Herrera Chalé, F.; Betancur Ancona, D.; Segura Campos, M. R. Compuestos bioactivos de la dieta con potencial en la prevención de patologías relacionadas con sobrepeso y obesidad; péptidos biológicamente activos. *Nutr. Hosp.* **2014**, *29* (1), 10–20.

[14] 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, 1202–1205.

se muestra en la siguiente figura. En esta clasificación también se incluyen algunos compuestos derivados, así como algunos micronutrientes, los cuales pueden influir en diferentes procesos metabólicos que están estrechamente relacionados con la salud humana.

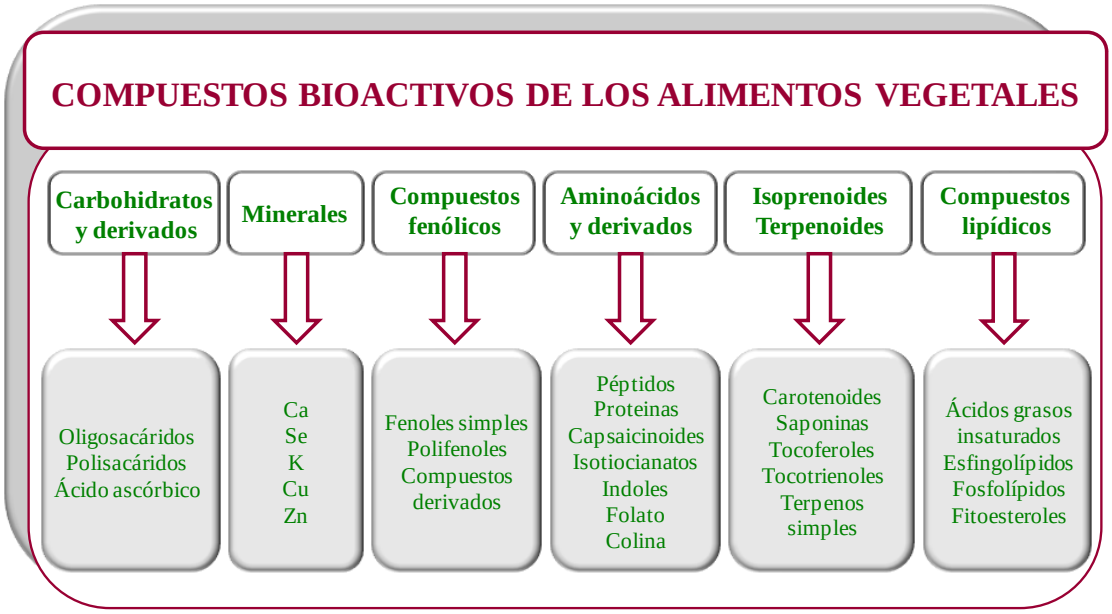


Figura 1. Compuestos bioactivos presentes en los alimentos de origen vegetal

Numerosos estudios realizados hasta la fecha, han propuesto diferentes funciones atribuidas a estos compuestos bioactivos procedentes en su mayor parte de productos vegetales, pero quizás la más conocida es su marcada actividad antioxidante. Algunas de estas funciones son las siguientes[15,16]:

- Efecto anticancerígeno
- Protección contra en daño en el ADN causado por expresiones genéticas anormales
- Modificación de la comunicación celular.

[15] 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*, 511–538.

[16] Denny, A.; Buttriss, J. *Plant foods and health: focus on plant bioactives*; EuroFir, 2005, Synthesis Report nº 4.

- Modificación del perfil hormonal.
- Modulación del perfil lipídico.
- Estimulación del sistema inmunitario.
- Efecto antiinflamatorio.
- Efecto hipocolesterolémico.
- Actividad antimicrobiana.
- Actividad antioxidante.

Dado el gran número y la diversidad de compuestos bioactivos identificados hasta la fecha, el estudio de su efecto sobre la salud es una tarea compleja, ya que como se explicó en el apartado 1, esto hace que este efecto esté influenciado por numerosos factores. La figura 2 esquematiza algunas de las actividades biológicas descritas, y los compuestos bioactivos asociados a las mismas[17–19].

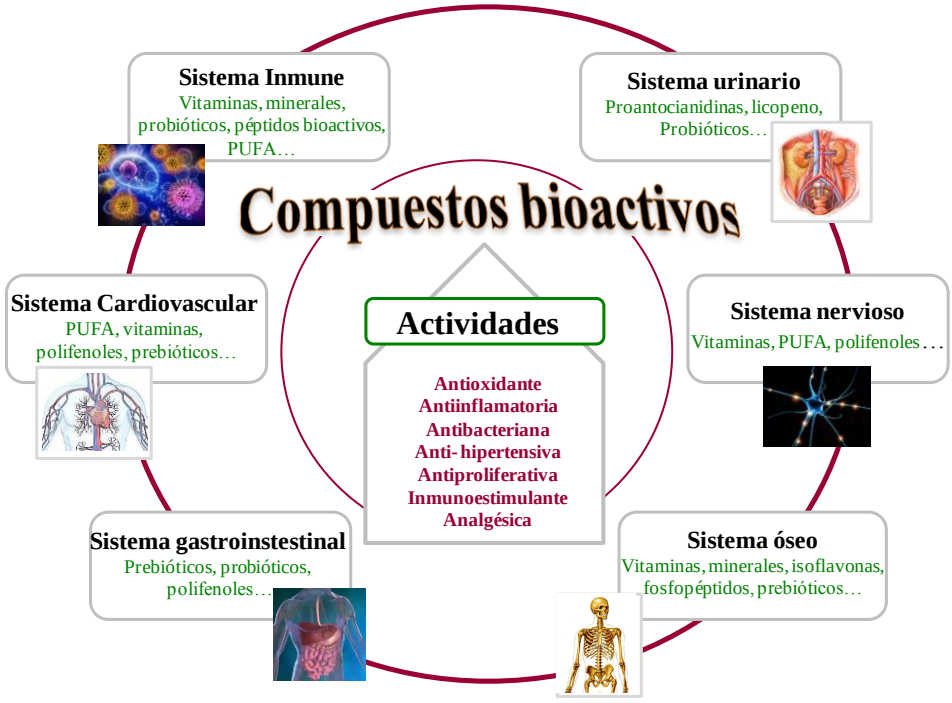


Figura 2. Algunas actividades biológicas y compuestos bioactivos asociados

[17] Ros, E. Health benefits of nut consumption. *Nutrients* **2010**, *2* (7), 652–682.
[18] Krueger, C. G.; Reed, J. D.; Feliciano, R. P.; Howell, A. B. Quantifying and characterizing proanthocyanidins in cranberries in relation to urinary tract health. *Anal. Bioanal. Chem.* **2013**, *405* (13), 4385–4395.
[19] Wang, J.; Tang, L.; Wang, J. Biomarkers of dietary polyphenols in cancer studies : current evidence and beyond. *Oxid. Med. Cell. Longev.* **2015**, 1–14.

consumida, y la biodisponibilidad de cada componente, así como por la variabilidad genética en respuesta a la dieta, y características clínicas de las personas consumidoras de estos compuestos[15].

A su vez, los niveles de los compuestos bioactivos dentro del vegetal se ven afectados por una gran cantidad de factores. En primera instancia, la concentración de estos compuestos dependerá de aspectos agronómicos como el tipo y zona de cultivo, tipo de suelo, clima, etc, los cuales afectan decisivamente a la composición de compuestos fenólicos[20]. En segundo lugar, el proceso de conservación y el tipo de procesado de estos alimentos hacen que la composición de compuestos bioactivos varíe considerablemente[21].

De todas las familias de compuestos bioactivos descritas, la presente tesis se ha centrado en el estudio de compuestos fenólicos y otros compuestos apolares como ácidos grasos, tocoferoles, fitoesteroles y fosfolípidos, los cuales se detallan a continuación.

2.1. Compuestos fenólicos

Los compuestos fenólicos constituyen uno de los grupos de sustancias bioactivas más numeroso y ampliamente distribuido en el reino vegetal. De hecho, se consideran metabolitos secundarios sintetizados por los vegetales tanto en su desarrollo normal como en respuesta a condiciones de estrés (polución, radiaciones UV, temperaturas extremas, parásitos)[22]. En la actualidad se conocen, más de 8000 estructuras fenólicas con diferentes actividades biológicas, encontradas principalmente en alimentos naturales como frutas, verduras, cereales, hierbas, especias, legumbres, frutos secos, aceitunas, aceite y chocolate,

[20] Dabbou, S.; Sifi, S.; Rjiba, I.; Esposto, S.; Taticchi, A.; Servili, M.; Montedoro, G. F.; Hammami, M. Effect of pedoclimatic conditions on the chemical composition of the Sigoise olive cultivar. *Chem. Biodivers.* **2010**, *7*, 898–908.

[21] Tierno, R.; Hornero-Méndez, D.; Gallardo-Guerrero, L.; López-Pardo, R.; Ruiz de Galarreta, J. I. Effect of boiling on the total phenolic, anthocyanin and carotenoid concentrations of potato tubers from selected cultivars and introgressed breeding lines from native potato species. *J. Food Compos. Anal.* **2015**, *41*, 58–65.

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

así como en bebidas de origen vegetal como té, café y vino. Por lo que, los compuestos fenólicos están integrados en una parte importante de la dieta humana[23].

Químicamente, esta familia de compuestos presenta una gran diversidad estructural pero todos ellos tienen en común la presencia de un anillo aromático unido a uno o más grupos hidroxilo (-OH). Una primera clasificación de los compuestos fenólicos podría basarse en el número de unidades de fenol que constituyen la molécula: fenoles simples o polifenoles. Por otro lado, en función del número de anillos fenólicos y de las diferentes variaciones estructurales que pueden formar parte de su esqueleto base, se pueden clasificar, a su vez, en diferentes clases o familias, como se muestra en la siguiente figura (figura 3)[24].

[23] Fantini, M.; Benvenuto, M.; Masuelli, L.; Frajese, G.; Tresoldi, I.; Modesti, A.; Bei, R. *In Vitro* and *in Vivo* antitumoral effects of combinations of polyphenols, or polyphenols and anticancer drugs: perspectives on cancer treatment. *Int. J. Mol. Sci.* **2015**, *16*, 9236–9282.

[24] Contreras-Gámez M. M.; Rodriguez-Pérez C.; García-Salas P. Polyphenols from the Mediterranean Diet: Structure, Analysis and Health Evidence. In *Occurrences, structure, biosynthesis, and health. Benefits based on their evidences of medicinal phythochemicals in vegetables and fruits*; Motohashi, N.; Nova Biomedical, 2014, vol. 2, pp 141-209.

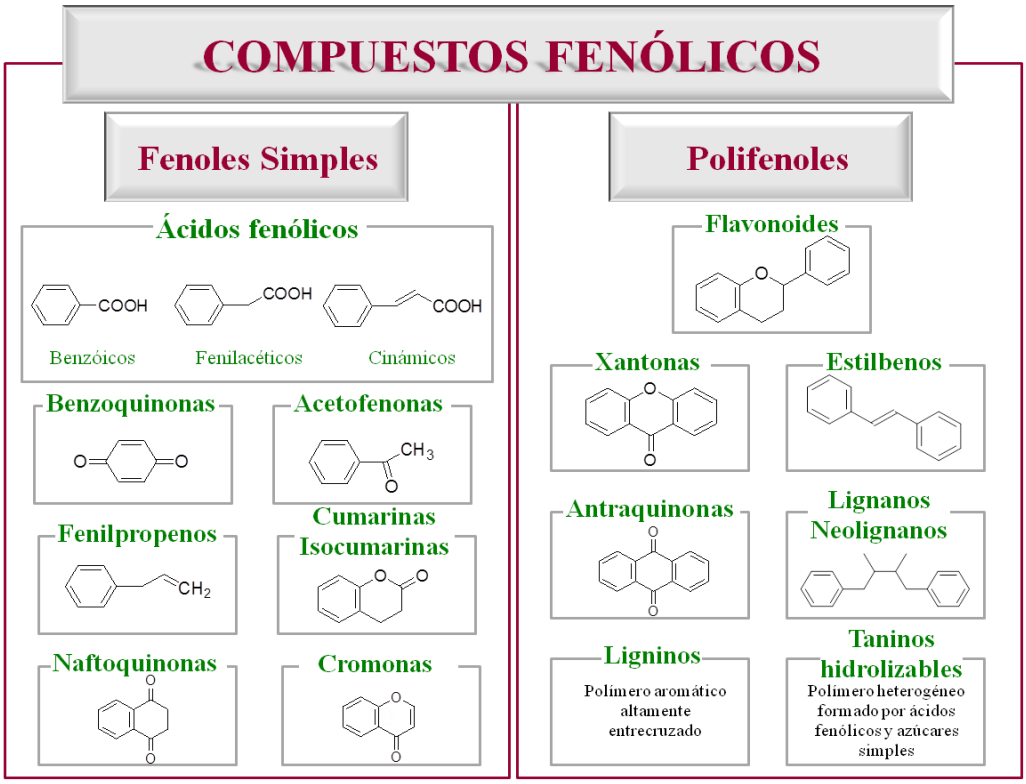


Figura 3. Clasificación de las principales familias de compuestos fenólicos y sus estructuras básicas

Los principales ácidos fenólicos son los ácidos hidroxibenzoicos e hidroxicinámicos que están presentes en la mayoría de los alimentos vegetales, mientras que los ácidos fenilacéticos se encuentran en menor medida.

Los flavonoides son la familia de polifenoles más abundante y ampliamente distribuida en la naturaleza, además de la más consumida en la dieta, constituyendo aproximadamente el 60 % del total de la misma. Su estructura común consiste en dos anillos aromáticos unidos por tres átomos de carbono que normalmente forman un heterociclo oxigenado. Los flavonoides pueden subdividirse en, al menos, otras 13 clases que difieren entre sí, en el nivel de oxidación y los sustituyentes de dicho heterociclo. Dentro de cada clase, los compuestos individuales se diferencian en los sustituyentes de los dos anillos aromáticos[24].

En la figura 4 se muestran las principales familias de flavonoides y sus estructuras básicas.

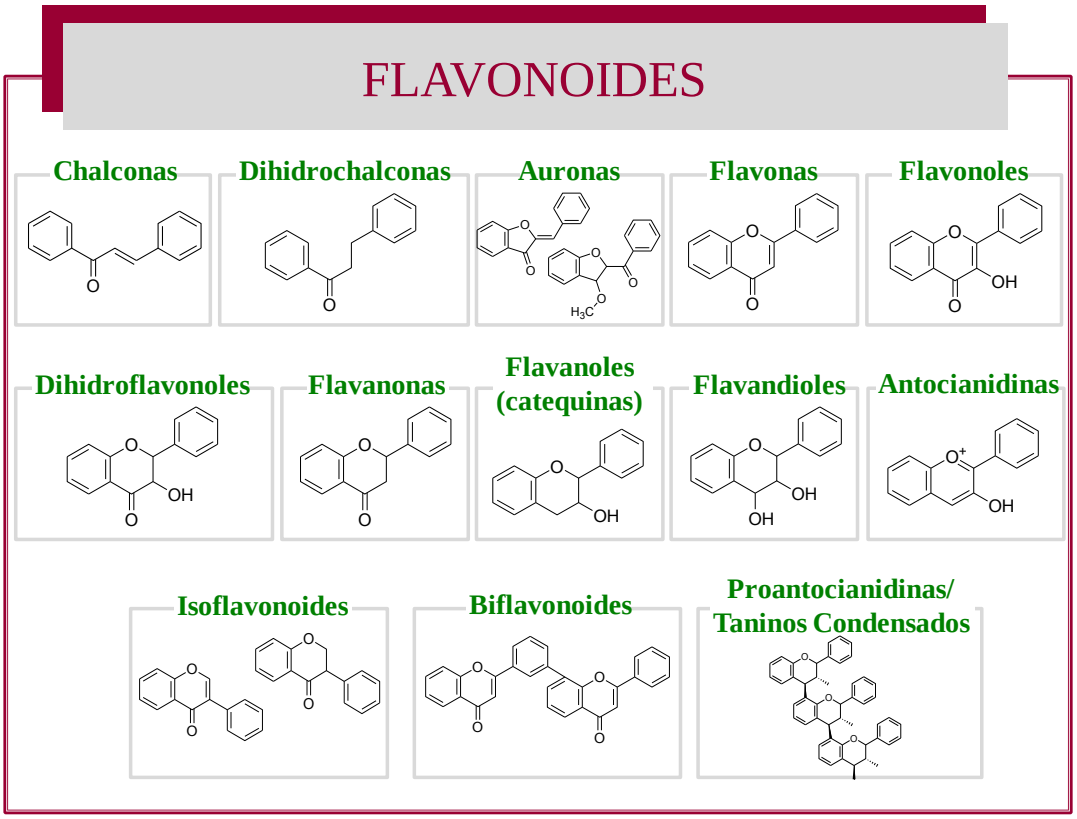


Figura 4. Clasificación de las principales familias de flavonoides

Uno de los grupos más abundantes y complejos de flavonoides, son las proantocianidinas que están constituidas por unidades de (epi)-catequina. Pueden también estar formadas por al menos una unidad de (epi)-galocatequina o (epi)-afzelequina, denominándose, prodelfinidinas y propelargonidinas, respectivamente[24].

En los vegetales, los compuestos fenólicos pueden aparecer como agliconas o estar conjugados a una o varias moléculas de glucosa u otro monosacárido a través de un grupo hidroxilo. En algunos casos se pueden encontrar uniones directas C-C entre el carbohidrato y un anillo aromático del compuesto fenólico. En consecuencia, la forma más común en la que se encuentran en la naturaleza es

como o-glucósidos, aunque también se pueden producir otras modificaciones de la estructura que dan lugar a derivados de ésteres o metil-ésteres[25].

En cuanto a su disposición en las plantas, la distribución de estos compuestos en los tejidos vegetales, a nivel celular y subcelular, no es uniforme lo que va a influir en las propiedades biológicas de cada parte de la planta. Los compuestos fenólicos insolubles se encuentran, en las paredes celulares, mientras que los solubles se encuentran en las vacuolas. Las capas exteriores contienen mayores cantidades de polifenoles que las interiores, ya que estos compuestos captan las radiaciones UV, impidiendo sus efectos nocivos en los tejidos internos. A su vez, los compuestos fenólicos desempeñan funciones fisiológicas y morfológicas importantes dentro de la planta, por ejemplo, actúan en el crecimiento y reproducción ya que las semillas acumulan importantes cantidades de fenoles en sus cubiertas que hacen de filtro para que el oxígeno no llegue al embrión, lo que influiría negativamente en su germinación.

Por otra parte, los polifenoles son en gran parte los responsables de las principales características organolépticas de los alimentos y bebidas procedentes de vegetales, como se muestra en la figura 5. Éstos, contribuyen en el color de los vegetales (incluyen pigmentos amarillos, naranjas, rojos y azules), en el sabor (sobre todo en el amargor y astringencia, que es el resultado de la interacción entre los taninos y las proteínas de la saliva), en el olor, y en la estabilidad oxidativa[26].

[25] Barba, F. J.; Esteve, M. J.; Frígola, A. Bioactive components from leaf vegetable products. In *Studies in Natural Products Chemistry*; Rahman, A.; Elsevier, **2014**, vol. 41, pp 321–346.
[26] Naczk, M.; Shahidi, F. Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1523–1542.

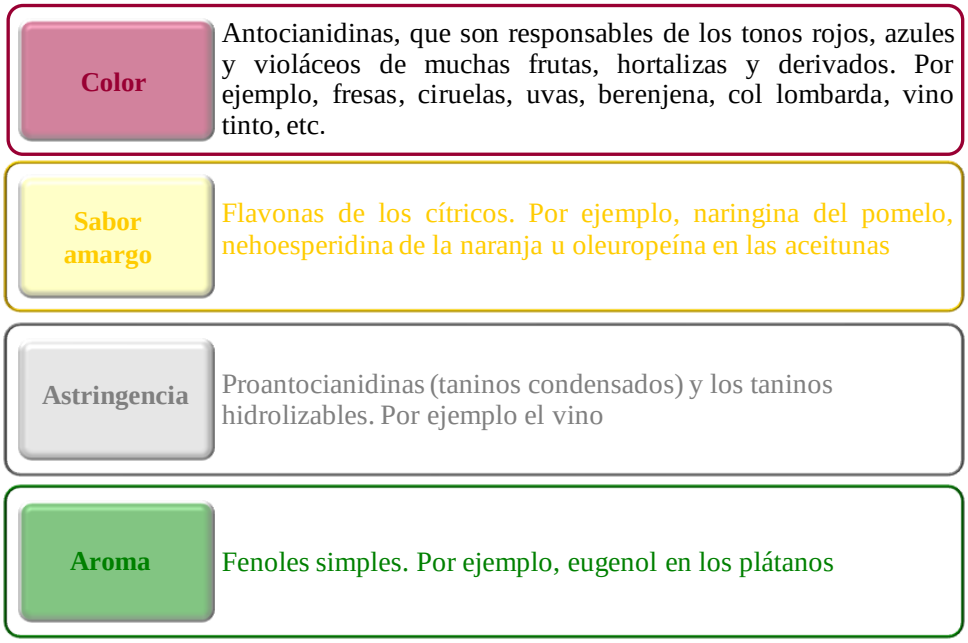


Figura 5. Propiedades organolépticas atribuidas a los compuestos fenólicos y algunos ejemplos

Los factores genéticos, las condiciones ambientales y las prácticas agronómicas juegan un papel importante en la composición fenólica de los vegetales. Los cultivos pueden estar afectados por numerosas variables antes de la cosecha, incluyendo factores endógenos como el genotipo (especie y variedad), la madurez de la cosecha, y la distribución de los compuestos en las distintas partes del vegetal; y factores exógenos, abióticos, como las condiciones climáticas (temperatura, lluvia, etc), condiciones agronómicas (riego, fertilización, etc), las condiciones y la composición del suelo (minerales y nutrientes orgánicos), así como, factores bióticos, por ejemplo, el estrés provocado por microorganismos patógenos, animales herbívoros o insectos polinizadores. Otro tipo de factores post-cosecha, como el proceso de recolección, transporte y condiciones de almacenamiento, también influyen en el contenido polifenólico de los vegetales[27,28].

[27] 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*, 853–876.

[28] Pérez-Gregorio, M. R.; Regueiro, J.; Simal-Gándara, J.; Rodrigues, a S.; Almeida, D. P. F. Increasing the added-value of onions as a source of antioxidant flavonoids: a critical review. *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 1050–1062.

Como se comentó previamente, muchos de los beneficios para la salud, derivados de la ingesta de frutas y verduras, están asociados a su composición fenólica, pues son los compuestos antioxidantes más abundantes en este tipo de alimentos. Estudios epidemiológicos sugieren que dietas ricas en polifenoles vegetales ofrecen protección contra el desarrollo de una amplia variedad de enfermedades crónicas como, cáncer, enfermedades cardiovasculares, diabetes, osteoporosis y enfermedades neurodegenerativas, entre otras[23,24,29]. Los posibles mecanismos de acción que pueden ejercer los compuestos fenólicos y la bioactividad asociada los mismos se pueden ver en la siguiente figura:

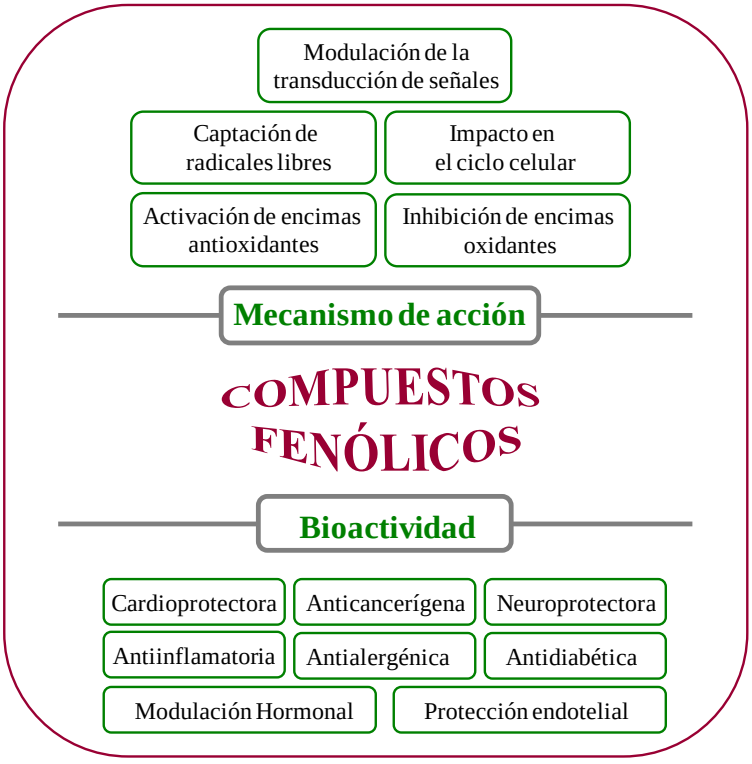


Figura 6. Esquema de los mecanismos de acción más conocidos y bioactividad asociada de los compuestos fenólicos

Con el fin de poder establecer los diferentes mecanismos de acción que expliquen estos efectos saludables, en los últimos años, se ha incrementado el estudio del papel protector de los compuestos fenólicos sobre las distintas

[29] Arts, I. C. W.; Hollman, P. C. H. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr.* **2005**, *81 (suppl.)*, 317S–325S.

enfermedades mencionadas, por lo que se han llevado a cabo numerosos estudios tanto *in vitro*, como en animales e incluso en humanos[30].

No obstante, los efectos protectores de los polifenoles *in vivo*, como en todos los compuestos bioactivos, dependen de su biodisponibilidad y capacidad de extracción de los alimentos, la absorción intestinal, el metabolismo, la acción biológica final en el cuerpo humano, y la interacción potencial con los tejidos diana[10].

2.2. Ácidos grasos

Los ácidos grasos son ácidos carboxílicos (RCOOH) formados por cadenas hidrocarbonadas de 4 a 36 átomos de carbono, cuya estructura es, generalmente, no ramificada y puede presentar enlaces saturados o insaturados. La coexistencia entre una parte alifática hidrofóbica (cadena hidrocarbonada) y un grupo funcional hidrofílico (grupo carboxilo), convierte a los ácidos grasos en moléculas anfipáticas. El carácter anfipático depende de la longitud de la cadena hidrocarbonada, siendo mayor cuanto menor es la longitud de la misma, por tanto, la solubilidad en agua decrece conforme aumenta la longitud de la cadena[31]. En la figura 7, se muestran dos ejemplos de ácidos grasos presentes en alimentos de origen vegetal.

[30] Chang, H. P.; Sheen, L.Y.; Lei, Y. P. The protective role of carotenoids and polyphenols in patients with head and neck cancer. *J. Chinese Med. Assoc.* **2015**, *78*, 89–95.
[31] Stillwell, W. Membrane Lipids: Fatty Acids. In *An Introduction to Biological Membranes*; Elsevier, 2013, pp 43–56.

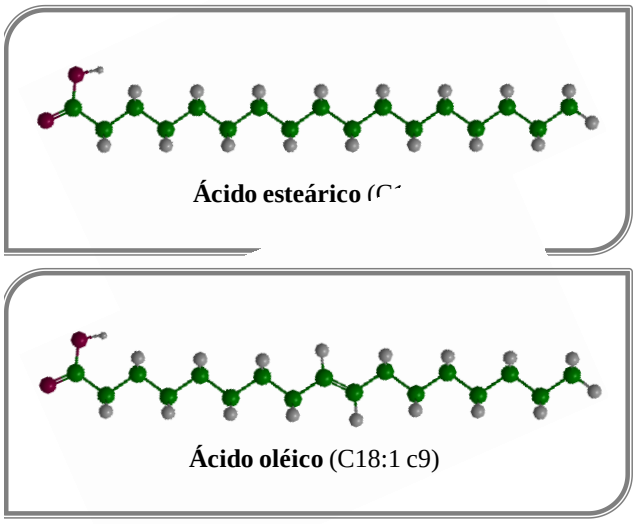


Figura 7. Estructura de dos ácidos grasos presentes en alimentos vegetales

La clasificación de los ácidos grasos se puede realizar en base a la longitud de la cadena alifática o en función del número de instauraciones de la misma[32]:

- Dependiendo de la longitud de la cadena, se denominan ácidos grasos de cadena corta (de 4 a 6 átomos de carbono), ácidos grasos de cadena media (de 8 a 12 átomos de carbono), ácidos grasos de cadena larga (de 14 a 18 átomos de carbono) y ácidos grasos de cadena muy larga (más de 20 átomos de carbono).

- Según el número de insaturaciones pueden ser: ácidos grasos saturados (no poseen dobles enlaces en su molécula); ácidos grasos monoinsaturados o MUFA (tienen un único doble enlace); y ácidos grasos poliinsaturados o PUFA (poseen más de un doble enlace).

Biológicamente, los ácidos grasos constituyen uno de los grupos fundamentales de los lípidos debido a que son el principal bloque estructural de los triglicéridos. Estos compuestos desempeñan un papel clave en las funciones celulares y por tanto, son vitales para el desarrollo y la salud humana[31–33]. Sin embargo, diversos estudios relacionados con los ácidos grasos que componen la alimentación

[32] Gil-Izquierdo, A. *Libro Blanco de los Omega 3*; Editorial Médica Panamericana, 2013.
[33] Wynn, J. Lipids , Fatty Acids. In Comprehensive Biotechnology; Moo-Young, M.; Elsevier, 2011; Vol. 1, pp 41–52.

demuestran que existe una relación entre consumo de ácidos grasos saturados y ácidos grasos *trans* y el aumento de las lipoproteínas de baja densidad (LDL) y consecuentemente de los niveles de colesterol en sangre, así como, el desarrollo de enfermedades cardiovasculares[34,35]. En contraposición, se ha demostrado que los ácidos grasos insaturados reducen el colesterol sérico, siendo este efecto más notable cuanto mayor número de insaturaciones presente el compuesto[36]. En este contexto, deben ser considerados, particularmente, los ácidos grasos esenciales ya que éstos no pueden ser sintetizados por el organismo y deben obtenerse de la dieta. Se trata de los ácidos grasos poliinsaturados con todos los dobles enlaces en posición *cis*, son los correspondientes a los derivados del acido α-linolénico (ALA, C18:3 ω3) y los derivados del ácido linoleico (C18:2 ω6), cuya principal fuente de obtención son las grasas de origen vegetal[32,37].

Los ácidos grasos ω-3 son componentes esenciales de las membranas celulares, donde son precursores de los eicosanoides (prostaglandinas, prostaciclinas, tromboxanos y leucotrienos). Numerosos trabajos de investigación indican que influyen positivamente en el desarrollo neurológico del feto y el recién nacido, en la visión, en enfermedades relacionadas con el sistema inmunitario, y enfermedades cardiovasculares, etc. Debido a su funcionalidad, la industria alimentaria los ha incorporado a diversos productos alimenticios como leche, zumo o galletas.

Por otra parte, a los ácidos grasos ω-6 se les atribuyen efectos beneficiosos sobre enfermedades como la dermatitis alérgica, cáncer, diabetes y esclerosis múltiple, a su vez, en equilibrio con los ω-3, actúan como protectores cardiovasculares. Sin

[34] Olagnero, G.; Marcenado, J. Alimentos funcionales : compuestos de naturaleza lipídica. *Diaeta* **2007**, *25* (120), 31–42.
[35] Lichtenstein, A. H. Dietary Trans fatty acids and cardiovascular disease risk: past and present. *Curr. Atheroscler. Rep.* **2014**, *16* (433), 1–7.
[36] Pedersen, J. I.; Kirkhus, B. Health aspects of saturated fatty acids. In Reducing Saturated Fats in Foods; Talbot, G.; Woodhead Publishing, 2011, pp 77–97.
[37] Vicente, A. R.; Manganaris, G. A.; Sozzi, G. O.; Crisosto, C. H. Nutritional Quality of Fruits and Vegetables. In *Postharvest Handlin*, 2014, pp 69–122.

embargo, un aporte excesivo de los mismos puede dar lugar a procesos protrombóticos y proinflamatorios[11,34,38].

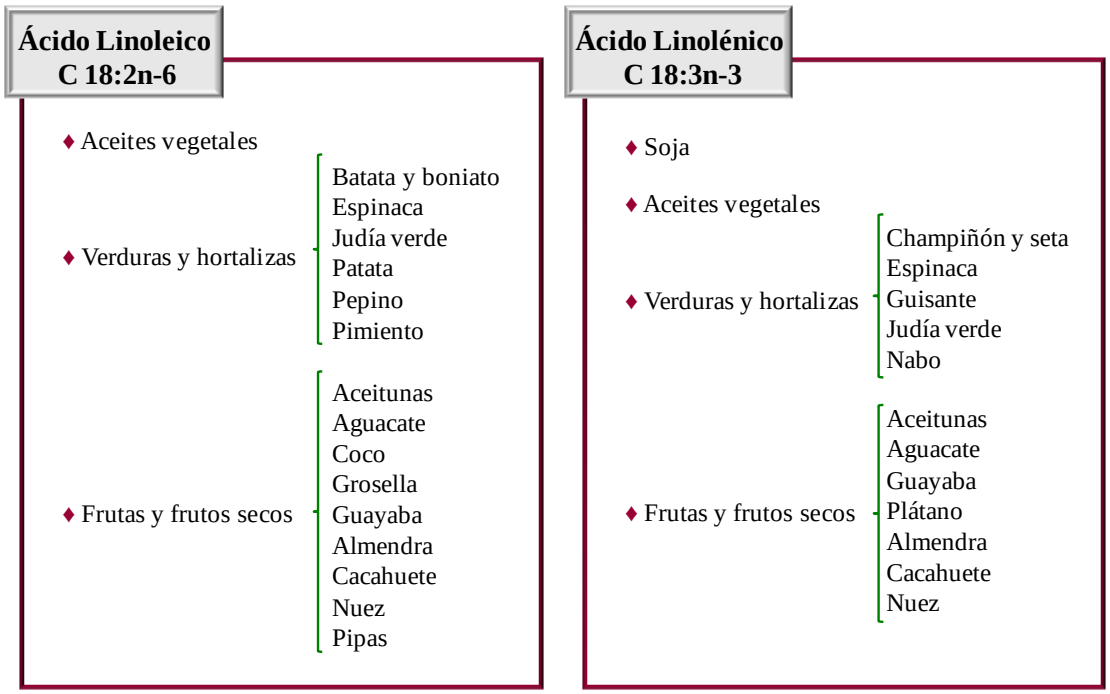


Figura 8. Algunas fuentes vegetales de ácido linoleico y ácido linolénico (adaptado de 32)

2.3. Fosfolípidos

Los fosfolípidos son lípidos anfipáticos que forman parte de todas las membranas celulares de plantas y animales, dispuestos como bicapas lipídicas.

Químicamente, los fosfolípidos vegetales son ésteres de ácido fosfatídico que pertenecen al grupo de lípidos derivados del glicerol, cuya estructura está compuesta por una molécula de glicerol a la que se unen dos ácidos grasos en las posiciones 1 y 2. Estos ácidos grasos pueden ser diferentes en cuanto a número de carbonos de la cadena alifática y/o grado de insaturación. Presentan una estructura similar a la de los triglicéridos con la diferencia de que el carbono 3 de la molécula de glicerol está unido a una molécula de ácido ortofosfórico, en lugar

[38] Calder, P. C. Nutritional benefits of omega-3 fatty. In *Food Enrichment with Omega-3 Fatty Acids*; Jacobsen, C.; Nielsen, N.S.; Frisenfeldt Horn, A.; Moltke Sørensen, A. D.; Woodhead Publishing, 2013, pp 3–26.

de a un tercer ácido graso (ver figura 9). El grupo fosfato puede estar, a su vez, unido a diferentes tipos de moléculas, lo que caracteriza a los diferentes tipos de fosfolípidos[39,40].

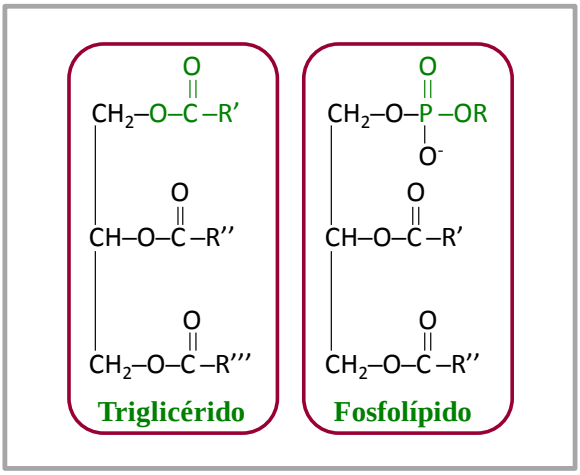


Figura 9. Comparación de la estructura general de un triglicérido y un fosfolípido

Los fosfolípidos más abundantes en las membranas celulares, son los conocidos como glicerofosfolípidos (derivados del glicerol): ácido fosfatídico, fosfatidiletanolamina (cefalina), fosfatidilinositol, fosfatidilcolina (lecitina) y fosfatidilserina, cuyas estructuras se pueden ver en la figura 9. Cuando en la estructura de los fosfolípidos, en lugar de la molécula de glicerol hay una molécula de esfingosina, se clasifican como esfingofosfolípidos[41], este grupo de fosfolípidos es menos abundante que el anteriormente descrito.

[39] Frank, A. W. Phospholipids. In *Chemistry of Plant Phosphorus Compounds;* Elsevier, **2013**, pp 159–304.
[40] Torres, J.; Durán, S. Fosfolípidos : propiedades y efectos sobre la salud. *Nutr. Hosp.* **2015**, *31* (1), 76–83.
[41] Küllenberg, D.; Taylor, L. a; Schneider, M.; Massing, U. Health effects of dietary phospholipids. *Lipids Health Dis.* **2012**, *11* (3), 1–16.

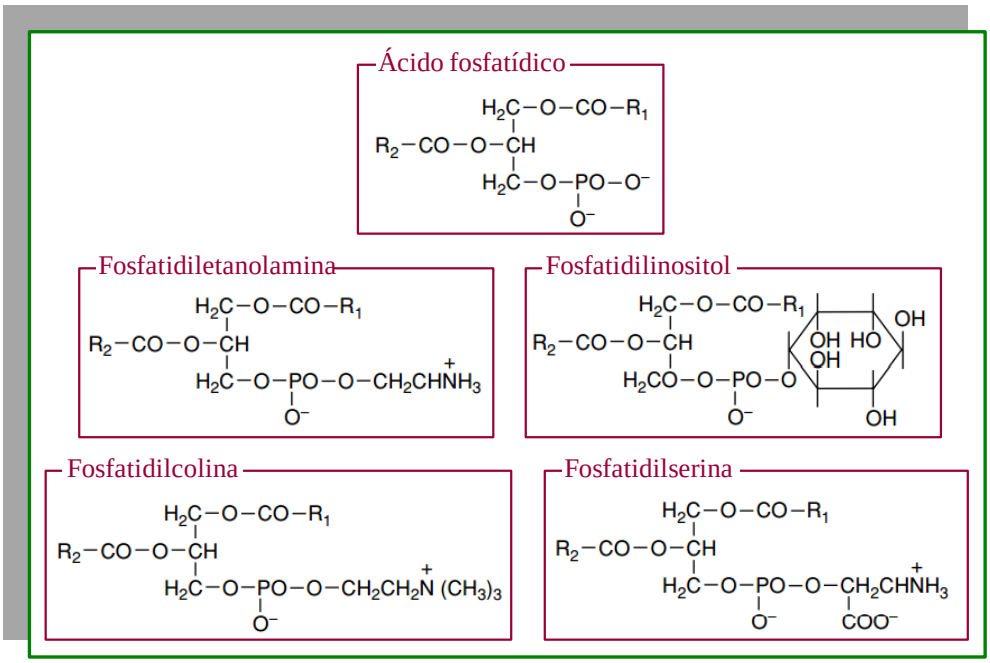


Figura 10. Estructura de los principales fosfolípidos

La composición de estos compuestos es diferente, según la fuente vegetal de la que procedan. Dentro de una misma especie, las clases de fosfolípidos son similares y generalmente, varían en los ácidos grasos unidos al grupo glicerol[42].

Los efectos beneficiosos de los fosfolípidos de la dieta[41] se conocen desde principios de 1900 y se les relaciona con la prevención de diferentes enfermedades como enfermedades coronarias, inflamación o cáncer. Los glicerofosfolípidos son capaces de interactuar con las membranas celulares afectando positivamente a la microestructura de la membrana y su función e influyendo, así, en una gran cantidad de procesos de señalización y actividades enzimáticas.

Utilizados como suplementos alimenticios, estos compuestos, podrían ser muy útiles para mejorar la gran variedad de condicionantes para una salud óptima, dado que no existen efectos secundarios descritos hasta el momento[41].

[42] Szuhaj, B. F. Phospholipids. In *Encyclopedia of Food Sciences and Nutrition;* Caballero, B.; Elsevier, 2003, pp 4519–4523.

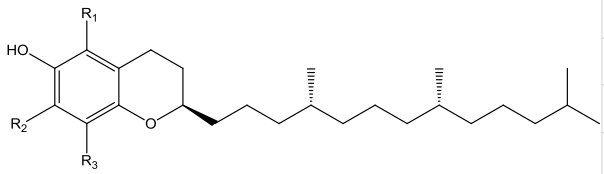
2.4. Tocoferoles

Tocoferoles es el nombre genérico que se le da a la familia de compuestos antioxidantes liposolubles, algunos de los cuales poseen actividad de "vitamina E". Los tocoferoles, a diferencia de otros antioxidantes, se sintetizan exclusivamente en los organismos fotosintéticos tales como plantas superiores y algas.

Estos compuestos tienen en común un anillo cromanol (alcohol aromático), cuyo grupo hidroxilo es capaz de donar un átomo de hidrógeno, lo que facilita su capacidad para la reducción de radicales libres y una cadena lateral hidrofóbica que posibilita su penetración en membranas biológicas. Cuando la cadena lateral es saturada se habla de tocoferoles y si presenta insaturaciones se denominan tocotrienoles, estos últimos también presentan actividad de "vitamina E".

Los principales tipos de tocoferoles (o tocotrienoles) derivan de la adición de un grupo metilo (-CH_3) en alguno de los carbonos disponibles del anillo cromanol, dando lugar cuatro formas primarias: α, β, γ, y δ-tocoferol[25,43], como se muestra en la siguiente tabla.

Tabla 1. Estructuras de los tocoferoles más característicos

Estructura principal	R_1	R_2	R_3	Nombre
	CH_3	CH_3	CH_3	α-tocoferol
	CH_3	H	CH_3	β-tocoferol
	H	CH_3	CH_3	γ-tocoferol
	H	H	CH_3	δ-tocoferol

Dependiendo de la cantidad de grupos metilo y del número de insaturaciones de la cadena lateral, los tocoferoles presentan una mayor o menor actividad.

[43] Hasanuzzaman, M.; Nahar, K.; Fujita, M. *Role of Tocopherol (Vitamin E) in Plants: Abiotic Stress Tolerance and Beyond*. In *Emerging Technologies and Management of Crop Stress Tolerance*; Ahmad, P.; Rasool, S.; Elsevier, 2014, Vol 2, pp 267–289.

Estos compuestos están ampliamente distribuidos en la naturaleza, siendo las principales fuentes dietéticas aceites vegetales y derivados, frutos secos, cereales, vegetales verdes y frutas[37,44]. La cantidad de estos compuestos varía dependiendo de la especie y partes de la planta. Durante la biosíntesis la concentración de tocoferoles aumenta bajo factores ambientales adversos y proporciona una mejor protección contra el daño oxidativo[43].

Los efectos beneficiosos de los tocoferoles en la salud humana están relacionados, como en la mayoría de los compuestos bioactivos, con su acción antioxidante. Por lo tanto, pueden disminuir el riesgo de padecer enfermedades crónicas relacionadas con el daño oxidativo, por ejemplo, pueden tener un efecto protector mediante la reducción del colesterol LDL debido a la inhibición de la biosíntesis del colesterol. También existen algunos estudios que relacionan algunos tocoferoles y tocotrienoles con la supresión de la proliferación de células tumorales[45,46].

2.5. Fitoesteroles

Los esteroles vegetales o fitoesteroles son compuestos derivados de las plantas que poseen estructuras parecidas y funciones similares al colesterol en los animales. Así, las membranas de las plantas, en lugar de contener colesterol, presentan diferentes tipos de esteroles vegetales que pueden existir en forma libre o formando parte de las mismas donde juegan importantes funciones

[44] Woollard, D.C.; Indyk, H. E. Tocopherols, properties and determination. In *Encyclopedia of Food Sciences and Nutrition*; Elsevier, 2003, pp 5789–5796.

[45] Qureshi, A. A.; Mo, H.; Packer, L.; Peterson, D. M. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *J. Agric. Food Chem.* **2000**, *48*, 3130–3140.

[46] Ryynänen, M.; Lampi, A. M.; Salo-Väänänen, P.; Ollilainen, V.; Piironen, V. A small-scale sample preparation method with HPLC analysis for determination of tocopherols and tocotrienols in cereals. *J. Food Compos. Anal.* **2004**, *17*, 749–765.

estructurales. También se pueden encontrar esterificados, representando productos de almacenamiento celular[47].

Estos compuestos, pertenecen a la familia de los triterpenos, con un anillo tetracíclico y una cadena lateral unida al carbono 17. Su estructura es similar a la del colesterol, pero incluye un grupo metilo o etilo en el carbono 24[47,48], como se puede ver en la figura 11.

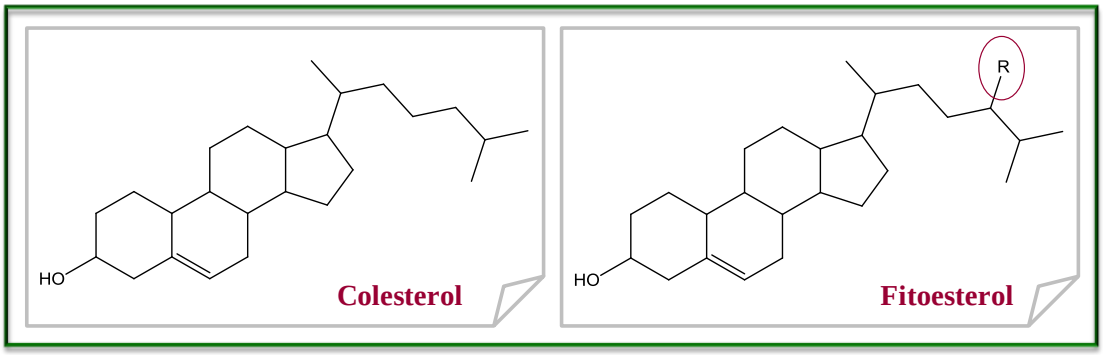


Figura 11. Comparación de la estructura del colesterol y la estructura general de los fitoesteroles

Dentro del grupo de los fitoesteroles se pueden encontrar dos categorías: los esteroles, que presentan un doble enlace en la posición 5 de su estructura y los estanoles, que no cuentan con dicho doble enlace. Actualmente, se encuentran descritos más de 200 tipos de fitoesteroles diferentes, de todos ellos los más característicos son el sitosterol o β-sitosterol, seguido por el campesterol y el estigmasterol, cuyas estructuras se muestran en la figura 12. En general, los estanoles son menos abundantes que los esteroles, siendo el más común el sitostanol[34].

[47] Jones, P. J. H.; Rideout, T. C. Plant sterols: nutritional aspect. In Comprehensive Biotechnology; Moo-Young, M.; Elsevier, 2005; Vol. 4, pp 535–542.
[48] Palou, A.; Catalina, O.; Segura, P.; Luisa, M.; Piña, B.; Oliver, P. *El libro blanco de los esteroles vegetales*; Unilever Foods S. A., 2005.

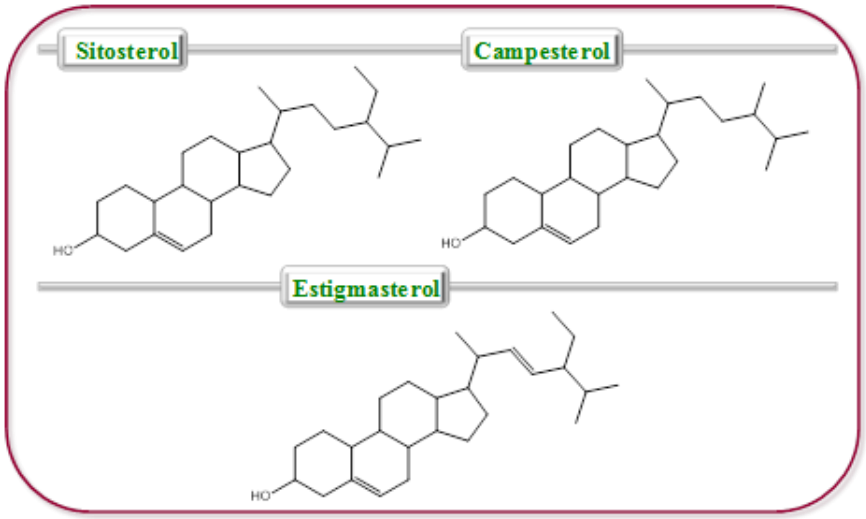


Figura 12. Estructura de los fitoesteroles más comunes

Los esteroles vegetales son constituyentes naturales de los alimentos, y normalmente se encuentran en los aceites vegetales, nueces, semillas, productos de granos, frutas y verduras[47].

Debido a que el cuerpo humano es incapaz de sintetizar esteroles vegetales, la ingesta mediante la dieta es la única fuente de estos compuestos bioactivos. Existen una gran cantidad de estudios experimentales que han demostrado el importante efecto hipocolesterolémico de los fitoesteroles, reduciendo las concentraciones de colesterol total y las de colesterol LDL, influyendo, de esta forma, en la prevención de enfermedades cardiovasculares. Asimismo, se han estudiado otras propiedades como posibles efectos anticancerígenos, potenciadores de la inmunidad y antiinflamatorios[11,48– 50] . De hecho, son comercializados una gran cantidad de alimentos funcionales que contienen estos compuestos.

[49] Hooper L., C. A. A rewiew of the health care potencial of bioactive compounds. *J. Sci. Food Agric*. **2006**, *86*, 1805–1813.
[50] Marangoni, F.; Poli, A. Phytosterols and cardiovascular health. *Pharmacol. Res. 61*, 193–199.

3. SELECCIÓN DE FUENTES VEGETALES PARA LA BÚSQUEDA DE COMPUESTOS BIOACTIVOS

Debido al potencial terapéutico atribuido a los componentes bioactivos, la búsqueda estos compuestos en alimentos naturales se ha intensificado en los últimos años con el fin de obtener principios activos inocuos y eficaces. En la industria farmacéutica y alimentaria, los productos de origen vegetal representan una importante fuente inagotable de nuevas moléculas bioactivas que pueden ser interesantes para el desarrollo de fármacos, nutracéuticos y alimentos funcionales[51,52].

Por otro lado, el procesamiento de los alimentos de origen vegetal genera grandes cantidades de subproductos y la eliminación de los mismos representa un costo para la industria alimentaria, además del potencial impacto medioambiental que conlleva. En los últimos 20 años numerosas investigaciones han revelado que muchos de estos subproductos podrían servir como fuente de compuestos bioactivos potencialmente valiosos. Sin embargo, a pesar de esto, la gran mayoría de los subproductos vegetales no están explotados como fuentes de estos compuestos[53].

En base a esto, en la presente tesis doctoral se ha estudiado la composición en compuestos bioactivos, especialmente compuestos fenólicos, de diferentes matrices vegetales. Seguidamente, se describen las características principales de cada uno de los vegetales que han sido objeto de estudio.

[51] Drago Serrano, M. E.; López López, M.; Saínz Espuñes, T. D. R. Componentes bioactivos de alimentos funcionales de origen vegetal. *Rev. Mex. Ciencias Farm.* **2006**, *37* (4), 58–68.

[52] Dudonne, S.; Vitrac, X.; Coutie, P.; Woillez, M.; Merillon, J. Comparative Study of Antioxidant Properties and Total Phenolic Content of 30 Plant Extracts of Industrial Interest Using DPPH , ABTS , FRAP. *J. Agric. Food Chem.* **2009**, *57*, 1768–1774.

[53] Wijngaard, H.; Hossain, M. B.; Rai, D. K.; Brunton, N. Techniques to extract bioactive compounds from food by-products of plant origin. *Food Res. Int.* **2012**, *46*, 505–513.

3.1. Mangostán

El mangostán (*Garcinia mangostana* L.) es el fruto de un árbol imperecedero tropical, originario del sudeste asiático, perteneciente a la familia Clusiaceae. Hoy en día, esta fruta se cultiva en las regiones tropicales de ambos hemisferios, occidental y oriental, con plantaciones comerciales en Tailandia, India, Malasia y Filipinas, otros países de Asia y Sudamérica son productores menores de la misma. Apreciada por su excelente sabor, en Asia, es llamada "la reina de las frutas"[54,55].

La estructura del mangostán es similar, en forma y tamaño, a la de la mandarina y se compone de pericarpio o corteza, pulpa o aril y semillas. El pericarpio se caracteriza por su grosor, suavidad y un color púrpura muy característico cuando la fruta está madura; la pulpa o aril consiste en segmentos de color blanco-rosáceos de exquisito sabor, donde se encuentran encajadas sus grandes semillas. En cuanto a las variedades de esta fruta, por lo general, solamente se diferencian en tamaño, sabor y grado de acidez ya que el fruto es bastante uniforme[56,57].

Durante siglos, el pericarpio del mangostán ha sido utilizado en la medicina tradicional asiática por sus efectos beneficiosos en el tratamiento de infecciones y heridas de la piel, así como en el tratamiento del dolor abdominal, inflamación, disentería y diarrea, entre otras afecciones[54,58].

Hoy día, tanto el extracto de la porción comestible como el de la piel se usan para la elaboración de suplementos alimenticios, hierbas medicinales, y/o cosméticos

[54] Pedraza-Chaverri, J.; Cárdenas-Rodríguez, N.; Orozco-Ibarra, M.; Pérez-Rojas, J. M. Medicinal properties of mangosteen (*Garcinia mangostana*). *Food Chem. Toxicol.* **2008**, *46*, 3227–3239.

[55] Obolskiy, D.; Pischel, I.; Siriwatanametano, N.; Heinrich, M. *Garcinia mangostana* L.: A Phytochemical and Pharmacological Review. *Phyther. Res.* **2009**, *23*, 1047–1065.

[56] Ketsa, S.; Paull, R. E. Mangosteen *(Garcinia mangostana L.)*.In *Postharvest Biology and Technology of Tropical and Subtropical Fruits*; Elhadi, Y.; Elsevier, 2011, pp 1–32e.

[57] Morton, J. F. *Fruits of Warm Climates*; 2004.

[58] Jung, H. A.; Su, B. N.; Keller, W. J.; Mehta, R. G.; Kinghorn, a. D. Antioxidant xanthones from the pericarp of *Garcinia mangostana* (Mangosteen). *J. Agric. Food Chem.* **2006**, *54*, 2077–2082.

naturales. Esto es debido a que diversos estudios demuestran el gran número de sustancias biológicamente activas que contiene esta fruta[59].

Como se muestra en la figura 13, tanto en la pulpa como en la piel del mangostán se han detectado ácidos fenólicos. Así mismo, se han encontrado otros polifenoles complejos como, flavonoides, xantonas, antocianinas y proantocianidinas, fundamentalmente en la piel[60,61].

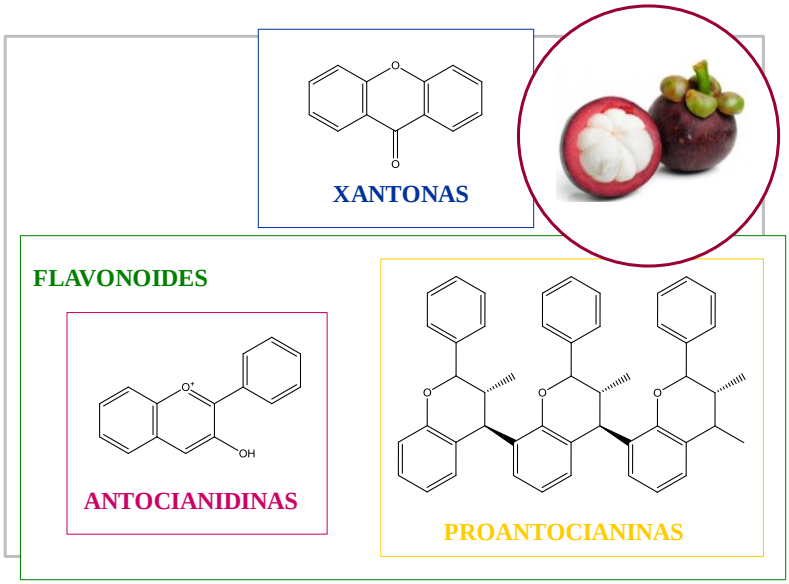


Figura 13. Principales familias de compuestos fenólicos del mangostán

De la composición de la fruta, lo más destacable es su contenido en xantonas y derivados, de las 200 xantonas conocidas en la naturaleza, más de 40 se encuentran en la misma.

[59] Pothitirat, W.; Chomnawang, M. T.; Supabphol, R.; Gritsanapan, W. Comparison of bioactive compounds content, free radical scavenging and anti-acne inducing bacteria activities of extracts from the mangosteen fruit rind at two stages of maturity. *Fitoterapia* **2009**, *80*, 442–447.

[60] Palakawong, C.; Sophanodora, P.; Toivonen, P.; Delaquis, P. Optimized extraction and characterization of antimicrobial phenolic compounds from mangosteen (Garcinia mangostana L.) cultivation and processing waste. *J. Sci. Food Agric.* **2013**, *93*, 3792–3800.

[61] Zarena, a. S.; Sankar, K. U. Phenolic acids, flavonoid profile and antioxidant activity in mangosteen (*Garcinia mangostana* L.) pericarp. *J. Food Biochem.* **2012**, *36*, 627–633.

Estudios recientes han demostrado las propiedades farmacológicas de la *Garcinia mangostana*, proporcionadas, en gran parte, por las mencionadas xantonas, como por ejemplo, efecto protector en enfermedades coronarias. A su vez, estudios desarrollados a partir de extractos del pericarpio de la fruta ponen de manifiesto propiedades anticancerígenas y citotóxicas[62–64].

En resumen, de las propiedades atribuidas al mangostán, cabe destacar: antiinflamatoria, antitumoral, antialergénica, antibacteriana, antiviral, anticolesterolémica, e incluso se ha encontrado actividad antimalaria[64–66].

3.2. Limón

El limón (*Citrus limon* L.) es el fruto en baya del limonero, árbol de hoja perenne y espinoso perteneciente a la familia de las Rutaceae y originario de Asia. Su desarrollo está favorecido por climas templados y tropicales, cultivándose actualmente en todo el mundo. El limón es el tercer cultivo cítrico más importante en el mundo (después de la naranja y la mandarina), siendo España, el tercer país productor de limón y cultivándose principalmente en el Sureste de

[62] Ho, C. K.; Huang, Y. L.; Chen, C. C. Garcinone E, a xanthone derivative, has potent cytotoxic effect against hepatocellular carcinoma cell lines. *Planta Med.* **2002**, *68*, 975–979.

[63] Suksamrarn, S.; Komutiban, O.; Ratananukul, P.; Chimnoi, N.; Lartpornmatulee, N.; Suksamrarn, A. Cytotoxic prenylated xanthones from the young fruit of *Garcinia mangostana*. *Chem. Pharm. Bull.* **2006**, *54* (3), 301–305.

[64] Pierson, J. T.; Dietzgen, R. G.; Shaw, P. N.; Roberts-Thomson, S. J.; Monteith, G. R.; Gidley, M. J. Major Australian tropical fruits biodiversity: Bioactive compounds and their bioactivities. *Mol. Nutr. Food Res.* **2012**, *56*, 357–387.

[65] Suksamrarn, S.; Suwannapoch, N.; Phakhodee, W.; Thanuhiranlert, J.; Ratananukul, P.; Chimnoi, N.; Suksamrarn, A. Antimycobacterial activity of prenylated xanthones from the fruits of *Garcinia mangostana*. *Chem. Pharm. Bull.* **2003**, *51* (7), 857–859.

[66] Leontowicz, H.; Leontowicz, M.; Drzewiecki, J.; Haruenkit, R.; Poovarodom, S.; Park, Y. S.; Jung, S. T.; Kang, S. G.; Trakhtenberg, S.; Gorinstein, S. Bioactive properties of Snake fruit (*Salacca edulis Reinw*) and Mangosteen (*Garcinia mangostana*) and their influence on plasma lipid profile and antioxidant activity in rats fed cholesterol. *Eur. Food Res. Technol.* **2006**, *223*, 697–703.

Valencia y Murcia, donde se localiza aproximadamente el 95% de las áreas de crecimiento de la fruta cítrica en España[67,68].

La estructura de la fruta es similar a la de otros cítricos y consiste en la piel y la parte comestible del interior (endocarpio o pulpa). La piel está formada por dos capas distintas; la capa exterior se denomina flavedo (epicarpio), y el interior, la parte blanca esponjosa, es el albedo (mesocarpio). En cuanto a las variedades, la más cultivada en España, así como en Argelia y Marruecos, es el limón 'Verna', cuya cosecha principal se produce en invierno. Sin embargo, en España, una gran cantidad de la fruta está disponible para la cosecha de verano, una temporada alta para la demanda de limón[69]. Las principales variedades de limón se resumen en siguiente tabla, junto con sus regiones de cultivo principales.

Tabla 2. Principales variedades de limón y zona de cultivo

Variedad	Región principal de cultivo
Bearss (Siciliano)	Florida (USA)
Eureka	California (USA), Australia, Argentina, Israel
Lamas	Turquia
Lisbon	California , Arizona (USA), Australia
Verna (Berna)	España

El limón es consumido fresco o procesado, siendo los principales productos de procesamiento el zumo y el aceite extraído de la piel o la flor ya que estas dos partes de la fruta son una fuente importante de aceites esenciales y compuestos

[67] González-Molina, E.; Domínguez-Perles, R.; Moreno, D. A.; García-Viguera, C. Natural bioactive compounds of *Citrus limon* for food and health. *J. Pharm. Biomed. Anal.* **2010**, *51*, 327–345.

[68] Ministerio de Agricultura, Alimentación y Medio Ambiente http://www.magrama.gob.es/es/buscador/resultados-busqueda (*accessed Oct 6, 2015*).

[69] Goodrich, R. Citrus fruits. Lemon. In *Encyclopedia of Food Sciences and Nutrition*; Trugo, L.; Finglas, P. M.; Elsevier**,** 2003, pp 1354–1359.

aromáticos cuyos extractos pueden ser utilizados para la fabricación de aditivos y fragancias[70]. No obstante, recientemente, las frutas cítricas y los subproductos derivados de las mismas, han recibido especial atención por sus beneficios para la salud en la nutrición humana.

Las propiedades saludables del limón radican principalmente en su elevada capacidad antioxidante de la que son responsables la gran de compuestos bioactivos que posee, entre ellos destacan los compuestos fenólicos[71,72]. Los polifenoles principales del limón son los flavonoides y dentro de éstos, los más abundantes son las flavanonas, aunque también posee flavonas y flavonoles. En menor cantidad se pueden encontrar ácidos fenólicos, ácidos hidroxicinámicos y cumarinas[67,70,73]. En la figura 14 se muestra un esquema de las estructuras de las principales familias de compuestos fenólicos presentes en limón. La distribución y concentración estos compuestos, como en todos los vegetales, va a depender de parte de la fruta es decir, piel, pulpa y hueso tendrán una composición fenólica diferente[67].

[70] Amenta, M.; Ballistreri, G.; Fabroni, S.; Romeo, F. V.; Spina, A.; Rapisarda, P. Qualitative and nutraceutical aspects of lemon fruits grown on the mountainsides of the Mount Etna: A first step for a protected designation of origin or protected geographical indication application of the brand name 'Limone dell´Etna'. *Food Res. Int.* **2015**, *74*, 250–259.

[71] Abad-García, B.; Berrueta, L. A.; Garmón-Lobato, S.; Urkaregi, A.; Gallo, B.; Vicente, F. Chemometric characterization of fruit juices from spanish cultivars according to their phenolic compound contents: I. Citrus fruits. *J. Agric. Food Chem.* **2012**, *60*, 3635–3644.

[72] Guimarães, R.; Barros, L.; Barreira, J. C. M.; Sousa, M. J.; Carvalho, A. M.; Ferreira, I. C. F. R. Targeting excessive free radicals with peels and juices of citrus fruits: Grapefruit, lemon, lime and orange. *Food Chem. Toxicol.* **2010**, *48*, 99–106.

[73] Russo, M.; Bonaccorsi, I.; Torre, G.; Sarò, M.; Dugo, P.; Mondello, L. Underestimated sources of flavonoids, limonoids and dietary fibre: Availability in lemon's by-products. *J. Funct. Foods* **2014**, *9*, 18–26.

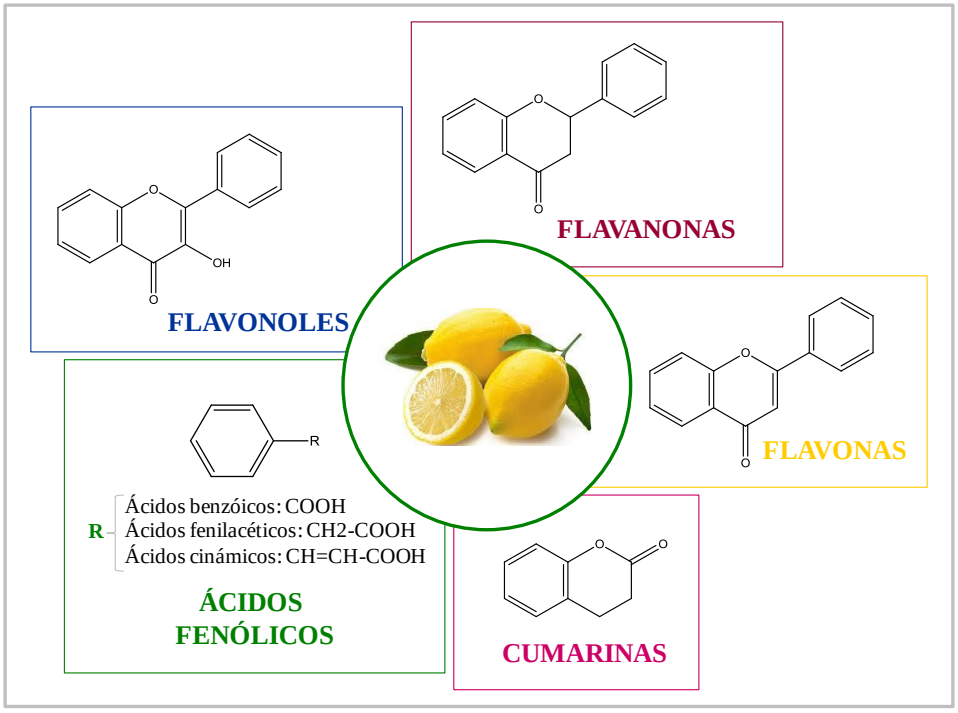


Figura 14. Principales familias de compuestos fenólicos presentes en limón

Diversos estudios indican que estos componentes del limón, especialmente los flavonoides son potencialmente beneficiosos para la salud humana ya que a ellos se les atribuyen diferentes funciones biológicas, incluyendo antivirales, antialérgicas, antiinflamatorias, y actividades anticancerígenas, además de las conocidas propiedades antioxidantes que influyen la prevención de enfermedades cardiovasculares y arteriosclerosis[74,75].

3.3. Berenjena

La berenjena (*Solanum melongena*), es el fruto de una planta herbácea, de la familia Solanaceae. Es originaria del sudeste asiático (India, China y Japón) aunque actualmente se cultiva en casi todo el mundo, sobre todo en países de la

[74] Del Río, J. A.; Fuster, M. D.; Gómez, P.; Porras, I.; García-Lidón, A.; Ortuño, A. Citrus limon: A source of flavonoids of pharmaceutical interest. *Food Chem.* **2004**, *84*, 457–461.
[75] Benavente-García, O.; Castillo, J. Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity. *J. Agric. Food Chem.* **2008**, *56*, 6185–6205.

cuenca mediterránea[76]. Entre las comunidades autónomas españolas con una mayor producción de berenjena se encuentran Andalucía, Murcia, Valencia, Cataluña y Baleares[77].

Es una planta que requiere mucha luz, soporta bien las temperaturas elevadas siempre que la humedad sea adecuada, y es muy sensible al frío. Sus semillas se siembran en invierno en invernaderos caldeados, y se trasplantan al suelo en primavera. Su temporada de recolección y mejor época de consumo se produce de octubre a abril y a veces también en septiembre y mayo[68].

La estructura interna de la berenjena inmadura es comparable a la de tomate, pero a medida que avanza la madurez, las paredes del fruto se fusionan con los tejidos internos desarrollados a partir de la placenta, formando una sola masa de tejido parenquimatoso en el que se encuentran inmersas pequeñas semillas, pardas y planas. El fruto comestible, es una baya con forma esférica, ovalada o alargada en la mayoría de los casos, con una piel lisa y brillante. La pulpa es de color blanco, de textura esponjosa, y con cierto sabor amargo[68, 78]. Existen gran cantidad de variedades de berenjena, las cuales varían según el color, tamaño y forma de la fruta. La variedad más común es la de color morado, pero existen, blanca, púrpura, negra, amarilla y roja, o de colores mezclados (blanco, morado y verde)[68,79].

A pesar de que su valor energético y nutritivo no es muy elevado en comparación con el de otras hortalizas, la berenjena, está entre los 10 vegetales que tienen la

[76] Concellón, A.; Zaro, M. J.; Chaves, A. R.; Vicente, A. R. Changes in quality and phenolic antioxidants in dark purple American eggplant (*Solanum melongena* L. cv. Lucía) as affected by storage at 0°C and 10°C. *Postharvest Biol. Technol.* **2012**, *66*, 35–41.

[77] Prohens J, N. F. Variedades tradicionales de berenjena en España. *vida Rural* **2001**, *46*, 17–32.

[78] Kashyap, V.; Vinod Kumar, S.; Collonnier, C.; Fusari, F.; Haicour, R.; Rotino, G. L.; Sihachakr, D.; Rajam, M. V. Biotechnology of eggplant. *Sci. Hortic.* **2003**, *97*, 1–25.

[79] Luthria, D.; Singh, A. P.; Wilson, T.; Vorsa, N.; Banuelos, G. S.; Vinyard, B. T. Influence of conventional and organic agricultural practices on the phenolic content in eggplant pulp: Plant-to-plant variation. *Food Chem.* **2010**, *121*, 406–411.

capacidad antioxidante más elevada[80]. Esta propiedad es debida a los altos niveles de compuestos bioactivos que posee, especialmente compuestos fenólicos. Como se puede ver en la figura 15, la familia principal de compuestos fenólicos que componen la pulpa de berenjena son ácidos hidroxicinámicos y derivados, entre los que se incluyen clorogénico, cafeico y p-cumárico[80,81]. En la piel de las variedades de berenjena pigmentada, se encuentran antocianidinas, sobre todo, nasunina, delfinidina y derivados. En menor medida, también han sido identificados otros flavonoides[82].

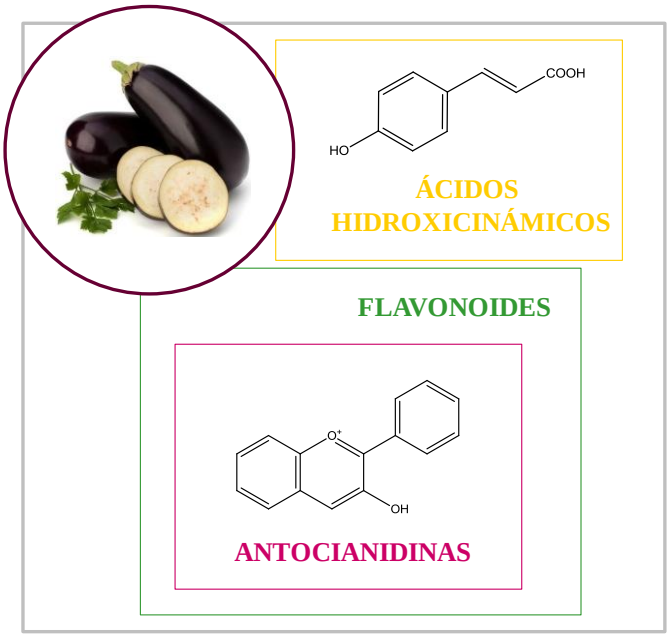


Figura 15. Principales familias de compuestos fenólicos identificadas en berenjena

[80] Whitaker, B. D.; Stommel, J. R. Distribution of hydrocinnamic acid conjugates in fruit of commercial eggplant (*Solanum melongena* L.) cultivars. *J. Agric. Food Chem.* **2003**, *51*, 3448–3454.

[81] Helmja, K.; Vaher, M.; Gorbatšova, J.; Kaljurand, M. Characterization of bioactive compounds contained in vegetables of the Solanaceae family by capillary electrophoresis. *Proc. Est. Acad. Sci. Chem.* **2007**, *56* (4), 172–186.

[82] Azuma, K.; Ohyama, A.; Ippoushi, K.; Ichiyanagi, T.; Takeuchi, A.; Saito, T.; Fukuoka, H. Structures and antioxidant activity of anthocyanins in many accessions of eggplant and its related species. *J. Agric. Food Chem.* **2008**, *56*, 10154–10159.

Los ácidos fenólicos son considerados por sus propiedades antitumorales y anticoleresterolémicas[83]. Por otro lado, a las antocianidinas se les reconocen efectos fisiológicos como antineoplástico, vaso-protector, antiinflamatorio y hepatoprotectivo, entre otros[84]. Así mismo, algunos estudios *in vivo* han demostrado que los compuestos fenólicos extraídos de berenjena poseen una actividad hipolipidémica significativa[85]. Además, se han encontrado extractos de berenjena capaces de inhibir procesos de inflamación relacionados con la aterosclerosis[86].

3.4. Chirimoya

La chirimoya (*Annona cherimola* Mill.) es el fruto comestible del chirimoyo, árbol perteneciente a la familia Anonaceae. Como todos los frutos de esta familia, éste no es simple, sino que se trata de un agregado de frutos adheridos sobre un sólo receptáculo que es producto de las pequeñas flores que se fecundan por separado[68].

El cultivo de la chirimoya es originario de los valles interandinos de Perú y Ecuador, no obstante, en las últimas décadas se ha extendido a varias zonas subtropicales en el mundo[87]. La principal cosecha de este fruto en la cuenca mediterránea es la costa de Granada y Málaga (España), la llamada "Costa Tropical" es por eso que, España está considerada uno de los principales productores, de esta fruta, a nivel mundial. La zona de producción, acondicionamiento y envasado de las chirimoyas están amparados por la

[83] Luthria, D. L.; Mukhopadhyay, S. Influence of sample preparation on assay of phenolc acids from eggplant. *J. Agric. Food Chem.* **2006**, *54*, 41–47.

[84] Todaro, A.; Cimino, F.; Rapisarda, P.; Catalano, A. E.; Barbagallo, R. N.; Spagna, G. Recovery of anthocyanins from eggplant peel. *Food Chem.* **2009**, *114*, 434–439.

[85] Sudheesh, S.; Presannakumar, G.; Vijayakumar, S.; Vijayalakshmi, N. R. Hypolipidemic effect of flavonoids from *Solanum melongena*. *Plant Foods Hum. Nutr.* **1997**, *51*, 321–330.

[86] Han, S. W.; Tae, J.; Kim, J. a.; Kim, D. K.; Seo, G. S.; Yun, K. J.; Choi, S. C.; Kim, T. H.; Nah, Y. H.; Lee, Y. M. The aqueous extract of *Solanum melongena* inhibits PAR2 agonist-induced inflammation. *Clin. Chim. Acta* **2003**, *328*, 39–44.

[87] Brito, B.; Rodríguez, M.; Samaniego, I.; Jaramillo, M. I.; Vaillant, F. Characterising polysaccharides in cherimoya (*Annona cherimola* Mill.) purée and their enzymatic liquefaction. *Eur. Food Res. Technol.* **2008**, *226*, 355–361.

Denominación de Origen "Chirimoya de la Costa Tropical de Granada-Málaga"[68].

El fruto tiene un tamaño similar al del pomelo, y se encuentra recubierto de una piel relativamente gruesa y frágil, de color verde, en la que se dibujan unas escamas que recuerdan a las de un reptil. La pulpa, de color blanco, posee una textura carnosa, blanda y de sabor dulce donde se encuentran numerosas semillas negras encamisadas, es decir, encerradas en los capelos, por lo que el desprendimiento no es directo aunque se desprenden con facilidad. Este fruto madura en invierno, una época óptima para disfrutar de su sabor y propiedades nutritivas[68]. Las dos variedades principales cultivadas en la "Costa Tropical" son, 'Fino de Jete' (más del 90 % de la superficie cultivada), que es la más ampliamente difundida en el mundo, y 'Campa' (aproximadamente un 5%)[88,89].

Es sabido que la chirimoya contiene numerosos nutrientes en su composición, sin embargo, existen muy pocos estudios acerca de los compuestos bioactivos que contiene esta fruta. Entre los compuestos fenólicos, destaca fundamentalmente la presencia de catequinas y derivados[89], y en frutas distintas de la misma familia también se han encontrado otros flavonoides[90]. A parte de estos compuestos fenólicos, que como ya se sabe están relacionados con la prevención de enfermedades asociadas al estrés oxidativo, estudios de investigación médica afirman que la *Annona cherimola* presenta diversos compuestos puros en sus semillas y tallos, como las acetogeninas, que han demostrado actividad antineoplásica contra líneas celulares de cáncer humano[91,92].

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[88] Alique, R.; Oliveira, G. S. Changes in sugars and organic acids in cherimoya (*Annona cherimola* Mill.) fruit under Controlled-Atmosphere storage. *J. Agric. Food Chem.* **1994**, *42*, 799–803.

[89] Barreca, D.; Laganà, G.; Ficarra, S.; Tellone, E.; Leuzzi, U.; Galtieri, A.; Bellocco, E. Evaluation of the antioxidant and cytoprotective properties of the exotic fruit *Annona cherimola* Mill. (Annonaceae). *Food Res. Int.* **2011**, *44*, 2302–2310.

[90] Huang, W. Y.; Cai, Y. Z.; Corke, H.; Sun, M. Survey of antioxidant capacity and nutritional quality of selected edible and medicinal fruit plants in Hong Kong. *J. Food Compos. Anal.* **2010**, *23*, 510–517.

[91] Loizzo, M. R.; Tundis, R.; Bonesi, M.; Menichini, F.; Mastellone, V.; Avallone, L.; Menichini, F. Radical scavenging, antioxidant and metal chelating activities of *Annona*

Otros estudios *in vivo* concluyen que extractos metanólicos de *Annona cherimola* puede ser considerados como un remedio natural prometedor para la hiperlipidemia y por lo tanto, ser preventivos de enfermedades relacionadas con la misma, como aterosclerosis y otras enfermedades cardiovasculares[93]. Estos efectos podrían estar relacionados con los compuestos bioactivos lipídicos de la chirimoya, como ácidos grasos, fosfolípidos y esteroles[94]. En este contexto, los subproductos derivados de esta fruta pueden representar una fuente potencial de ingredientes bioactivos naturales que pueden ser alternativas seguras para la fabricación de nutracéuticos, alimentos funcionales o alimentos antioxidantes[95].

En la siguiente figura, se muestran las estructuras básicas de algunos compuestos bioactivos encontrados en chirimoya.

cherimola Mill. (cherimoya) peel and pulp in relation to their total phenolic and total flavonoid contents. *J. Food Compos. Anal.* **2012**, *25*, 179–184.

[92] Quispe-Mauricio, A.; Riva, D. C.; Rojas-Camayo, J.; Curzo, D. Z.; Margarita, C.; Rivera, P.; Wolach, A. J. V. Efecto citotóxico de las semillas de *Annona cherimola* en cultivos de cáncer de cérvix, mama y leucemia mieloide crónica. *Acta Médica Peru.* **2009**, *26* (3), 156–161.

[93] Adarsh Verma, M.; Ajay Kumar, P.; Raja Shekar, K.; Kranthi Kumar, A. Pharmacological Screening of *Annona cherimola* for Antihyperlipidemic Potential. *J. Basic Clin. Pharm.* **2011**, *2* (2), 63–69.

[94] Gutiérrez, M.; Sola, M. M.; Vargas, A. M. Fatty acid composition of phospholipids in mesocarp of cherimoya fruit during ripening. *Food Chem.* **2005**, *90*, 341–346.

[95] Ayala-Zavala, J. F.; Vega-Vega, V.; Rosas-Domínguez, C.; Palafox-Carlos, H.; Villa-Rodriguez, J. A.; Siddiqui, M. W.; Dávila-Aviña, J. E.; González-Aguilar, G. A. Agro-industrial potential of exotic fruit byproducts as a source of food additives. *Food Res. Int.* **2011**, *44*, 1866–1874.

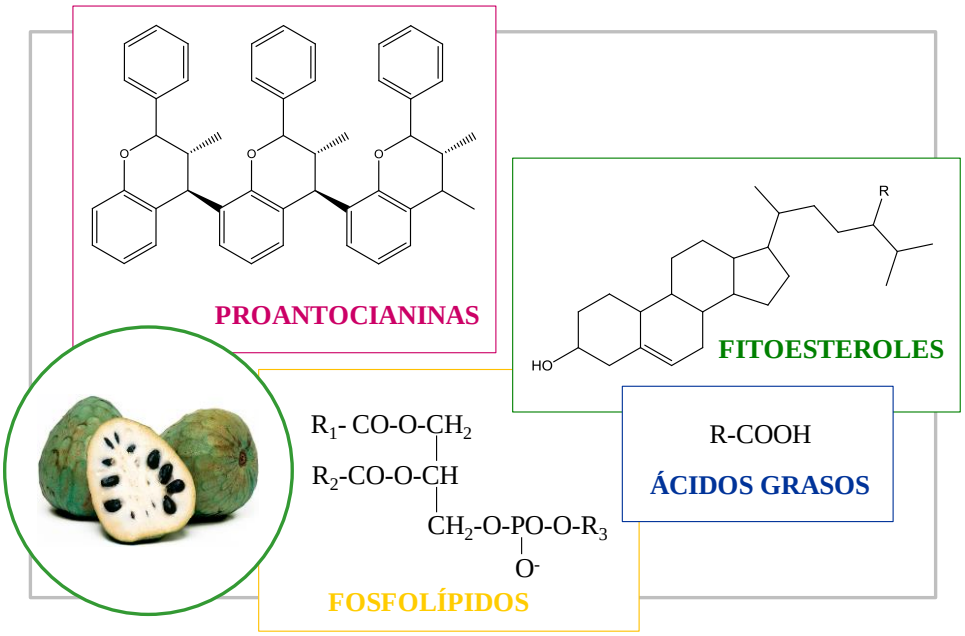


Figura 16. Principales familias de compuestos bioactivos identificadas en chirimoya

3.5. Granada

La granada (*Punica granatum*) es la fruta carnosa del granado, árbol caduco de la familia de las Lythraceae, pequeña familia de árboles y arbustos, cuyos frutos tienen semillas prismáticas y rugosas. Desde su origen, en países de Asia central, su cultivo se ha ido extendiendo al este de India y China y al oeste de países mediterráneos como Turquía, Egipto, Túnez, Marruecos y España, entre otros. Actualmente, la producción de granada se centra en Afganistán, India, China, Japón, Rusia y el sur de Europa[96,97]. España se considera como el productor más importante de Europa, cuya producción proviene, principalmente, de Valencia, Andalucía y Murcia[68].

La fruta se puede dividir en tres partes: piel, pulpa (alrededor del 30% del peso de la fruta) y semillas (alrededor del 20%). Se trata de una baya globular con una corteza coriácea, cuyo interior está dividido en varios lóbulos mediante una membrana blanquecina. Dentro de los lóbulos se encuentran numerosas semillas

[96] Erkan, M.; Kader, A. A. Pomegranate (*Punica granatum* L.).In *Postharvest Biology and Technology of Tropical and Subtropical Fruits*; Yahia, E.; Elsevier, 2011, pp 287–313e.
[97] Faria, A.; Calhau, C. Pomegranate in Human Health: An Overview. In *Bioactive Foods in Promoting Health*; Watson, R. R.; Preedy, V. R.; Elsevier, 2010, pp 551–563.

angulares contenidas en un saco de pulpa roja y jugosa, llamado sarcotesta; a los pequeños granos comestibles de la fruta se les llama arilos. Las condiciones óptimas para su cultivo son los climas áridos y semiáridos, donde los inviernos son suaves y los veranos son calurosos y secos durante las últimas etapas de desarrollo del fruto[96,97]. Entre las diferentes variedades los arilos varían en tamaño y las semillas en dureza y la piel en grosor. En general, las variedades cultivadas en distintas partes del mundo presentan diferencias en cuanto a morfología y propiedades físico-químicas. Las variedades más producidas en España son: 'Grano Elche' y 'Mollar de Játiva' o 'Mollar de Valencia'[68].

Muchos estudios demuestran que la granada es uno de los alimentos más saludables debido a la gran cantidad de componentes bioactivos que presenta[98,99]. Algunos de los efectos beneficiosos para la salud de esta fruta se deben a su contenido en compuestos fenólicos como ácidos fenólicos, taninos, flavonoles, y antocianinas. De los taninos hidrolizables, destaca la punicalagina que ha demostrado poseer actividades farmacológicas notables. Diversos trabajos de investigación han destacado que el zumo de granada es una de las principales fuentes frutales de antocianinas, especialmente 3-glucósidos y 3,5-diglucósidos de delfinidina, cianidina, y pelargonidina[100].

De la totalidad de la fruta, aproximadamente el 70 % es pulpa, motivo por el cual es muy usada para la fabricación de zumos lo que da lugar a la generación de productos de desecho como semillas y piel. En este sentido, el estudio de los subproductos de la granada está en auge, de hecho, trabajos recientes han puesto de manifiesto el uso de las semillas de granada como fuente de compuestos

[98] Shukla, M.; Gupta, K.; Rasheed, Z.; Khan, K. A; Haqqi, T. M. Bioavailable constituents/metabolites of pomegranate (*Punica granatum* L) preferentially inhibit COX2 activity ex vivo and IL-1beta-induced PGE2 production in human chondrocytes in vitro. *J. Inflamm.* **2008**, *5* (9), 1–10.

[99] Gundogdu, M.; Yilmaz, H. Organic acid, phenolic profile and antioxidant capacities of pomegranate (*Punica granatum* L.) cultivars and selected genotypes. *Sci. Hortic.* **2012**, *143*, 38–42.

[100] Gómez-Caravaca, A. M.; Verardo, V.; Toselli, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Caboni, M. F. Determination of the major phenolic compounds in pomegranate juices by HPLC-DAD-ESI-MS. *J. Agric. Food Chem.* **2013**, *61*, 5328–5337.

bioactivos, principalmente lipídicos[101]. El aceite extraído de las semillas de granada se compone de un 65-80 % de ácidos grasos conjugados, el más importante es el ácido punícico (CLnA), cuya bioactividad influye en el metabolismo de los lípidos y por tanto en la obesidad y las enfermedades cardiovasculares[102, 103]. A estos efectos beneficiosos se suman, propiedades antiinflamatorias, y anticancerígenas, entre otras[103–105], que también se podrían asociar, además de a los ácidos grasos poliinsaturados, a otros compuestos lipídicos presentes en estas semillas, como son los esteroles, los tocoferoles y los fosfolípidos[101, 106, 107]. Un esquema sobre los compuestos bioactivos más significativos de la granada y sus estructuras básicas se muestra en la figura 17.

[101] Fernandes, L.; Pereira, J. A.; López-Cortés, I.; Salazar, D. M.; Ramalhosa, E.; Casal, S. Lipid composition of seed oils of different pomegranate (*Punica granatum* L.) cultivars from Spain ´. *Int. J. Food Stud.* **2015**, *4*, 95–103.

[102] Abbasi, H.; Rezaei, K.; Rashidi, L. Extraction of essential oils from the seeds of pomegranate using organic solvents and supercritical CO_2. *J. Am. Oil Chem. Soc.* **2008**, *85*, 83–89.

[103] Arao, K.; Wang, Y. M.; Inoue, N.; Hirata, J.; Cha, J.Y.; Nagao, K.; Yanagita, T. Dietary effect of pomegranate seed oil rich in 9cis, 11trans, 13cis conjugated linolenic acid on lipid metabolism in obese, hyperlipidemic OLETF rats. *Lipids Health Dis.* **2004**, *3*, 1–4.

[104] Gasmi, J.; Sanderson, J. T. Growth inhibitory, antiandrogenic, and pro-apoptotic effects of punicic acid in LNCaP human prostate cancer cells. *J. Agric. Food Chem.* **2010**, *58*, 12149–12156.

[105] Coursodon-Boyiddle, C. F.; Snarrenberg, C. L.; Adkins-Rieck, C. K.; Bassaganya-Riera, J.; Hontecillas, R.; Lawrence, P.; Brenna, J. T.; Jouni, Z. E.; Dvorak, B. Pomegranate seed oil reduces intestinal damage in a rat model of necrotizing enterocolitis. *AJP Gastrointest. Liver Physiol.* **2012**, *303*, G744–G751.

[106] Garima, P.; Akoh, C. C. Antioxidant capacity and lipid characterization of six georgia-grown pomegranate cultivars. *J. Agric. Food Chem.* **2009**, *57* (2), 9427–9436.

[107] Eikani, M. H.; Golmohammad, F.; Homami, S. S. Extraction of pomegranate (*Punica granatum* L.) seed oil using superheated hexane. *Food Bioprod. Process.* **2012**, *90*, 32–36.

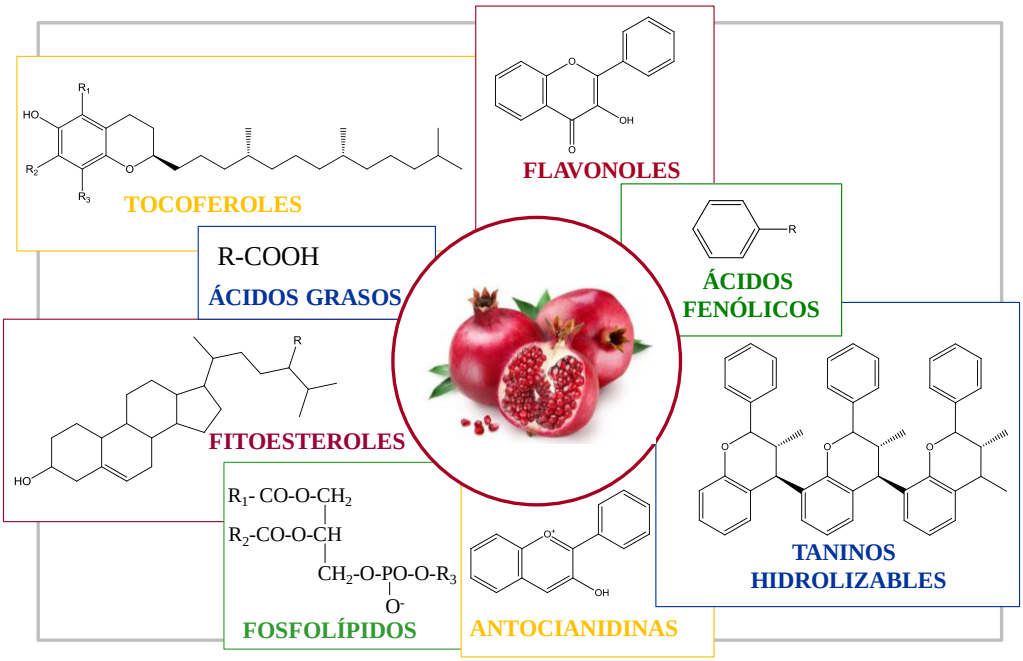


Figura 17. Principales familias de compuestos bioactivos en granada

4. PRETRATAMIENTO Y TRATAMIENTO DE LA MUESTRA

Los procesos de pretratamiento y tratamiento de la muestra pueden ser considerados como una etapa decisiva en cualquier estudio analítico ya que van a influir de manera considerable en el resultado final del análisis. En estos procesos se persiguen varios objetivos[108]:

- Mejorar la estabilidad de la muestra y a su vez de los componentes de la misma.
- Aumentar la eficacia del método de extracción de los analitos.
- Eliminar, en la medida de lo posible, las impurezas de la matriz que puedan interferir en el proceso analítico.

En definitiva, se trata de optimizar la matriz para mejorar la detección, la separación y/o la cuantificación, y lograr la máxima eficiencia y reproducibilidad en el análisis.

[108] Watson, R. R. *Polyphenols in plant*; Elsevier, 2014.

Por lo general, para muestras sólidas, como es el caso de matrices vegetales, en la etapa previa al tratamiento de la muestra propiamente dicho, éstas son sometidas a un triturado con el fin de aumentar su superficie y, posteriormente, realizar un proceso de secado. Una vez hecho esto, se procede al tratamiento de la muestra que va a consistir en la extracción de los compuestos de interés.

4.1. Pretratamiento de la muestra: etapa de secado

Los alimentos de origen vegetal, tienen una vida útil muy corta debido a que más del 80 % de su composición es agua, la reducción de este contenido en agua mediante el secado de la muestra va a permitir mantener la estabilidad de los componentes bioactivos del alimento hasta que sean analizados.

A la hora de seleccionar la tecnología de secado más adecuada, uno de los factores clave a tener en cuenta es la temperatura del proceso, lo más adecuado es elegir una técnica que permita trabajar a temperaturas lo más reducidas posible, lo que va a permitir poder garantizar la estabilidad de los ingredientes bioactivos, a pesar de que en tales condiciones el tiempo invertido y coste del proceso son mayores[109].

Sistemas comunes de secado, como, secado por aire caliente o secado en estufa, a pesar de ser los más económicos implican tiempos elevados y un tratamiento térmico a altas temperaturas dando lugar a una posible degradación de los compuestos de interés, así como generar un problema de descomposición de la muestra por oxidación. Por otra parte, este proceso de tratamiento térmico puede dar lugar a productos derivados de la reacción de Maillard que van a interferir en la actividad antioxidante de los vegetales[110].

[109] Jiang, H.; Zhang, M. Fruit and vegetable powders. In *Handbook of Food Powders*; Bhandari, N. Bansal, M. Z.; Schuck, P.; Woodhead Publishing Limited, 2013, pp 532-552.

[110] Mrkìc, V.; Cocci, M.; Dalla Rosa, M.; Sacchetti, G. Effect of drying conditions on bioactive compounds and antioxidant activity of broccoli (*Brassica oleracea* L.). *J. Sci. Food Agric.* **2006**, *86*, 1559–1566.

Otras técnicas más actuales como la desecación por radiación y aplicación de un campo electromagnético tienen el inconveniente de que el calor generado puede ocasionar la desestabilización de los compuestos de interés; así mismo, en las tecnologías de secado indirecto o por conducción, la temperatura del proceso se acerca al punto de ebullición del agua, por tanto, no es recomendable para la conservación de compuestos bioactivos ya que la mayoría de ellos se degradan a estas temperaturas de trabajo[111,112].

En definitiva, la elección de la técnica será un compromiso entre la naturaleza del producto inicial, la estabilidad de los compuestos bioactivos durante el proceso y otros factores como el volumen de extracto seco requerido, los costes y la capacidad de los medios existentes[111]. En base a esto, las técnicas de deshidratación más convenientes para el secado de productos vegetales, a pesar de ser las más costosas, son las tecnologías de secado directo o por convección y la liofilización, que permiten la obtención de deshidratados conservando las propiedades de sus componentes mediante el control de la temperatura y otros factores.

Dentro de las tecnologías de secado por convección se encuentran la tecnología de spray drying, que es usada para el secado de productos en estado líquido, y los secadores de lecho fluidizado o secadores Batch que permiten la deshidratación de productos como polvos, gránulos aglomerados y pastillas con un tamaño de partícula promedio entre 50 y 500 micras.

La liofilización se considera el mejor método de secado para la eliminación de agua en materiales sólidos sensibles al calor, tales como frutas y verduras, con el fin de obtener productos secos. La deshidratación del material se consigue mediante bajas temperaturas y alto vacío, asegurando de esta manera la alta calidad del producto final. El proceso se desarrolla en dos etapas, la primera consiste en congelar la muestra a una temperatura de 233,15 K o inferior, y

[111] 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.
[112] Berk, Z. *Food Process Engineering and Technology*; Elsevier, 2013.

posteriormente, el material congelado se somete a un vacío de unos 10 mm de Hg, con lo cual, el hielo que se formó durante la primera etapa, sufre un proceso de sublimación quedando la muestra libre de humedad.

El control y la optimización de los parámetros de liofilización son esenciales para lograr una operación viable y eficiente, y van a depender en gran parte del producto a procesar.

El principal inconveniente de esta técnica es que requiere una inversión importante en equipamiento y altos costes de energía (3 veces mayor que en técnicas de secado comunes), el proceso es relativamente lento debido a la baja tasa de transferencia de calor (4-10 horas por ciclo de secado), y se pueden causar daños a productos si se dan cambios de pH cuando se concentran solutos como consecuencia de que el agua pura se convierte en hielo[109]. No obstante, en comparación con otros métodos de deshidratación, la liofilización posee características importantes como se muestra en la tabla 3, de las cuales cabe destacar: pérdida mínima de nutrientes, excelente capacidad de rehidratación debido al desarrollo de la estructura porosa en el producto, lo que va a permitir una mejor extracción de los compuestos a estudiar y por supuesto mayor conservación de los compuestos bioactivos, como por ejemplo, los polifenoles, que son susceptibles a la degradación enzimática durante el secado por aire debido a la actividad oxidasa, y otros compuestos termolábiles[113].

[113] Ratti, C. Freeze drying for food powder production. In *Handbook of Food Powders*; Bhandari, N. Bansal, M. Z.; Schuck, P.; Woodhead Publishing Limited, 2013, pp 57–84.

Tabla 3. Comparación de las características de la deshidratación convencional frente a la liofilización

DESHIDRATACIÓN	LIOFILIZACIÓN
• Alimentos específicos • Temperatura entre 37 y 93°C • Presión atmosférica • Baja rehidratación del producto final • Cambios estructurales • Diferencia en las propiedades sensoriales • Pardeamiento del color • Pérdida significativa de propiedades nutricionales • Bajo coste	• Gran variedad de alimentos • Baja temperatura • Alta presión de vacío • Rápida rehidratación del producto final • Sin cambios estructurales • Sin variación en las propiedades organolépticas • Color normal • Conservación de propiedades nutricionales • Coste elevado

Los productos liofilizados tienen un contenido residual de humedad (<2%) y suelen ser materiales higroscópicos por lo que su almacenamiento se debe llevar a cabo en condiciones adecuadas. No se recomienda ni su refrigeración ni su congelación y los envases deben de prevenir el contacto con la humedad, el oxígeno (para evitar el enranciamiento) y la luz (afecta al color y la oxidación del alimento).

4.2. Tratamiento de la muestra: extracción de compuestos bioactivos

Una vez que se ha realizado el pretratamiento a la muestra (liofilización), se procede al tratamiento de la misma para el análisis de compuestos bioactivos. Concretamente, en la preparación de muestras para el análisis cromatográfico, las técnicas de extracción se utilizan, tanto para separar, los analitos objeto de estudio de otros componentes de la muestra que no interesan o que deben ser eliminados para evitar interferencias como para la concentración de analitos específicos en cantidades de volumen más pequeñas[114].

Debido a la complejidad de las matrices vegetales y a la gran diversidad estructural de estos compuestos, el tipo de procedimiento de extracción

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

seleccionado juega un papel decisivo en la determinación cualitativa y cuantitativa de los compuestos bioactivos. Particularmente, los compuestos fenólicos, como se comentó en el apartado 2.1, pueden tener múltiples grupos hidroxilo que pueden conjugarse con azúcares, ácidos o grupos alquilo, por lo que la polaridad varía significativamente de un compuesto a otro y en consecuencia, es difícil desarrollar un único método para la extracción óptima de todos los polifenoles. Por lo tanto, la optimización del procedimiento de extracción seleccionado es esencial para un análisis de compuestos fenólicos lo más exacto posible.

Para el estudio los compuestos bioactivos, se pueden emplear diversos sistemas de extracción, como extracción sólido-líquido (SLE), extracción y microextracción en fase sólida (SPE/SPME), extracción con fluidos supercríticos (SFE), con fluidos presurizados (PLE), asistida por microondas (MAE) y métodos de filtración (ver figura 18).

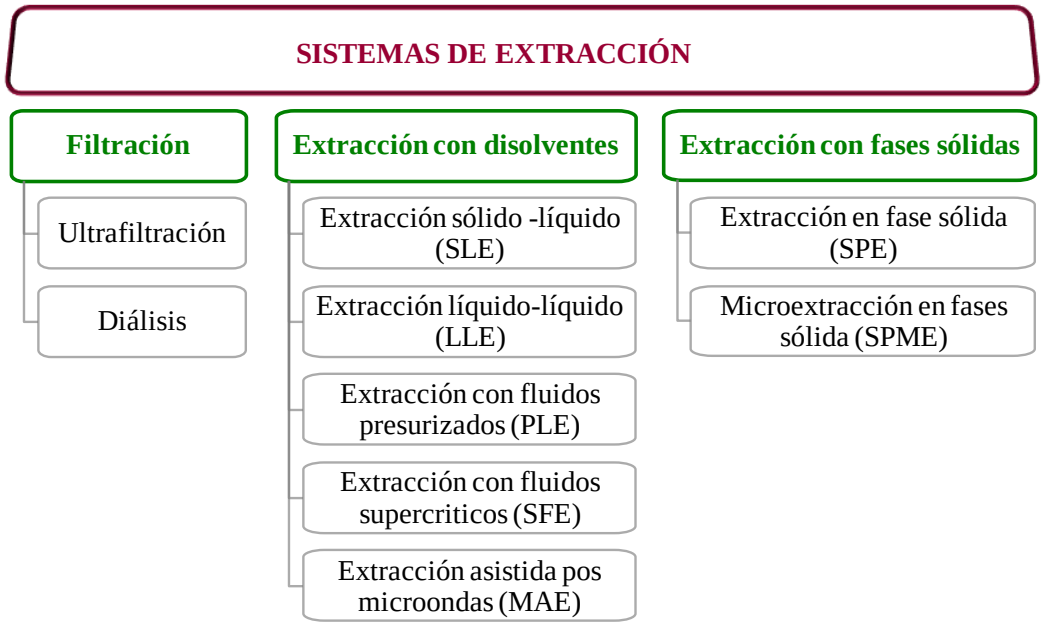


Figura 18. Diagrama de los distintos sistemas de extracción

La aplicación de estas técnicas en la extracción de compuestos fenólicos se verá con más detalle en el capítulo 1 de la presente memoria. No obstante, cabe destacar que los sistemas de extracción con disolventes son de los más utilizados,

y dentro de ellos, los más económicos y relativamente sencillos son la LLE y la SLE, cuando los productos de partida son líquidos o sólidos respectivamente[114]. Este tipo de extracciones se basan en la separación de una o más especies procedentes de una muestra dependiendo de las diferencias de solubilidad relativa entre los componentes que van a ser separados y los demás constituyentes de la matriz[115].

En los trabajos de investigación llevados a cabo en la presente tesis, la técnica utilizada ha sido la SLE. La eficiencia de una SLE está influenciada por los siguientes parámetros[115]:

- Naturaleza del extractante: polar-polar y apolar-apolar
- Temperatura durante el proceso: Al aumentar mejora la solubilidad (se rompen fuerzas intermoleculares dando lugar a un aumento en la movilidad de las moléculas disminuyendo a la vez su viscosidad), sin embargo, como se comentó en el apartado anterior a altas temperaturas aumenta la posibilidad de degradación de los componentes bioactivos.
- Agitación: mejora la penetración del extractante en el sólido mediante el movimiento enérgico de las moléculas de disolvente.
- Presión: al aumentar mejora la penetración del disolvente en los poros de la matriz.
- Tamaño de partícula del soluto: a menor tamaño de partícula, mejor penetración del disolvente en los poros de la matriz.
- Tiempo: necesario para que tenga lugar el proceso de extracción

Normalmente, se usa la técnica de ultrasonidos como una alternativa económica y simple para mejorar la eficacia de las técnicas de extracción con disolventes. El mecanismo de ultrasonidos se atribuye a eficacias mecánicas y de cavitación que

[115] 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*, 1821–1835.

pueden resultar en la interrupción de la pared celular, la reducción de tamaño de partícula, y mejora la transferencia de masa a través de la membrana celular[116].

Los disolventes de extracción más comunes para la separación de compuestos polares (compuestos fenólicos) en matrices vegetales son disolventes orgánicos como metanol, etanol, acetona o acetonitrilo, puros, disueltos en agua o acidificados[26, 117]. El metanol es ampliamente utilizado en la extracción de compuestos fenólicos en vegetales, por ejemplo para el análisis polifenólico de judía o membrillo se realizó la extracción con 100 % de metanol[118, 119]. A menudo, se juega con diferentes proporciones de metanol/agua para separar compuestos en un rango lo más amplio posible de polaridad, como es el caso de estudios en alcachofa y berenjena[120,121]. Otros estudios demuestran que para la extracción de antocianinas, lo más usual es utilizar metanol o etanol acidificados como extractantes; de ambos métodos, la extracción con metanol es la más eficiente, de hecho, se ha encontrado que en extracciones de antocianinas en pulpa de uva, la extracción con metanol es 20% más eficaz que con etanol[122].

[116] Wang, X.; Wu, Y.; Chen, G.; Yue, W.; Liang, Q.; Wu, Q. Optimisation of ultrasound assisted extraction of phenolic compounds from Sparganii rhizoma with response surface methodology. *Ultrason. Sonochem.* **2013**, *20*, 846–854.

[117] Dinelli, G.; Bonetti, A.; Minelli, M.; Marotti, I.; Catizone, P.; Mazzanti, A. Content of flavonols in Italian bean (*Phaseolus vulgaris* L.) ecotypes. *Food Chem.* **2006**, *99*, 105–114.

[118] Aparicio-Fernández, X.; Yousef, G. G.; Loarca-Pina, G.; De Mejia, E.; Lila, M. A. Characterization of polyphenolics in the seed coat of black jamapa bean (*phaseolus vulgaris* L.). *J. Agric. Food Chem.* **2005**, *53*, 4615–4622.

[119] Costa, R. M.; Magalhães, A. S.; Pereira, J. A.; Andrade, P. B.; Valentão, P.; Carvalho, M.; Silva, B. M. Evaluation of free radical-scavenging and antihemolytic activities of quince (*Cydonia oblonga*) leaf: A comparative study with green tea (*Camellia sinensis*). *Food Chem. Toxicol.* **2009**, *47*, 860–865.

[120] Mulinacci, N.; Prucher, D.; Peruzzi, M.; Romani, A.; Pinelli, P.; Giaccherini, C.; Vincieri, F. F. Commercial and laboratory extracts from artichoke leaves: estimation of caffeoyl esters and flavonoidic compounds content. *J. Pharm. Biomed. Anal.* **2004**, *34*, 349–357.

[121] Singh, A. P.; Luthria, D.; Wilson, T.; Vorsa, N.; Singh, V.; Banuelos, G. S.; Pasakdee, S. Polyphenols content and antioxidant capacity of eggplant pulp. *Food Chem.* **2009**, *114*, 955–961.

[122] Castañeda-Ovando, A.; Pacheco-Hernández, M. de L.; Páez-Hernández, M. E.; Rodríguez, J. A.; Galán-Vidal, C. A. Chemical studies of anthocyanins: A review. *Food Chem.* **2009**, *113*, 859–871.

Los disolventes utilizados para la extracción de compuestos lipídicos suelen ser éter de petróleo o dietiléter, aunque en algunos casos se utiliza hexano. Sin embargo, el uso de un solo disolvente no polar podría limitar la extracción a compuestos lipídicos que sean más polares. Cuando se pretende realizar un estudio de compuestos lipídicos diferentes (ácidos grasos, esteroles, tocoferoles, fosfolípidos, etc) se pueden utilizar mezclas de disolventes más y menos polares con objeto de asegurar una completa recuperación de los compuestos. En este sentido, el método Folch es uno de los más utilizados para la extracción de los lípidos totales, siendo el disolvente una mezcla de cloroformo/metanol[123,124].

5. EVALUACIÓN DE LA CAPACIDAD ANTIOXIDANTE

Idealmente, para la caracterización de un alimento vegetal desde el punto de vista de sus propiedades antioxidantes, se deberían cuantificar cada uno de los componentes antioxidantes que "a priori" se conoce que el alimento posee, y posteriormente evaluar la capacidad antioxidante como la suma de cada uno de estos compuestos teniendo en cuenta las interacciones entre los mismos. Esto, resultaría analíticamente complejo y muy costoso debido a la gran cantidad y diversidad estructural de los compuestos antioxidantes presentes en los vegetales[125].

De forma general, para la evaluación de la capacidad antioxidante, se puede medir lo siguiente[125]:

[123] Folch, J.; Lees, M.; Stanley, S. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. **1957**, *226* (1), 497–509.

[124] Akoh, C. C.; Min, D. B. *Food Lipids: Chemistry, Nutrition, and Biotechnology; CRC Press*, 2008.

[125] Análisis de antioxidantes : ¿qué y cómo se deben medir? http://www.portalantioxidantes.com/ (*accessed Sep 15, 2015*).

▪ El contenido de los compuestos antioxidantes más relevantes en el vegetal, por ejemplo, el contenido de licopeno en tomate, o la concentración de algún compuesto fenólico en particular.

▪ El contenido total de alguna familia de compuestos, como el contenido total de polifenoles o de flavonoides.

▪ La capacidad antioxidante del alimento, es decir, la medida de la capacidad que tendrían todos los compuestos antioxidantes presentes en mismo.

Por otro lado, a la hora de seleccionar un método que refleje de forma completa el perfil antioxidante de un alimento, éste debería reunir las siguientes características[126,127]:

• Utilizar una fuente de radicales biológicamente relevante.

• Asegurar que el sustrato y el modo de inducir la oxidación sean relevantes como fuentes de daño oxidativo.

• Ser sencillo.

• Tener un mecanismo y un punto final definido.

• Poseer una instrumentación más o menos disponible.

• Tener una buena reproducibilidad.

• Ser adaptable para medir antioxidantes hidrofílicos y lipofílicos.

• Ser adaptable para la realización de análisis rutinarios a gran escala.

Sin embargo, actualmente, no existe ningún método que reúna tales características por varias razones:

♦ En primer lugar, los mecanismos de acción en los que se basan los métodos de capacidad antioxidante pueden ser muy diferentes. Los dos mecanismos más comunes están esquematizados en la figura 19[127]:

[126] Frankel, E. N.; Meyer, A. S. The problems of using one- dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.

[127] Prior, R.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Agric. Food Chem.* **2005**, *53*, 4290–4302.

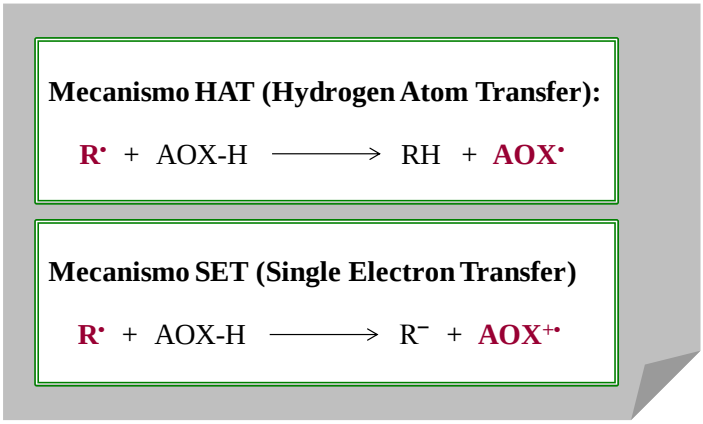


Figura 19. Principales mecanismos de acción de los antioxidantes

Ambos mecanismos están basados en la reacción directa de los antioxidantes con los radicales libres. En el mecanismo HAT el antioxidante estabiliza los radicales libres mediante la transferencia directa de un átomo de hidrógeno, mientras que en el SET esto se realiza mediante la de transferencia de un electrón desapareado. Otro proceso de acción antioxidante es la quelación de metales libres que intervienen en las reacciones de formación de especies reactivas de oxígeno (ROS)[128].

♦ Por otro lado, en un mismo alimento puede haber mezclas de diferentes antioxidantes con distintos mecanismos de acción, además, entre estos compuestos, se pueden establecer reacciones sinérgicas o inhibitorias.

Por estos motivos, para la determinación de la capacidad antioxidante, se suele utilizar más de un método, de esta manera, el conjunto de los resultados dará una idea, más acertada, de la actividad antioxidante de todas las moléculas presentes en el alimento que intervengan en la misma[129].

[128] Jovanovic, S. V.; Simic, M. G.; Steenken, S.; Hara, Y. Iron complexes of gallocatechins. Antioxidant action or iron regulation? *J. Chem. Soc. Perkin Trans.* **1998**, *2*, 2365–2370.
[129] Jayawardena, N.; Watawana, M. I.; Waisundara, V. Y. Evaluation of the Total Antioxidant Capacity, Polyphenol Contents and Starch Hydrolase Inhibitory Activity of Ten Edible Plants in an In vitro Model of Digestion. *Plant Foods Hum. Nutr.* **2015**, *70*, 71–76.

En definitiva, los métodos de determinación de la capacidad antioxidante son muy variados, aunque por lo general son métodos de inhibición donde se emplea una especie generadora de radicales y una sustancia que detecta esas especies, que será la muestra a analizar, y que debido a su capacidad antioxidante es capaz de inhibir la generación de dichos radicales. Estos métodos pueden ser clasificados de acuerdo con las estrategias de detección y cuantificación empleadas, en la siguiente tabla se muestran los principales métodos de capacidad antioxidante[126,127,130]:

[130] Tan, J. B. L.; Lim, Y. Y. Critical analysis of current methods for assessing the *in vitro* antioxidant and antibacterial activity of plant extracts. *Food Chem.* **2015**, *172*, 814–822.

Tabla 4. Métodos más usados para la determinación de la capacidad antioxidante

MECANISMO	MÉTODO	SUSTANCIA OXIDANTE	PATRÓN DE REFERENCIA	FUNDAMENTO
HAT	**ORAC**	Radical peroxilo	Trolox	El radical peroxilo oxida a la fluoresceina con lo que la fluorescencia decrece. La medida se basa en el mantenimiento de la fluorescencia en presencia de la sustancia antioxidante
	TRAP	Radical peroxilo	Trolox	El radical peroxilo oxida a una proteína produciendo fluorescencia. La medida se basa en el retardo en la aparición de la fluorescencia en presencia de la sustancia antioxidante
SET	**FRAP**	Fe^{3+}-TPTZ	$FeSO_4$	El Fe^{3+} es reducido a Fe^{2+}. La medida se basa en la absorbancia del Fe^{2+}, cuya concentración va a depender de la sustancia antioxidante
	TEAC	$ABTS^{\bullet+}$	Trolox	La sustancia antioxidante reacciona con el radical generado que es coloreado. La medida se basa en la reducción de la absorbancia
	DPPH	$DPPH^{\bullet+}$	Trolox	La sustancia antioxidante reacciona con el radical coloreado. La medida se basa en la reducción de la absorbancia
	Compuestos fenólicos totales	Mo^{6+}	Ácido gálico	El Mo^{6+} es reducido a Mo^{5+}. La medida se basa en la absorbancia del Mo^{5+}, cuya concentración va a depender de la concentración total de polifenoles en la muestra

A continuación, se explicarán detalladamente los métodos de capacidad antioxidante utilizados durante el desarrollo de la presente tesis doctoral.

5.1. Método Folin-Ciocalteu para la determinación de compuestos fenólicos totales.

El método para la determinación de compuestos fenólicos totales se basa en la medida de la capacidad que tienen los polifenoles para reducir el Mo^{6+}, del complejo molibdotugstato que caracteriza el reactivo de Folin-Ciocalteu (FCR), a Mo^{5+}. Como consecuencia de esa reducción, el reactivo que era, inicialmente, de color amarillo pasa a color azul intenso y es medido espectrofotométricamente a 765 nm[125]. Normalmente, el patrón de referencia utilizado es ácido gálico.

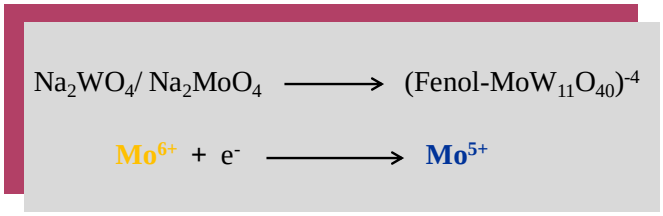


Figura 20. Reacción producida entre el FCR y los compuestos fenólicos[127]

Los métodos que emplean dicho reactivo, pueden variar en cuanto a las concentraciones de reactivo empleadas, tipo de base para alcalinizar el medio, y/o tiempo de incubación necesario para asegurar una concentración medible del reactivo reducido[125,127]. Es importante tener en cuenta que el reactivo, puede ser reducido no sólo por los polifenoles, sino también por azúcares, aminas aromáticas, ácidos orgánicos, etc y todas las matrices vegetales tienen algunos de estos interferentes[127, 131] . Para reducir la respuesta de los mismos, se han desarrollado diferentes metodologías como la purificación parcial de los compuestos fenólicos mediante una extracción en fase sólida, la corrección de los polifenoles totales mediante en cálculo de la actividad reductora del ácido

[131] Lester, G. E.; Lewers, K. S.; Medina, M. B.; Saftner, R. A. Comparative analysis of strawberry total phenolics via Fast Blue BB vs. Folin-Ciocalteu: Assay interference by ascorbic acid. *J. Food Compos. Anal.* **2012**, *27*, 102–107.

ascórbico o el tratamiento del extracto fenólico con agentes oxidantes que oxiden a los interferentes previamente[132].

El método original fue desarrollado por Folin y Ciocalteu para la determinación indirecta de la concentración de proteína total mediante la medida del contenido de tirosina y triptófano[133] y posteriormente, la aplicación del mismo fue ampliada para determinar compuestos fenólicos totales en extractos de alimentos vegetales. Los primeros ensayos realizados en vegetales fueron los llevados a cabo por Swain y Hillis en ciruela y por Singleton y Rossi en muestras de vino[134,135], siendo, recientemente, adaptado para la medida de antioxidantes lipofílicos[132,136].

En la actualidad, es el método más utilizado para la determinación de polifenoles totales en alimentos como alternativa a la medida individual de los compuestos antioxidantes, cuando éstos son en su mayoría compuestos fenólicos, como es el caso de vegetales. No obstante, debido a que existen otros compuestos antioxidantes diferentes de los fenólicos, para dar una idea más general de la capacidad antioxidante del alimento y evitar sub-estimaciones, es conveniente combinar estos resultados con los de otros métodos de capacidad antioxidante más generales[125,127].

[132] Sánchez-Rangel, J. C.; Benavides, J.; Heredia, J. B.; Cisneros-Zevallos, L.; Jacobo-Velázquez, D. A. The Folin-Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. *Anal. Methods* **2013**, *5*, 5990–5999.

[133] Folin, O.; Ciocalteau, V. Tyrosine and Tryptophane determinations in Proteins. *J. Biol. Chem.* **1927**, *73* (2), 627–648.

[134] Swain, T.; Hillis, W. E. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric.* **1959**, *10* (1), 63–68.

[135] Singleton V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

[136] Berker, K. I.; Ozdemir Olgun, F. A.; Ozyurt, D.; Demirata, B.; Apak, R. Modified Folin-Ciocalteu antioxidant capacity assay for measuring lipophilic antioxidants. *J. Agric. Food Chem.* **2013**, *61*, 4783–4791.

5.2. Método ORAC (Oxygen Radical Absorbance Capacity).

El método ORAC mide la capacidad que tienen todos los antioxidantes presentes en una muestra para neutralizar radicales peroxilo mediante la donación de átomos de hidrógeno. El radical peroxilo, generado a partir de la molécula orgánica, AAPH (2,2'-Azobis-(2-aminopropano)-dihidrocloruro) oxida a la fluoresceína de forma que ésta pierde su fluorescencia, produciéndose, de este modo, un descenso de la misma. En presencia de sustancias antioxidantes, el descenso de la fluorescencia se hace más lento[137].

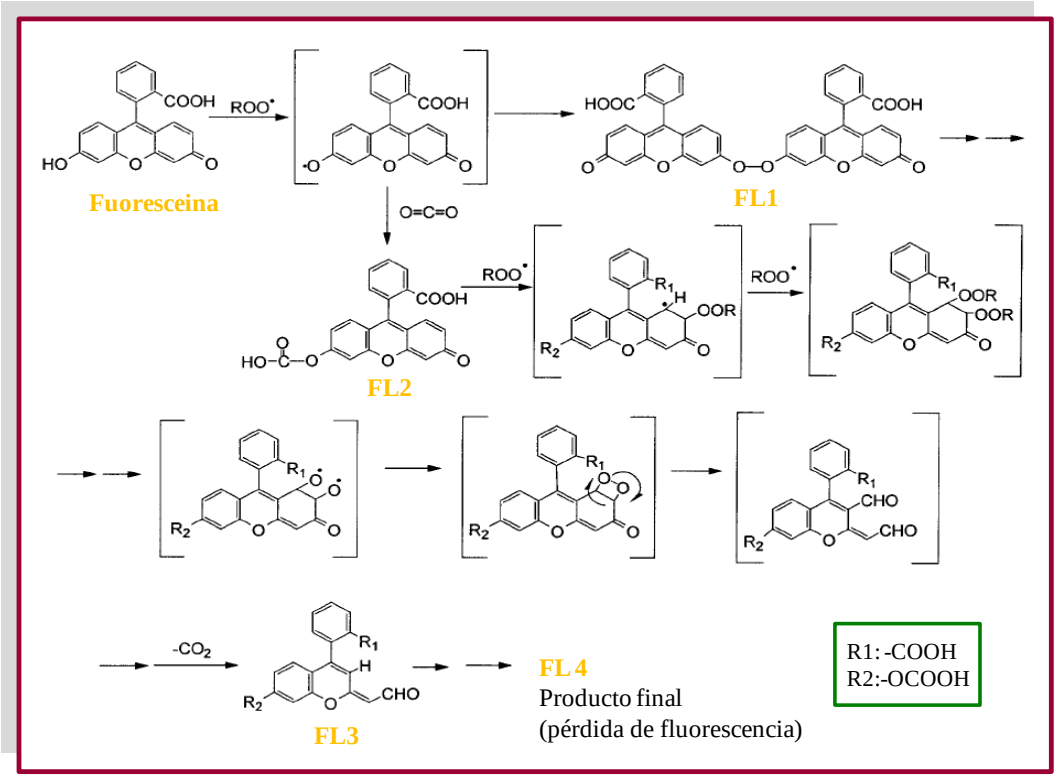


Figura 21. Reacción de oxidación de la fluoresceína en presencia de AAPH (Adaptado de 134)

El ensayo ORAC mide el valor de capacidad antioxidante como equivalentes de trolox e incluye tanto el tiempo como el grado de inhibición de la oxidación, por lo tanto, da una idea de la capacidad antioxidante total, pudiéndose comparar los

[137] Ou, B.; Hampsch-woodill, M.; Prior, R. L. Development and validation of an improved Oxygen Radical Absorbance Capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem*. **2001**, *49*, 4619–4626.

resultados obtenidos en alimentos de diversa naturaleza. Además ofrece una alta sensibilidad, reproducibilidad y precisión, lo que lo convierte en uno de los métodos más empleados para la evaluación de la capacidad antioxidante[125].

Este método comenzó siendo utilizado para la evaluación de la capacidad de captación de radicales libres en muestras biológicas y fue posteriormente adaptado a otro tipo de matrices como extractos de plantas, suplementos dietéticos, nutracéuticos y alimentos de origen vegetal, como frutas, hortalizas, vinos, etc. Actualmente es la opción más usada en el análisis de alimentos y fármacos, ya que permite analizar muestras con múltiples ingredientes y cinéticas de reacción complejas. Además, aunque el método ORAC básico se limita a sustratos hidrófilos debido a la baja solubilidad de las sondas fluorescentes en entornos hidrófobos, este ensayo se puede adaptar fácilmente para medir la actividad antioxidante de compuestos lipofílicos[130].

En comparación con los ensayos basados en SET como DPPH o TEAC, el ORAC ofrece resultados diferentes, mostrando generalmente una actividad antioxidante más alta para compuestos fenólicos y más baja para otros compuestos antioxidantes, como por ejemplo ácido ascórbico. A esto se suma que el ORAC es más sensible y puede detectar la actividad antioxidante de compuestos como naringenina y hesperidina, que muestran muy poca actividad antioxidante con los ensayos DPPH o TEAC. Este aspecto se puede atribuir, como se comentó anteriormente, a la medición combinada del tiempo y grado de inhibición. No obstante, cabe señalar que el aumento de la sensibilidad del método ORAC no significa que necesariamente los valores sean más altos en comparación con el TEAC, ya que esto sigue siendo dependiente de la composición química de las muestras[130]. Por ejemplo, está descrito que en algas, el ensayo ORAC muestra una capacidad antioxidante menor en comparación con el ensayo TEAC[138]. Así mismo, el ORAC está poco correlacionado con el ensayo

[138] Balboa, E. M.; Conde, E.; Moure, A.; Falqué, E.; Domínguez, H. *In vitro* antioxidant properties of crude extracts and compounds from brown algae. *Food Chem.* **2013**, *138*, 1764–1785.

FRAP, hecho que refuerza aún más la diferencia en los resultados observada entre métodos con distinto mecanismo de reacción[139].

5.3. Método TEAC (Trolox Equivalent Antioxidant Capacity).

El TEAC es un método espectrofotométrico, originalmente descrito por Miller *et al.*[140], donde el radical $ABTS^{\bullet+}$ se genera a partir de su precursor, el ácido 2,2'-azinobis (3- etilbenzotiazolín)-6-sulfónico (ABTS, ver figura 22). Este radical, se puede obtener a través de una reacción química (con dióxido de manganeso o persulfato potásico), enzimática (con peroxidasa o mioglobulina) o también electroquímica, la forma más usual es utilizando persulfato potásico. El compuesto obtenido es de color verde-azulado, estable y con un espectro de absorción ultravioleta-visible (UV-Vis) a 734 nm[126,127].

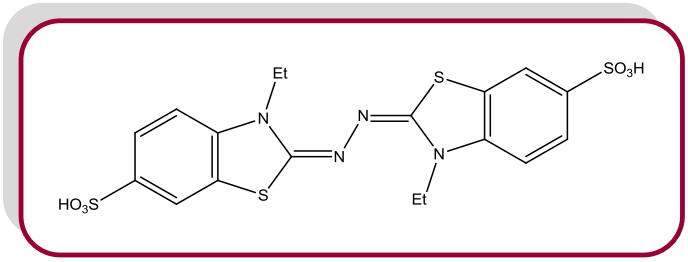


Figura 22. Estructura del Ácido 2,2'-azinobis (3- etilbenzotiazolín)-6-sulfónico (ABTS

La generación del $ABTS^{\bullet+}$ mediante persulfato potásico se lleva a cabo mediante una reacción estequiométrica (1:0.5), a temperatura ambiente, en ausencia de luz, y en un tiempo de 12 a 16 horas. Es un radical artificial que no mimetiza bien la situación *in vivo* y termodinámicamente puede ser reducido por compuestos que tengan un potencial redox menor (potencial redox del $ABTS^{\bullet+}$: 0.68V), pudiendo reaccionar con el mismo, muchos compuestos, por ejemplo los compuestos fenólicos. La medida se basa en el descenso de la absorbancia del radical, en

[139] Floegel, A.; Kim, D. O.; Chung, S. J.; Koo, S. I.; Chun, O. K. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J. Food Compos. Anal.* **2011**, *24*, 1043–1048.

[140] Miller, N. J.; Rice-Evans, C.; Davies, M.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407–412.

presencia de la sustancia antioxidante, por lo tanto, el punto final de la reacción lo marca la sustancia antioxidante objeto de estudio. Al igual que en el método ORAC, los resultados se calculan como equivalentes de trolox[126,127].

El método TEAC se realiza a menudo en muestras de alimentos por su sencillez, rapidez y reproducibilidad, entre otras ventajas. Es un ensayo viable en un amplio rango de valores de pH ya que el radical es soluble en disolventes acuosos y orgánicos, además de ser independiente de la fuerza iónica, esto hace posible su uso en la determinación de la capacidad antioxidante tanto hidrofílica como lipofílica[127,130].

Los resultados obtenidos mediante el ensayo TEAC se correlacionan positivamente con el ensayo DPPH, sin embargo, existen estudios que demuestran que el primero ofrece mejores estimaciones de la capacidad antioxidante en alimentos, particularmente frutas, verduras y bebidas derivadas de las mismas, concluyéndose que el ensayo TEAC es más adecuado que el DPPH cuando se trabaja con alimentos vegetales que contienen compuestos antioxidantes hidrofílicos, lipofílicos, y altamente pigmentados[139].

Es muy común encontrar estudios en los que se utilizan métodos basados en SET simultáneamente, por ejemplo DPPH, TEAC y Folin-Ciocalteu, sin embargo hacer esto resulta redundante ya que se estaría dando información sobre una sola faceta de la capacidad antioxidante de la muestra[141]. Este hecho no es incorrecto, pero estos resultados no se deben extrapolar a la capacidad antioxidante total ya que no se tienen en cuenta los compuestos antioxidantes que actúan bajo otros mecanismos[130].

[141] Chandra, S.; Khan, S.; Avula, B.; Lata, H.; Yang, M. H.; Elsohly, M. A.; Khan, I. A. Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-based Complement. Altern. Med.* **2014**, *2014*, 1–9.

5.4. Método FRAP (Ferric Reducing Antioxidant Power).

El método FRAP original fue desarrollado por Benzie y Strain para medir el poder reductor en plasma y posteriormente el ensayo fue adaptado para su uso en la determinación de capacidad antioxidante en vegetales[142,143]. Este método se basa en la reducción del complejo férrico-2,4,6-tripiridil-s-triazina (TPTZ) incoloro al complejo ferroso de color azul (figura 23), es decir, se basa en el poder que tiene una sustancia antioxidante para reducir el Fe^{3+} a Fe^{2+} que es menos antioxidante.

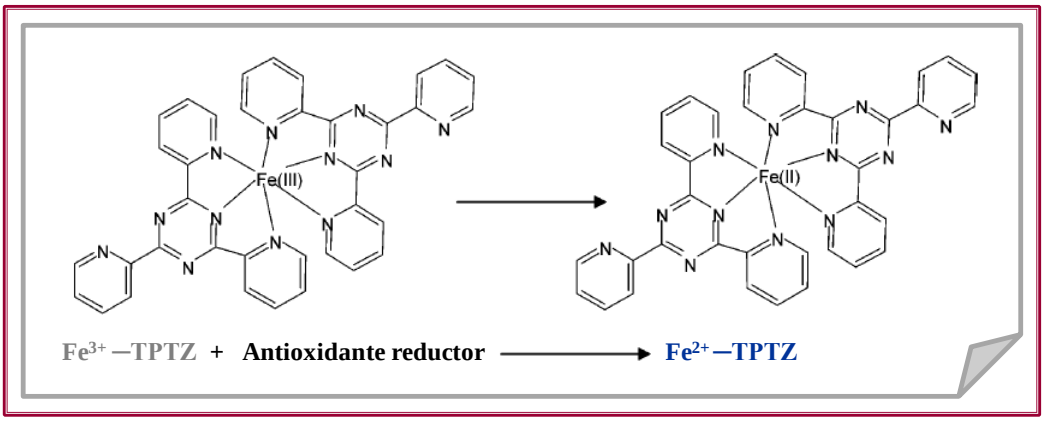


Figura 23. Reacción de reducción ocurrida en el método FRAP

La cantidad de complejo reducido, medida espectrofotométricamente a 593 nm, va a depender de las sustancias antioxidantes capaces de donar electrones[126,144], pudiendo ser reducido por sustancias con potenciales redox menores a 0,7V (potencial redox del complejo Fe^{3+} -TPTZ). Debido a que el potencial redox del Fe^{3+}-TPTZ es comparable con el del $ABTS^{\bullet+}$ se pueden analizar compuestos similares con ambos métodos aunque las condiciones de la reacción sean distintas.

[142] Benzie, I. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

[143] Benzie, I. F. F.; Szeto, Y. T. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **1999**, *47*, 633–636.

[144] Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396–3402.

Este método, al igual que el TEAC se basan en la hipótesis de que las reacciones redox tienen lugar tan rápido que se completan en pocos minutos, sin embargo este hecho no es del todo cierto. Se ha demostrado, que los resultados obtenidos mediante el FRAP pueden variar considerablemente dependiendo del tiempo de análisis. En este sentido, algunos compuestos fenólicos que se unen al hierro o se descomponen en otros con una reactividad más baja o diferente, se analizan mejor con tiempos de reacción cortos, sin embargo, algunos polifenoles reaccionan más lentamente y requieren tiempos de reacción más largos para su detección[127]. Existen evidencias de que en el análisis mediante FRAP de polifenoles derivados de alimentos, la absorbancia aumenta lentamente en el caso de compuestos como ácido cafeico, ácido tánico, ácido ferúlico, ácido ascórbico, y quercetina, incluso después de varias horas de tiempo de reacción. Por lo tanto, un único punto de absorción como punto final, puede que no represente la reacción completa[144].

A pesar de esto, el FRAP es un método sencillo y reproducible que se puede aplicar no sólo para el estudio de la capacidad antioxidante en extractos de alimentos vegetales y bebidas, sino también en el estudio de antioxidantes puros presentes en la dieta, ofreciendo resultados comparables a los obtenidos con otras metodologías más complejas[144,145 147,148]. No obstante, como se ha comentado anteriormente, es conveniente que este método sea usado junto a otro basado en un mecanismo diferente para poder ofrecer resultados que reflejen de la forma más completa posible la capacidad antioxidante total de una muestra.

[145] Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables - the millennium ' s health. *Int. J. food Sci. Technol.* **2001**, *36*, 703–725.

6. TÉCNICAS SEPARATIVAS PARA LA DETERMINACIÓN DE COMPUESTOS BIACTIVOS EN MATRICES VEGETALES

Actualmente, la Química Analítica dispone de un amplio número de técnicas avanzadas que permiten la separación de los componentes de una muestra basándose en las propiedades físico-químicas de los mismos. La separación es, en la mayoría de los casos, el paso previo al análisis estructural y a la cuantificación de los diferentes compuestos. Para la identificación de cada uno de los compuestos bioactivos de muestras complejas como son los extractos vegetales, las técnicas cromatográficas son las más usadas[146,147].

En general, en cualquier técnica cromatográfica, los componentes de una muestra son transportados mediante una fase que se mueve en una dirección definida (fase móvil) a través de una fase que está inmóvil (fase estacionaria). El contacto entre las dos fases es similar a un equilibrio dinámico, en el que los componentes a separar presentarán una repartición diferencial debido a las interacciones entre la fase estacionaria y la fase móvil. Es decir, cuando existe una fuerte interacción entre un compuesto determinado y la fase estacionaria, la migración del componente se ralentizará, si la interacción es mínima, el compuesto se moverá a la misma velocidad que la fase móvil. La cromatografía, ofrece información básica sobre los componentes de la muestra separados, como el tiempo de retención (característico de la estructura molecular de un compuesto) y la señal de intensidad (relacionada con la cantidad de compuesto)[148].

Las diferencias entre las distintas técnicas cromatográficas residen en la fase móvil aplicada (gas o líquido), en el tipo de retención y en el mecanismo de flujo.

[146] Alarcón-Flores, M. I.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. Determination of Phenolic Compounds in Artichoke, Garlic and Spinach by Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Food Anal. Methods* **2014**, *7*, 2095–2106.

[147] Loizzo, M. R.; Bonesi, M.; Pugliese, A.; Menichini, F.; Tundis, R. Chemical composition and bioactivity of dried fruits and honey of *Ficus carica* cultivars Dottato, San Francesco and Citrullara. *J. Sci. Food Agric.* **2014**, *94*, 2179–2186.

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

Las técnicas cromatográficas propiamente dichas son, la **cromatografía líquida de alta resolución** (HPLC), que se utiliza para la identificación de compuestos no volátiles y es muy adecuada para el análisis de moléculas tanto de bajo, como de alto peso molecular; y la **cromatografía de gases** (GC), apropiada para la separación de compuestos volátiles aunque también puede usarse para el análisis de compuestos no volátiles siendo necesario un proceso previo de derivatización con el fin de aumentar la volatilidad y la estabilidad térmica, consiguiéndose, así, la detección de los mismos.

Otras técnicas separativas importantes en la actualidad son las técnicas electroforéticas, en concreto la **Electroforesis Capilar** (CE), adecuada para la determinación de compuestos no volátiles, que estén permanente o temporalmente cargados[149].

Para completar el proceso de identificación, el sistema de separación debe estar seguido de un detector. Los sistemas de detección acoplados a estas técnicas pueden ser de índole muy diversa, siendo los más usados, la detección espectrofotométrica UV-Vis y la espectrometría de masas (MS). La cromatografía, combinada con MS está considerada como la técnica más eficiente ya que ofrece alta sensibilidad y selectividad[149] (este tema será abordado en profundidad más adelante).

A continuación, se detallan las técnicas separativas, así como los detectores utilizados en el desarrollo del trabajo experimental de la presente tesis doctoral.

[149] Kornél Nagy, K. V. Separation methods. In *Medical Applications of Mass Spectrometry*; Vékey, K.; Telekes, A.;Vertes, A.; *Elsevier*, 2008, pp 61–92

6.1. Cromatografía de gases

La Cromatografía de Gases (GC), debido a sus características y gran versatilidad, es uno de los mayores descubrimientos de la química analítica en la segundad mitad del siglo pasado[150,151]. El origen de la GC se remonta a 1905 cuando W. Ramsey separó una mezcla de gases y vapores procedentes de sólidos adsorbentes, esta técnica fue descrita como adsorción gas-sólido, y fue precursora de la cromatografía gas-sólido que precedió al descubrimiento de la cromatografía en columna líquido-sólido descubierta por Tswett (1906). La GC propiamente dicha, es decir, donde se utiliza un gas como fase móvil nació en 1952 cuando James y Martin, aprovechando un estudio realizado por Martin y Synge (1941), desarrollaron una nueva técnica de separación para analizar una mezcla de 17 ácidos grasos[152,153]. Actualmente, esta técnica puede ser utilizada para la separación y posterior análisis cualitativo y cuantitativo de una gran cantidad de muestras, desde las más simples a las más complejas, con la limitación de que los compuestos objeto de estudio deben ser volátiles y térmicamente estables[154].

En GC, la fase móvil es un gas, llamado gas portador, mediante el cual eluyen los componentes de una mezcla a través de la columna que contiene la fase estacionaria. El gas portador es un gas inerte generalmente helio, nitrógeno, hidrógeno o argón de elevada pureza, con un caudal conocido y controlado, que no interacciona con el analito y su única misión es la de transportar la muestra. La fase estacionaria suele ser un líquido que recubre la pared interna de la columna, por lo que recibe el nombre de cromatografía gas-líquido (GLC), este

[150] Ahuja, S. High-pressure liquid chromatography. In *Separation Science and Technology*; Elsevier, 2003, Vol. 4, pp 153–208.
[151] Walter G. J.; Colin, F. P. Milestones in the Development of Gas Chromatography. In *Gas Chromatography;* Colin, F. P.; Elsevier, 2012, pp 1–17.
[152] Martin, A. J. P.; Synge, R. L. M. A new form of chromatogram employing two liquid phases. *Biochem. J.* **1941**, *35* (12), 1358–1368.
[153] James, A. T.; Martin, A. J. P. Gas-liquid partition chromatography; the separation and micro-estimation of volatile fatty acids from formic acid to dodecanoic acid. *Biochem. J.* **1952**, *50*, 679–690.
[154] Marriott, P. J. Gas chromatography. In *Journal of Chromatography Library*; Heftmann, E.; Elsevier, 2004, Vol 69, pp 319–368.

tipo de cromatografía ha tenido un desarrollo continuado desde que fue descubierta por Martin y Singe[154–156].

Para la realización de una separación por cromatografía de gases, la muestra se inyecta, a través de un septum, en la cámara de inyección donde se vaporiza y se arrastra hasta el principio de la columna. La temperatura inicial de la columna se fija en 40º C por debajo del punto de ebullición del disolvente, y éste condensa en la cabeza de la columna lo que hace que los solutos vayan quedando atrapados lentamente en la porción de disolvente condensado, y formen una banda estrecha al principio de la columna. Los analitos, en fase gaseosa y cada uno a una velocidad determinada, pasan a través de la columna, arrastrados por el gas portador, y por equilibrios sucesivos entre fase móvil y estacionaria. La columna debe estar suficientemente caliente con el fin de que los analitos alcancen una presión de vapor suficiente para que se eluyan en un tiempo razonable. Finalmente, los analitos separados llegan al detector cuya respuesta aparece en forma de cromatograma en el registrador de señal. El detector debe estar a una temperatura más alta que la columna, de forma que los analitos se mantengan en forma gaseosa[155].

Las partes esenciales de un cromatógrafo de gases pueden verse en la siguiente figura:

[155] Skoog, D. A; Holler, F. J.; Nieman, T. A. *Principios de Análisis Instrumental*; McGraw-Hill, 2001.

[156] Tsao, R.; Deng, Z. Separation procedures for naturally occurring antioxidant phytochemicals. *J. Chromatogr. B.* **2004**, *812*, 85–99.

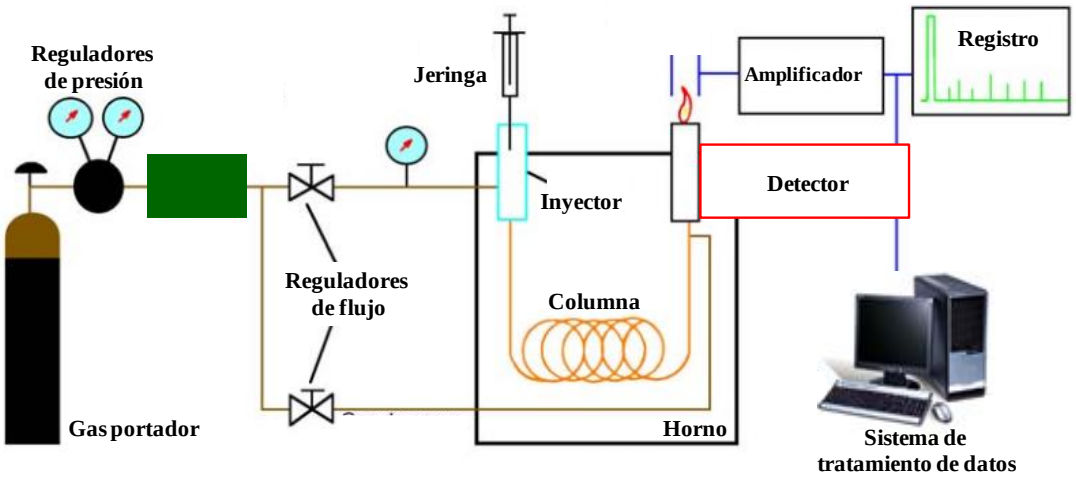


Figura 24. Diagrama de un cromatógrafo de gases

A pesar de la alta resolución y la sensibilidad que ofrece la GC, esta técnica es poco usada para la determinación de compuestos bioactivos derivados de vegetales, debido a la falta de volatilidad que presentan la mayoría de ellos, especialmente cuando se pretenden identificar compuestos más polares. En la mayoría de los casos, se utiliza para la separación de fitoquímicos antioxidantes procedentes de aceites esenciales de plantas o aceites extraídos de vegetales[156].

No obstante, y a pesar de la ausencia de volatilidad, la GC ha sido empleada en numerosas ocasiones para la separación e identificación de diferentes compuestos fenólicos en matrices vegetales. Por lo general, para tal fin suelen utilizarse columnas capilares y espectrometría de masas como sistema de detección[115, 157 , 158] . Los métodos para el análisis de compuestos fenólicos mediante GC ofrecen alta resolución y bajos límites de detección, pero requieren un gran trabajo previo de derivatización que se realiza normalmente mediante metilación o trifluoroacetilación, etapa que es inevitable para aumentar la volatilidad de estos compuestos y mejorar la estabilidad térmica de la muestra[115].

[157] De Rijke, E.; Out, P.; Niessen, W. M. A; Ariese, F.; Gooijer, C.; Brinkman, U. A. T. Analytical separation and detection methods for flavonoids. *J. Chromatogr. A* **2006**, *1112*, 31–63.

[158] Robbins, R. J. Phenolic acids in foods : an overview of analytical methodology. *J. Agric. Food Chem*. **2003**, *51*, 2866–2887.

Esta técnica ya se usó para el análisis de flavonoides a principios de 1960, en el primer artículo sobre este tema los flavonoides, previamente derivatizados, fueron separados utilizando una columna de polímero de silicona SE-30, con un sistema de detección de conductividad térmica[159]. Después de la introducción de la cromatografía líquida (LC), el análisis de flavonoides mediante GC se hizo menos prominente, pero recientemente se está volviendo a retomar debido la evolución de la GC de alta temperatura y a la mejora de los procedimientos de derivatización[157]. El procedimiento analítico para el análisis de flavonoides en alimentos vegetales consiste, en la mayoría de los casos, en la hidrólisis de los flavonoides y posterior conversión de los mismos en derivados de trimetilsililéter (TMS), sin embargo, para flavonoides con más de un sustituyente hidroxilo la metilación puede producir varios derivados, lo cual dificulta su cuantificación. En general, los flavonoides son separados en una columna capilar no polar, DB-5 o DB-1, en modo split o splitless, mediante un programa lineal de temperatura de hasta 300 ºC y en un tiempo de 30-90 min. Los agentes derivatizantes más comunes son BSTFA (N, O-bis-(trimetilsilil)-trifluoroacetamida) y TBDMS (N-(tert-butildimetilsilil)-N-metiltrifluoroacetamida). Como sistema de detección, normalmente, se usa el analizador de impacto electrónico acoplado a MS (EI-MS) cuya temperatura de la fuente de ionización es de hasta 250 ºC[157].

La GC también se ha utilizado para la identificación de ácidos fenólicos en vegetales, siendo los agentes derivatizantes mas utilizados BSA (N,O-bis-(trimetilsilil) acetamida), MSTFA (N-metil-N-(trimetilsilil) trifluoroacetamida) y BSTFA (N,O-bis- (trimetilsilil)-trifluoroacematida). En estudios recientes, las columnas más comunes son columnas capilares de sílice fundida DB-5 o SPB-1, cuyas dimensiones suelen ser de 25 a 30 m de longitud, diámetro interno de 0,25 a 0,5 mm y espesor de la fase estacionaria de 0,25 µm. La mayoría de los métodos han sido realizados con un gradiente de temperatura que suele ir de 2 a 40 °C/min[158].

[159] Narasimhachari, N.; Rudloff, E. V. Gas-liquid chromatography of some flavonoid compounds and hydroxy diphenyls. *Can. J. Chem.* **1962**, *40*, 1123–1129.

En la tabla 5 se resumen algunos estudios de flavonoides y ácidos fenólicos en alimentos vegetales mediante esta técnica. No obstante, la LC es la técnica más utilizada para el análisis de compuestos fenólicos en matrices vegetales.

Tabla 5. Algunos estudios de compuestos fenólicos en matrices vegetales mediante GC (Adaptado de 157,158160,161)

Muestra	Técnica analítica	Derivatización	Familia de compuestos fenólicos identificados
Tejidos vegetales	GC-MS	TMS	Ácidos fenólicos
Futas y frutos secos	GC-MS	TBDMS	Flavonoides
Alimentos de soja	GC-MS	TMS	Flavonoides
Ginkgo bilova	GC-MS	TMS	Flavonoides
Arándanos	GC-MS	BSTFA+TCMS	Ácidos fenólicos
Extractos de hierbas	GC-MS	TMS	Flavonoides
Hojas de papaya	GC-MS	BSTFA+TCMS	Ácidos fenólicos y flavonoides
Garcinia mangostana	GC-MS	BSA	Ácidos fenólicos

Por otro lado, la cromatografía de gases es la técnica más utilizada para el análisis de ácidos grasos en aceites derivados de vegetales. Estos compuestos poseen una polaridad relativamente alta, por lo que se hace necesaria su conversión en compuestos más volátiles como son los ésteres metílicos de ácidos grasos (FAMEs). La generación de FAMEs se puede hacer directamente en un procedimiento que combina la extracción y la derivatización de los lípidos mediante transesterificación de los aceites vegetales con metilato de sodio, NaOH o KOH. Las columnas capilares con fases estacionarias polares, son las más utilizadas ya que permiten la separación según el grado de insaturación y número de carbonos[160]. El primer estudio de los ácidos grasos, mediante esta técnica, en aceites vegetales de girasol, soja, semillas de lino, maíz y frutos

[160] Ruiz-Rodriguez, A.; Reglero, G.; Ibañez, E. Recent trends in the advanced analysis of bioactive fatty acids. *J. Pharm. Biomed. Anal.* **2010**, *51*, 305–326.

secos, data de 1959[161]. Estudios más actuales muestran la composición de ácidos grasos en aceites extraídos de semillas de frutas. Por ejemplo, la cuantificación de los FAMEs en aceite procedente de las semillas de 25 variedades de granada fue realizada con GC y detector de ionización de llama (FID). Para esta separación se utilizó una columna capilar (FFAP, 25 m x 0.25 mm, 0.22 μm), y un gradiente de temperatura de 170 a 190ºC[162]. Otros trabajos recientes fueron, por ejemplo, la cuantificación de 10 ácidos grasos en semillas de frutas de la familia Annonaceae, o en siete variedades de semillas de manzana donde se identificaron 9 compuestos, para ambos estudios se utilizó un detector FID[163,164]. Uno de los últimos trabajos se ha realizado en diferentes tipos de aceites vegetales como uva, coco, calabaza y oliva, entre otros. En este estudio se ha utilizado una columna HP-88 (100m × 0.25 mm), con un espesor de la fase estacionaria de 0.2 μm y de nuevo un detector FID. Más adelante, en la tabla 6, se resumen algunos de los estudios, de la composición de ácidos grasos, realizados en vegetales en los últimos años.

Otro tipo de modalidad cromatográfica utilizada en el análisis de ácidos grasos es la cromatografía de gases "fast" (fast-GC), esta técnica representa una evolución de la cromatografía de gases tradicional ya que posee la gran ventaja de reducir los tiempos de análisis aumentando la eficiencia cromatográfica. En la fast-GC la separación puede hacerse en un rango de pocos minutos, obteniéndose picos con una anchura de aproximadamente 1-3 segundos. El comienzo de la fast-GC se remonta a 1960, sin embargo no fue hasta hace unos años cuando comienza a utilizarse en análisis de matrices vegetales[154,160]. En definitiva, con la fast-GC se utilizan columnas, instrumentos y condiciones cromatográficas particulares que

[161] Craig, B. M.; Murty, N. L. Quantitative fatty acid analysis of vegetable oils by gas-liquid chromatography. *J. Am. Oil Chem. Soc.* **1959**, *36*, 549–552.
[162] Fadavi, A.; Barzegar, M.; Hossein Azizi, M. Determination of fatty acids and total lipid content in oilseed of 25 pomegranates varieties grown in Iran. *J. Food Compos. Anal.* **2006**, *19*, 676–680.
[163] Moreno Luzia, D. M.; Jorge, N. Soursop (*Annona muricata* L.) and sugar apple (*Annona squamosa* L.). *Nutr. Food Sci.* **2012**, *42* (6), 434–441.
[164] Bada, J.C.; León-Camacho, M.; Copovi, P.; Alonso L. Characterization of apple seed oil with Denomination of Origin from Asturias, Spain. *Grasas y aceites* **2014**, *65* (2).

permiten un análisis de 3 a 10 veces más rápido garantizando una resolución elevada. Un análisis mediante fast-GC se realiza modificando parámetros analíticos como la longitud y el diámetro interno de la columna capilar, el espesor de la fase estacionaria, el tipo de gas portador, la velocidad linear y la temperatura del horno. Las columnas utilizadas en este tipo de análisis tienen un diámetro interno de 0.1 a 0.18 mm, el espesor de la película es muy fino y la velocidad de flujo de la fase móvil se reduce hasta los cm/s[165,166]. Mondello *et al.* utilizó esta técnica para analizar la composición de ácidos grasos de grasas comestibles, entre las que se encuentra el aceite de oliva, la columna utilizada fue una supelcowax-10 con unas dimensiones de 10m x 0.10 mm y 0.10 µm de espesor de la fase estacionaria[167]. Más recientemente, Destaillats y Cruz-Hernández publicaron un estudio muy interesante sobre la separación de FAMEs complejos utilizando una columna de este tipo altamente polar (BPX-70; 10 m × 0,1 mm × 0,2 µm), entre las muestras analizadas en este trabajo se encuentra la manteca de cacao[168].

Para el análisis de fitoesteroles, también prevalece la cromatografía de gases. Aunque la separación de los esteroles y estanoles sin derivatización es posible, la resolución y la sensibilidad mejoran mediante una derivatización previa, transformándolos en derivados de trimetilsililo (TMS) o derivados de acetato. Los agentes de derivatización generalmente utilizados son: N-metil-N-trimetilsililtrifluoroacetamida (MSTFA) en piridina anhidra y bis (trimetilsilil)-trifluoroacetamide (BSTFA) con un 1% de trimetilclorosilano (TMCS). Las columnas capilares son las que dan mejores rendimientos, ya que permiten la separación de los esteroles casi completamente, lo cual es casi imposible con

[165] Cramers, C. A.; Janssen, H. G.; Van Deursen, M. M.; Leclercq, P. A. High-speed gas chromatography: An overview of various concepts. *J. Chromatogr. A* **1999**, *856*, 315–329.
[166] Maštovská, K.; Lehotay, S. J. Practical approaches to fast gas chromatography-mass spectrometry. *J. Chromatogr. A* **2003**, *1000*, 153–180.
[167] Mondello, L.; Tranchida, P. Q.; Costa, R.; Casilli, A.; Dugo, P.; Cotroneo, A.; Dugo, G. Fast GC for the analysis of fats and oils. *J. Sep. Sci.* **2003**, *26*, 1467–1473.
[168] Destaillats, F.; Cruz-Hernández, C. Fast analysis by gas-liquid chromatography. Perspective on the resolution of complex fatty acid compositions. *J. Chromatogr. A* **2007**, *1169*, 175–178.

columnas de relleno. La mayoría de las determinaciones de esteroles se han llevado a cabo con fases estacionarias no polares, sin embargo, el uso de una fase estacionaria ligeramente polar, de alta estabilidad térmica, ofrece una mejor resolución de los picos. Los detectores más utilizados son FID o MS que junto con un sistema de ionización por impacto electrónico (EI) proporciona una resolución, identificación y cuantificación más efectiva[169,170].Uno de los trabajos más antiguos disponibles sobre la determinación de fitoesteroles en matrices vegetales, concretamente en semillas de calabaza, fue publicado en 1977, en el cual se identificaron 14 compuestos mediante GC-MS[171]. Posteriormente fueron publicados numerosos trabajos en diferentes matrices como cereales, semillas de frutas y frutos secos ya que estas matrices son una fuente importante de estos compuestos bioactivos[170]. Estudios más recientes, se muestran esquematizados en la tabla 7. Por ejemplo, en semillas de granada, donde Caligiani y colaboradores identificaron 5 fitoesteroles de tres variedades diferentes mediante GC-MS utilizando un método con gradiente de temperatura de 80 a 280 ºC[172]. Bada *et al*, realizó un trabajo similar en distintas variedades de semillas de manzana, esta vez utilizando un detector FID[164]. Otro trabajo de los últimos años fue la determinación de estos compuestos en 10 variedades de pulpa de banana, cuya separación fue realizada en una columna DB-1 (30 m × 0.32 mm, 0.25 μm), la detección se llevó a cabo mediante FID[173].

[169] Aparicio, R.; Aparicio-Ruíz, R. Authentication of vegetable oils by chromatographic techniques. *J. Chromatogr. A* **2000**, *881*, 93–104.

[170] Lagarda, M. J.; García-Llatas, G.; Farré, R. Analysis of phytosterols in foods. *J. Pharm. Biomed. Anal.* **2006**, *41*, 1486–1496.

[171] Bastić, M.; Bastić, L.; Jovanović, J. A.; Spiteller, G. Sterols in pumpkin seed oil. *J. Am. Oil Chem. Soc.* **1977**, *54* (11), 525–527.

[172] Caligiani, A.; Bonzanini, F.; Palla, G.; Cirlini, M.; Bruni, R. Characterization of a Potential Nutraceutical Ingredient: Pomegranate (*Punica granatum* L.) Seed Oil Unsaponifiable Fraction. *Plant Foods Hum. Nutr.* **2010**, *65*, 277–283.

[173] Vilela, C.; Santos, S. a O.; Villaverde, J. J.; Oliveira, L.; Nunes, A.; Cordeiro, N.; Freire, C. S. R.; Silvestre, A. J. D. Lipophilic phytochemicals from banana fruits of several *Musa* species. *Food Chem.* **2014**, *162*, 247–252.

Tabla 6. Algunos estudios sobre ácidos grados en matrices vegetales mediante GC

Muestra	Técnica analítica	Derivatización	Columna	Referencias
Determinación de ácidos grasos				
Pulpa de chirimoya	GC-FID y GC-MS	MeOH/BF_3	DB-3; HP1	94
Aceite de grosella negra	GC-FID	MeOH/$CHCl_3$	DB-Wax (30 m x 0.25 mm, 0.25µm)	174
Semillas de granada	GC-FID	MeOH/NaOH	FFAP (25 m x 0.25 mm, 0.22 µm)	162
Manteca de cacao	Fast-GC-FID	Transesterificación	BPX-70 (10 m × 0,1 mm, 0,2 µm)	168
Aceite de girasol	GC-MS	HCL/MeOH, BF_3/MeOH	SPB^{TM}-1 (30 m × 0.32 mm, 0.25 µm)	175
Frutos secos	GC-MS	BF3/MeOH	Carbowax (60 m x 0.25 mm, 0.2 µm)	176
Semillas de frutas Annonaceae	GC-FID	Transmetilación	CP-Sil 88 (50m x 0.25mm, 0.20mm)	163
Semillas de manzana	GC-FID	MeOH/KOH	P-2380 (60m × 0.25 mm, 0.2 µm)	164
Aceites vegetales	GC-FID	MeOH/NaOH	HP-88 (100m × 0.25 mm, 0.2 µm)	177

[174] Stránský, K.; Zarevúcka, M.; Wimmer, Z. Gas chromatography analysis of blackcurrant oil in relation to its stability. *Food Chem.* **2005**, *92*, 569–573.

[175] Kanya, T. C. S.; Rao, L. J.; Sastry, M. C. S. Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. *Food Chem.* **2007**, *101*, 1552–1557.

[176] Charef, M.; Yousfi, M.; Saidi, M.; Stocker, P. Determination of the fatty acid composition of Acorn (*Quercus*), *Pistacia lentiscus* seeds growing in Algeria. *JAOCS, J. Am. Oil Chem. Soc.* **2008**, *85*, 921–924.

[177] Orsavova, J.; Misurcova, L.; Ambrozova, J.; Vicha, R.; Mlcek, J. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *Int. J. Mol. Sci.* **2015**, *16*, 12871–12890.

Tabla 7. Algunos estudios sobre fitoesteroles en matrices vegetales mediante GC

Muestra	Técnica analítica	Derivatización	Columna	Referencias
Determinación de fitoesteroles				
Frutos secos y semillas	GC-MS	TMS	RTX-5MS (30 m × 0.25 mm, 0.25 μm)	178
Aceites de semillas	GC-MS	Piridina/HMS/TMCS	HP5MS (30 m × 0.25 mm, 0.25 μm)	179
Aceites vegetales	GC-MS	Piridina/Ácido acético	DB-5MS (30 m × 0.25 mm, 0.25 μm)	180
Semillas de granada	GC-MS	BSTFA	DB5	172
Aceites vegetales	GC-FID	Sin derivatización	DB-5 (30m × 0.25 mm, 1 μm)	181
Pulpa de banana	GC-MS	BSTFA/TMCS	DB-1 (30 m × 0.32 mm, 0.25 μm)	173
Semillas de manzana	GC-FID	EtOH/KOH	HP-5 (30 m × 0.32 mm, 0.25)	164

[178] Phillips, K. M.; Ruggio, D. M.; Ashraf-Khorassani, M. Phytosterol composition of nuts and seeds commonly consumed in the United States. *J. Agric. Food Chem.* **2005**, *53*, 9436–9445.

[179] Cheikh-Rouhou, S.; Besbes, S.; Lognay, G.; Blecker, C.; Deroanne, C.; Attia, H. Sterol composition of black cumin (*Nigella sativa* L.) and Aleppo pine (*Pinus halepensis* Mill.) seed oils. *J. Food Compos. Anal.* **2008**, *21*, 162–168.

[180] Mitei, Y. C.; Ngila, J. C.; Yeboah, S. O.; Wessjohann, L.; Schmidt, J. Profiling of phytosterols, tocopherols and tocotrienols in selected seed oils from botswana by GC-MS and HPLC. *J. Am. Oil Chem. Soc.* **2009**, *86*, 617–625.

[181] Hassanien, M. F. R. Tocol and phytosterol composition of edible oils in the egyptian market. *J. Food Process. Preserv.* **2012**, *36*, 531–538.

6.2. Cromatografía líquida de alta resolución

En general, la LC se define como una técnica separativa en la que la separación de los componentes de una mezcla se basa en la distribución de los mismos entre dos fases, una fase estacionaría, y una fase móvil, que en este caso es un líquido que fluye a través de la primera[182]. El desarrollo de la cromatografía líquida comienza en 1906, cuando Tswett y Day realizaron una serie de experimentos usando columnas abiertas con rellenos sólidos variados, dando lugar a lo se conoce como fase estacionaria[183–185]. Sin embargo, no fue hasta 1930 cuando Kuhn y Lederer describieron la cromatografía líquida propiamente dicha, momento a partir del cual, la técnica no deja de desarrollarse hasta día de hoy[186,187]. La modalidad de cromatografía clásica o gravitatoria, denominada así porque la fase móvil recorre el relleno por la simple acción de la gravedad, presentaba inconvenientes importantes como: su lentitud, escasa eficacia y dificultad, ya que se hacía necesaria la intervención prácticamente constante del operador. Además, una vez finalizadas las etapas de separación y recolección de fracciones, se tenía que aplicar una detección discontinua (off-line) a cada fracción del eluído ya que no proporcionaba directamente el cromatograma[188].

Posteriormente y hasta principios de los años 70, los esfuerzos científicos, en este tema, se centraron en crear una LC "rápida", con el objetivo de aumentar la resolución y el tiempo de separación. Esto dio lugar a la disminución del tamaño de las partículas de la fase estacionaria, lo que propició la necesidad de utilizar un sistema de bombeo a alta presión para conseguir un flujo razonable de la fase

[182] Yashin, Y. I.; Yashin, A. Y. Liquid Chromatography. In Chemical Analysis of Food: Techniques and Applications; Picó, Y.; Elsevier 2012, pp 285–310.
[183] Tswett, M. On a new category of adsorption phenomena and their application to biochemical analysis. *Proc. Warsaw Soc. Nat. Sci., Biol. Sect. XIV* **1903**.
[184] Tswett, M. Zur Kenntnis der Phaeophyceenfarbstoffe. *Ber. dtsch. Bot.* **1906**, *24*, 235–244.
[185] M. Tswett. Adsorptions analyse und chromatographische Methode. *Ber. Deutsch. Bot. Ges.* **1906**, *24*, 384–385.
[186] Kuhn, R.; Zerlegung, E. L. Des Carotins in seine Komponenten. (Über das Vitamin des Wachstums, I. Mitteil.). *Ber. dtsc. Chem. Ges* **1931**, *641*, 349–1356.
[187] Cela, R. Lorenzo, R. A.;. Casais, M. C. Cromatografía líquida en columna. En *Técnicas de separación en Química Analítica*; Síntesis, 2002.
[188] Valcárcel Cases, M.; Gómez Hens, A. *Técnicas analíticas de separación*; Reverte, 1988.

móvil, dando lugar a lo que hoy se conoce como cromatografía líquida de alta resolución[182].

La Cromatografía Líquida de Alta Resolución (HPLC) es una técnica de separación en la que una mezcla de compuestos se distribuye entre las dos fases, en función de las distintas afinidades que presente cada compuesto de la mezcla por las mismas. La fase estacionaria es generalmente un sólido poroso, o bien una fina capa de sustancia líquida ligada a un soporte sólido, contenido en el interior de un tubo habitualmente metálico, llamado columna cromatográfica, parte clave del cromatógrafo de líquidos. La fase móvil es un líquido, ya sea un disolvente único o una mezcla, a veces con un pH modificado mediante adición de ácidos, bases o sistemas tampón.

La presión elevada de la fase móvil líquida permite:

- Reducir el tamaño de partícula de la fase estacionaria, con lo que se podría aumentar espectacularmente la eficacia separativa.
- Reducir la duración de una separación cromatográfica, de 5 a 50 veces, en relación con la modalidad a baja presión.
- Una detección continua del eluido, es decir, un instrumento que separa y suministra información cualitativa y cuantitativa.

Estas ventajas conllevan una serie de inconvenientes como complicaciones técnicas en comparación con la modalidad clásica, y un notable aumento del coste de adquisición y mantenimiento del instrumento. No obstante, estos aspectos pasan a un segundo plano debido a la enorme potencialidad que presenta esta técnica separativa en comparación con la GC ya que la LC no está restringida a la volatilidad ni la estabilidad térmica de la muestra pudiendo ser analizados una gran cantidad de compuestos (compuestos iónicos, muy polares, termolábiles, no volátiles, muestras líquidas, etc)[150].

Un instrumento moderno de HPLC consta de los siguientes componentes principales:

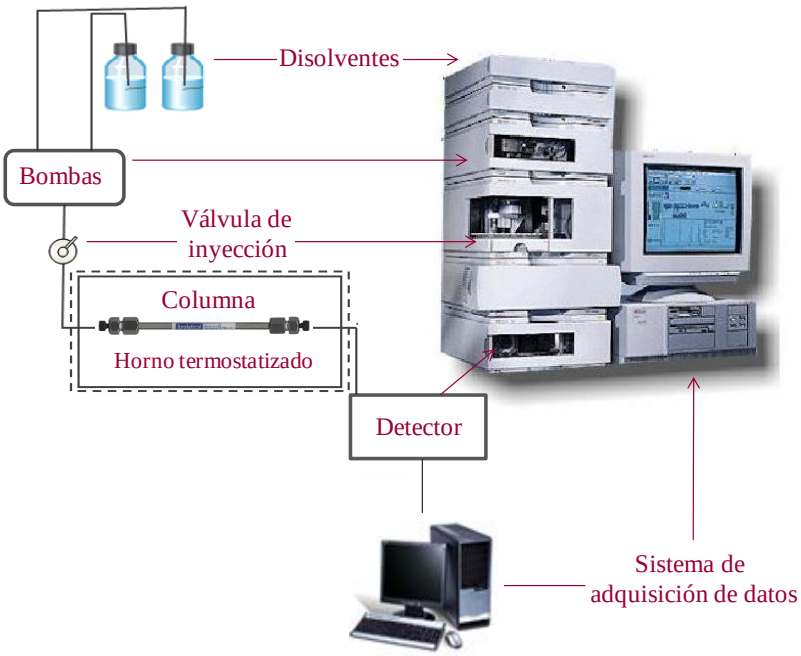


Figura 25. Esquema simplificado de un equipo de HPLC junto a uno comercial

En definitiva, en una separación por HPLC, la fase móvil impulsada por la bomba transporta una banda de muestra a través de la columna cromatográfica, donde los analitos interaccionan con la fase estacionaria de tal forma que aquellos que sean más afines con la fase móvil serán menos retenidos por dicha fase y eluirán antes, mientras que aquellos que tengan más afinidad por la fase estacionaria avanzarán más lentamente a través de la columna y eluirán más tarde. Una vez eluido cada compuesto debe ser detectado, para ello, se colocan a la salida de la columna cromatográfica uno o varios detectores que proporcionarán una respuesta al paso de los analitos (absorbancia, emisión fluorescente, conductividad, etc). El procesado de esta señal da lugar al cromatograma, en el que representa la respuesta obtenida por el detector frente al tiempo. Cada analito, por tanto, estará representado por un pico que poseerá un determinado tiempo de retención (t_R). La intensidad de cada pico será

directamente proporcional al factor de respuesta y la concentración del analito correspondiente en la muestra.

Los diferentes modos de LC se diferencian según la naturaleza de la fase estacionaria, siendo el más ampliamente utilizado la cromatografía de partición o reparto que a su vez puede tener dos modalidades de trabajo:

- Cromatografía en fase normal, en la que la fase estacionaria presenta una naturaleza fuertemente polar y la fase móvil es de naturaleza no polar (o poco polar). Por tanto los analitos apolares serán poco retenidos por la fase estacionaria eluyendo en primer lugar.
- Cromatografía en fase reversa, donde la fase estacionaria es no polar y la fase móvil, es polar. En esta modalidad el proceso de separación ocurre a la inversa que en la anterior. En el caso de análisis de matrices vegetales, este tipo de cromatografía es la alternativa más utilizada ya que la mayoría de sus componentes bioactivos son de naturaleza hidrofílica.

La mayoría de los investigadores coinciden en que la cromatografía líquida de alta resolución, es el sistema más utilizado para la separación e identificación de fitoquímicos, especialmente para compuestos fenólicos. La versatilidad de esta técnica es debida, en parte, a los diferentes modos de separación y diferentes sistemas de detección acoplables a la misma[115,156,189].

El análisis de compuestos fenólicos en matrices vegetales, mediante HPLC, depende de varios factores que incluyen la purificación de la muestra, las fases móviles, el tipo de columna y el detector[189]. La elección de la columna apropiada es uno de los factores críticos en la identificación de los polifenoles ya que la polaridad de la fase móvil va a influir en su detección. Normalmente, como se comentó anteriormente, la separación cromatográfica se realiza en fase reversa y se utilizan columnas de sílice modificada C18, la longitud de las mismas va de 10 a 30 cm, el diámetro interno suele ser de 3.9 a 4.6 mm y el tamaño de partícula

[189] Khoddami, A.; Wilkes, M. A.; Roberts, T. H. Techniques for analysis of plant phenolic compounds. *Molecules* **2013**, *18*, 2328–2375.

de la fase estacionaria desde 3 a 10 µm. Sin embargo, en técnicas más avanzadas como la Cromatografía Líquida de Ultra-alta Presión (UHPLC), Cromatografía Líquida de Alta Temperatura (HTLC) o Cromatografía Líquida de dos Dimensiones (LC×LC) se pueden utilizar columnas cuya longitud puede ir de 3-25 cm, diámetro interno de 1 a 4.6 mm y tamaño de partícula de 1.7 a 10 µm. Otro tipo de fases estacionarias que se usan en menor medida son: C8, sílice, Sephadex y poliamida[189]. En cuanto a las fases móviles, se utilizan sistemas binarios de disolventes que consisten, generalmente, en agua acidificada (disolvente A) o con algún aditivo como acetato o tampón formiato, y un disolvente orgánico polar (disolvente B) siendo los más comunes metanol o acetonitrilo puros o acidificados, aunque también se pueden utilizar otros como etanol, tetrahidrofurano o propanol. Los métodos basados en una elución en gradiente predominan sobre los basados en elución isocrática.

La mayoría de los análisis de este tipo se realizan a temperatura ambiente, pero en algunos casos, se recomiendan temperaturas de hasta 40 ◦C, para reducir el tiempo de análisis y aumentar la reproducibilidad. El tiempo de análisis es otro de los factores claves para la detección de los polifenoles, éstos normalmente pueden variar de 10 a 150 min a una velocidad de flujo de entre 0.8-1.5 mL/min aunque existen trabajos donde se utilizan flujos menores. Sin embargo, en ocasiones, para una separación más exhaustiva y con una alta reproducibilidad, los tiempos pueden ser de hasta 2 h[115,156,157,189]. Un ejemplo de la diferencia entre los tiempos de análisis podría ser el trabajo de Huck *et al*. donde se llevó a cabo la separación de cinco agliconas (quercitrina, myricetina, quercetina, kaempferol y acacetina), presentes en extractos de plantas, en sólo 5 minutos, mientras que en el estudio de Kinoshita *et al.* se necesitaron 340 min para la identificación de isoflavonas en salsas de soja[190,191].

[190] Kinoshita, E.; Sugimoto, T.; Ozawa, Y.; Aishima, T. Differentiation of soy sauce produced from whole soybeans and defatted soybeans by pattern recognition analysis of HPLC profiles. *J. Agric. Food Chem.* **1998**, *46*, 877–883.

[191] Huck, C.; Buchmeiser, M.; Bonn, G. Fast analysis of flavonoids in plant extracts by liquid chromatography-ultraviolet absorbance detection on poly-(carboxylic acid)-coated silica

Actualmente, existe una gran cantidad de trabajos de revisión bibliográfica disponibles sobre el análisis de compuestos fenólicos en matrices vegetales mediante HPLC[192]. En la tabla 8, se recogen algunos de los trabajos publicados en los últimos años.

Otros compuestos bioactivos presentes en la fracción lipídica de los vegetales, que suelen analizar mediante HPLC, son los tocoferoles y los fosfolípidos. Las separaciones por HPLC de tocoferoles se pueden realizar tanto en fase normal como en fase reversa, aunque las columnas de fase reversa son más utilizadas por tener una mejor estabilidad y durabilidad. Sin embargo, la mayoría de estas columnas no son capaces de separar los isómeros ß y γ de los tocoferoles y tocotrienoles. Además de proporcionar mayor selectividad en la determinación de compuestos liposolubles y su capacidad para separar los isómeros ß y γ, las ventajas de las columnas de fase normal sobre las de fase inversa incluyen: (a) la posibilidad de trabajar con diferentes disolventes orgánicos, lo que permite una alta solubilidad de los lípidos; (b) la capacidad de tolerar altas cargas de lípidos ya que son fáciles de lavar usando disolventes no polares y (c) proporcionan una alta selectividad añadiendo modificadores polares en la fase móvil. Por lo tanto, para muestras de vegetales cuya composición es compleja se suelen utilizar columnas de fase normal. Generalmente, las fases móviles más utilizadas consisten en un disolvente orgánico, generalmente hexano junto con una variedad de modificadores orgánicos, como, éter dietílico, di-isopropil éter, terc-butil-metil éter, metanol, isopropanol, entre otros[193,194].

and electrospray ionization tandem mass spectrometric detection. *J. Chromatogr. A* **2002**, *943*, 33–38.

[192] Robards, K. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. *J. Chromatogr. A* **2003**, *1000*, 657–691.

[193] Kamal-Eldin, A.; Görgen, S.; Pettersson, J.; Lampi, A. M. Normal phase high-performance liquid chromatography of tocopherols and tocotrienols. *J. Chromatogr. A* **2000**, *881*, 217–227.

[194] Ahsan, H.; Ahad, A.; Siddiqui, W. A. A review of characterization of tocotrienols from plant oils and foods. *J. Chem. Biol.* **2015**, *8*, 45–59.

Por otro lado, el análisis de fosfolípidos en aceites vegetales mediante HPLC, normalmente implica el uso de columnas de gel de sílice, y los métodos analíticos pueden estar basados tanto en elución isocrática como en gradiente. En cuanto a las fases móviles, entre las más utilizadas se encuentran: cloroformo/metanol, acetonitrilo, ácido fosfórico/metanol al 85%, o acetonitrilo/metanol/agua[42,195].

[195] Cert, A.; Moreda, W.; Pérez-Camino, M. C. Chromatographic analysis of minor constituents in vegetable oils. *J. Chromatogr. A* **2000**, *881*, 131–148.

Tabla 8. Algunos estudios publicados en los últimos años sobre análisis de compuestos fenólicos en matrices vegetales mediante HPLC

Muestra	Familia de compuestos fenólicos identificados	Técnica analítica	Columna	Disolventes	Flujo (mL/min)	Ref.
Varios vegetales	Ácidos fenólicos y flavonoides	HPLC-MS	C18 (150 mm x 4.6 mm)	H_2O/CH_3COOH; $CH_3COOH/AcN/MeOH$	0.8	196
Té verde	Catequinas	HPLC-UV	C18 (250 mm x 4.6 mm; 5 µm)	$H_2O/CH_3COOH/AcN$; AcN/CH_3COOH	1	197
Semillas de frutas	Ácidos fenólicos	HPLC-UV	RP-18 (250 mm x 4 mm; 5 µm)	H_2O/CH_3COOH; MeOH	1	198
Pulpa de berenjena	Ácidos fenólicos y flavonoides	HPLC-MS/MS	C18 (150 mm x 3 mm; 3µm)	$MeOH/H_2O/CH_2O_2$; $MeOH/H_2O/AcN$	0.3	121
Piel de berenjena	Antocianidinas	HPLC-UV	RP-18 (100 mm x 3 mm)	H_2O/CH_2O_2; $H_2O/AcN/MeOH/CH_2O_2$	0.8	84
Tomate	Compuestos fenólicos y otros metabolitos	HPLC-MS	C18 (150 mm x 4.6 mm; 1.8 µm)	H_2O/CH_3COOH; AcN	1.8	199
Chirimoya	Procianidinas	HPLC-UV	C18 (250 mm x 4.6 mm; 5 µm)	H_2O/MeOH	1	89
Pepino	Ácidos fenólicos y flavonoides	HPLC-MS/MS	C18 (150 mm x 4.6 mm; 1.8 µm)	H_2O/CH_3COOH; AcN	0.8	200
Zumo de frutas cítricas	Flavonoides, ácidos hidroxicinámicos	HPLC-UV	C18 (150 mm x 4.6 mm; 3µm)	H_2O/CH_3COOH; MeOH	0.8	71

[196] Huang, Z.; Wang, B.; Eaves, D. H.; Shikany, J. M.; Pace, R. D. Phenolic compound profile of selected vegetables frequently consumed by African Americans in the southeast United States. *Food Chem.* **2007**, *103*, 1395–1402.

[197] Chen, Q.; Zhao, J.; Chaitep, S.; Guo, Z. Simultaneous analysis of main catechins contents in green tea (Camellia sinensis (L.)) by Fourier transform near infrared reflectance (FT-NIR) spectroscopy. *Food Chem.* **2009**, *113*, 1272–1277.

[198] K.L. Nyam, C.P. Tan, O.M. Lai, K. Long, Y. B. C. M. Physicochemical properties and bioactive compounds of selected seed oils. *LWT - Food Sci. Technol.* **2009**, *42*, 1396–1403.

[199] Gómez-Romero, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Metabolite profiling and quantification of phenolic compounds in methanol extracts of tomato fruit. *Phytochemistry* **2010**, *71*, 1848–1864.

[200] Abu-Reidah, I. M.; Arráez-Román, D.; Quirantes-Piné, R.; Fernández-Arroyo, S.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC–ESI-Q-TOF-MS for a comprehensive characterization of bioactive phenolic compounds in cucumber whole fruit extract. *Food Res. Int.* **2012**, *46*, 108–117.

Muestra	Familia de compuestos fenólicos identificados	Técnica analítica	Columna	Disolventes	Flujo (mL/min)	Ref.
Zumo de granada	Antocianidinas	HPLC-MS	SB-C18 (100 mm x 3 mm; 2.7µm)	H_2O/CH_2O_2; AcN	0.8	100
Zumo de granada	Ácidos fenólicos, flavonoides y taninos	HPLC-MS	SB-C18 (100 mm x 3 mm; 2.7µm)	H_2O/CH_3COOH; AcN	0.8	100
Pimiento	Ácidos fenólicos, alcoholes fenólicos, flavonoides y lignanos	HPLC-MS	C18 (150 mm x 4.6 mm; 1.8 µm)	H_2O/CH_3COOH; AcN	0.8	201
Piel y pulpa de mango	Ácidos fenólicos, taninos, flavonoides y xantonas	HPLC-MS/MS	C18 (150 mm x 4.6 mm; 5µm)	CH_2O_2; MeOH	0.4	202
Fruta de la pasión, chirimoya, fresa y limón	Ácidos fenólicos, taninos y flavonoides	HPLC-MS/MS	C18 (250 mm x 3 mm; 5 µm)	H_2O/CH_2O_2; AcN	0.4	203
Zumo de caqui	Ácidos fenólicos y flavonoides	HPLC-MS	C18 (150 mm x 4.6 mm; 1.8 µm)	H_2O/CH_3COOH; MeOH	0.8	204

[201] Morales-Soto, A.; Gómez-Caravaca, A. M.; García-Salas, P.; Segura-Carretero, A.; Fernández-Gutiérrez, A. High-performance liquid chromatography coupled to diode array and electrospray time-of-flight mass spectrometry detectors for a comprehensive characterization of phenolic and other polar compounds in three pepper (*Capsicum annuum* L.) samples. *Food Res. Int.* **2013**, *51* (2), 977–984.

[202] Ramirez, J.; Zambrano, R.; Sepúlveda, B.; Simirgiotis, M. Antioxidant Properties and Hyphenated HPLC-PDA-MS Profiling of Chilean Pica Mango Fruits (*Mangifera indica* L. Cv. piqueño). *Molecules* **2013**, *19*, 438–458.

[203] Spínola, V.; Pinto, J.; Castilho, P. C. Identification and quantification of phenolic compounds of selected fruits from Madeira Island by HPLC-DAD–ESI-MSn and screening for their antioxidant activity. *Food Chem.* **2015**, *173*, 14–30.

[204] Jiménez-Sánchez, C.; Lozano-Sánchez, J.; Marti, N.; Saura, D.; Valero, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Characterization of polyphenols, sugars, and other polar compounds in persimmon juices produced under different technologies and their assessment in terms of compositional variations. *Food Chem.* **2015**, *182*, 282–291.

6.3. Electroforesis capilar

La electroforesis capilar (CE) es una técnica basada en el movimiento diferencial o migración de iones en disolución por atracción o repulsión en un campo eléctrico, lo que se define como proceso electroforético. En la práctica, en una disolución que contiene iones se introducen un electrodo positivo (ánodo) y uno negativo (cátodo) y se aplica un voltaje a ambos, los iones de diferente carga, aniones con carga negativa y cationes con carga positiva, se mueven a través de la disolución hacia el electrodo de carga opuesta[205,206].

Las primeras técnicas separativas basadas en principios electroforéticos fueron desarrolladas por Tiselius[207], el cual usaba un tubo en U que contenía disoluciones libres o no soportadas que eran separadas por delimitadores que se movían por el voltaje aplicado. Estudios posteriores sobre esta técnica fueron llevados a cabo en columnas y en soportes planos de papel, sílice o geles, humedecidos con disoluciones acuosas. Estas separaciones por electroforesis convencional, que fueron denominadas como electroforesis en zona o en gel, siguen teniendo una amplia aplicación en la identificación o cuantificación de macromoléculas, especialmente proteínas, sin embargo son técnicas lentas y laboriosas, poco reproducibles y no permiten la automatización.

La separación electroforética fue descrita como electroforesis libre, por primera vez en 1967 cuando Hjerten utilizó capilares de 3 mm de diámetro interno y realizó la detección de los compuestos separados por absorción UV[208]. A partir de este momento, la electroforesis se fue haciendo popular hasta la aparición del primer instrumento comercial a finales de 1980[206]. En la actualidad, la CE se desarrolla en estrechos capilares, normalmente de 25 a 100 µm de diámetro

[205] Butler, J. M. Capillary Electrophoresis: Principles and Instrumentation. In *Advanced Topics in Forensic DNA Typing: Methodology*; Elsevier, 2012, pp 141–165.
[206] Bosserhoff, A.; Hellerbrand, C. Capillary Electrophoresis. In *Molecular Diagnostics*; Patrinos, G. P.; Ansorge, W. J.; Elsevier, 2010, pp 59-73.
[207] Tiselius, A. A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans. Faraday Soc.* **1937**, *33*, 524–531.
[208] Hjertén, S. Free zone electrophoresis. *Chromatogr. Rev.* **1967**, *9*, 122–219.

interno, rellenos de una disolución de separación denominada medio electroforético, que suele ser una disolución reguladora.

La instrumentación necesaria en CE, es bastante simple como se puede ver en la siguiente figura:

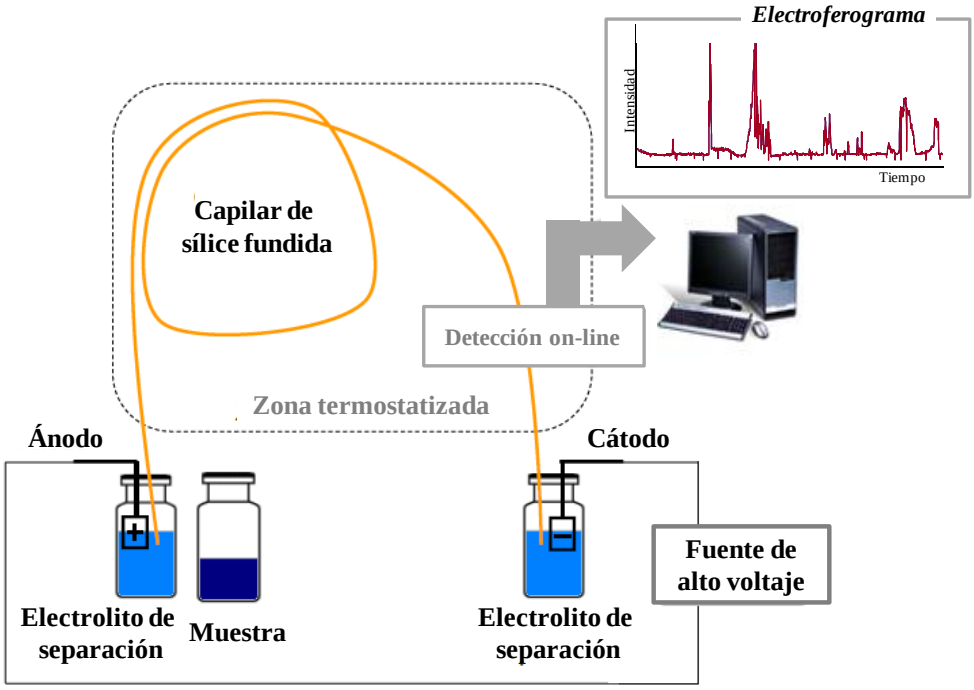


Figura 26. Esquema básico de un instrumento de CE

El proceso se separación consiste en introducir los extremos del capilar en dos viales rellenos de disolución de separación, que contienen, a su vez, cada uno un electrodo y ambos están conectados a una fuente de alto voltaje capaz de llegar hasta 30 kV. La muestra se inyecta dentro del capilar sustituyendo temporalmente uno de los viales iniciales, usualmente el del ánodo, por un vial que contiene la muestra a separar, la inyección se realiza aplicando un potencial eléctrico (inyección electrocinética), o bien, una presión externa durante unos segundos (inyección hidrodinámica). A continuación, se vuelve a reemplazar el vial de muestra por el inicial que contiene la disolución de separación y se aplica

un potencial eléctrico a lo largo del capilar, lo que origina la separación[209,210]. Una vez separados, los analitos pueden ser detectados mediante detección óptica (UV-Vis, fluorimétrica, fosforimétrica, quimioluminiscente o infrarroja), habitualmente a través de la ventana del capilar que se encuentra cerca del extremo opuesto a donde se hizo la inyección (normalmente cerca del cátodo), esto sería detección on-line; o mediante otros sistemas de detección como la espectrometría de masas o las técnicas eléctricas, colocando el detector al final del capilar por lo que sería detección off- line[210]. Los datos producidos por el detector son procesados por un ordenador dando como resultado un electroferograma que representa la respuesta del detector, en función del tiempo.

Aunque HPLC se mantiene como la técnica de separación dominante para el análisis de compuestos bioactivos, la electroforesis capilar es una alternativa especialmente adecuada para la separación y cuantificación de compuestos cargados de bajo o medio peso molecular. La CE en comparación con HPLC tiene varias ventajas[156]:

- Tiempos de análisis cortos.
- Elevadas eficacias, debido al frente no parabólico.
- Requerimiento de pequeños volúmenes de muestra y reactivos.
- Gran variedad de aplicaciones.
- Facilidad de automatización

La CE es una técnica muy versátil debido a los distintos modos de separación disponibles, de hecho, los diferentes mecanismos de separación empleados hacen que los modos sean complementarios entre sí. Una de las grandes ventajas de esta técnica es que para cambiar de modo puede bastar simplemente con variar la composición de la disolución reguladora empleada, es decir, dos modos electroforéticos pueden ser utilizados en análisis sucesivos sin cambiar el capilar.

[209] Fernández Gutiérrez, A.; Segura Carretero, A. *Electroforesis capilar: aproximación según la técnica de detección*; Universidad de Granada, 2005.
[210] González de Buitrago, J. M. Electroforesis. En *Técnicas y Métodos de Laboratorio Clínico*; Elsevier, 2010, pp 211–217.

Este hecho contrasta con HPLC, donde para cambiar de modo normalmente es necesario cambiar tanto la columna, como la fase móvil[209].

De todos los modos de CE, la electroforesis capilar en zona es el más utilizado para la separación de compuestos bioactivos en vegetales, particularmente compuestos fenólicos[156].

La **electroforesis capilar en zona** (CZE) fue la primera modalidad que se desarrolló, a partir de los estudios de Jorgenson y Luckas[211]. El principio de la separación en esta técnica, se basa en las diferencias que existen en la relación carga/masa (*m/z*) de los diferentes componentes de una muestra, estas diferencias derivan en movilidades electroforéticas diferentes y, por lo tanto, en distintas velocidades de migración de las especies iónicas presentes en el medio electroforético que contiene el capilar. En base a esto, las especies neutras no podrán ser separadas permitiéndose solamente la separación de mezclas de aniones y cationes. Como se explicó anteriormente, la muestra es introducida desde el ánodo y el detector está al lado del cátodo. Por tanto, el orden de elución, como se muestra en la siguiente figura (figura 27), será: cationes, sustancias neutras y aniones[212].

[211] Jorgenson, J. W.; Lukacs, K. D. Free-zone electrophoresis in glass capillaries. *Clin. Chem.* **1981**, *27* (9), 1551–1553.

[212] Morzunova, T. G. Structure of chemical compounds, methods of analysis and process control. Capillary electrophoresis in pharmaceutical. *Pharm. Chem. J.* **2006**, *40* (3), 158–170.

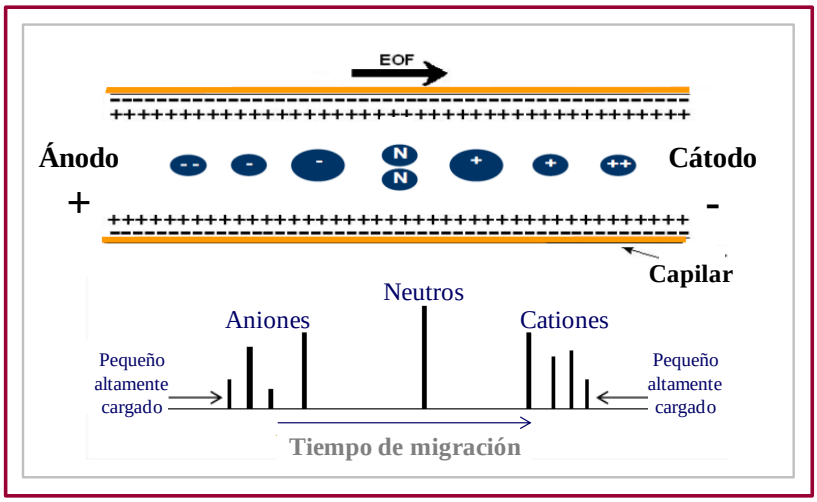


Figura 27. Representación esquemática de una separación y de un electroferograma por CZE

El orden de elución, dado por la relación *m/z*, se debe a que los cationes se mueven a través del capilar en la misma dirección que el flujo electrosmótico (EOF) pero más rápido que éste, las moléculas neutras, se moverán empujadas sólo por el EOF, eluyendo después de los cationes, pero sin separarse. Por último, los aniones, al poseer carga negativa, se moverán hacia el ánodo en sentido opuesto al EOF, pero generalmente éste es mayor que las velocidades electroforéticas de los mismos, por lo que éstos se desplazan hacia el cátodo lentamente, y eluyen en último lugar.

La resolución entre picos y el tiempo de separación van a depender de las dimensiones del capilar, así como del voltaje seleccionado y de la temperatura. Para evitar el efecto Joule que provocaría un solapamiento entre picos lo ideal es usar capilares de diámetro interno de 50-75 µm, un voltaje máximo de 30 kV, y una temperatura de entre 10 y 50 ºC. Otros parámetros como el tiempo de inyección y las características del medio electroforético (pH, fuerza iónica, capacidad reguladora) también van a influir en la eficacia, resolución y sensibilidad de la separación. En CZE a menudo se añaden modificadores al

tampón, tales como disolventes orgánicos para aumentar la selectividad y la resolución[213].

Desde su inicio, la CZE ha demostrado su versatilidad permitiendo el desarrollo de distintos métodos para la separación de antioxidantes fenólicos en distintas matrices vegetales. Uno de los primeros trabajos con esta técnica, fue el descrito por Kulomaa *et al.*, donde se estudiaron diferentes bebidas derivadas de plantas permitiendo la identificación de catequinas, flavonoides y ácidos fenólicos, entre otros compuestos antioxidantes, en muestras de vino[214]. Un método más reciente para el estudio de esta misma matriz, es el desarrollado para la separación de las antocianinas, donde se relaciona el contenido de estos compuestos con el envejecimiento del vino tinto[215]. Otro estudio sobre antocianinas en fruta, mediante esta técnica, fue descrito por Da Costa *et al.*, en muestras de grosella negra, donde se observa que la resolución y las formas de pico de las antocianinas están críticamente influenciados por las características del tampón de separación. En este estudio, la separación óptima fue lograda en un capilar de sílice fundida con tampón fosfato/acetonitrilo al 30% (v/v) a pH 1,5[216]. La composición fenólica en la piel extractos de tomate, ajo, berenjena y patata mediante CZE también fue realizado con esta técnica, donde se observa que mediante un detector electroquímico se ofrece una selectividad y sensibilidad superiores ya que sólo pueden ser detectados analitos electroactivos[81].

[213] Watzig, H. Validation of analytical methods using capillary electrophoresis. In *Separation Science and Technology;* Ahuja, S.; Jimidar, M. I.Elsevier, 2008, Vol 9, pp 225–244.
[214] Kulomaa, A.; Sirrn, H.; Riekkola, M. L. Identification of antioxidative compounds in plant beverages by capillary electrophoresis with the marker index technique. *J. Chromatogr. A* **1997**, *781*, 523–532.
[215] Sáenz-López, R.; Fernández-Zurbano, P.; Tena, M. T. Analysis of aged red wine pigments by capillary zone electrophoresis. *J. Chromatogr. A* **2004**, *1052*, 191–197.
[216] Da Costa, C. T.; Horton, D.; Margolis, S. A. Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography-mass spectrometry and capillary electrophoresis. *J. Chromatogr. A* **2000**, *881*, 403–410.

Uno de los trabajos de búsqueda bibliográfica más completos sobre el uso de electroforesis capilar para la determinación de compuestos fenólicos en matrices vegetales es el publicado por Hurtado-Fernández *et al.* 2010[217].

A continuación, en la tabla 9 se resumen algunos de los estudios de los últimos años realizados con esta técnica en muestras vegetales.

[217] Hurtado-Fernández, E.; Gómez-Romero, M.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material. *J. Pharm. Biomed. Anal.* **2010**, *53*, 1130–1160.

Tabla 9. Algunos estudios publicados en los últimos años sobre análisis de compuestos fenólicos en matrices vegetales mediante CE

Muestra	Familia de compuestos fenólicos identificados	Técnica analítica	Voltaje (KV)	Capilar	Buffer de separación	pH	Ref.
Bayas	Ácidos fenólicos y flavonoides	CE-UV	20	Sílice fundida (39 cm x 50 μm)	Tetraborato sódico	9.3	218
Piel de uva	Ácidos fenólicos y flavonoides	CE-ED	12	Sílice fundida (75 cm x 25 μm, 360 μm)	Borato	9	219
Semillas de guaraná	Proantocianidinas	CE-UV	18	Sílice fundida (40 cm x 75 μm, 375 μm)	Borato + (2-hidroxipropil)-γ-CD	8.5	220
Tomate, pimiento, berenjena y patata	Ácidos fenólicos y flavonoides	CE-UV	25	Sílice fundida (50 cm x 75 μm)	Tetraborato sódico	8.5	81
Piel de naranja	Flavonoides	CE-IT-MS	25	Sílice fundida (100 cm x 10 μm, 50 μm)	Ácido bórico	9.5	221
Té	Flavonoides	CE-AD	15	Sílice fundida (60 cm x 25 μm, 360 μm)	Fosfato sódico dihidrogenado + tetraborato sódico	7.6	222

[218] Ehala, S.; Vaher, M.; Kaljurand, M. Characterization of phenolic profiles of Northern European berries by capillary electrophoresis and determination of their antioxidant activity. *J. Agric. Food Chem.* **2005**, *53*, 6484–6490.

[219] Wu, T.; Guan, Y.; Ye, J. Determination of flavonoids and ascorbic acid in grapefruit peel and juice by capillary electrophoresis with electrochemical detection. *Food Chem.* **2007**, *100*, 1573–1579.

[220] Kofink, M.; Papagiannopoulos, M.; Galensa, R. Enantioseparation of catechin and epicatechin in plant food by chiral capillary electrophoresis. *Eur. Food Res. Technol.* **2007**, *225*, 569–577.

[221] Sawalha, S. M. S.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Quantification of main phenolic compounds in sweet and bitter orange peel using CE–MS/MS. *Food Chem.* **2009**, *116*, 567–574.

[222] Chi, L.; Li, Z.; Dong, S.; He, P.; Wang, Q.; Fang, Y. Simultaneous determination of flavonoids and phenolic acids in Chinese herbal tea by beta-cyclodextrin based capillary zone electrophoresis. *Microchim. Acta* **2009**, *167*, 179–185.

Muestra	Familia de compuestos fenólicos identificados	Técnica analítica	Voltaje (KV)	Capilar	Buffer de separación	pH	Ref.
Vino	Flavonoides	CE-UV	25	Sílice fundida (40 cm x 50 μm)	Borato	9	223
Aceites vegetales	Ácidos fenólicos	CE-UV	30	Sílice fundida (40 cm x 50 μm)	Tetraborato sódico	9.15	224
Aguacate	Ácidos fenólicos	CE-UV	30	Sílice fundida (50 cm x 50 μm)	Tetraborato sódico	9.4	225
Frutas y zumos de frutas	Proantocianidinas	CE-UV	25	Sílice fundida (50 cm x 75 μm)	Tetraborato sódico	9.5	226

[223] Nicolaou, I. N.; Kapnissi-Christodoulou, C. P. Analysis of polyphenols using capillary zone electrophoresis - Determination of the most effective wine sample pre-treatment method. *Electrophoresis* **2010**, *31*, 3895–3902.

[224] Bakar, N. B. A.; Makahleh, A.; Saad, B. In vial liquid–liquid microextraction-capillary electrophoresis method for the determination of phenolic acids in vegetable oils. *Anal. Chim. Acta* **2012**, *742*, 59–66.

[225] Hurtado-Fernández, E.; Contreras-Gutiérrez, P. K.; Cuadros-Rodríguez, L.; Carrasco-Pancorbo, A.; Fernández-Gutiérrez, A. Merging a sensitive capillary electrophoresis–ultraviolet detection method with chemometric exploratory data analysis for the determination of phenolic acids and subsequent characterization of avocado fruit. *Food Chem.* **2013**, *141*, 3492–3503.

[226] Navarro, M.; Núñez, O.; Saurina, J.; Hernández-Cassou, S.; Puignou, L. Characterization of fruit products by Capillary Zone Electrophoresis and Liquid Chromatography using the compositional profiles of polyphenols: Application to Authentication of Natural Extracts. *J. Agric. Food Chem.* **2014**, *62*, 1038–1046.

7. SISTEMAS DE DETECCIÓN

Una vez finalizada la separación de los componentes de la muestra, por algunas de las técnicas comentadas anteriormente, es requerida la presencia de un detector, que produzca una señal medible cuando los analitos eluyan de la columna cromatográfica o capilar, permitiendo así la caracterización y cuantificación de los mismos. Generalmente, las condiciones que debe cumplir un detector son las siguientes[155,227]:

- Presentar una sensibilidad adecuada para los compuestos de interés.
- Proporcionar límites de detección bajos. Para ello es necesaria la combinación de una alta sensibilidad con una baja fluctuación en la señal de fondo. En ausencia de analito, la señal debería ser nula, sin embargo esto no es instrumentalmente factible. Para que la señal de fondo no perturbe la señal analítica conviene que ésta sea lo más pequeña y constante posible.
- La respuesta del detector ante un pequeño cambio en la concentración de analito debe ser rápida.
- Las señales proporcionadas deben ser reproducibles y estables en el tiempo, es decir, que den lugar a resultados fiables.
- Poseer un amplio intervalo de respuesta lineal, es decir, que el intervalo de concentración de analito dentro del cual el detector responde con cambios de señal sea lo más amplio posible.
- Ser selectivo cuando el análisis lo requiera.
- La presencia del detector no debe perjudicar a la eficacia de la separación.

No existe un detector que reúna todas estas características, sin embargo, todas las condiciones expuestas anteriormente influyen, en gran medida, a la hora de seleccionar el sistema de detección que se acopla a cualquier técnica separativa. A su vez, a la hora de elegir un detector también hay que tener en cuenta la

[227] Scott, R. P. W. Performance Criteria of Liquid Chromatography. Detectors. In *Journal of Chromatography Library*; Elsevier,1986, pp 7–48.

naturaleza y propiedades de los compuestos que se quieren determinar y qué tipo de información se desea obtener (estructural, cualitativa o cuantitativa).

Los sistemas de detección se clasifican en tres categorías: detectores ópticos, electroquímicos y "otros", la siguiente figura muestra los ejemplos más representativos de cada categoría.

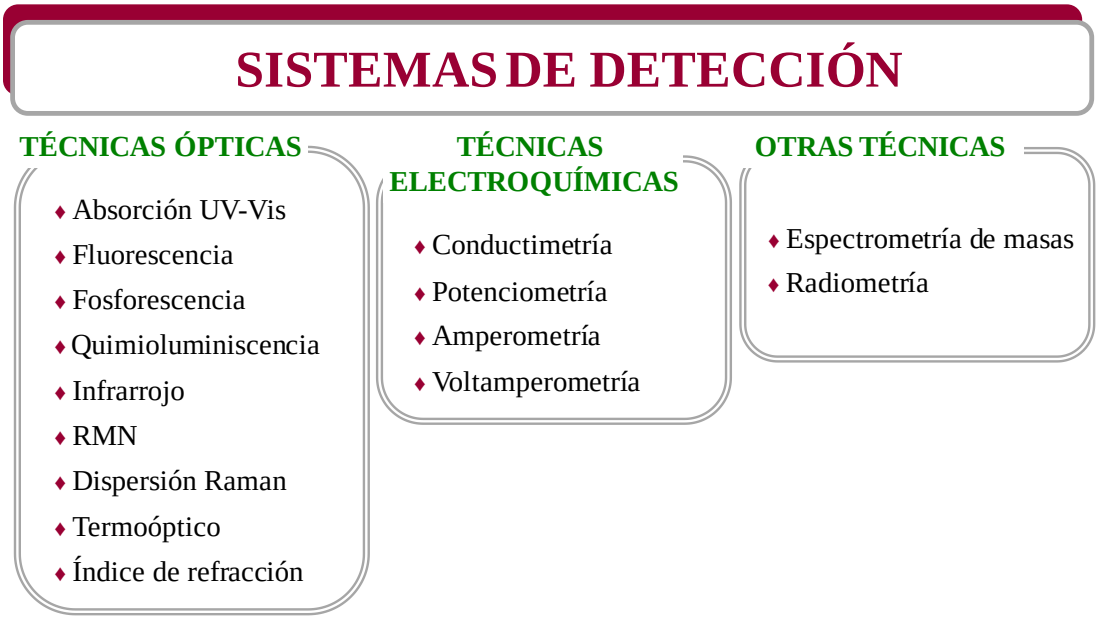


Figura 28. Principales sistemas de detección

En los distintos trabajos experimentales que se recogen en la presente memoria, se ha empleado la detección espectrofotométrica ultravioleta-visible (UV-Vis) acoplada a electroforesis capilar y cromatografía líquida; fluorescencia, detector evaporativo de dispersión de luz (ELSD), acoplados a cromatografía líquida; e ionización de llama (FID) con cromatografía de gases. También, se ha utilizado la espectrometría de masas trabajando con analizadores de masas como, cuadrupolo (Q) acoplado a cromatografía de gases; tiempo de vuelo (TOF) acoplado a electroforesis capilar y cromatografía líquida; y cuadrupolo-tiempo de vuelo (Q-TOF) acoplado a cromatografía líquida.

7.1. Detector de espectroscopia de absorción UV-Vis

La detección mediante espectroscopía de absorción UV-Vis, se basa en la interacción entre la radiación UV-Vis (zona del espectro electromagnético comprendida entre 200 y 800 nm, aprox.) y la materia, lo que origina la transición de electrones (excitación) a causa de la absorción de determinadas longitudes de onda de dicha radiación por parte de las moléculas de analito. Este detector podría ser catalogado como el "detector universal" debido a la gran cantidad de moléculas que absorben en esa región del espectro electromagnético. Además, la detección UV-Vis es muy robusta lo que lo convierte en una herramienta muy útil para análisis cuantitativos. Sin embargo, su sensibilidad es menor que la de otros detectores y presenta el inconveniente de no proporcionar información estructural, por lo tanto, no permite identificar de forma inequívoca compuestos si no se dispone de patrones comerciales, como es el caso de algunos compuestos fenólicos. Aún así, su sencillez, rapidez y relativo bajo coste hacen que su uso esté ampliamente extendido[148].

Los detectores de absorción UV-Vis pueden ser de tres tipos: (a) de longitud de onda fija, (b) de longitud de onda variable o (c) diodo array. El **detector de diodo array** (DAD) es el más moderno y potente de los tres ya que permite obtener los espectros completos de absorción UV-Vis de muestras que pasan a través de la celda de detección. Este detector consiste en la conducción de la luz mediante un sistema de diodos alineados en un chip de circuitos integrados que se ponen en el plano de imagen de un espectrómetro para dejar que un rango de longitudes de onda se detecte simultáneamente[228] (ver figura 29).

[228] Poole, C. F. Instrumental Aspects of Liquid Chromatography. In *The Essence of Chromatography*; Elsevier, 2003, pp 431–497.

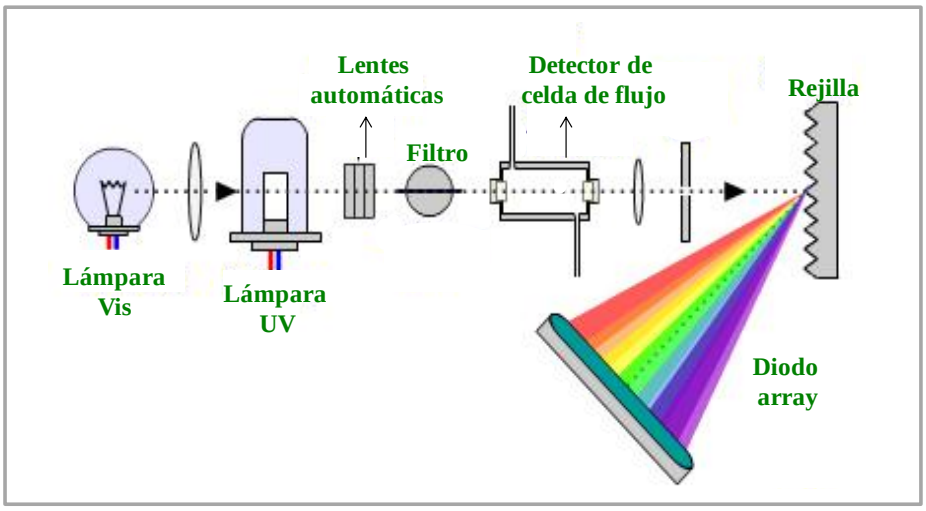


Figura 29. Representación esquemática de un DAD

Esta técnica de detección es el sistema estándar para la determinación de compuestos fenólicos ya que los múltiples enlaces conjugados presentes en los mismos, los convierten en cromóforos que presentan bandas de absorción en la región UV e incluso en el visible como es el caso de las antocianidinas y algunos flavonoles[229,230]. En general, como se puede ver en la tabla 10, los compuestos fenólicos presentan un espectro con dos bandas de absorción comunes, una a 300-380 nm y otra a 240-280 nm, a parte de las bandas de absorción características de cada familia.

[229] Harnly, J. M.; Bhagwat, S.; Lin, L. Z. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal. Bioanal. Chem.* **2007**, *389*, 47–61.
[230] Bernal, J.; Mendiola, J. A.; Ibáñez, E.; Cifuentes, A. Advanced analysis of nutraceuticals. *J. Pharm. Biomed. Anal.* **2011**, *55*, 758–774.

Tabla 10. Bandas de absorción características de diversas familias de compuestos

Compuestos	Bandas UV (nm)
Acidos benzoicos	270-280
Acidos cinamicos	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	250-270/350-380
Flavanonas	270-295
Flavanoles	270-280
Antocianidinas	240-280/450-560
Isoflavonas	245-270/300-340
Proantocianidinas	≈280

Esta información, es de gran ayuda en análisis cualitativos para acotar la búsqueda del compuesto, al proporcionar información sobre la familia de compuestos fenólicos a la que podría pertenecer.

7.2. Detector de fluorescencia

El detector de fluorescencia, al igual que el anterior está basado en la interacción entre la radiación y la materia. En este caso, compuestos que poseen ciertos grupos funcionales se excitan mediante la absorción de energía de ciertas longitudes de onda y posteriormente se relajan emitiendo una radiación con una longitud de onda mayor que la absorbida. La detección por fluorescencia de grupos químicos específicos va a ser función de las longitudes de onda de excitación y emisión seleccionadas. La radiación emitida se mide

perpendicularmente a la luz incidente responsable de la excitación, con objeto de evitar interferencias. Los detectores modernos, pueden ser programados para cambiar las longitudes de onda de excitación y emisión durante la separación, con lo que se consigue mantener una selectividad y sensibilidad óptimas en todo el cromatograma[148,228].

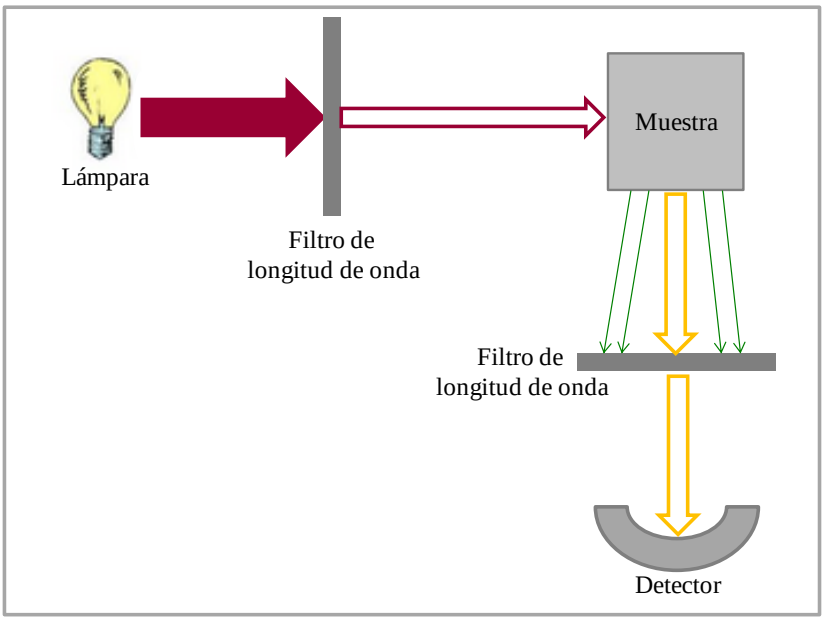


Figura 30. Representación esquemática de un detector de fluorescencia

La fluorescencia es un fenómeno que ocurre a intensidades elevadas, por lo que ofrece más sensibilidad, y permite la detección a niveles de trazas. No obstante, este sistema de detección puede ser usado para la detección de compuestos no fluorescentes, tras la realización de una reacción de derivatización.

Los detectores de fluorescencia son más selectivos que los de absorbancia y los detectores evaporativos de dispersión de luz (ELSD), además de poseer una especificidad muy alta, lo que es una ventaja para la determinación de especies que presentan fluorescencia en muestras complejas como son las muestras de origen vegetal. Por este motivo, estos detectores, acoplados a HPLC son los más utilizados para la determinación de tocoferoles en vegetales[193,195196,198].

7.3. Detector evaporativo de dispersión de luz

El sistema de detección ELSD se basa en la diferencia de volatilidad entre los eluyentes y la muestra. En un primer paso, se produce nebulización de los compuestos que eluyen de la columna junto con la fase móvil, mediante un gas (normalmente N_2). Posteriormente, se produce el secado de las gotas de nebulización en una cámara a alta temperatura, evaporándose así el disolvente y registrándose, en continuo, la luz dispersada por las partículas sólidas (no volátiles) residuales. La señal obtenida se amplifica mediante un tubo fotomultiplicador o fotodiodo colocado a 90-120º de la dirección de la luz[231,232].

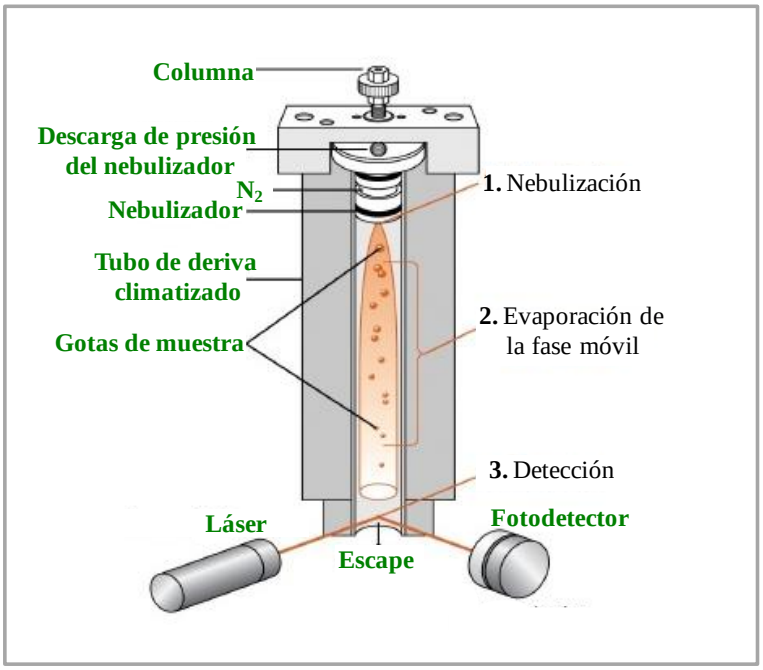


Figura 31. Representación esquemática de un ELSD y su funcionamiento

Este detector es sensible a todos los compuestos sólidos y a los que son menos volátiles que la fase móvil, por lo tanto, su respuesta dependerá de la

[231] Dreux, M.; Lafosse, M. Evaporative Light Scattering Detection of carbohydrates in HPLC. In *Carbohydrate analysis: high performance liquid chromatography and capillary* electrophoresis; El Rassi, Z.; Elsevier, 1995, pp 515–540.

[232] Lafosse, M.; Herbreteau, B. Carbohydrate analysis by LC and SFC using evaporative light scattering detection. In *Journal of Chromatography Library*; El Rassi, Z.; Elsevier, 2002; Vol. 66, pp 1101–1134.

composición de la fase móvil y de la cantidad de analito, siendo independiente de la estructura de la molécula. La reproducibilidad va a depender de que el flujo del gas nebulizador y la temperatura de evaporación sean constantes.

Éste es un modo de detección relativamente barato alternativo a los detectores de absorción, en el caso de que los compuestos de interés no absorban en el UV-Vis[231,232]. En base a esto, la mayor parte de las aplicaciones de ELSD han sido desarrolladas sobre todo para la detección de compuestos lipídicos en diversas matrices entre las que cabe destacar: alimentos, bebidas y productos naturales[233–236].

En los últimos años, el detector ELSD acoplado a HPLC, ha representado una alternativa útil en el análisis de fosfolípidos en alimentos entre los que se incluyen productos naturales. El uso de este detector permite el análisis de estos compuestos sin la necesidad de una reacción de derivatización previa, a su vez, permite trabajar con una amplia gama de disolventes, así como con elución en gradiente. Además, como se ha comentado anteriormente la señal es independiente del grado de saturación y de la longitud de la cadena. Por otra parte, HPLC-ELSD permite la cuantificación de las diferentes clases de fosfolípidos, sin embargo, para la determinación de los ácidos grasos de los fosfolípidos se suele emplear el detector de espectrometría de masas por su alta especificidad y poder de separación[236].

[233] Christie, W. W. Detectors for high-performance liquid chromatography of lipids with special reference to evaporative light-scattering detection. *Adv. Lipid Methodol.* **1992**, 239–271.
[234] Chase, G. W.; Akoh, C. C.; Eitenmiller, R. R. Analysis of tocopherols in vegetable oils by high-performance liquid chromatography: Comparison of fluorescence and evaporative light-scattering detection. *J. Am. Oil Chem. Soc.* **1994**, *71* (8), 877–880.
[235] Bravi, E.; Perretti, G.; Montanari, L. Fatty acids by high-performance liquid chromatography and evaporative light-scattering detector. *J. Chromatogr. A* **2006**, *1134*, 210–214.
[236] Restuccia, D.; Spizzirri, U. G.; Puoci, F.; Cirillo, G.; Vinci, G.; Picci, N. Determination of phospholipids in food samples. *Food Rev. Int.* **2012**, *28*, 1–46.

7.4. Detector de ionización de llama

El detector de ionización de llama (FID) es quizás, el detector más ampliamente utilizado en GC para el análisis de compuestos lipídicos. Consiste en un quemador donde el gas procedente de la columna (gas portador y analito) llega a la llama producida por una mezcla de hidrógeno y aire. La mayoría de los compuestos orgánicos, cuando se pirolizan a altas temperaturas, producen iones y electrones que al ser conductores eléctricos facilitan el paso de la corriente eléctrica a través de la llama. Entre el extremo del quemador y un electrodo colector situado por encima de la llama, se aplica una diferencia de potencial, de unos centenares de voltios, la corriente generada se utiliza como señal de detección, pero al ser demasiado baja, es aumentada mediante un amplificador operacional de alta impedancia antes de ser registrada[154,228].

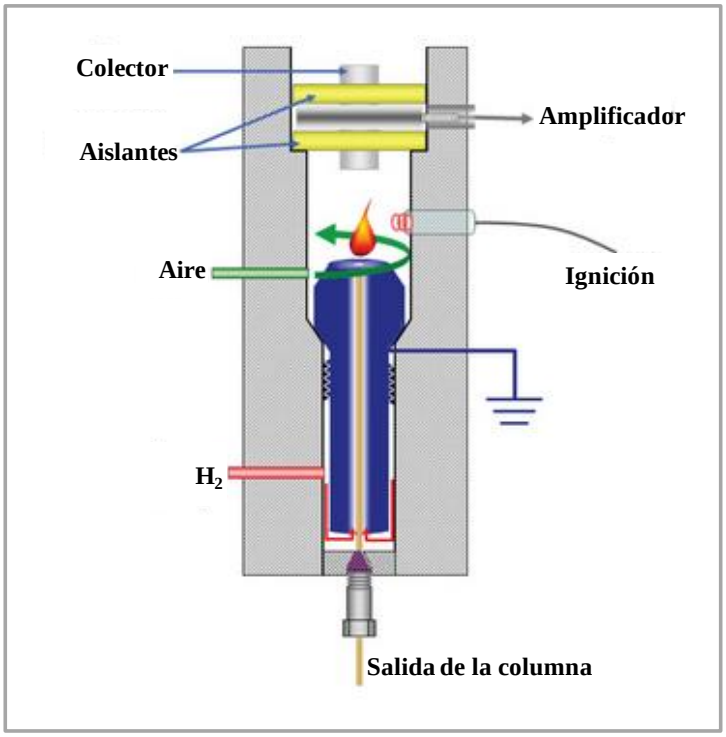


Figura 32. Esquema de un detector FID

En la práctica, a este detector, se le puede considerar de respuesta universal ya que por su alta selectividad hacia compuestos que tienen enlaces C-H en su estructura, son muy pocos los compuestos que no dan señal en él. Presenta poca

sensibilidad a grupos como carbonilo, aminas, alcoholes y halógenos y es totalmente insensible a gases no combustibles como H_2O, CO_2, SO_2 y óxidos de nitrógeno. Este hecho, más que limitar el ámbito de aplicación de este detector, lo hace más selectivo permitiendo el análisis de muestras contaminadas con alguno de los compuestos mencionados. Posee una buena sensibilidad, amplio intervalo de respuesta lineal, excelente estabilidad de la línea de base, robustez y sencillez. También son muy fiables, con un volumen muerto insignificante y un tiempo de respuesta rápido. Estos detectores se pueden utilizar con todos los gases portadores y son fáciles de calibrar[237].

Para el análisis de ácidos grasos el detector FID junto con MS, es el más ampliamente utilizado (ver tabla 6), de hecho en cuanto a las técnicas analíticas más utilizadas en la determinación de estos compuestos, un gran porcentaje de los trabajos utiliza GC-FID y en menor medida GC-MS[160,230].

7.5. Detector de espectrometría de masas

Desde la construcción del primer espectrómetro de masas en 1912, el desarrollo de esta técnica ha sido continuo, hasta llegar a convertirse en una de las herramientas más potentes para aplicaciones tanto cuantitativas como cualitativas debido a su elevada selectividad y a la información estructural que proporciona. Actualmente, esta técnica puede ser usada en el estudio de cualquier molécula, prácticamente sin limitaciones de masa[238].

La base de esta técnica es la ionización de moléculas en fase gas, la aceleración de las mismas en un campo eléctrico y posterior separación, en función de su *m/z*, es requisito indispensable que los iones estén en fase gas antes de que puedan ser separados y posteriormente detectados. A veces, el proceso de ionización suministra suficiente energía para que las moléculas se rompan en

[237] Martínez-Castro I., Sanz J., D. M. V. Gas chromatography. *J. Inorg. Nucl. Chem.* **2003**, *25* (3), 329–330.

[238] 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*, 210–230.

diversos fragmentos[239]. La corriente total de todos los iones generados y las masas de éstos es registrada dando lugar a un cromatograma que representa la corriente iónica total frente al tiempo de elución, es el denominado TIC (Total Ion Current o Total Ion Chromatogram). También se puede hacer un seguimiento de un ión seleccionado, si se busca un compuesto o una clase de compuestos determinados (EIC o EIE, Extract Ion Chromatogram o Extract Ion Electropherogram).

Existen diversos tipos de espectrómetros de masas, pero todos incluyen los siguientes elementos básicos: una fuente de ionización, un acelerador de iones mediante un campo eléctrico, un analizador que separe los iones de acuerdo a su *m/z* y un detector de los iones junto con un procesador adecuado de la señal (Figura 33). El acelerador de iones, el analizador y el detector deben estar a una presión inferior a 10^{-4} - 10^{-8} Torr para evitar colisiones entre los iones de interés y otras sustancias como radicales o moléculas neutras[239,240].

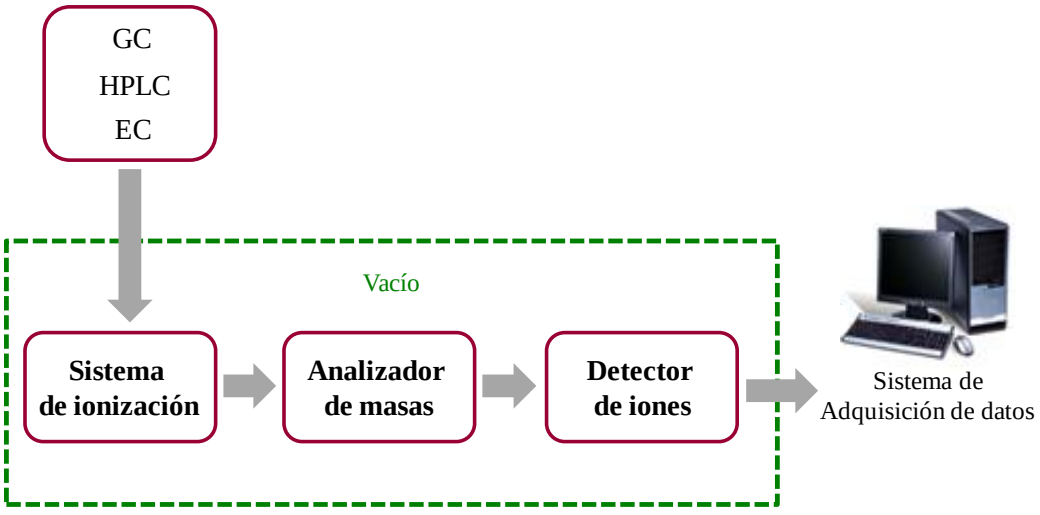


Figura 33. Representación esquemática de las partes de un MS

[239] J. Throck Watson, O. D. S. Introduction to Mass Spectrometry. **2007**, 1–44.
[240] Somogyi, A. Mass spectrometry instrumentation and techniques. In *Medical Applications of Mass Spectrometry*. Vékey, K.; Telekes, A.; Vertes, A.; Elsevier, 2008, pp 93–140.

La MS se puede utilizar directamente sin necesidad de una técnica separativa precedente (experimentos de infusión directa)[240]. No obstante, el hecho de que este sistema de detección se acople a una técnica separativa presenta la ventaja de que proporciona una segunda dimensión de separación ya que tras separar los compuestos según su tiempo de migración o retención, se produce en el espectrómetro de masas una separación en función de la *m/z*. Mediante el acoplamiento, se combinan la rapidez del análisis, el alto poder de resolución, y un consumo pequeño de muestra, proporcionados por las técnicas separativas, con la selectividad del análisis y la información estructural que proporciona la MS[241,242].

Para el análisis de compuestos fenólicos, normalmente se utiliza una sola etapa de MS en combinación con la detección UV-Vis con el fin de confirmar la identidad de los mismos, con la ayuda de datos de referencia como son la masa del compuesto y la longitud de onda a la que absorbe (tabla 10). Cuando se persigue la identificación de compuestos desconocidos, lo ideal es usar la espectrometría de masas en tándem (MS/MS) debido su mayor selectividad y a que la información obtenida es de mayor alcance, la detección MS/MS también suele usarse junto con la UV-Vis[157,242].

El mayor problema que surge a la hora de acoplar las diferentes técnicas separativas con MS está relacionado con la forma de introducir el volumen de muestra líquido procedente del cromatógrafo en la fuente de ionización, ya que las sustancias para ser analizadas deben estar en fase gaseosa. Para ello, existen diversos tipos de interfases que hacen de unión entre el equipo de separación y el detector, siendo éste el compartimento donde se producen las especies cargadas.

[241] Tomer, K. B. Separations combined with mass spectrometry. *Chem. Rev.* **2001**, *101*, 297–328.

[242] Liu, E. H.; Qi, L. W.; Cao, J.; Li, P.; Li, C. Y.; Peng, Y. B. Advances of modern chromatographic and electrophoretic methods in separation and analysis of flavonoids. *Molecules* **2008**, *13*, 2521–2544.

La clasificación de los diferentes tipos de sistemas de ionización se realiza en función del grado de fragmentación que provoquen en la estructura del compuesto a estudiar, éstos pueden ser: sistemas de ionización fuertes, como impacto electrónico (EI) y otros más suaves entre los que se pueden encontrar la ionización por electrospray (ESI), ionización química a presión atmosférica (APCI), fotoionización a presión atmosférica (APPI), ionización química (CI), desorción/ionización láser asistida por matriz (MALDI), entre otras. La característica común a todas las técnicas de ionización es que se basan en la transferencia de cierta energía al analito[240,243].

El analizador de masas es la parte del instrumento donde se produce la discriminación de los iones en función de su *m/z* al ser sometidos a campos eléctricos y/o magnéticos constantes. Los analizadores más comunes son: cuadrupolo (Q), trampa de iones (IT), triple cuadrupolo (QQQ), tiempo de vuelo (TOF), transformada de fourier-resonancia de ión ciclotrónica (FT-ICR) y orbitrap (OT), también existen acoplamientos entre varios de los mencionados[240]. La diferencia entre ellos reside en la exactitud al determinar la masa molecular de los analitos (error entre la masa exacta determinada y el valor teórico), la capacidad o incapacidad para determinar distribuciones isotópicas, el poder hacer MS/MS (de gran utilidad para determinar estructuras químicas), la velocidad de barrido, el rango de masas en el que pueden medir, y la resolución (el valor de la masas dividido entre la diferencia de masa entre dos iones muy próximos)[244].

A modo de resumen, en la siguiente figura se muestran los sistemas de ionización y analizadores más usuales en el acoplamiento de MS con las diferentes técnicas analíticas de separación.

[243] Gelpí, E. Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments. *J. Mass Spectrom.* **2002**, *37*, 241–253.
[244] Marshall, A. G.; Hendrickson, C. L. High-resolution mass spectrometers. *Annu. Rev. Anal. Chem.* **2008**, *1*, 579–599.

Figura 34. Sistemas de ionización y analizadores de masas más utilizados
(■ Utilizados en la realización de la tesis)

A continuación, se detallan las características de los sistemas de ionización y los analizadores empleados en el desarrollo de la parte experimental de esta tesis doctoral. Para los acoplamientos HPLC-MS y EC-MS se han utilizado la ionización por electrospray, mientras que para GC-MS se usó la ionización por impacto electrónico. En cuanto a los analizadores de masas, éstos han sido, Q, TOF y Q-TOF.

7.5.1. Sistemas de ionización

► ***Ionización por electrospray***

Esta técnica de ionización ha revolucionado el campo de los acoplamientos entre técnicas analíticas de separación que trabajan en fase líquida y MS. Es utilizada, normalmente, para el análisis de moléculas polares, no volátiles y termolábiles, de hecho, es la más común en el análisis de compuestos fenólicos[230].

En el proceso de formación del electrospray, llevado a cabo a presión atmosférica, intervienen diversos mecanismos al mismo tiempo. La muestra en estado líquido, procedente de la columna, pasa a través de un fino capilar de acero inoxidable cuyo extremo se encuentra a un potencial eléctrico elevado (3-6 kV), este potencial unido al pequeño radio de curvatura al final del capilar crean un fuerte campo eléctrico. Gracias a ese campo eléctrico, que produce reacciones de oxidación-reducción, y con la ayuda de un gas nebulizador, normalmente N_2,

que fluye a través de un tubo coaxial al capilar principal, la muestra es dispersada, produciéndose, unas micro-gotas cargadas eléctricamente. Como se observa en la figura 35, las micro-gotas van aumentando su densidad de carga eléctrica a la vez que el disolvente que las acompaña se va evaporando (desolvatación). Como consecuencia, el área superficial de las gotas se reduce y los iones que se encuentran en la superficie se ven forzados a aproximarse entre sí debido al campo electrostático que se aplica entre la salida del capilar y la entrada al equipo MS (±2-5 kV)[228,245]. Llega un momento en que la repulsión de los iones de signo contrario se hace mayor que la tensión superficial que mantiene unidas las micro-gotas, alcanzando el denominado límite de Rayleigh, lo que hace que las gotas se vuelvan inestables y sufran un proceso de "explosión" (explosiones de Coulomb) formándose de nuevo pequeñas gotas cargadas y posterior desolvatación. Este proceso se repite sucesivamente hasta que se forman los iones cargados (con una o más cargas) en fase gaseosa, los cuales, debido al voltaje aplicado, serán atraídos a la entrada del espectrómetro de masas[240,245].

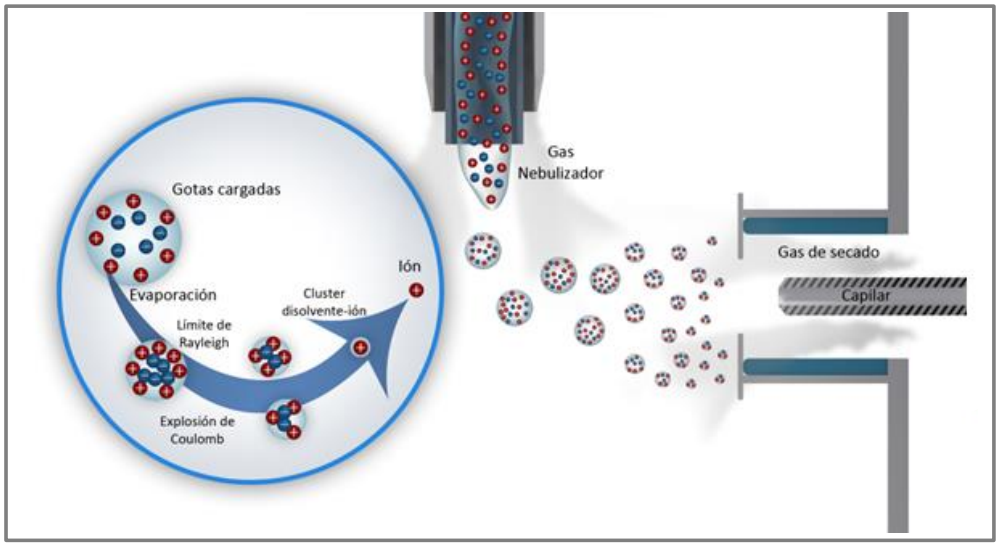


Figura 35. Proceso de formación del electrospray

[245] Crotti, S.; Isak, I.; Traldi, P. Advanced Spectroscopic Detectors for Identification and Quantification: Mass Spectrometry. In *Liquid Chromatography: Fundamentals and Instrumentation;* Fanali, S.; Haddad, P. R.; Poole, C.; Schoenmakers, P. Lloyd, D. K.; Elsevier, 2013, pp 307–331.

El hecho de que en el proceso de ionización se puedan formar iones mono o multicargados, es una de las características más importantes de esta técnica. Un incremento en el número de cargas en una molécula disminuirá el valor de su *m/z*, que como se explicó anteriormente, es el parámetro utilizado para el análisis por MS. De este modo, mediante la detección de iones multicargados, se van a poder detectar compuestos con pesos moleculares muy altos empleando analizadores de masas que trabajan con un intervalo limitado de valores *m/z*.

La ionización se puede llevar a cabo en modo positivo o negativo, aspecto importante para el análisis de polifenoles. En el modo positivo, se podrán formar iones múltiplemente protonados $[M+nH]^{n+}$. Del mismo modo, es posible también la formación de aductos con iones sodio, litio, potasio, amonio, etc. En el modo negativo, se observa normalmente la pérdida de protones de las moléculas, pudiéndose formar también iones múltiplemente desprotonados $[M-nH]^{n-}$ [228,245].

El diseño de la interfase ESI es distinto dependiendo de la técnica separativa a la que se acople. En el acoplamiento HPLC-ESI-MS, el principal problema es el excesivo flujo de fase móvil que eluye de la columna (0,5-3 mL/min) y que no puede introducirse directamente en el espectrómetro de masas. Las interfases ESI empleadas en el acoplamiento HPLC-MS admiten flujos comprendidos entre 1 µL/min y 1 mL/min, sin embargo, el flujo recomendado a la entrada del espectrómetro de masas, depende del analizador y oscila entre 0,2-0,5 mL/min. Para solucionar el problema de los flujos elevados el diseño de la interfase ESI no se modifica, sino que se suelen emplear divisores de flujo entre la salida de la columna cromatográfica y la entrada de la interfase, con el inconveniente de una disminución de la sensibilidad producida por la eliminación de parte de la muestra. Otra solución sería utilizar columnas cromatográficas más estrechas con flujos de 0,2 mL/min, que sí se pueden introducir directamente en el espectrómetro de masas.

►*Impacto electrónico*

El sistema de ionización por impacto electrónico (EI) permite el estudio de moléculas neutras y volátiles, motivo por el cual es uno de los sistemas utilizados en el acoplamiento GC-MS. Esto es debido a que este tipo de acoplamiento requiere sistemas especiales de conexión ya que el efluente que emerge de la columna cromatográfica está a presión atmosférica y debe introducirse en el interior del espectrómetro de masas que trabaja a alto vacío[192].

El fundamento de esta técnica, que se muestra esquemáticamente en la figura 36, consiste en el bombardeo de las moléculas gaseosas que entran en la fuente de ionización, por electrones emitidos mediante un filamento calentado (generalmente tungsteno o renio) y acelerados con un potencial de 70 eV que se aplica entre el filamento y la fuente de iones. El haz de electrones debe estar bien enfocado y debe mantenerse en un margen estrecho de energía. La colisión entre los electrones y las moléculas neutras dan lugar a la ruptura de las mismas originándose iones cargados positivamente. Para que se produzca la fragmentación, la energía media de los electrones debe superar el potencial de ionización de la molécula neutra. Dado que la energía de los electrones (70eV) es mayor que la energía de ionización de las moléculas (la mayoría de los compuestos orgánicos: 7-20eV) no sólo se produce la eliminación de un electrón de la molécula, sino también un ión molecular excitado. El ión molecular es un ión radical ($M^{\bullet+}$) que tiene una relación *m/z* correspondiente a la masa molecular del analito[228,240].

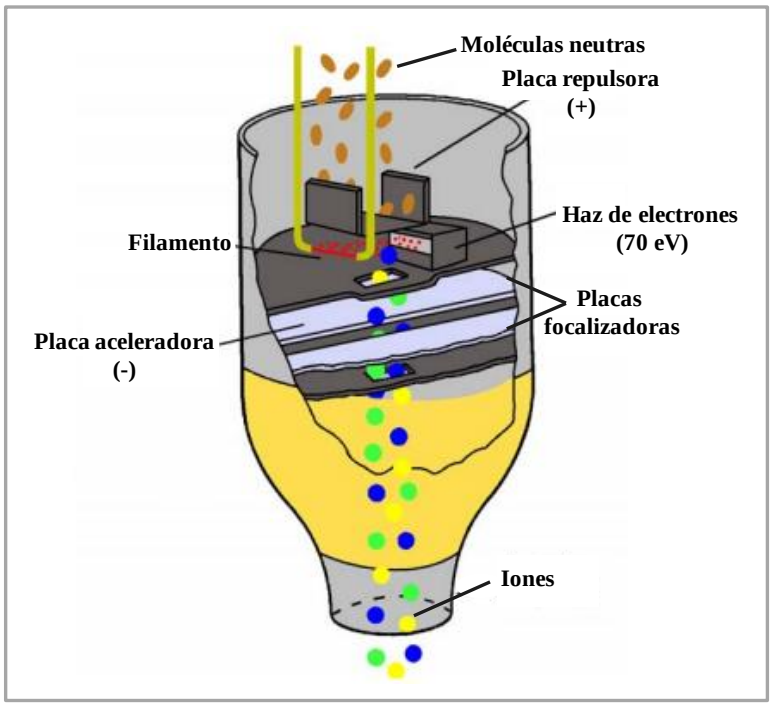


Figura 36. Sistema de ionización EI

La presión con la que el analito entra en la fuente debe ser lo suficientemente baja (menos de 104-105 Torr) para evitar colisiones ion-molécula (es decir, el recorrido libre medio ha de ser mayor que la dimensión de la cámara de ionización). Dependiendo de su estructura y de la cantidad de energía absorbida, los iones pueden atravesar el analizador intactos o fragmentarse generando iones más pequeños. Este proceso de fragmentación tiene lugar en la mayoría de los casos, donde el ion molecular M^+ puede ser poco abundante e incluso no estar presente en el espectro de masas[240].

Normalmente, esta técnica se utiliza para compuestos de bajo peso molecular (1-800 Da) ya que éstos poseen una volatilidad más alta, sin embargo, hay otros compuestos, como hidrocarburos fluorados o algunos complejos con metales de transición, que tienen pesos más altos (1000 Da) y siguen siendo lo suficientemente volátiles para el análisis mediante IE[240].

7.5.2. Analizadores de masas

Una vez que en la interfase se ha llevado a cabo la producción de los iones, éstos son dirigidos hacia el analizador de masas. Los analizadores de masas permiten la detección y cuantificación de los analitos objeto de estudio con un grado de sensibilidad y selectividad bastante elevado, proporcionando información sobre su masa molecular.

A continuación, se exponen los analizadores de masas utilizados en la parte experimental de la presente tesis.

► ***Analizador de Cuadrupolo***

Actualmente, el analizador de cuadrupolo (Q), es posiblemente el analizador más usado, sobre todo en análisis de rutina, ya que proporciona alta sensibilidad, reproducibilidad y precisión para la cuantificación, además de un rango de masas bastante aceptable (40-4000 uma). Este analizador, como su nombre indica, está constituido por cuatro barras (polos) metálicas, paralelas y organizadas simétricamente alrededor de un eje central imaginario (ver figura 37). A las barras opuestas se les aplica una corriente continua de igual signo y opuesto a las otras dos, a la vez que se aplica otro potencial de corriente alterna asociado a una radiofrecuencia. A continuación, los iones son introducidos en el campo cuadrupolar por lo que comienzan a oscilar en un plano perpendicular al cuadrupolo debido a la atracción y repulsión provocada por las barras. De esta forma, la trayectoria que describen los iones va a depender directamente de su *m/z*. Este analizador actúa como un filtro de masas, es decir, en función de los voltajes aplicados al cuadrupolo (continuo y alterno) sólo lo atravesarán iones con una *m/z* determinada, mientras que los demás se desestabilizan y terminan estrellándose contra las barras o contra las paredes aislantes. 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[240].

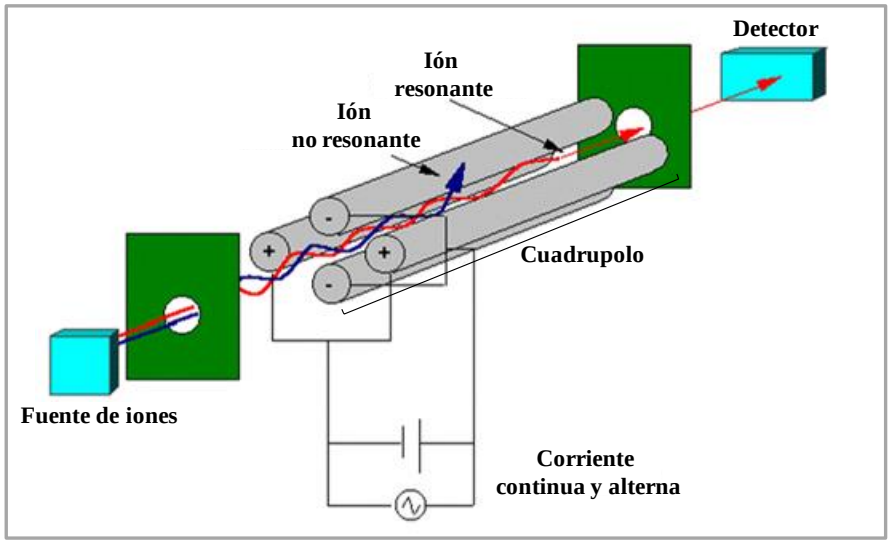


Figura 37. Esquema de un analizador de masas cuadrupolo (Q)

La resolución de un Q depende del número de oscilaciones que describen los iones conforme atraviesan el cuadrupolo. A mayor número de oscilaciones, mayor será la resolución que se alcance, por lo que los iones más pesados serán los que se analizarán con mayor precisión, a expensas de un mayor tiempo de análisis. Este tipo de analizadores trabajan a bajos voltajes y la energía cinética aplicada para hacer que los iones atraviesen el analizador es del orden de algunos eV. Esto elimina los problemas que implica el uso de alto voltaje y facilita su acoplamiento a sistemas separativos[240].

El Q, se utiliza para una gran cantidad de estudios sobre compuestos bioactivos en vegetales. Por ejemplo, Orban *et al.* utilizó este analizador acoplado a GC mediante un sistema de ionización APCI para el análisis de esteroles en muestras de uva[246], Onofrejová *et al.* lo utilizó para el análisis de compuestos fenólicos en algas, esta vez acoplado a HPLC con una fuente de ionización ESI. Los esteroles y tocoferoles de muestras de granada también han sido determinados mediante el uso de este analizador[172].

[246] Orbán, N.; Kozák, I. O.; Drávucz, M.; Kiss, A. LC-MS method development to evaluate major triterpenes in skins and cuticular waxes of grape berries. *Int. J. Food Sci. Technol.* **2009**, *44*, 869–873.

►*Analizador de Tiempo de Vuelo*

La separación de los iones mediante un analizador de tiempo de vuelo (TOF) se basa en la distinta velocidad que éstos adquieren en su interior en función de su *m/z*. Todos los iones generados en la fuente de ionización tienen la misma energía cinética, por lo que su velocidad es inversamente proporcional al cuadrado de su masa.

Una vez que los iones son extraídos de la cámara de nebulización son focalizados hacia el tubo de vuelo donde se aceleran mediante un campo electrostático que les aporta una elevada energía cinética (ver figura 38).

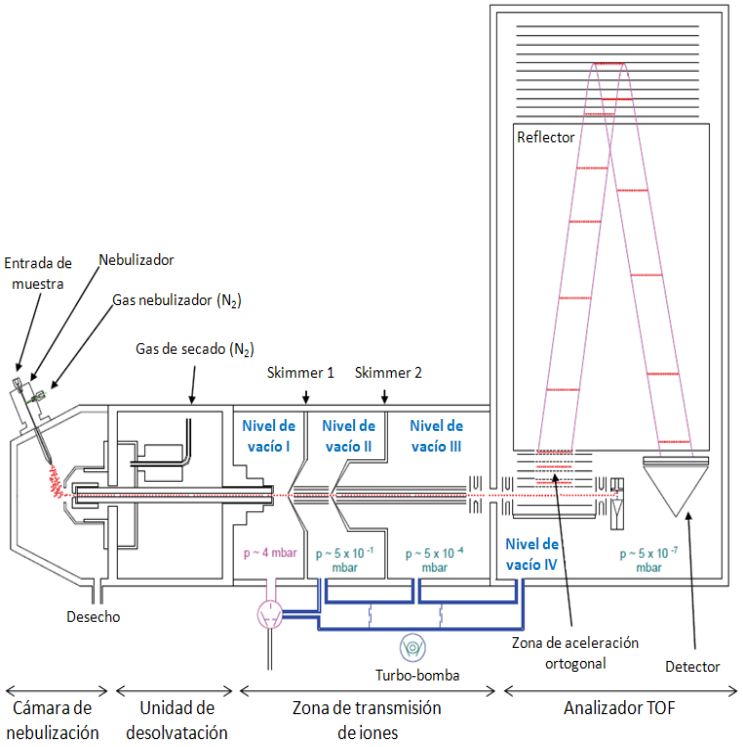


Figura 38. Esquema de las partes de las que consta un espectrómetro de masas TOF

Los iones formados en la cámara de nebulización atraviesan la unidad de desolvatación, que separa las zonas que están a presión atmosférica de la primera zona de alto vacío, y que consta de un calentador del gas de secado y un capilar de vidrio. A través de esta primera unidad se llega a la zona de transmisión o transferencia óptica que consta de tres módulos que están a alto vacío, separados

entre sí por dos skimmers. Los dos hexapolos son los que transfieren los iones hasta la zona de alto vacío, mientras que las lentes enfocan o dirigen dichos iones. A continuación los iones llegan a la siguiente unidad, que es el analizador TOF, éste, a su vez, consta de tres zonas. La primera de ellas es la zona de aceleración ortogonal donde los iones son acelerados hacia el tubo de vuelo aplicando un campo eléctrico intermitente. Los iones "volarán" a lo largo del tubo de vuelo hasta alcanzar la segunda zona, el reflector, que permite corregir la dispersión en la energía cinética de los iones, aumentando de esta forma la resolución. El último componente del analizador TOF es un detector de impacto electrónico que consiste en una serie de placas a alto voltaje que convierten el impacto de los iones en señales eléctricas. En el detector hay millones de pequeños poros internamente recubiertos con una capa semiconductora, de forma que cada uno de ellos trabaja como un multiplicador de electrones independiente.

En definitiva, los iones, que tienen la misma energía cinética pero diferentes valores de *m/z* adquieren distinta velocidad de forma que no todos llegarán al extremo contrario a la vez, es decir, los iones de mayor *m/z* "volarán" a menor velocidad que los de menor *m/z*. La resolución entre los iones de diferente *m/z* será mayor cuanto mayor sea la longitud del tubo (ya que habrá una mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en las energías cinéticas de los iones formados en la fuente.

El analizador TOF ofrece multitud de ventajas, es rápido, sensible, y gracias a su elevada resolución permite obtener valores de masa muy exactos. Posibilita la determinación de la fórmula molecular del compuesto aplicando un método analítico bidimensional que combina la determinación de masas exactas con el análisis de la distribución isotópica, dando óptimos resultados en un rango muy amplio sin requerir tediosos procesos rutinarios de re-calibración. Entre las especificaciones más interesantes del TOF se encuentran, que puede analizar un rango de masas de 50-3.000 *m/z*, y que presenta una resolución de entre 10.000 y 15.000 FWHM. Además, usándolo para cuantificación, presenta una exactitud de 3 ppm con calibración interna y 5 ppm si se realiza una calibración externa.

Para la caracterización de compuestos fenólicos el detector TOF es de gran utilizad, entre otras cosas por el hecho de que éste proporciona valores de masa exacta ya que en algunos casos, no se dispone de patrones comerciales para su identificación. Este detector acoplado a HPLC, con un sistema de ionización ESI, ha sido utilizado en el análisis de los compuestos fenólicos de gran cantidad de matrices vegetales como pepino[200], pimiento[247], judía[248] o tomate[199], entre otros.

► ***Cuadrupolo-tiempo de vuelo***

La diferencia entre el analizador cuadrupolo-tiempo de vuelo (QTOF) y el TOF reside en que se introduce un cuadrupolo donde se pueden seleccionar determinados iones para ser, posteriormente, fragmentados en una celda de colisión con la ayuda de un gas de colisión, normalmente N_2. Los iones fragmentados se separan en el analizador TOF en función de su relación *m/z* de la misma forma que se ha descrito anteriormente. A parte de eso, está formado básicamente por las mismas partes que un analizador TOF, con la diferencia de que se sustituyen los skimmers de la zona de trasmisión de iones por funnels (anillos concéntricos apilados en forma de embudo) que evitan la pérdida de iones durante la transmisión iónica, lo que permite aumentar la sensibilidad.

[247] Morales-Soto, A.; Gómez-Caravaca, A. M.; García-Salas, P.; Segura-Carretero, A.; Fernández-Gutiérrez, A. High-performance liquid chromatography coupled to diode array and electrospray time-of-flight mass spectrometry detectors for a comprehensive characterization of phenolic and other polar compounds in three pepper (*Capsicum annuum* L.) samples. *Food Res. Int.* **2013**, *51*, 977–984.

[248] Abu-Reidah, I. M.; Arráez-Román, D.; Lozano-Sánchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phytochemical Characterisation of Green Beans (*Phaseolus vulgaris* L) by Using High-performance Liquid Chromatography Coupled with Time-of-flight Mass Spectrometry. *Phytochem. Anal.* **2013**, *24*, 105–116.

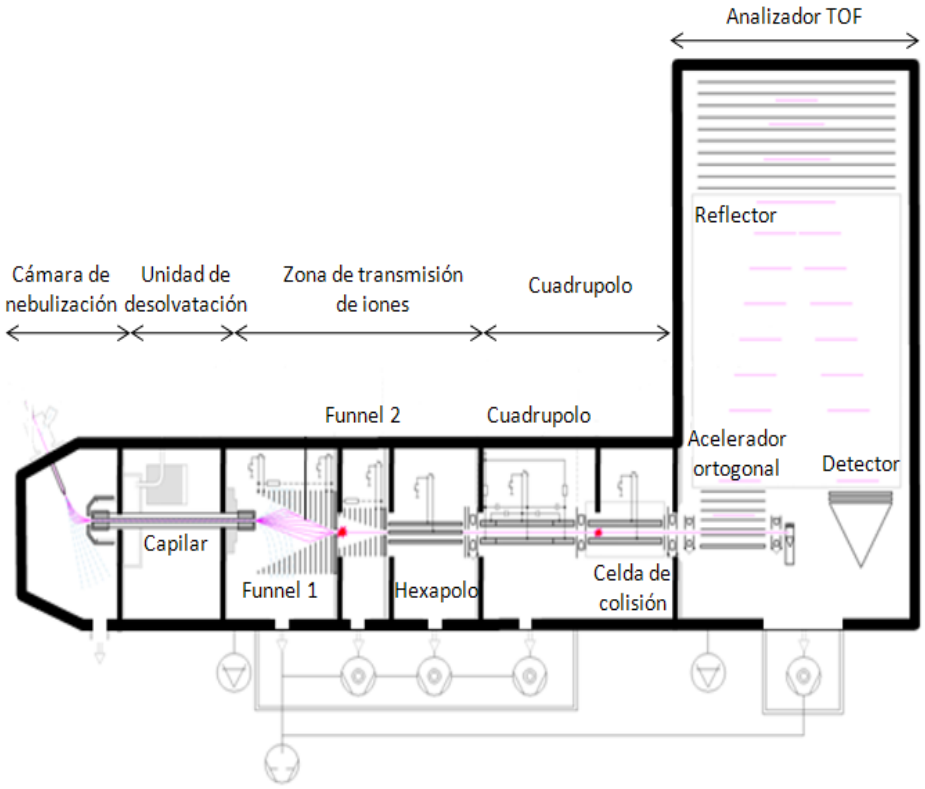


Figura 39. Esquema de las partes de las que consta un espectrómetro de masas QTOF

El analizador QTOF ofrece la posibilidad de realizar análisis de MS/MS, por lo tanto presenta una ventaja adicional que se une a la exactitud de masas y a la distribución isotópica, para llevar a cabo la identificación de compuestos.

En cuanto a las principales especificaciones del QTOF, presenta un rango de masas de 50-20.000 *m/z*, una resolución de entre 17.500 y 20.000 FWHM, la posibilidad de llevar acabo análisis de MS/MS, y una exactitud de 3 ppm con calibración interna y 5 ppm con calibración externa tanto de iones precursores como de fragmentos[249].

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

Normalmente, para estudios sobre los compuestos fenólicos en vegetales en los que se utiliza un detector QTOF, éste va acoplado a HPLC mediante un sistema de ionización ESI. Algunos de los trabajos publicados en los últimos años se han realizado en muestras como té, alcachofa, melón o mango [250 - 253]

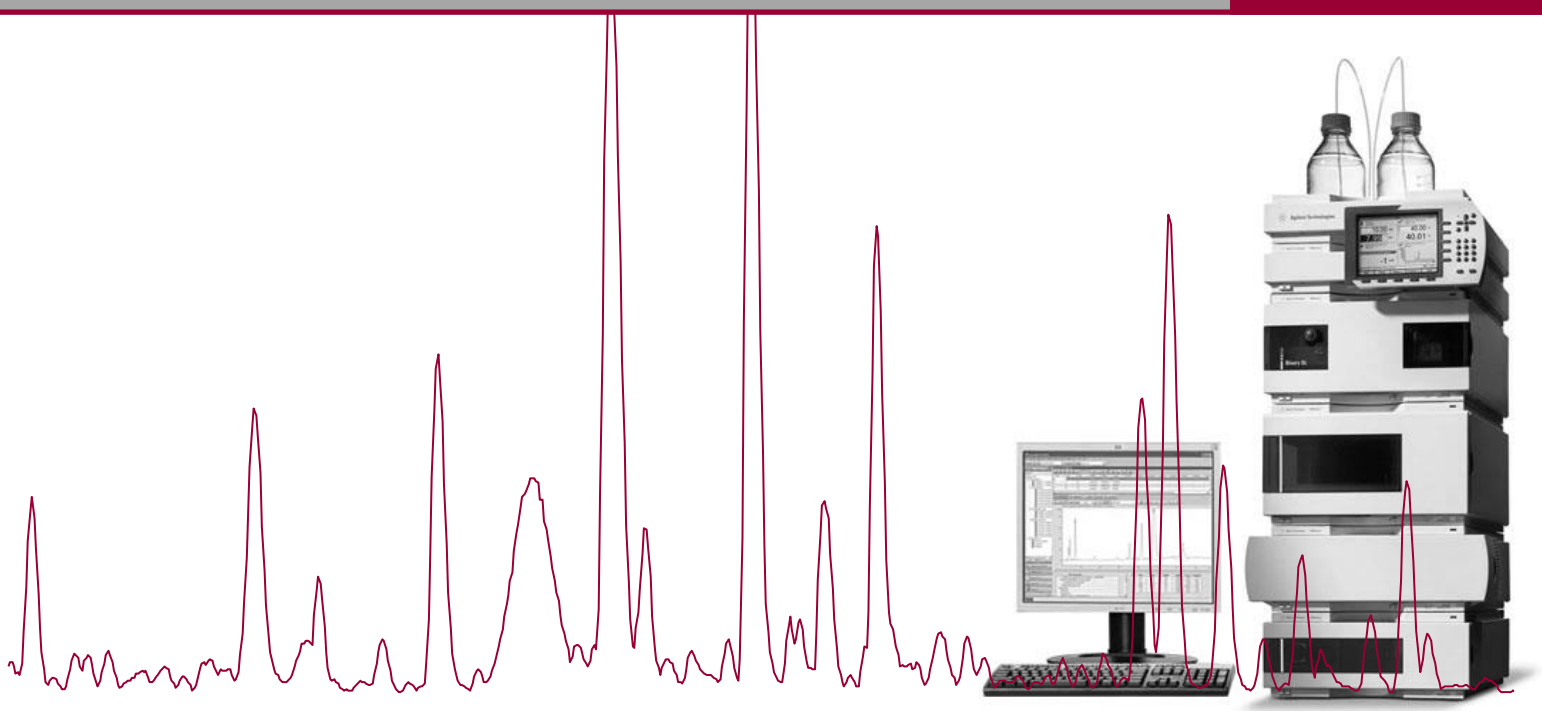
[250] Rodríguez-Pérez, C.; Quirantes-Piné, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Comparative characterization of phenolic and other polar compounds in Spanish melon cultivars by using high-performance liquid chromatography coupled to electrospray ionization quadrupole-time of flight mass spectrometry. *Food Res. Int.* **2013**, *54*, 1519–1527.

[251] Abu-Reidah, I. M.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Extensive characterisation of bioactive phenolic constituents from globe artichoke (*Cynara scolymus* L.) by HPLC–DAD-ESI-QTOF-MS. *Food Chem.* **2013**, *141*, 2269–2277.

[252] Cádiz-Gurrea, M. L.; Fernández-Arroyo, S.; Segura-Carretero, A. Pine bark and green tea concentrated extracts: antioxidant activity and comprehensive characterization of bioactive compounds by HPLC–ESI-QTOF-MS. *Int. J. Mol. Sci.* **2014**, *15*, 20382–20402.

[253] 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.

PARTE EXPERIMENTAL



CAPÍTULO 1

Phenolic compound extraction systems for fruit and vegetable samples

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Review

Phenolic-Compound-Extraction Systems for Fruit and Vegetable Samples

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[†] These two authors contributed equally to this work.

Abstract

This paper reviews the phenolic-compound-extraction systems used to analyse fruit and vegetable samples over the last 10 years. Phenolic compounds are naturally occurring antioxidants, usually found in fruits and vegetables. Sample preparation for analytical studies is necessary to determine the polyphenolic composition in these matrices. The most widely used extraction system is liquid-liquid extraction (LLE), which is an inexpensive method since it involves the use of organic solvents, but it requires long extraction times, giving rise to possible extract degradation. Likewise, solid-phase extraction (SPE) can be used in liquid samples. Modern techniques, which have been replacing conventional ones, include: supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). These alternative techniques reduce considerably the use of solvents and accelerate the extraction process.

Keywords: phenolic compounds; liquid-liquid extraction; solid-phase extraction; supercritical fluid extraction; pressurized liquid extraction; microwave-assisted extraction; ultrasound-assisted extraction

1. Introduction

Today's society is characterized by having many unhealthy dietary habits. Not only snacking but also the inadequate intake of healthy foods triggers a major dietary imbalance, this being a major cause of chronic diseases such as obesity, diabetes mellitus, cardiovascular disease, hypertension, stroke, and several types of cancer. Therefore, it is vital to ascertain the composition and nutritional value of these products. To prevent the above-mentioned diseases, epidemiological studies recommend the consumption of whole fruits, vegetables, and legumes [1,2].

In recent decades, fruit and vegetable consumption has attracted growing interest because many epidemiological and biochemical studies have consistently demonstrated a clear and significant positive association between intake of these natural food products, consumed regularly as part of the Mediterranean diet, and reduced rates of heart disease, common cancers, and other degenerative diseases, as well as aging. The protection that fruits and vegetables provide against these maladies has been attributed to the presence of several antioxidants, especially to antioxidative vitamins, including ascorbic acid (vitamin C), α-tocopherol (vitamin E) and β-carotene (provitamin A). Nevertheless, recent studies seem to indicate that (poly) phenolic substances are the main phytochemicals with antioxidant properties found in higher plants [3, 4, 5].

Polyphenols, widely distributed in plants, contribute to fruit organoleptic and nutritive quality in terms of colour, taste, aroma, and flavour [6], also being involved in astringent and bitter tastes. It is known that, amongst other factors, such as maturity stage or light exposure, phenolic composition varies with the cultivar. In addition, the phenolic profile has already been revealed to be a useful parameter for the discrimination of the different fruit parts [7].

The intake of these compounds is an important health-protecting factor. These bioactive compounds retard or inhibit lipid autoxidation by acting as radical scavengers and, consequently, are essential antioxidants that protect against the

propagation of the oxidative chain [8]. Evidence for their role in the prevention of degenerative diseases is emerging. Experimental studies on animal and human cell lines have demonstrated that polyphenols can play a role in preventing cancer and cardiovascular diseases, when taken daily in adequate amounts [9].

The determination of phenolic compounds in fruits, vegetables, and other foods has been of increasing interest in recent years [10]. Therefore, the objective of the present review is to show the classification of the polyphenolic compounds, taking into account different aspects related to these compounds. Moreover, our aim is to examine the various methods used for preparing and/or treating samples to determine the phenolic content in fruits and vegetables, including the different factors that affect the content in plant bioactive compounds, such as light, temperature, mineral nutrition, pathogens, mechanical damage, plant-growth regulators, and other factors [11].

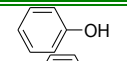
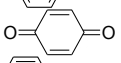
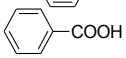
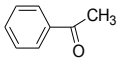
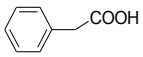
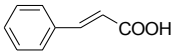
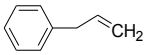
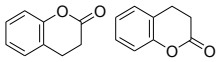
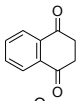
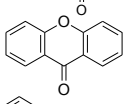
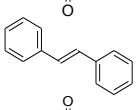
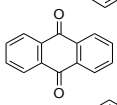
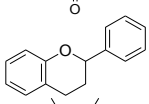
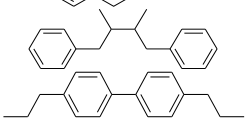
2. Classification and Properties of Phenolic Compounds

Polyphenols have been a feature of plants since their early appearance. These compounds, also called secondary metabolites, are indeed crucial for many important functional aspects of plant life, including structural roles in different supportive or protective tissues, involvement in defence strategies, and signalling properties, particularly in the interactions between plants and their environment. Collectively, higher plants synthesise several thousand different known phenolic compounds, and the number of these which have been fully characterized is continually increasing [12].

The term “polyphenol” includes more than 8,000 compounds with great structural diversity (although each has at least one aromatic ring with one or more hydroxyl groups). They can be divided into 10 different classes depending on their basic chemical structure. Table 1 shows the main families of phenolic compounds, most of which are found in nature associated with mono- or polysaccharides (glycosides) or functional derivatives such as esters or methyl

esters. Moreover, the main sources where phenolic compounds are found have been classified.

Table 1. Classification of families of phenolic compounds

Carbon numbers	*Class*	*Basic structure*	*Sources*
C_6	Simple phenols		
	Benzoquinones		
C_6-C_1	Benzoic acids		Cramberry, cereals
C_6-C_2	Acetophenones		Apple, apricot, banana, cauliflower
	Phenylacetic acids		
C_6-C_3	Cinamic acids		Carrot, citrus, tomato, spinach, peach, cereals, pear, eggplant
	Phenilpropenes		
	Coumarins		Carrot, celery, citrus, parsley
	Chromones		
C_6-C_4	Naphthoquinones		Nuts
C_6-C_1-C_6	Xanthones		Mango, mangosteen
C_6-C_2-C_6	Stilbenes		Grapes
	Anthraquinones		
C_6-C_3-C_6	Flavonoids		Widely distributed
$(C_6$-$C_3)_2$	Lignans, neolignans		Sesame, rye, wheat, flax.
$(C_6$-$C_1)_n$	Hidrolysable tannins	Heterogeneus polymer composed of phenolic acids and simple sugars	Pomegranate, raspberry
$(C_6$-$C_3)_n$	Lignins	Highly crosslinked aromatic polymer	

The most abundant polyphenols in the diet are phenolic acids (benzoic and cinnamic acids), and flavonoids (30 and 60% of the total, respectively) [13, 14, 15]. On the one hand, phenolic acids occur in different forms in plants, including aglycones (free phenolic acids), esters, glycosides, and/or bound complexes. These different forms of phenolic acids show variable suitability for different extraction conditions and vary in their susceptibility to degradation [16]. On the other hand, the common structure of flavonoids consists of two aromatic rings linked by three carbons that usually form an oxygenated heterocycle. In plants, flavonoids can be found as aglycones, although they are usually found as glycosides contributing to the colour (blue, scarlet, orange) of leaves, flowers, and fruits. Phenolic compounds are found not only in fruits and vegetables but also can be found in legumes, cereals, nuts, medicinal plants, spices, and beverages (e.g. tea, wine, and beer). Furthermore, flavonoids can be subdivided in 13 classes: chalcones, dihydrochalcone, auron, flavones, flavonols, dihydroflavonol, flavanones, flavanols (catechins), flavandioles or leucoanthocyanidins, anthocyanidins (its glycoside is called anthocyanin), isoflavononas, flavonoids, and condensed tannins or proanthocyanidins [13, 17].

According to the epidemiological studies, the intake of phenolic compounds is inversely correlated with the risk of coronary heart disease [18, 19]. In the human body, these phytochemicals are thought to provide health benefits by several mechanisms, including: (1) free-radical scavenging; (2) protection and regeneration of other dietary antioxidants (*i.e.* vitamin E); and (3) chelating of pro-oxidant metal ions. The species and levels of phenolic compounds vary dramatically among plants, and their different structures or levels are likely to have different functional properties [5, 20]. Besides the general properties of the compounds, a number of polyphenolic compounds, especially catechins, have been found to be potent antioxidants and to be effective in preventing cancer [21] while tannins have been reported to exert other physiological effects; e.g. they can reduce blood pressure, accelerate blood clotting, lower serum-lipid levels, modulate immunoresponses and cause liver necrosis [22].

As mentioned above, it is impossible to separate the close relationship between the structure and properties of polyphenolics. The structure of phenolic compounds is a key determinant of their radical scavenging and metal-chelating activity. For example, in the case of phenolic acids, the antioxidant activity depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group. Thus, the antioxidant activity of phenolic acids increases the higher the degree of hydroxylation [14].

As a result, it is important to analyse the composition of phenolic compounds in fruits and vegetables before their health-promoting properties can be adequately studied. The analysis of phenolic compounds in plant samples is difficult because of the great variety of their structure and the lack of appropriate standards [5, 20].

3. Extraction Systems for Phenolic Compounds

Extraction is one of the most important steps in sample pretreatment. Generally, it is a separation process where the distribution of the analyte (in this case, a phenolic compound) between two immiscible phases is made in order to arrive at the appropriate distribution coefficient [23]. The extraction procedure is sequential and systematically carried out using an aqueous organic solvent to extract phenolic compounds in fruit and vegetable samples. This traditional method is called liquid-liquid extraction (LLE) and different extraction solvents have been mentioned in the literature such as ethanol, acetone or methanol, or a mixture with water [16]. Soxhlet system is used to extract the lipidic fraction from food and other solid samples, using suitable solvents. Although it is not specific for phenolic compounds extraction, usually the extraction yields are compared to those obtained with another type of polyphenol extraction systems [24].

The ultimate goal of sample preparation is to eliminate or reduce potential matrix interferences [25]. The extraction must be performed with the most adequate solvent and under ideally predetermined analytical conditions of temperature and

pH. Moreover, it is essential to take account the polyphenolic structure because these compounds may have multiple hydroxyl groups that can be conjugated to sugars, acids or alkyl groups. Thus, the polarities of phenolic compounds vary significantly and it is difficult to develop a single method for optimum extraction of all phenolic compounds. Hence, the optimisation of the extraction procedure is essential for an accurate assay of phenolic compounds from different food matrices.

In the end, the effort amounts to lowering costs and reducing sampling time during the above mentioned conventional extraction. In any case, the extraction stage is extremely important, as its outcome will determine the release of analytes from the vegetable matrix into the medium, and this in turn will allow the quantitative determination of the extract [13].

For this reason, modern extraction and isolation techniques will be described as alternative techniques to considerably reduce solvent consumption and accelerate the extraction process. These modern techniques include: supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). These will be explained after the LLE description [26].

3.1. Liquid-Liquid Extraction (LLE)

Solubility of phenolics is governed by their chemical nature in the plant, which may vary from simple to very highly polymerized. Plant materials may contain varying quantities of phenolic acids, phenylpropanoids, anthocyanins, and tannins, among others. There is a possibility of interaction of phenolics with other plant components such as carbohydrates and proteins that may lead to the formation of complexes that may be quite insoluble. Likewise, the solubility of phenolics is affected by the polarity of solvent(s) used. Therefore, it is very difficult to develop an extraction procedure suitable for the extraction of all plant phenolics. The phenolic extracts from plant materials are always a diversified

mixture of plant phenolics soluble in the solvent system used. Additional steps may be required to remove the unwanted phenolics and non-phenolic substances such as waxes, terpenes, fats, and chlorophylls [14, 27].

The extraction methods for simple phenolic compounds (benzoic acids, benzoic aldehydes, cinnamic acids, and catechins) from solid or semi-solid materials have been focused on maceration using organic solvents. The current official analytical method for extracting phenolic compounds is liquid-liquid extraction (LLE) for liquid samples. This method requires expensive and hazardous organic solvents, which are undesirable for health and disposal reasons, and they require a long time per analysis, giving rise to possible degradations. The process of degradation can be triggered both by external and internal factors. Light, together with air and temperature, are the most important factors that facilitate degradation reactions. The extraction temperature usually needs to be high in order to minimise the duration of the process. For these reasons, these traditional extraction sample methods have been replaced by other methodologies which are more sensitive, selective, fast, and environmentally friendly [4, 28]. In any case, LLE is still used as the standard preconcentration step for phenol determination in water because it is a cheap and easy method.

Table 2. LLE methods

Sample	Reference	Solvent	Number of polyphenols identified
Bean (*Phaseolus vulgaris* L.)	[19]	Methanol (100%)	8
Bean (*Phaseolus vulgaris* L.)	[37]	Methanol/water (80:20v:v), HCl 2N	6
Bean (*Phaseolus vulgaris* L.)	[11]	Acetonitrile HCl 0,1 N	3
Bean (*Phaseolus vulgaris* L.)	[2]	Methanol/water (80:20v:v), HCl 2N	17
Bayberry (*Myrica rubra Sieb. et Zucc*)	[18]	Ethyl acetate	10
Artichoke (*Cynara scolymus* L.)	[19]	Methanol/water (82:18 v:v)	3
Mustard greens (*Brassica juncea*)	[20]	Methanol/water (80:20 v:v)	3
Kale (*Brassica oleracea var. acephala*)	[20]	Methanol/water (80:20 v:v)	3

Sample	Reference	Solvent	Number of polyphenols identified
Okra (*Hibiscus esculentus* L.)	[20]	Methanol/water (80:20 v:v)	1
Potato (*Solanum tuberosum* L.)	[20]	Methanol/water (80:20 v:v)	2
Green Onion (*Allium fistulosum*)	[20]	Methanol/water (80:20 v:v)	1
Purslane (*Portulaca oleracea* L.)	[20]	Methanol/water (80:20 v:v)	3
Collard greens (*Brassica oleracea* L.)	[20]	Methanol/water (80:20 v:v)	2
Purple hull-peas (*Vigna unguiculata*)	[20]	Methanol/water (80:20 v:v)	1
Bean (*Phaseolus vulgaris* L.)	[30]	Acetone 80%	4
Bean (*Phaseolus vulgaris* L.)	[31]	Methanol/water (85:15v:v), HCl 1M	7
Parsley flakes (*Petroselinum crispum* L.)	[25]	Methanol	1
Quince (*Cydonia oblonga* L.)	[5]	Methanol (100%)	18
Tree tomato (*Cyphomandra betacea* L.)	[1]	Acetone 70%	8
Naranjilla (*Solanum quitoense* L.)	[1]	Acetone 70%	2
Artichoke (*Cynara Scolymus* L.)	[32]	Methanol/water (50:50 v:v)	15
Garlic (*Allium sativum* L.)	[38]	Methanol/water (50:50 v:v)	2
Onion (*Allium cepa* L.)	[38]	Methanol/water (50:50 v:v)	2
Bean (*Phaseolus vulgaris* L.)	[16]	Methanol/water (85:15 v:v)	12
Papaya (*Carica papaya* L.)	[33]	Methanol (100%)	12
Eggplant (*Solanum melongena* L.)	[34]	Methanol/water (80:20 v:v)	18
Eggplant (*Solanum melongena* L.)	[17]	Methanol (100%)	4
Red lettuce (*Lactuca sativa* L.)	[17]	Methanol (100%)	4
Red onion (*Allium fistulosum* L.)	[17]	Methanol (100%)	10
Bean (*Phaseolus vulgaris* L.)	[17]	Methanol (100%)	9
Pistachio (*Pistacia vera* L.)	[17]	Methanol (100%)	2
Cucumber (*Cucumis sativus* L.)	[35]	DMSO	11

Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, and their combinations have also been used for the extraction of phenolics, often with different proportions of water. For example, phenolic compounds can be efficiently extracted from legumes using an ethanol/water (70:30 v:v) system (see Table 2) [36].

Generally, LLE is used at room temperature to avoid the degradation of phenolic compounds, but there are many studies such as Costa *et al.*, Aparicio-Fernández *et al.* or Magalhães *et al.* using temperatures around 20 to 40 ºC. When hydrolysis of phenolic compounds is carried out, the temperature is usually 80-95 ºC for acid hydrolysis or 45 ºC for basic hydrolysis [2, 16, 20, 37, 38]. Otherwise, extraction times depend on several factors such as maceration time, centrifugation time or the time spent on the evaporation of solvents.

Anthocyanins are usually extracted from plant material with an acidified organic solvent, most commonly methanol. This solvent system destroys the cell membranes, simultaneously dissolves the anthocyanins, and stabilizes them. However, the acid may bring about changes in the native form of anthocyanins by breaking down their complexes with metals and co-pigments [14]. An example is described by Ross *et al.*, where aglycone forms of glycoside flavonoids are obtained by acid hydrolysis of the bean extracts, using a methanol/water (85:15 v:v) system [16].

3.2. Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) is an increasingly useful sample-preparation technique. With SPE, many of the problems associated with liquid-liquid extraction, such as incomplete phase separations, less-than-quantitative recoveries, use and disposal of large and expensive quantities of organic solvents, can be avoided, although the cost of the equipment required for SPE is higher than for LLE. This technique is used most often to prepare liquid samples and extract semivolatile or nonvolatile analytes, but can also be used with solids that are pre-extracted into solvents. They are available in a wide variety of chemistries, adsorbents, and sizes so that it is necessary to select the most suitable product for each application and sample. For phenolic determination in grapes or wines and other beverages, different solid phases have been tested for SPE. Polymers of styrene-divinylbenzene provided good results, while C_{18}-based phases afforded less satisfactory results for polar phenolics [10]. The particular

case of phenolic extraction from olive-oil samples has been extensively studied. It is well known that the C_{18} phase is less suitable for the isolation of polar components from a nonpolar matrix than is the normal-phase SPE [27].

3.3. Supercritical Fluid Extraction (SFE)

Usually, phenolic compounds are extracted from plant samples by SPE coupled with other techniques, such as supercritical fluid extraction (SFE). SFE is a relatively recent technique which presents various advantages over traditional methods, such as the use of low temperatures and reduced energy consumption and high product quality due to the absence of solvents in the solute phase. However, this technique is limited to compounds of low or medium polarity. The literature offers descriptions of extraction methods for polyphenols by SFE, the main characteristics of which are the need for high percentages of organic modifiers; this usually means that the process takes place under subcritical conditions.

Supercritical carbon dioxide (SC-CO_2) is the most widely used solvent for SFE due to its particular characteristics, such as moderate critical conditions (31.1 ºC and 73.8 MPa) and ready availability. It is also nontoxic, inflammable and chemically stable. However, SFE using CO_2 as the extracting solvent is of no use for phenolic compounds because of the low polarity of CO_2 in comparison to most phenols [4, 39].

Generally, for this extraction procedure, several steps are followed: samples are loaded onto the sorbent of the SPE cartridge, which is inserted into the SPE/SFE extraction cell. The supercritical fluid used can be carbon dioxide, which must go through the SPE cartridge filled with the hydrolysed sample. Thus, analytes (phenolic compounds) are quantitatively trapped by a trapping solvent (for example, methanol) at laboratory temperature (the trapping solvent is cooled naturally during the extraction by the expansion of CO_2). Finally, the extracts are

evaporated to dryness, dissolved in the mobile phase, and injected directly into the HPLC/ESI-MS system [28].

Castro-Vargas *et al.* compared different extraction systems for guava seed samples, the results of which are presented below. The yield of the SFE process in terms of phenolic fraction is also lower than the value achieved by Soxhlet extraction with ethanol (SE-EtOH), although the total extraction yields for SFE with CO_2/EtOH are typically higher. This behaviour is explained by the non-polar characteristic of the carbon dioxide, which increases the extraction of low-polarity compounds, compared with polar ones (found particularly in the phenolic fraction) [39].

In SFE the yield results (phenolic and total) increase directly with solvent polarity and the use of EtOH as a co-solvent is particularly useful to enhance the phenolic fraction yield. At constant temperature, the rise in pressure increases the yield due to density enhancement. At constant pressure, the phenolic and the total yield decrease with rising temperatures due to the solvent density reduction. Lastly, it bears mentioning that SFE is of enormous interest today, with more than 200 references in the literature dealing with this topic in the last two years (2007-2009). The range of applications of SFE includes not only its use in sample preparation but also new and recent advances in different areas such as pharmaceutical, environmental science, and food science. With regard to the present results, readers are encouraged to treat the information as a tool to develop new processes at the laboratory and pilot scale, to discover new ways for sample preparation, to learn how to deal with SFE optimisation and, certainly, to be able to develop emerging technologies that can fulfil the requirements of environmentally clean processes [40].

3.4. Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE) uses organic solvents at high pressures and temperatures above their normal boiling point. It is the newer modern method for

isolation of analytes from solid samples [26]. In general, with PLE, a solid sample is packed into a stainless steel extraction cell and extracted with a suitable solvent under high temperatures (40-200 ºC) and pressure (500-3000 p.s.i.) for short periods of time (5-15 min). The sample extract is purged into a collection vial with the aid of a compressed gas.

The procedure described by Alonso-Salces *et al.*, is based on polyphenol extraction in apple samples. Previously, freeze-dried samples are mixed with diatomaceous earth as a dispersion agent in order to reduce the solvent volume used for the extraction. The extracts are filtered, evaporated to dryness, reconstituted in methanol-aqueous hydrochloric acid 0.1% (30:70 v/v) and filtered again prior to injection into the HPLC system. These authors also examined different parameters such as percentages of methanol in the solvent, temperature, pressure, and static extraction time.

In Luthria *et al.*, all extractions were carried out with either one or two solvent mixtures, ethanol- water (50:50, v/v) and/or acetone-water (50:50, v/v), using a pressurized liquid extractor [25**]**

According to Liazid *et al.*, PLE has been shown to be effective as a method for extracting polyphenols, while rapid methods, taking 10 min, have been developed that use high temperatures (150 ºC) to accelerate the process [4].

Briefly, Dobiáš *et al.* developed a new modern method for isolating analytes from solid samples, based on pressurised fluid extraction (PFE). In this case, the extraction process is carried out at higher temperature and higher pressure and the main advantages of this method involve low solvent consumption and a short extraction times [23].

3.5. Microwave-Assisted Extraction (MAE)

Microwave technology is commonly known for its use as heat treatment. For example, it is used as a heat process for commercial fruit products to achieve a fast but mild pasteurization of these products. At the same time, the use of

microwaves serves to determine the stability of total polyphenol content after the treatment. As Picouet *et al.* conclude, significant losses occur during storage until the decrease of polyphenolic content is finished [41]. This technology is also used to speed up the drying process in wine and fresh grape samples, improving their pre-treatment and being a useful protocol to examine phenolic compounds [42].

Recently, microwave-assisted extraction (MAE), also called microwave-assisted process (MAP), has been applied in the development of extraction methods for organic compounds from soil, sediment, seed, and food matrices. These studies show that the extraction is more effective when microwave energy is used. The study by Sutivisedsak *et al.* demonstrates the utility of microwave-assisted extraction in determining the total phenolic contents of eight common bean types, using the Folin- Ciocalteau colorimetric method.

As occurs with SFE and PLE systems, MAE makes it possible to perform extractions in the absence of light. Phenolic compounds are very sensitive to this factor, giving these techniques a great advantage. This is important because, for example, resveratrol can be found in two isomeric forms (its *cis* and *trans* configurations), but only one of these, *trans*-resveratrol, presents biological activity. Light can catalyse the transformation from the active to the inactive form. In addition, the short extraction times that these techniques present (less than 1 h) reduce the adverse effects of enzymatic activity. Another important factor to be taken into account in the MAE is the temperature of the extraction. According to Liazid *et al.*, there is a clear relationship between the chemical structure and the stability of phenolic compounds that are studied under different conditions of MAE. Moreover, it has been shown that those that have a greater number of hydroxyl-type substituents are more easily degraded under these temperature conditions [4, 43].

The main advantage of MAE is the possibility that several samples could be simultaneously extracted quicker than with Soxhlet extraction, and that similar recoveries to those of SFE were achieved. However, care must be taken when

working with flammable solvents or in the case of samples that contain constituents which couple strongly with microwave radiation to cause a rapid rise in temperature and thereby lead to potentially hazardous situations [44].

3.6. Ultrasound-Assisted Extraction (UAE)

Ultrasonic radiation is a powerful aid in accelerating various steps of the analytical process. This energy is of great help in the pre-treatment of solid samples as it facilitates and speeds up operations such as the extraction of organic and inorganic compounds, homogenization, and various others. Ultrasound-assisted leaching is an effective way to extract analytes from different matrices in shorter times than with other extraction techniques [23]. For example, ultrasound-assisted systems have been widely used to extract capsaicinoids in hot peppers [45].

Ultrasonic extraction (USE) is considered one of the simplest extraction techniques because it is easy to perform in common laboratory equipment (*i.e.* ultrasonic bath). In this method, the crushed sample is mixed with the suitable solvent and placed into the ultrasonic bath, where the working temperature and extraction time are set [26].

The application of ultrasound-assisted extraction (UAE) in food-processing technology is of interest for facilitating the extraction of components from plant materials. The higher yield achieved in these UAE processes is of major interest from an industrial standpoint, since the technology is an add-on step to the existing process with minimum alteration, application in aqueous extraction where organic solvents can be replaced with solvents generally recognised as safe (GRAS), reduction in solvent usage, and shorter extraction time. The use of ultrasonic means for extraction purposes in high-cost raw materials is an economical alternative to traditional extraction processes, this being a demand by industry for a sustainable development.

Ultrasound can enhance existing extraction processes and enable new commercial extraction opportunities and processes. The main targets have been polyphenols and carotenoids and in both aqueous and solvent extraction systems. The ultrasound extraction trials have demonstrated improvements in extraction yield ranging from 6 to 35% [46].

Many studies have examined the stability of the analytes during ultrasound-assisted process. Herrero *et al.* evaluated the phenolic-compound decomposition when phenolics were subjected to solid-liquid, subcritical water or microwave-assisted extraction, and sonication was performed in order to assess the type of energy that provides a lower degradation of the analytes. The method was applied to two types of strawberries in order to demonstrate the applicability of the proposed method, which is much faster and results in less analyte degradation than do others [23].

Therefore, in recent years it has been shown that UAE offers lower phenolic compound recovery when compared to pressurized hot-water extraction methods. Vilkhu *et al.* proposed supercritical carbon dioxide extraction as a better method than ultrasound-assisted extraction of polyphenolic compounds from grape seeds. It was believed that the lower catechin (used as a measure of phenolic content) recovery from the ultrasound method could be due to the insufficient power of the solvent used (aqueous methanol) or due to the degradation of samples during extraction process. These authors focused on the efficiency of supercritical fluid extraction (SFE) rather than other methods used in the experiment. The results of catechin recovery using different extraction methods compared to a control (solvent extraction only) was not available and, consequently, it was not possible to determine whether ultrasound treatment (although having a lower recovery compared to SFE method) contributed to the increase in catechin recovery relative to a control. Most importantly, though, the frequency of ultrasound and other extraction conditions (e.g. temperature) was not stated, so that it is not known whether suitable frequencies or application conditions were used [46].

4. Conclusions

In this review, the advantages and disadvantages of different extraction systems for phenolic compounds are discussed. The most widely used extraction system is liquid-liquid extraction (LLE), which is an inexpensive method, since it involves the use of organic solvents, but it involves long extraction times, which give rise to possible degradations. Consequently, new techniques such as SFE, SPE, PLE, MAE, and UAE have been developed.

Normally, extraction efficiency increases at higher extraction temperatures, but the working temperature affects the stability of the phenolic compounds, which also depends on their chemical structure. Thus, factors that influence the extraction processes (temperature, polyphenolic structure, pressure, sample characteristics, and other factors) are discussed using examples.

Acknowledgements

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CAPÍTULO 2

Antioxidant capacity of 44 cultivars of fruits and vegetables grown in Andalusia (Spain)

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Abstract

Human diet has an important role in protection against oxidative stress. This health-protecting factor has been partly attributed to the compounds with antioxidant capacity, fruits and vegetables being major sources of dietary antioxidants. Horticultural production in Andalusia includes a large variety of fruits and vegetables which are available not only in Spain but also in other European Union countries.

The aim of the present study was to evaluate the antioxidant capacity of the edible portion of 44 fruits and vegetables grown in Andalusia and commonly consumed by both Spanish and other European consumers. The samples, collected at different times according to the harvesting period of each crop, were submitted to three in vitro assays: TEAC (trolox equivalent antioxidant capacity), FRAP (ferric ion reducing antioxidant power), and ORAC (oxygen radical absorbance capacity).

The samples showing the highest antioxidant capacity were red 'California' pepper (4.550-13.810 mmol eq. Trolox/100 g), 'Fino' lemon (11.560-14.340 mmol eq. FeSO4/100 g), and red onion (2.921-13.830 mmol eq. Trolox/100 g) in the TEAC, FRAP, and ORAC assays, respectively. The results also showed that 'Vivaldi' potato was the sample with the lowest antioxidant capacity in all the methods assessed (0.035-0.590 mmol eq. Trolox/100 g in TEAC, 0.200-0.399

mmol eq. FeSO4/100 g in FRAP and 0.238-0.286 mmol eq. Trolox/100 g in ORAC assay).

In conclusion, this study provides a comprehensive comparison of the antioxidant capacity among different cultivars of fruits and vegetables grown in Andalusia that can be acquired year round. This information reveals that the Andalusian cultivars analyzed are good sources of antioxidants.

Keywords: Vegetal samples, Antioxidant capacity, TEAC, FRAP, ORAC

1. Introduction

The intake of fruits and vegetables has been associated with a notable health-protecting factor against diseases caused by oxidative stress, including coronary heart disease, cancer, and neurodegenerative pathologies (Fu *et al.*, 2011; Hervert-Hernández, García, Rosado, & Goñi, 2011). These beneficial effects have been attributed partly to the compounds having antioxidant capacity and an ability to overcome oxidative stress by neutralizing the overproduction of oxidant species (Podsedek, 2007; Rahman, 2008).

A balanced diet is essential to acquire optimal health benefits from exogenous antioxidants. Many authors have reported that the additive and synergistic effects provided from the complex mixture of phytochemicals present in fruits and vegetables cannot be achieved through micronutrient supplements (Liu, 2003). Therefore, the consumption of fruits and vegetables as sources of antioxidants is recommended (Chu, Sun, Wu, & Liu, 2002; Halvorsen *et al.*, 2002; Chun *et al.*, 2005). In this sense, horticultural production in Spain in general, and Andalusia in particular, includes a large variety of fruits and vegetables, such as tomato, pepper, onion, melon, watermelon, and grape (Abu-Reidah *et al.*, 2012; Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010; Morales-Soto, Gómez-Caravaca, García-Salas, Segura-Carretero, & Fernández-Gutiérrez, 2013; Rodríguez- Pérez, Quirantes-Piné, Fernández-Gutiérrez, & Segura-Carretero, 2013). According to data from the Spanish Ministry of Agriculture, Food, and Environment, about 91% of the total production is exported abroad, mainly to European Union members such as Germany and the United Kingdom (FEPEX, 2013). This makes Andalusian fruit and vegetable production one of the motors of the regional economy and particularly vital for its economic resilience.

For an assessment of the antioxidant capacity provided by different fruits and vegetables included in the diet, different *in vitro* method can be employed (García-Alonso, De Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2004). The methods to assess antioxidant capacity can be classified according to

different issues. On the one hand, the classification is related to the mechanism of reaction between the antioxidant compounds and the oxidant species. In this way, the methods based on single electron transfer (SET) can be distinguished from those based on hydrogen atom transfer (HAT). On the other hand, there are direct competition methods where natural antioxidants compete for the radical with respect to the free-radical scavenger, and there are indirect methods based on monitoring the decay of a free radical due to the addition of the antioxidant-containing sample. However, a review of all the methods indicates that a consensus for an official standardized method has not been reached and therefore more than one in vitro chemical-based assay should be applied (García-Salas *et al.*, 2013; López-Alarcón & Denicola, 2013). Hence, the advantages and disadvantages of each methodology need to be taken into account. In general, the direct methods are more suitable than the indirect ones because they are more sensitive, but they are also more time consuming. Therefore, for routine tests, most studies use only indirect assays as an easy handling option (Roginsky & Lissi, 2005).

Most of the previous research papers dealing with antioxidant capacity of fruit and vegetable samples have focused on the most commonly consumed species such as apple or cucumber (Saura-Calixto & Goñi, 2006). Also, many attention-grabbing articles concern the large number of fruits and/or vegetables included in the present study, but in most cases the samples were purchased in American markets (Zhou & Yu, 2006). Regarding Spanish samples, few articles have reported the antioxidant capacity of tropical fruits, which have been studied mainly in papers from Asian countries (Fu *et al.*, 2011; Guo *et al.*, 2003). The study by García-Alonso *et al.* (2004) examines the antioxidant capacity of 28 Spanish fruits, including not only the commonly consumed fruits, but also some fruits such as fig, quince or kiwi. In the study, only two different antioxidant capacity methods were employed (García-Alonso *et al.*, 2004). Most papers focus either on the fruits in the optimal state for harvest or on the variation in activity values according to the stage of ripeness. However, consumers can

acquire fruits and vegetables throughout the year, and there is little information on the antioxidant capacity provided by the fruits and vegetables available year round (Borochov-Neori *et al.*, 2009; Ferreyra, Viña, Mugridge, & Chaves, 2007).

The main purpose of the present study was to evaluate the antioxidant capacity of the edible portion from a total of 44 fruits and vegetables, including several selected cultivars grown in Andalusia. Three different *in vitro* methods were applied: TEAC (trolox equivalent antioxidant capacity), FRAP (ferric ion reducing antioxidant power) and ORAC (oxygen radical absorbance capacity). As the sampling included the different harvest seasons of every cultivar, the results offer the consumer an idea of the beneficial properties provided by the Andalusian-grown fruits and vegetables available on the market, whatever the season, thereby promoting their consumption.

2. Material and methods

2.1. Chemicals and reagents

The reagents used to measure the antioxidant capacity, AAPH (2,2′-azobis-2-methyl-propanimidamide, dihydrochloride), TPTZ (1,3,5-triphenyltetrazolium chloride), ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate)], trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid), fluorescein, potassium persulfate, and ferric sulfate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated sodium phosphate, trihydrated sodium acetate, sodium acetate, ferric chloride, hydrochloric acid, and acetic acid were obtained from Panreac (Barcelona, Spain).

2.2. Instrumentation

Fluorescence (ORAC) and absorbance (FRAP and TEAC) measurements were made with a Synergy Mx Monochromator-Based Multi- Mode Microplate reader (Bio-Tek Instruments Inc.) by using 96-well polystyrene microplates.

2.3. Samples

The fruits and vegetables were chosen according to their socio- economic importance in Andalusia (Spain). The vegetables included were: asparagus (*Asparagus officinalis* L.), artichoke (*Cynara scolymus* L.), carrot (*Daucus carota* L.), eggplant (*Solanum melongena* L.), fava bean (Vicia faba L.), garlic (*Allium sativum* L.), green bean (*Phaseolus vulgaris* L.), lettuce (*Lactuca sativa* L.), onion (*Allium cepa* L.), pepper (*Capsicum annuum* L.), potato (*Solanum tuberosum* L.), tomato (*Solanum lycopersicum* L.), and zucchini (*Cucurbita pepo* L.). The selected fruits are also described as follows: avocado (*Persea americana* Mill.), custard apple (*Annona cherimola* Mill.), grape (*Vitis vinifera* L.), lemon (*Citrus limon* (L.) Burm. f.), loquat (*Eriobotrya japonica* (Thunb.) Lindl.), mango (*Mangifera indica* L.), melon (*Cucumis melo* L.), persimmon (*Diospyros kaki*), pomegranate (*Punica granatum* L.), quince (*Cydonia oblonga* Mill.), and watermelon (*Citrullus lanatus* (Thunb.) Mansf.).

Of these, the most representative cultivars from south-eastern Spain were selected for each species, depending on their availability and their nutritional properties. Between one and three cultivars of each fruit/vegetable were included in this study, for a total of 29 vegetable cultivars and 15 fruit cultivars. These samples were collected at different times according to the harvesting period of each crop (from two to five different acquisition times). All the fruit and vegetable cultivars used and the seasons in which these are commercially available are shown in Fig. 1.

About 1 to 1.5 kg of each vegetable was collected over the entire year of 2011. The edible portion of each fresh sample was separated from the seeds and from the skin (where necessary). Then, samples were ground, homogenized, stored at -25 °C and placed on a lyophilizer shelf (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK), which was pre-cooled to -50 °C for 1 h at 1 mbar. Before the lyophilization, the percentage of moisture of each sample was determined. Then, samples were kept at -18 °C until analyzed.

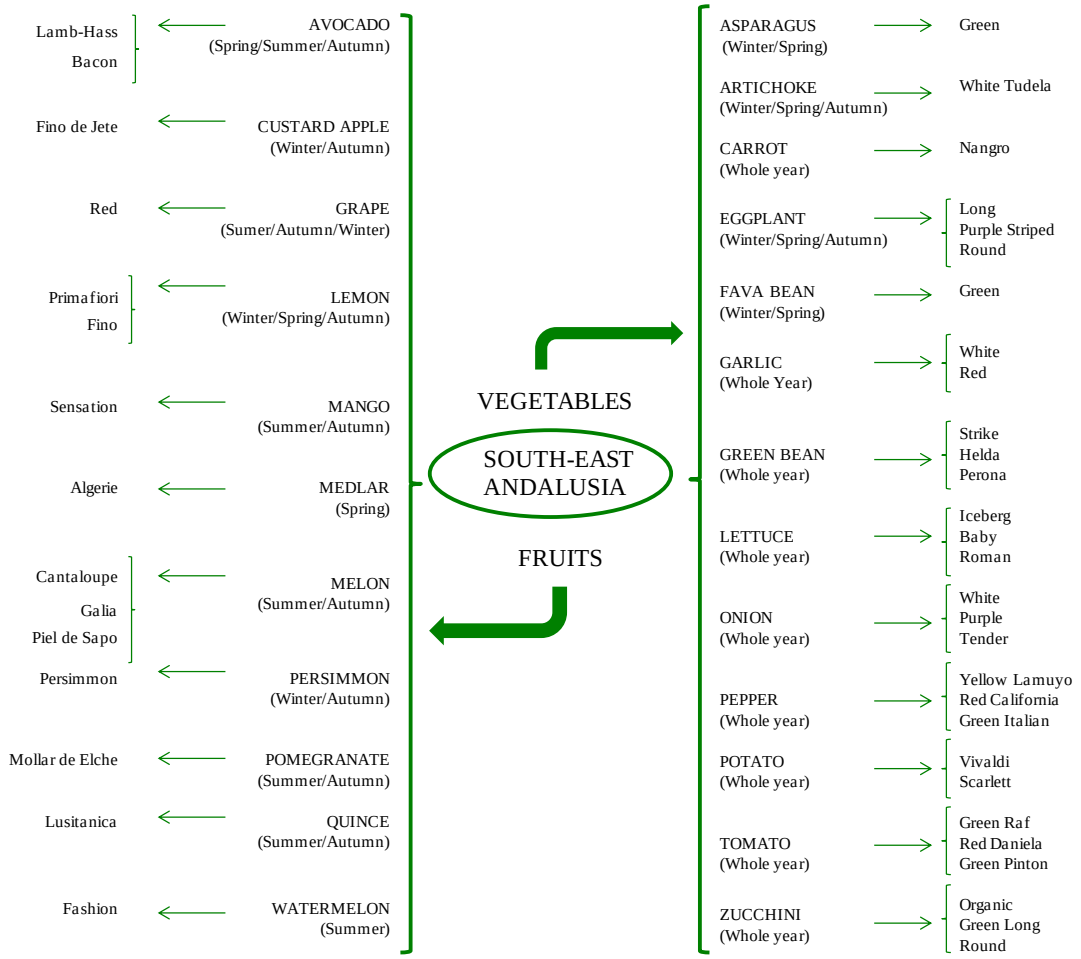


Fig. 1. Outline of the cultivars grown and harvested throughout the year.

2.4. Preparation of extracts

The extraction was conducted as follows: 500 mg of dried vegetable was placed in a test tube. Then, 15 mL methanol/water (80:20 v/v) was added and the sample was sonicated for 20 min and centrifuged at 984 ×g for 10 min. The supernatant was removed, evaporated, and reconstituted in 2 mL methanol/water (80:20 v/v). Finally, the extract solution was filtered through a 0.22 μm micro-filter (Millipore, Bedford, MA, USA) before analysis.

2.5. Antioxidant capacity assays

Three different methods were applied to determine the antioxidant capacity of the samples: TEAC and FRAP, based on single electron transfer (SET); and ORAC, based on hydrogen atom transfer (HAT). All the experiments were performed in triplicate.

2.5.1. Trolox equivalent antioxidant capacity (TEAC) assay

The trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the radical cation of 2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulphonate) (ABTS) by antioxidants, was based on the method of Miller, Riceevans, Davies, Gopinathan, and Milner (1993) as described by Laporta, Pérez-Fons, Mallavia, Caturla, and Micol (2007) (Laporta *et al.*, 2007; Miller *et al.*, 1993). Briefly, the ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and keeping the mixture in darkness at room temperature for 12 to 24 h before use. For the antioxidant assay with vegetable extracts, the $ABTS^{+\bullet}$ solution was diluted with water until reaching an absorbance value of 0.70 (± 0.02) at 734 nm. For the spectrophotometric assay, 300 μL of the $ABTS^{+\bullet}$ solution and 30 μL of the extract were mixed for 45 s and measured immediately after 5 min (absorbance did not change significantly up to 10 min). The readings were performed at 734 nm and 25 °C. The result of each sample was then compared with a standard curve made from the corresponding readings of trolox (0.5-30 μM in the microplate wells).

2.5.2. Ferric-reducing ability power (FRAP) assay

The FRAP assay was conducted following the method described by Benzie and Strain (1999). Briefly, 40 μL of the extracts was mixed on a 96-well plate with 250 μL of freshly prepared FRAP reagent. Samples were incubated for 10 min at 37 °C, and then, absorbance at 593 nm was recorded for 4 min on the microplate

reader. The final absorbance of each sample was compared with those from the standard curve made from $FeSO_4 \cdot 7H_2O$ (12.5-200 μM in the microplate wells).

2.5.3. Oxygen radical absorbance capacity (ORAC) assay

To assay the capacity of the extracts to scavenge peroxyl radicals, a validated ORAC method was used (Ou, Hampsch-Woodill, & Prior, 2001) as modified by Laporta *et al.* (2007). In the final assay mixture (210 μL total volume), fluorescein and 2,2′-azobis-(2- methylpropionamine)-dihydrochloride (AAPH) were used at 40 nM and 133 mM, respectively. Several dilutions of trolox (0.5-15 μM in the microplate wells) were used to draw the calibration curve. A freshly prepared AAPH solution was used for each experiment. The temperature of the incubator was set at 37 °C and the fluorescence was recorded every minute after the addition of AAPH. The final ORAC values were calculated using a regression equation between the trolox concentration and the net area of the fluorescence decay curve (area under curve, AUC).

2.6. Statistical analysis

Conventional statistical methods were used to calculate means and standard deviations of three simultaneous assays carried out with the different methods. The Origin software (version Origin Pro 8 SR0, Northampton, MA, USA) was used for all statistical analyses.

3. Results and discussion

In the present study, TEAC, FRAP, and ORAC assays were used to evaluate the antioxidant capacities of a large variety of fruits and vegetables from Andalusia and commonly consumed not only by Spanish but also by other European consumers. Because of the different acquisition times of the 44 fruit and vegetable cultivars, 154 analyses were made for fruit samples and 339 analyses

in the case of the vegetable samples. Taking into account the triplicates, a total of 1479 analyses were carried out in this work.

The antioxidant capacity data of the three different assays are summarized in Tables 1 and 2 for the fruit and vegetable cultivars, respectively. The data are presented as mean ± standard deviation (SD) for all the samples collected at different times. Both tables also include the moisture percentages found before the lyophilization process. The results were calculated for 100 g of each sample (dry weight) and expressed as mmol equivalents (eq.) of trolox for TEAC and ORAC assays, and mmol eq. of ferric sulfate ($FeSO_4$) in the case of the FRAP assay.

Table 1. Antioxidant capacity results (TEAC, FRAP, and ORAC methods) per 100 g dry weight and % moisture for fruit samples ([a] "n.a." when the value is not available)

Fruit	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
AVOCADO					
Lamb-Hass Avocado 1	March	1.580±0.046	1.310±0.115	1.630±0.033	76
Lamb-Hass Avocado 2	June	2.140±0.122	1.090±0.009	2.140±0.017	
Bacon Avocado 1	June	3.130±0.112	2.020±0.055	0.814±0.033	77
Bacon Avocado 2	October	1.281±0.007	1.330±0.060	0.198±0.013	
CUSTARD APPLE					
Fino de Jete Custard Apple 1	January	2.000±0.209	1.700±0.127	0.830±0.017	95
Fino de Jete Custard Apple 2	February	2.309±0.114	2.880±0.104	0.967±0.017	
Fino de Jete Custard Apple 3	March	2.530±0.101	2.597±0.007	1.305±0.033	
Fino de Jete Custard Apple 4	March	2.299±0.214	2.634±0.045	1.139±0.083	
GRAPE					
Red Grape 1	August	3.960±0.117	13.160±0.248	2.417±0.050	79
Red Grape 2	September	3.230±0.159	16.380±0.315	3.577±0.083	
Red Grape 3	November	4.440±0.336	13.964±0.151	2.652±0.100	
Red Grape 4	December	4.600±0.675	13.829±0.447	2.706±0.117	
LEMON					
Fino Lemon 1	September	5.314±0.231	14.340±0.588	2.910±0.067	90
Fino Lemon 2	November	5.920±0.384	11.560±0.650	3.719±0.133	
Fino Lemon 3	December	3.280±0.160	12.502±0.862	2.922±0.050	
Primafiori Lemon 1	May	4.695±0.073	13.310±0.100	2.080±0.017	91
Primafiori Lemon 2	August	4.751±0.409	12.820±0.171	n.a.	
Primafiori Lemon 3	October	5.380±0.159	12.066±0.088	2.440±0.100	

Fruit	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
Primafiori Lemon 4	November	3.950±0.242	11.550±0.099	2.394±0.033	
MANGO					
Sensation Mango 1	August	3.855±0.110	4.059±0.079	0.450±0.017	85
Sensation Mango 2	September	4.160±0.185	4.170±0.035	0.690±0.033	
Sensation Mango 3	September	3.620±0.178	3.820±0.066	0.684±0.013	
Sensation Mango 4	October	3.220±0.288	3.520±0.108	0.691±0.017	
LOQUAT					
Algerie Loquat 1	May	0.230±0.054	0.560±0.031	n.a.	84
Algerie Loquat 2	May	0.610±0.061	0.660±0.024	0.380±0.017	
Algerie Loquat 3	June	0.451±0.107	0.585±0.015	0.610±0.002	
MELON					
Cantaloupe Melon 1	June	2.660±0.117	3.340±0.026	0.470±0.033	93
Cantaloupe Melon 2	July	0.940±0.085	2.294±0.065	0.423±0.017	
Cantaloupe Melon 3	July	1.571±0.049	2.064±0.026	0.423±0.033	
Cantaloupe Melon 4	October	1.345±0.061	1.230±0.039	0.475±0.013	
Galia Melon 1	June	1.241±0.141	1.958±0.036	0.275±0.017	90
Galia Melon 2	June	1.172±0.119	1.910±0.066	0.220±0.012	
Galia Melon 3	July	1.610±0.135	2.120±0.058	n.a.	
Galia Melon 4	October	1.030±0.091	2.380±0.028	0.533±0.033	
Sapo Melon 1	June	1.042±0.081	2.170±0.030	0.190±0.012	90
Sapo Melon 2	July	1.170±0.073	1.908±0.016	0.402±0.033	
Sapo Melon 3	July	0.910±0.031	1.730±0.032	0.420±0.017	
Sapo Melon 4	October	0.998±0.088	1.778±0.034	0.398±0.002	
PERSIMMON					
(Persimmon) Persimmon 1	March	0.630±0.033	0.712±0.012	n.a.	
(Persimmon) Persimmon 2	October	0.411±0.019	0.730±0.009	0.313±0.012	86
(Persimmon) Persimmon 3	November	0.330±0.023	0.660±0.004	0.252±0.017	
POMEGRANATE					
Mollar de Elche Pomegranate 1	August	3.770±0.378	4.830±0.079	1.344±0.008	93
Mollar de Elche Pomegranate 2	September	5.770±0.168	5.442±0.215	1.100±0.067	
Mollar de Elche Pomegranate 3	October	5.522±0.209	5.720±0.226	0.914±0.033	
QUINCE					
Lusitanica Quince 1	July	0.773±0.079	1.623±0.091	0.455±0.010	85
Lusitanica Quince 2	August	0.708±0.028	1.258±0.044	0.344±0.003	
Lusitanica Quince 3	October	1.170±0.085	2.410±0.068	0.581±0.005	
Lusitanica Quince 4	November	0.280±0.037	0.920±0.029	0.386±0.010	
WATERMELON					
Fashion Watermelon 1	June	0.330±0.010	0.619±0.017	n.a.	92
Fashion Watermelon 2	July	0.687±0.065	0.860±0.026	0.550±0.010	
Fashion Watermelon 3	July	0.586±0.002	0.706±0.010	0.535±0.017	
Fashion Watermelon 4	August	0.637±0.061	0.668±0.012	0.360±0.017	
Fashion Watermelon 5	August	1.130±0.096	0.816±0.032	0.433±0.003	

Table 2. Antioxidant capacity results (TEAC, FRAP, and ORAC methods) per 100 g weight, and % moisture for vegetable samples ([a] "n.a." when the value is not available)

Vegetable	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
ASPARAGUS					
Green Asparagus 1	February	3.401±0.063	5.130±0.188	n.a.	
Green Asparagus 2	Fefruary	3.02±0.064	3.860±0.281	2.983±0.063	94
Green Asparagus 3	March	3.116±0.253	4.462±0.387	3.167±0.203	
Green Asparagus 4	March	3.660±0.331	4.811±0.182	2.882±0.031	
Green Asparagus 5	March	3.92±0.105	4.825±0.178	2.974±0.156	
ARTICHOKE					
White Tudela Artichoke 1	February	0.670±0.034	1.103±0.028	0.853±0.002	
White Tudela Artichoke 2	March	0.786±0.040	1.08±0.033	0.453±0.031	84
White Tudela Artichoke 3	May	1.450±0.064	2.94±0.111	0.811±0.078	
White Tudela Artichoke 4	November	1.003±0.008	1.719±0.018	0.988±0.047	
CARROT					
Nangro Carrot 1	May	1.480±0.069	1.426±0.013	0.630±0.013	
Nangro Carrot 2	June	0.440±0.027	0.390±0.002	n.a.	91
Nangro Carrot 3	September	0.529±0.047	1.440±0.030	0.369±0.008	
Nangro Carrot 4	November	0.170±0.054	0.540±0.049	0.354±0.008	
EGGPLANT					
Purple-Striped Eggplant 1	March	3.450±0.29	6.432±0.041	n.a.	
Purple-Striped Eggplant 2	April	2.924±0.166	6.348±0.079	2.089±0.094	
Purple-Striped Eggplant 3	May	3.238±0.131	5.140±0.121	0.800±0.016	93
Purple-Striped Eggplant 4	October	2.973±0.182	6.580±0.199	0.798±0.047	
Purple-Striped Eggplant 5	December	2.780±0.040	5.550±0.215	1.013±0.016	
Round Eggplant 1	February	1.420±0.002	2.300±0.090	n.a.	
Round Eggplant 2	April	1.20±0.012	2.215±0.071	1.278±0.094	
Round Eggplant 3	September	1.316±0.077	1.894±0.007	0.595±0.031	92
Long Eggplant 1	February	4.294±0.051	7.980±0.536	n.a.	
Long Eggplant 2	March	3.63±0.113	8.660±0.190	n.a.	
Long Eggplant 3	September	5.14±0.056	5.310±0.167	0.383±0.031	93
Long Eggplant 4	October	4.886±0.367	7.629±0.430	0.519±0.002	
Long Eggplant 5	October	4.342±0.240	7.803±0.487	0.594±0.011	
FAVA BEAN					
Green Fava Bean 1	January	5.39±0.158	3.945±0.040	1.292±0.031	
Green Fava Bean 2	June	4.818±0.142	4.340±0.118	2.530±0.016	62
Green Fava Bean 3	December	4.715±0.111	4.059±0.088	2.248±0.078	
Green Fava Bean 4	December	4.05±0.075	3.130±0.053	2.281±0.031	
GARLIC					
White Garlic 1	May	0.13±0.067	0.386±0.018	2.338±0.047	
White Garlic 2	August	0.55±0.169	0.580±0.057	4.308±0.313	67
White Garlic 3	September	0.304±0.236	0.544±0.017	1.962±0.063	

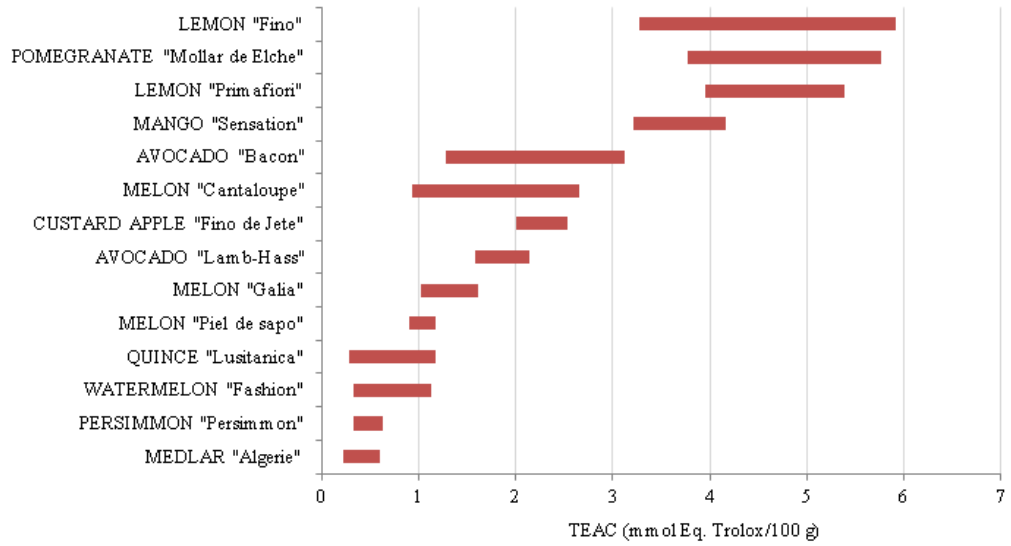
Vegetable	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
White Garlic 4	October	0.240±0.034	0.466±0.025	0.832±0.047	
Red Garlic 1	June	0.363±0.030	0.830±0.026	n.a.	
Red Garlic 2	August	0.59±0.029	0.712±0.016	3.840±0.109	69
Red Garlic 3	September	0.398±0.011	0.723±0.018	3.640±0.234	
Red Garlic 4	October	0.28±0.021	0.550±0.014	3.680±0.469	
GREEN BEAN					
Strike Green Bean 1	April	1.09±0.120	1.520±0.039	0.512±0.0313	93
Strike Green Bean 2	September	1.48±0.108	1.720±0.038	0.995±0.047	
Perona Green Bean 1	February	0.506±0.024	1.000±0.047	0.550±0.031	
Perona Green Bean 2	August	0.59±0.015	0.927±0.016	0.860±0.047	93
Perona Green Bean 3	September	0.27±0.039	0.480±0.025	0.600±0.003	
Perona Green Bean 4	October	0.492±0.052	0.650±0.008	0.668±0.031	
Helda Green Bean 1	February	0.230±0.021	0.360±0.016	n.a.	
Helda Green Bean 2	August	0.310±0.031	0.377±0.018	n.a.	93
Helda Green Bean 3	September	0.690±0.029	0.330±0.030	0.610±0.009	
Helda Green Bean 4	October	0.322±0.011	0.692±0.008	0.532±0.031	
LETTUCE					
Roman Lettuce 1	January	0.468±0.046	0.446±0.003	0.475±0.047	
Roman Lettuce 2	February	0.280±0.051	0.427±0.009	0.521±0.016	95
Roman Lettuce 3	May	0.930±0.070	1.772±0.045	0.968±0.016	
Roman Lettuce 4	November	1.020±0.029	1.965±0.047	0.870±0.013	
Iceberg Lettuce 1	January	0.080±0.012	0.375±0.016	0.497±0.063	
Iceberg Lettuce 3	April	0.403±0.029	0.523±0.001	0.629±0.047	97
Iceberg Lettuce 4	November	1.670±0.018	2.084±0.106	0.616±0.016	
Iceberg Lettuce 5				2.024±0.094	
Baby Lettuce 1	March	3.390±0.178	5.975±0.577	1.988±0.078	
Baby Lettuce 2	October	2.450±0.190	5.360±0.020	1.777±0.078	95
Baby Lettuce 3	December	2.733±0.129	6.360±0.217	2.002±0.063	
ONION					
White onion 1	April	3.190±0.070	3.420±0.194	1.597±0.016	
White onion 2	May	2.454±0.092	6.860±0.085	1.658±0.016	90
White onion 3	July	2.857±0.231	3.250±0.198	2.060±0.031	
White onion 4	September	5.630±0.164	3.270±0.153	0.520±0.011	
Purple Onion 1	January	5.63±0.233	6.170±0.143	3.833±0.094	
Purple Onion 2	March	5.033±0.067	5.556±0.389	2.921±0.188	91
Purple Onion 3	August	5.126±0.009	5.709±0.346	13.797±0.141	
Purple Onion 4	October	3.950±0.354	5.090±0.234	13.830±0.031	
Tender Onion 1	February	3.130±0.242	3.050±0.186	1.597±0.016	
Tender Onion 2	March	2.360±0.141	2.360±0.290	1.658±0.016	89
Tender Onion 3	September	2.450±0.411	3.184±0.455	1.679±0.031	
Tender Onion 4	November	2.870±0.105	3.280±0.643	1.584±0.011	
PEPPER					

Vegetable	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
Yellow Lamuyo Pepper 1	January	11.160±0.627	13.830±0.559	2.769±0.094	
Yellow Lamuyo Pepper 2	August	10.263±0.554	10.909±0.325	1.653±0.031	89
Yellow Lamuyo Pepper 3	September	4.94±0.471	5.530±0.261	1.248±0.005	
Yellow Lamuyo Pepper 4	October	9.448±0.537	8.688±0.202	2.430±0.078	
Red California Pepper 1	February	4.550±0.190	7.962±0.165	0.330±0.016	
Red California Pepper 2	March	5.261±0.147	7.200±0.126	1.478±0.006	89
Red California Pepper 3	August	10.796±0.203	12.110±0.244	1.477±0.005	
Red California Pepper 4	September	13.810±0.159	11.269±0.367	1.500±0.016	
Green Italian Pepper 1	February	4.556±0.307	4.996±0.674	n.a.	
Green Italian Pepper 2	March	5.310±0.004	6.118±0.050	1.457±0.016	
Green Italian Pepper 3	August	3.760±0.405	4.068±0.116	1.573±0.016	92
Green Italian Pepper 4	September	3.380±118	5.850±0.190	1.881±0.078	
Green Italian Pepper 5	September	4.140±0.183	3.840±0.104	2.290±0.047	
POTATO					
Vivaldi Potato 1	February	0.590±0.023	0.399±0.008	0.286±0.006	
Vivaldi Potato 2	April	0.420±0.016	0.200±0.019	0.2622±0.0006	77
Vivaldi Potato 3	July	0.035±0.013	0.216±0.013	0.238±0.003	
Vivaldi Potato 4	December	0.450±0.020	0.287±0.017	0.283±0.005	
Scarlet Potato 1	January	0.276±0.016	0.410±0.022	0.729±0.011	
Scarlet Potato 2	February	0.270±0.004	0.524±0.012	0.750±0.031	77
Scarlet Potato 3	March	0.590±0.020	0.680±0.006	0.729±0.031	
Scarlet Potato 4	November	0.426±0.025	0.700±0.013	0.550±0.008	
TOMATO					
Green Raf Tomato 1	January	1.528±0.140	5.994±0.161	n.a.	
Green Raf Tomato 2	March	1.476±0.082	5.473±0.084	0.965±0.031	
Green Raf Tomato 3	May	1.380±0.081	6.000±0.068	0.834±0.063	93
Green Raf Tomato 4	July	1.781±0.112	6.720±0.552	1.222±0.031	
Green Raf Tomato 5	October	1.950±0.112	4.610±0.454	1.340±0.031	
Red Daniela Tomato 1	Fefruary	1.686±0.151	3.030±0.062	0.844±0.016	
Red Daniela Tomato 2	May	1.950±0.126	3.563±0.054	1.016±0.047	93
Red Daniela Tomato 3	August	1.834±0.1185	5.000±0.240	1.254±0.031	
Red Daniela Tomato 4	October	1.280±0.153	4.635±0.171	1.188±0.031	
Green Pinton Tomato 1	January	1.264±0.067	4.220±0.047	n.a.	
Green Pinton Tomato 2	March	1.050±0.038	4.065±0.182	0.788±0.008	
Green Pinton Tomato 3	May	1.266±0.017	3.818±0.272	0.772±0.013	93
Green Pinton Tomato 4	July	1.810±0.082	2.910±0.288	1.034±0.016	
Green Pinton Tomato 5	October	1.501±0.050	3.438±0.185	1.250±0.047	
ZUCCHINI					
Green Zuccini 1	February	2.218±0.175	1.780±0.007	1.420±0.047	
Green Zuccini 2	March	2.520±0.071	0.796±0.019	1.072±0.009	80
Green Zuccini 3	June	2.363±0.166	0.830±0.010	1.083±0.031	
Green Zuccini 4	September	1.030±0.062	0.780±0.024	1.020±0.016	

Vegetable	Harvest Date (2011)	TEAC (mmol eq Trolox/100 gr)	FRAP (mmol eq FeSO4/100 gr)	ORAC (mmol eq Trolox/100 g)	Moisture %
Round Zucchini 1	April	1.090±0.070	0.660±0.014	0.607±0.019	80
Round Zucchini 2	May	0.997±0.032	0.310±0.031	0.896±0.025	
Round Zucchini 3	July	0.440±0.031	0.360±0.043	0.480±0.031	
Round Zucchini 4	September	0.578±0.004	0.540±0.062	0.499±0.006	
Organic Zucchini 1	March	1.640±0.098	3.090±0.076	1.728±0.002	80
Organic Zucchini 2	June	1.822±0.152	2.760±0.025	1.477±0.063	
Organic Zucchini 3	September	1.942±0.128	2.920±0.079	1.391±0.023	
Organic Zucchini 4	December	2.160±0.047	2.660±0.045	1.656±0.063	

The TEAC, FRAP, and ORAC results are also represented graphically in Figs. 2, 3, and 4, respectively. These graphs presented the results as a range of antioxidant capacity related to the availability period of the samples in the market. This information broadens the under- standing of the beneficial effects provided by consuming fruits and vegetables grown in Andalusia.

A



B

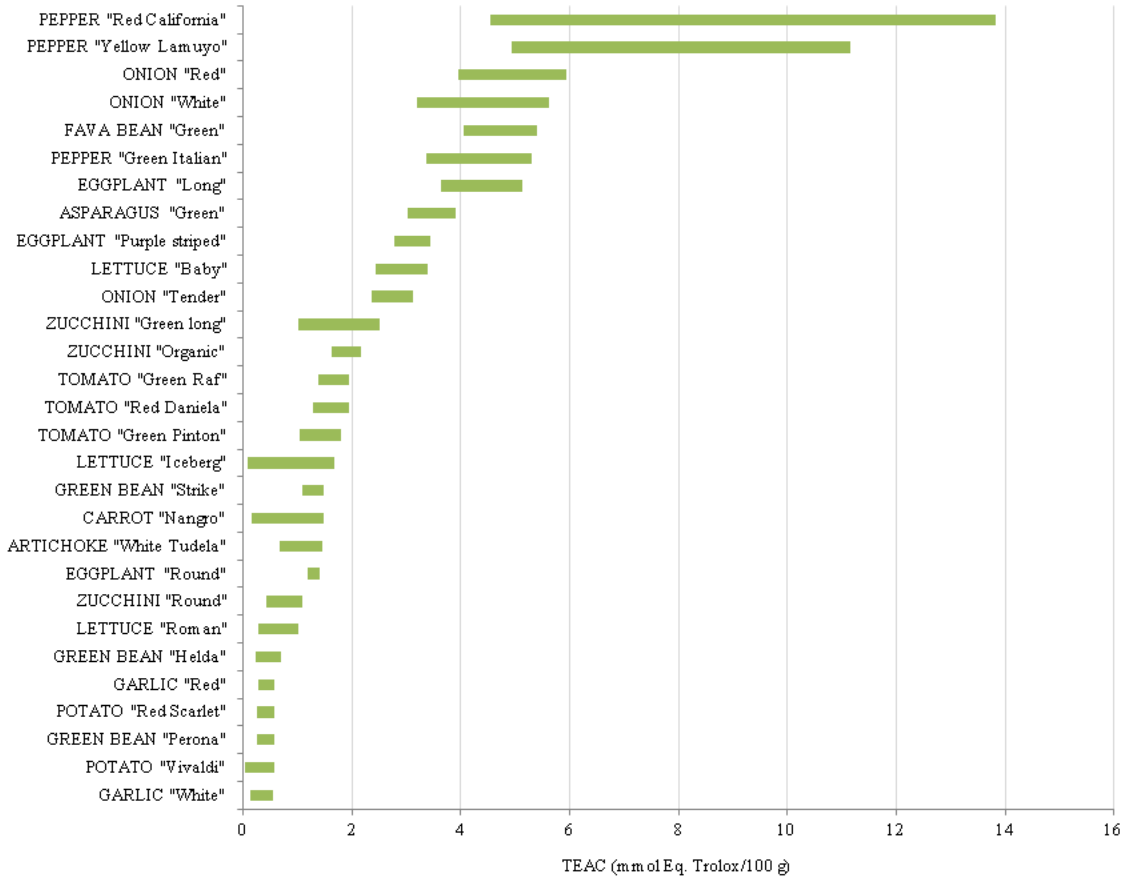
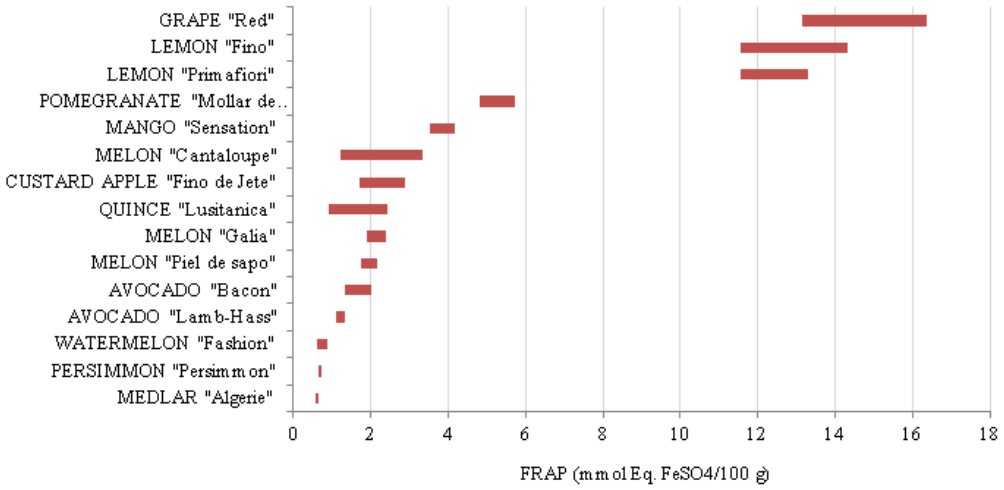


Fig. 2. Antioxidant capacity of fruit cultivars (A) and vegetable cultivars (B) determined by using TEAC assay

A



B

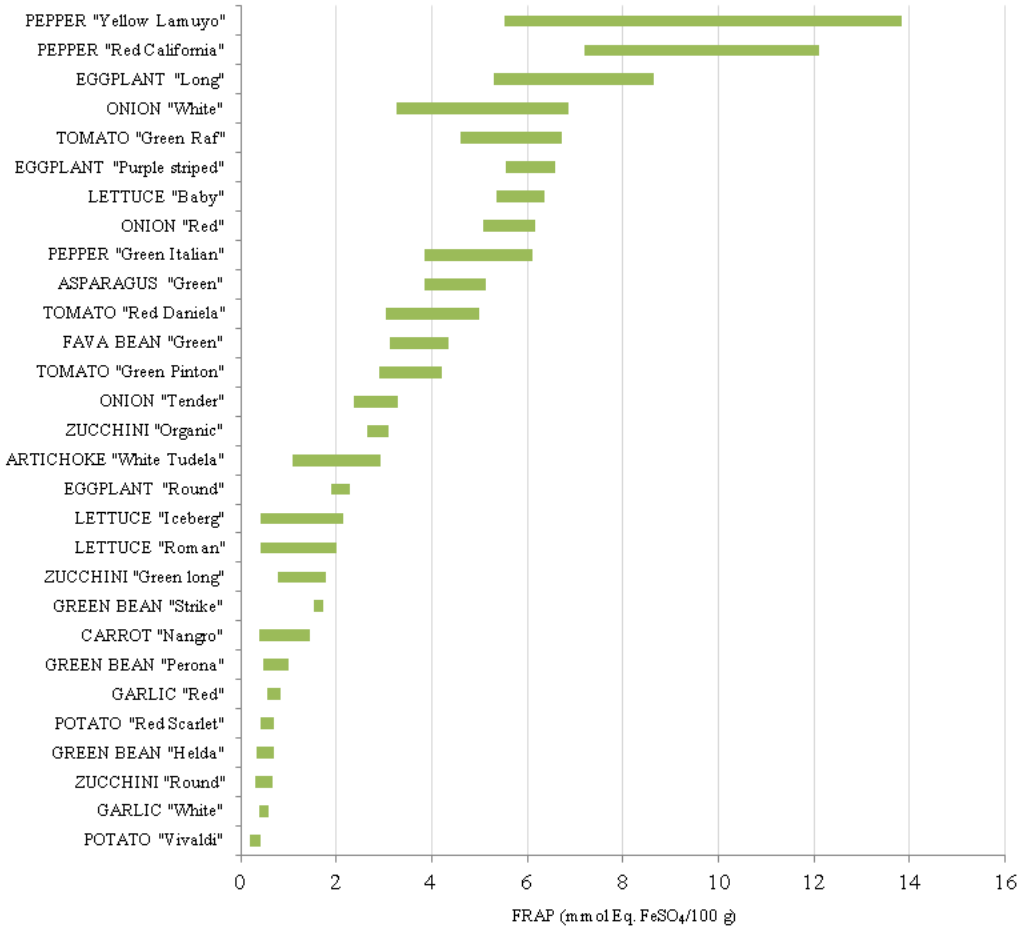
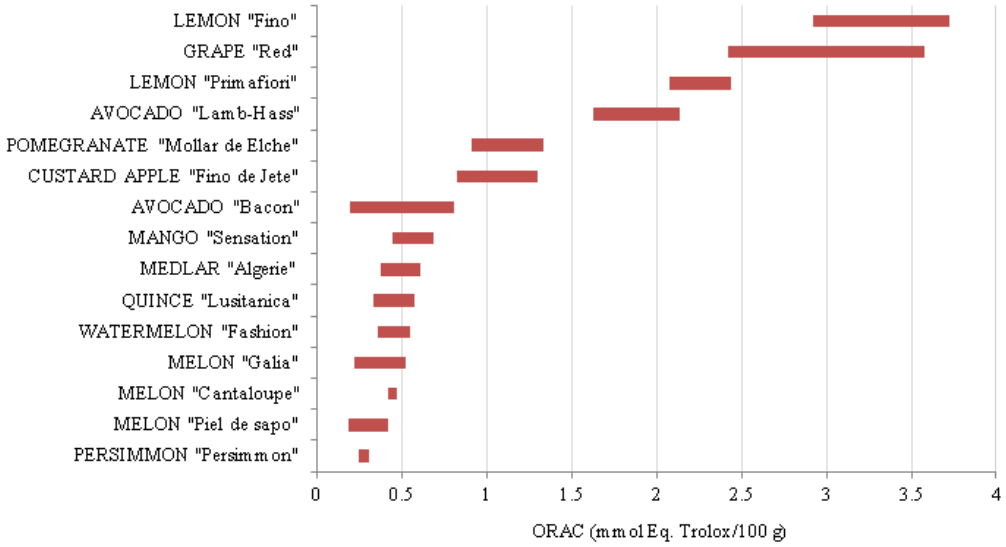


Fig. 3. Antioxidant capacity of fruit cultivars (A) and vegetable cultivars (B) determined by using FRAP assay.

A



B

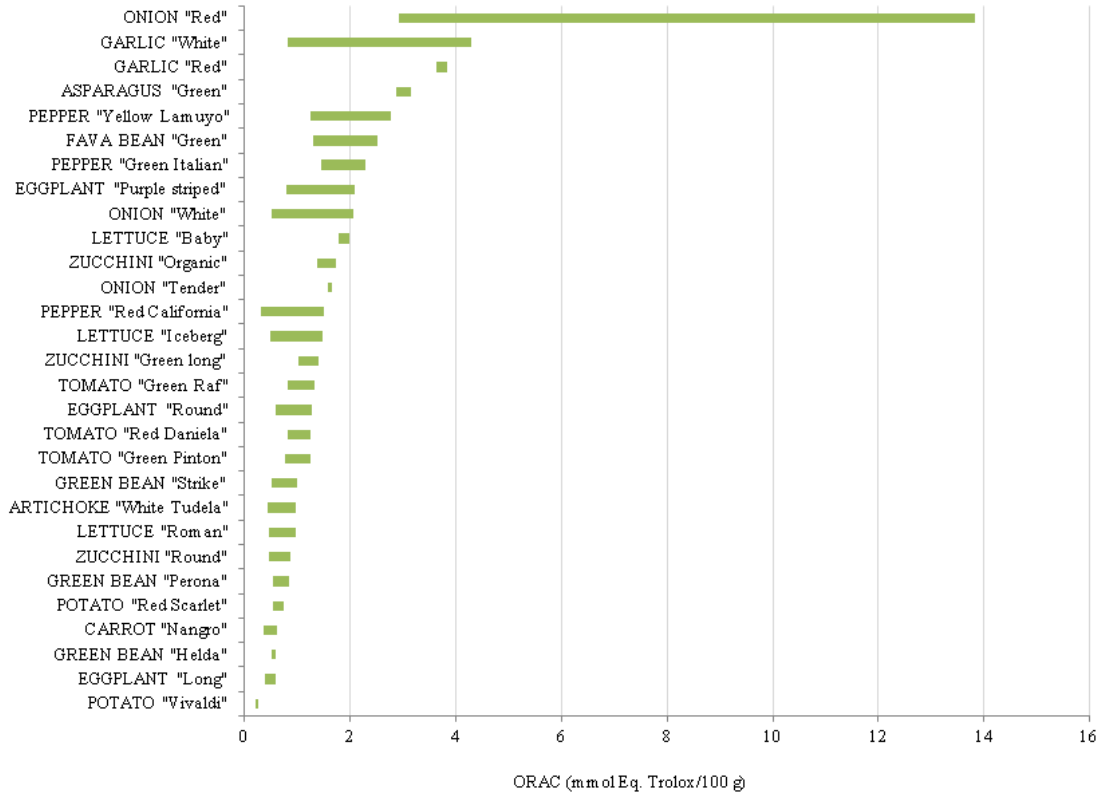


Fig. 4. Antioxidant capacity of fruit cultivars (A) and vegetable cultivars (B) determined by using ORAC assay.

It is noteworthy that the results varied depending on the assay used; that is, the antioxidant values based on electron transfer (TEAC and FRAP assays) differed from those based on hydrogen atom transfer (ORAC assay).

Generally, in the cases in which the edible portion consists only of the pulp, antioxidant capacity values were much lower than the results in other studies that included skin and/or seeds. In fact, several papers have studied the antioxidant capacity of by products from industries such as wine production, and these wastes have proved to be excellent sources of antioxidants (Arvanitoyannis, Ladas, & Mavromatis, 2006). Some studies have revealed that genotype is the parameter which most influences the phytochemical composition and, therefore, the antioxidant capacity. Therefore, the results of antioxidant capacity from different cultivars of the same species are quite similar (Rodríguez-Pérez *et al.*, 2013). The antioxidant capacity varied according to the genotype and the method used; in addition, it was also strongly influenced by the physiological state of the plants. As reflected in Figs. 2, 3, and 4, the antioxidant capacity ranged widely for some cultivars such as red and yellow peppers, and red onion. This fact discloses that the antioxidant capacity varied significantly among the samples collected at different times of the harvesting period. Several examples of these differences among samples are specifically compared in the following subsections with the seasonal timetable of fruits and vegetables from the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA). The results for the fruits and vegetables are explained separately.

3.1. Antioxidant capacity of the fruits

For fruits, the FRAP values varied from 0.560 to 16.380 mmol eq. $FeSO_4$/100 g, TEAC values varied from 0.230 to 5.920 mmol eq. Trolox/100 g, and ORAC values varied from 0.190 to 3.719 mmol eq. Trolox/100 g. Generally, the ORAC values were lower than those of the other two assays. As shown in Figs. 3A and 4A, lemon, ‘red’ grape, and pomegranate showed the highest antioxidant capacity in all the methods assessed. According to TEAC and ORAC (Figs. 2A

and 4A, respectively), the values found for the two lemon cultivars were relatively similar, while the antioxidant values shown by the FRAP assay was much higher than by the other methods (Fig. 3A). In all the methods assessed, the antioxidant capacity of 'Fino' lemon was slightly higher than that of 'Primafiori' lemon. For pomegranate and 'red' grape, studies on their antioxidant capacity are limited since most research papers deal with juice extracts (El Kar, Ferchichi, Attia, & Bouajila, 2011; Elfalleh *et al.*, 2011). The FRAP and TEAC values from pomegranate 'Mollar de Elche' cultivar varied from 4.830 to 5.720 mmol eq. $FeSO_4$/100 g, and from 3.770 to 5.770 mmol eq. Trolox/100 g, respectively. Concerning the differences of antioxidant capacity among the different acquisition times of pomegranate samples, it is outstanding that TEAC and FRAP values were similar for the samples collected in September and October, and slightly lower for the samples collected in August than the others (Table 1). This might be explained by the fact that August corresponds to an early harvest time for this fruit (MAGRAMA, 2013). Fu *et al.* (2011) studied a large set of samples by using FRAP and TEAC assays, and their results agree with our data in that the pomegranate showed a high antioxidant capacity (Fu *et al.*, 2011). However, the ORAC results cannot be compared to data from the literature because few studies use this assay to analyze the edible portion in pomegranate and 'red' grape.

The least bioactive fruit samples differed according to the method used. In the TEAC and FRAP assays, the samples that registered the lowest antioxidant capacity were loquat, persimmon, and watermelon, in this order. However, 'Galia' and 'Piel de sapo' melon cultivars together with 'Bacon' avocado presented the lowest antioxidant capacity in the ORAC assay. The total phenolics of loquat extracts have been widely reported (Gruz, Ayaz, Torun, & Strnad, 2011). Nevertheless, little complementary information is available on ORAC, TEAC or FRAP values for loquat samples. Another sample showing a low antioxidant capacity in the TEAC assay was quince; this information cannot be compared to data from other studies because the antioxidant capacity reported in

literature refers mostly to jams and/or is from the DPPH assay (Silva *et al.*, 2004; Wojdylo, Oszmianski, Teleszko, & Sokól-Letowska, 2013). In this study, persimmon registered TEAC values of 0.330 to 0.630 mmol eq. Trolox/100 g, and FRAP values of 0.660 to 0.730 mmol eq. $FeSO_4$/100 g. These results are consistent with the literature (Guo *et al.*,2003). Little data is available from the ORAC assay in persimmon. The watermelon cultivar analyzed, 'Fashion' registered TEAC values of 0.330 to 1.130 mmol eq. Trolox/100 g, FRAP values of 0.619 to 0.860 mmol eq. $FeSO_4$/100 g, and ORAC values of 0.360 to 0.550 mmol eq. Trolox/100 g. These values showed a low antioxidant capacity, which agrees with the results reported by other authors (Fu *et al.*, 2011). As shown in Table 1, the watermelon samples collected in July were similar to each other in all the methods employed. The lowest TEAC value was found with the first sample of 'Fashion' watermelon. This is because it was collected in June, a date close to the early harvest date (MAGRAMA, 2013). Of 'Cantaloupe', 'Galia' and 'Piel de sapo' melon cultivars analyzed in the present study, the cultivar that showed the lowest antioxidant capacity was 'Piel de sapo'. For this cultivar, TEAC values varied from 0.910 to 1.170 mmol eq. Trolox/100 g, FRAP varied from 1.730 to 2.170 mmol eq. $FeSO_4$/100 g, and ORAC values varied from 0.190 to 0.420 mmol eq. Trolox/100 g. Guo *et al.* (2003), using an ORAC assay, also characterized melon as one of the fruits with the least antioxidant capacity. The low antioxidant capacity values of melon and watermelon could be due to the fact that most of the compounds contributing to antioxidant capacity in these fruits are found in the seeds and skin (Guo *et al.*, 2003). For 'Bacon' avocado, TEAC values ranged from 1.281 to 3.130 mmol eq. Trolox/100 g, FRAP values were from 1.330 to 2.020 mmol eq. $FeSO_4$/100 g, and ORAC values were lower than the other methods (from 0.198 to 0.814 mmol eq. Trolox/100 g). Concerning the differences of antioxidant capacity between the acquisition dates of avocado samples, 'Bacon' cultivar was collected in March, June, and October. The best results were found with the'Bacon' avocado samples collected in June for all the methods. In the case of the other avocado cultivar, 'Lamb-Hass', the

antioxidant capacity values were higher for the samples of June than March in TEAC and ORAC assays. These results may have occurred because the harvesting period of avocado is from May to November, these two months being the early and the late harvest times, respectively (MAGRAMA, 2013).

3.2. Antioxidant capacity of the vegetables

For the vegetables analyzed in the present study, as a whole, TEAC values ranged from 0.035 to 13.810 mmol eq. Trolox/100 g, FRAP values varied from 0.200 to 13.830 mmol eq. $FeSO_4$/100 g, and ORAC values varied from 0.238 to 13.830 mmol eq. Trolox/100 g.

The 'red' and 'yellow' pepper cultivars showed the highest anti-radical capacity in both the TEAC and FRAP assays (Figs. 2B and 3B, respectively). The third-highest TEAC and FRAP values corresponded to 'red' onion and 'long' eggplant, respectively. Bahorun, Luximon-Ramma, Crozier, and Aruoma (2004) studied a set of 10 Mauritian vegetables, proposing that pepper belonged to a group having a medium level of total phenol content while onion belonged to a group having a high level. In our study, TEAC and FRAP values were the opposite, in the sense that the pepper samples showed more antioxidant capacity than 'white' onion. This disagreement with the literature could be because the results of Bahorun *et al.* (2004) refer to a hot pepper rather than a sweet type (Bahorun *et al.*, 2004).

Antioxidant values for 'red' onion ranged from 3.950 to 5.950 mmol eq. Trolox/100 g (TEAC), and from 5.090 to 6.170 mmol eq. $FeSO_4$/100 g (FRAP). These data are similar to those of Gorinstein *et al.* (2009), according to which red onion had the highest antioxidant capacity. However, Li *et al.* (2012) reported that antioxidant capacity of 'red' onion was lower than in other highly pigmented (red or purple) vegetables (Gorinstein *et al.*, 2009; Li *et al.*, 2012).

With respect to ORAC results, the samples that showed the highest antioxidant capacity were 'red' onion, 'white' and 'red' cultivars of garlic, and 'green'

asparagus, in that order (Fig. 4B). Despite the garlic cultivars had high ORAC values, the antioxidant capacity in these samples according to the TEAC and FRAP assays was low (from 0.130 to 0.590 mmol eq. Trolox/100 g and from 386 to 0.830 mmol eq. $FeSO_4$/100 g, respectively). According to the ORAC results, the antioxidant capacity of 'white' garlic is not clearly higher than that of 'red' garlic because the range shown by 'white' garlic is wider than that of the 'red' garlic. This reveals that the antioxidant capacity of 'white' garlic varies greatly among the samples collected at different times in the same year. 'Green' asparagus showed TEAC values ranging from 3.020 to 3.920 mmol eq. Trolox/100 g. These data agree with the results reported by Tiveron *et al.* (2012), where the antioxidant activity of Brazilian vegetables was reported and asparagus showed considerable antioxidant activity (Tiveron *et al.*, 2012). Regarding the different collection times of asparagus, very little variation was detected among the samples (Table 1). It can be highlighted that the early collection time of February corresponded to the lowest values of antioxidant capacity in TEAC and FRAP assays (MAGRAMA, 2013).

The antioxidant capacity of fava bean can also be highlighted, ranking the fifth and sixth highest in the TEAC (from 4.050 to 5.390 mmol eq Trolox/100 g) and ORAC (from 1.292 to 2.530 mmol eq. Trolox/100 g) results, respectively. Regarding the harvest and the intake of fava beans, the most suitable date is January (MAGRAMA, 2013). This fact could explain why the highest TEAC value appeared in the sample collected during this month. The vegetable with the lowest antioxidant capacity was 'Vivaldi' potato in all the methods assessed. While the ORAC values for this potato ranged from 0.238 to 0.286 mmol eq. Trolox/100 g, the antioxidant capacity of 'Scarlet' potato measured with the same method varied from 0.550 to 0.750 mmol eq. Trolox/100 g. A lower antioxidant capacity was expected for 'Vivaldi' than for red 'Scarlet' as a high antioxidant capacity has been previously reported for the latter. Hu, Tsao, Liu, Alan Sullivan, and McDonald (2012) suggested that purple/red potato cultivars have a greater antioxidant capacity than white or yellow cultivars, indicating that

anthocyanins are major antioxidants (Hu *et al.*, 2012). The third-highest FRAP results corresponded to long eggplant (5.310-8.660 mmol eq. $FeSO_4$/100 g). However, this eggplant cultivar showed the second lowest antioxidant capacity in the ORAC assay (0.383-0.594 mmol eq. Trolox/100 g). These ORAC values are similar to those reported by Stangeland, Remberg, and Lye (2009), eggplant values being just lower than sweet potato (Stangeland *et al.*, 2009). Eggplant is available all year, except for summer (MAGRAMA, 2013). This could explain the low values found with the FRAP and ORAC methods when the sample was harvested in September.

3.3. Structure-antioxidant capacity relationships

Typical phenolics that possess antioxidant capacity are known to be mainly phenolic acids and flavonoids. Phenolic acids are a major class of phenolic compounds, widely occurring in the plant kingdom, especially in fruits and vegetables (Wojdylo, Oszmianski, & Czemerys, 2007). Flavonoids are the main polyphenols present in a wide variety of plant sources. In general, antioxidant capacity of these compounds has been attributed to their electron-donating ability. Structure-activity relationship (SAR) studies have shown that structure and substitution pattern of hydroxyl groups are essential for effective free-radical scavenging capacity (Sekher Pannala, Chan, O'Brien, & Rice-Evans, 2001).

Among fruits, grape, pomegranate and lemon showed higher antioxidant values than did others such as persimmon and loquat. The high antioxidant capacity of grape samples may have occurred because of their content of phenolic compounds belonging to four phenolic classes (anthocyanins, flavonols, hydroxycinnamic acids, and flavan-3-ols) (Guerrero *et al.*, 2009). In pomegranate, it has been demonstrated that punicalagin isomers (ellagitannins in which gallagic and ellagic acids are linked to a glucose molecule) are the leading bioactive constituents responsible for the high antioxidant capacity of this fruit (Mena *et al.*, 2011). In the case of lemon samples, the results might be explained by the flavonoid family compounds (quercetin and apigenin derivatives), mostly

reported in this fruit (García-Salas *et al.*, 2013). The low antioxidant capacity shown by the persimmon, loquat, and melon cultivars can be explained by their poor flavonol content (García-Alonso *et al.*, 2004; Sakakibara, 2003). Specifically, melon Spanish samples have a predominant group of phenolic antioxidants, which is composed by phenolic acids (Rodríguez-Pérez *et al.*, 2013).

The vegetables with the strongest antioxidant capacities according to the FRAP, TEAC, and ORAC assays were pepper and onion, perhaps due to their phenolic content. According to the literature, most of the metabolites identified in pepper samples were phenolic acids (hydroxybenzoic, hydroxycinnamic, and phenylacetic acids) such as vanillic and caffeic acid derivatives, and flavonoids such as quercetin, luteolin, and naringenin glycosides (Morales-Soto *et al.*, 2013). Regarding onion samples, quercetin and kaempferol as well as their glycosides have been reported to be the most abundant flavonoids in the acid hydrolyzed onion samples, and therefore, the compounds that probably influence more strongly the antioxidant capacity of this vegetable (Price & Rhodes, 1997). The major flavonoids of onion bulb are quercetin-3,4-O-diglucoside and quercetin-4-monoglucoside (Nuutila, Puupponen-Pimiä, Aarni, & Oksman-Caldentey, 2003). On the contrary, potato samples showed the lowest antioxidant capacity. Sakakibara (2003) demonstrated that root vegetables such as potato contained only simple phenols such as cinnamic and benzoic acid derivatives (Sakakibara, 2003).

4. Conclusions

The antioxidant capacity of 44 fruits and vegetables from Andalusia (Spain) was evaluated using three different methods (TEAC, FRAP, and ORAC). The results varied depending on the assay used and the sample analyzed, with lemon, pomegranate, grape, avocado, pepper, onion, garlic, asparagus, and eggplant being the fruits and vegetables which registered the highest antioxidant capacity.

Loquat, persimmon, melon, watermelon, and potato had the lowest antioxidant values.

In conclusion, this work compares the antioxidant capacity of different fruit and vegetable cultivars. The results reveal that several Andalusian cultivars are a good source of antioxidant compounds. Since the samples were collected at different times of the year taking into account the agricultural export abroad, the information compiled in this study offers a preliminary characterization of the antioxidant capacity of fruits and vegetables available year round on the European Market.

Acknowledgments

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CAPÍTULO 3

Garcinia mangostana fruit: Determination of the total antioxidant activity in extracts and phenolic compound characterization using CE-MS(TOF)

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Functional Plant Science and Biotechnology

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Garcinia mangostana Fruit: Determination of the Total Antioxidant Activity in Extracts and Phenolic Compound Characterization using CE-MS(TOF)

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Abstract

Xanthones are an important family of polyphenols present in *Garcinia mangostana* fruit. Products of *G. mangostana* have begun to be commercialized as a dietary supplement because of their potent antioxidant properties. Interest in their beneficial health effects such as antioxidant characteristics, lipid profile and anticarcinogenic activity, etc., has encouraged scientific research to determine compounds responsible for these properties. The antioxidant capacity determination and the phenolic compound characterization in extracts of *G. mangostana* were investigated. High antioxidant capacity was determined in whole fruit dried extract by ORAC, FRAP and $ABTS^{\bullet+}$ assays. The values obtained by FRAP, ORAC and ABTS assays were 1222 and 4088 and 3590 μM TE/g, respectively. Phenolic compound identification in these extracts was carried out by capillary electrophoresis coupled to ionization by electrospray-mass spectrometry (CE-ESI-MS) with a time of flight analyzer (Micro TOF), and a sensible, fast and efficient method was developed. The CE-ESI parameters were optimized to obtain good separation and suitable sensitivity. The CE parameters were: 80 mM ammonium acetate, pH 10.5, 30 kV, 15 s for injection and fused-silica capillary of 50 μm i.d. and 100 cm in length. The ESI-MS parameters were also optimized: drying gas flow of 4L/min, 300°C, nebulization gas pressure at 4 psi, sheath liquid composition and flow (60:40)

isopropanol/water + 0.1% triethylamine to 0.18 mL/h. Spectrometric determination was achieved in negative polarity and the mass range was between 100-800 *m/z*. Considering the scarcity of commercial standards and the scanty bibliography references, some of the most important xanthones from *G. mangostana* fruit have been identified using CE-ESI-MS (micro TOF), such as Garcinine C, Garcinone E, Mangostenone C and β-mangostin.

Keywords: ABTS, capillary electrophoresis, FRAP, mangosteen, ORAC.

Abbreviations: AAPH, 2,2'-azobis-(2-amidinopropane)-dihydrochloride; **AUC**, area under the curve; **AWA**, acetone/water/acetic acid; **CE**, capillary electrophoresis; **CUPRAC**, cooper reduction capacity assay; **DPPH**, 2, 2-diphenyl-1-picrylhydrazyl; **ESI**, electrospray ionization; **FC**, Folin-Ciocalteau; **FRAP**, ferric ion reducing antioxidant power; **HAT**, hydrogen transfer mechanism; **MS**, mass spectrometry; **ORAC**, oxygen radical absorbance capacity; **SET**, single electron transfer; **TEAC**, trolox equivalent antioxidant capacity; **TOFMS**, time of flight-mass spectrometry; **TPTZ**, 2, 4, 6-tripiridil-s-triazine; **TRAP**, total reducing ability of plasma; **TROLOX**, 6-Hydroxy- 2, 5, 7, 8-tetramethylchroman-2-carboxylic acid.

INTRODUCTION

Oxidative stress has been associated with the development of many chronic and degenerative diseases, including cancer (Ames *et al.* 1995), heart disease (Diaz *et al.* 1997) and neuronal degeneration (Scalbert *et al.* 2005) and is involved in the process of aging (Ames *et al.* 1993). A diet rich in fruits and vegetables is considered as an excellent source of antioxidants (Block *et al.* 1992; Ness and Powles 1997). Vitamins C and E, polyphenols and carotenoids are considered to be responsible for most of the antioxidant activity in food (Wu *et al.* 2004). But, in terms of disease prevention, a clinical trial with whole fruits and vegetables rather than using vitamin C, E or carotenoid supplements are more likely to give positive (Wu *et al.* 2004).

Garcinia mangostana L. (Clusiaceae), commonly known as mangosteen, is a tropical evergreen tree. The fruit has been used in traditional medicine for the treatment of skin infections, wounds, and diarrhea, is recognized to improve cardiovascular health (Peres *et al*. 2000; Leontowicz *et al*. 2006), and has anti-inflammatory, antitumor, antioxidant and antibacterial activity (Suksamrarn *et al*. 2003; Moongkarndi *et al*. 2004; Suksamrarn *et al*. 2006). The major secondary metabolites of mangosteen have been found to be prenylated xanthone derivatives. Alpha and gamma-mangostins exhibited a powerful antioxidant activity (Jung *et al.* 2006). Studies on extracts or xanthones obtained from the pericarp of the fruit or isolated from the young fruit have been assayed to determine the anticancer properties (Peres *et al*. 2000; Itoh *et al.* 2008) and these have demonstrated cytotoxic properties against three human cancer cell lines (Ho *et al.* 2002; Suksamrarn *et al.* 2006). Pericarp extract from *G. mangostana* exhibited potent antileukemic activity (Chiang *et al.* 2004) and the most abundant xanthone from *G. mangostana*, α-mangostin, showed a possible cancer chemopreventive activity (Chin *et al*. 2008).

Recently, products manufactured from *G. mangostana* have begun to be used as dietary supplements in the United States, because of their potent antioxidant properties. Mangosteen have a high concentration of bioactive compounds, high

antioxidant potential and positively affect plasma lipid profile and plasma antioxidant activity in rats fed cholesterol-containing diets (Leontowicz *et al.* 2006). Mangosteen fruit juice has become a major botanical dietary supplement, and was ranked as one of the top-selling "botanicals" on the market in 2005 (Nutrition Business Journal 2006).

The antioxidant capacity of mangosteen has been assayed from pericarp or pulp extracts or xanthones isolated from the pericarp of fruit (Jung *et al*. 2006), however little additional information has been reported.

A number of methods have been developed to measure the efficiency of dietary antioxidants, either as pure compounds, botanicals or in foods, food extracts, as well as to determine the antioxidant activity of plasma as an index of the antioxidant status *in vivo*. It is clear that no one antioxidant capacity assay will truly reflect the "total antioxidant capacity" of a particular sample. Total antioxidant capacity needs to reflect both lipophilic and hydrophilic capacity, and at least for physiological activity it needs to reflect and differentiate the mechanisms of the antioxidant defence (Prior *et al*. 2005). Many of the frequently cited assays can be broadly categorised as hydrogen transfer mechanism (HAT) such as ORAC (oxygen radical absorbance capacity; Cao *et al*. 1995) and TRAP (total reducing ability of plasma; Ghiselli *et al*. 1995) assays and mechanisms based on single electron transfer (SET), including FC (Folin-Ciocalteau; George *et al*. 2005), TEAC (Trolox equivalent antioxidant capacity; Miller *et al*. 1993), FRAP (ferric ion reducing antioxidant power; Benzie and Strain 1999), 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sanchez-Moreno *et al.* 1998), and the copper reduction capacity assay (CUPRAC; Apak *et al.* 2004) between others. These methods measure the radical scavenging capacity or the reducing ability, respectively.

Certain methods were suggested to be evaluated for standardization at the "First International Congress on Antioxidant methods" congress in Orlando in 2004; ORAC, the Folin-Ciocalteau assay and TEAC were suggested to be used in the

routine quality control and measurement of antioxidant capacity of food, dietary supplements and other botanicals (Prior *et al*. 2005).

Many studies have shown that mangosteen is a rich source of phenolic xanthones and actually more than 40 xanthones have been isolated and identified (Nilar *et al*. 2005; Ee *et al*. 2006; Suksamrarn *et al*. 2006). In this sense, a few chromatographic methods have been proposed for the identification of phenolic compounds in *G. mangostana* (Haruenkit *et al*. 2007; Ji *et al*. 2007). Due to its high efficiency, flexibility, very high resolution and rapidity of the method, capillary electrophoresis (CE) has gained widespread interest as a favorable technique for the analysis of phenolic compounds (Arráez-Román *et al.* 2006; Gómez Romero *et al*. 2007; Carrasco-Pancorbo *et al.* 2007; Arráez Román *et al.* 2008). It has become an alternative or complement to chromatographic separations for the analysis of phenolic compounds because it needs no derivatization step, requires only small amounts of sample and buffer and has proved to be a high-resolution technique. The advantages of mass spectrometry (MS) detection include the capacity to determine molecular weights and provide structural information. Also time of flight-mass spectrometry (TOF-MS) provides excellent mass accuracy over a wide dynamic range, if modern detector technology is chosen. The latter, moreover, allows measurements of the isotopic pattern, providing important additional information for the determination of elemental composition. Thus, the on-line coupling of CE with TOF-MS yields a powerful technique for the analysis of phenolic compounds.

The goal of this study was to determine, by different assays, the antioxidant capacity of whole fruit (pericarp and pulp) and describe the first application of CE-ESI-TOF-MS for the identification of phenolic compounds in *G. mangostana*.

MATERIALS AND METHODS

Reagents and materials

The reagents used for antioxidant capacity assays were 2,2'-azobis-(2-amidinopropane)-dihydro-chloride (AAPH), 6-hydroxy-2,5,7,8-tetra-methyl-chroman-2-carboxylic acid (Trolox) and fluorescein (sodium salt), 2,2'-azinobis-(3-etilbenzotiazolin)-6-sulfonic, TPTZ (2,4,6-tripiridil-s-triazine), all obtained from Sigma Aldrich (Steinheim, Germany). Other reagents (hexane, acetone and dichloromethane, acetic acid, sodium phosphate dihydrate, sodium acetate trihydrate, clorhidric acid, ferric cloride) were purchased from Panreac (Barcelona Spain), and the 96-well polystyrene microplate for fluorescent measures from Biogen Científica (Madrid, Spain).

All chemicals used for characterization of phenolic compounds by CE-ESI-TOF-MS were of analytical reagent grade and used as received. Ammonium hydroxide was purchased from Fluka (Buchs, Switzerland) and ammonium acetate from Merck (Darmstadt, Germany). 2-propanol HPLC grade, used in the sheath flow, methanol, and sodium hydroxide, used for capillary cleaning procedures before each analysis, were obtained from Panreac and triethylamine from Sigma-Aldrich. Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA). CE buffers were prepared by weighing ammonium acetate at the concentrations indicated and adjusting the pH when necessary by adding 0.5 M ammonium hydroxide. The buffers were stored at 4°C and warmed to room temperature before use. All solutions were filtered through 0.45 µm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary column.

Sample extraction

1. Antioxidant capacity methods

The Prior *et al.* (2003) procedure of sample separation was followed. Mangosteen (pericarp and pulp) dried extract sample (1 g) was initially extracted in a 15-mL screw-cap tube with 10 mL of hexane: dichloromethane (1:1), followed by centrifugation to 9000 rpm for 15 min and removal of the hexane layer; this extraction was repeated twice. The residue was extracted with 10 mL AWA (acetone/water/acetic acid; 70:29.5:0.5, v/v/v). After the addition of solvent, the tube was vortexed for 30 s, followed by sonication at 37 °C for 5 min. The tube was kept at room temperature for 10 min, centrifuged at 9000 rpm for 15 min and the supernatant was removed. The samples were extracted one more time with 10 mL AWA using the same procedure, and the supernatants were combined. The supernatants were transferred to a 25 mL volumetric flask, and AWA was added to make up a final volume of 25 mL. The hydrophilic solution from the extracted sample was then diluted as appropriate to be applied to different antioxidant methods. Each sample was extracted in triplicate and assayed in duplicate.

2. Characterization of phenolic compound

Ten kinds of extraction procedures were used to compare which gave mainly phenolic compounds. The extraction procedure which was selected due to its ability to release a major number of compounds for characterization is as follows: 0.5 g of the dried sample were weighted and extracted with 5 mL methanol: H_2O (50:50, v/v), followed by centrifugation to 4000 rpm for 4 min and filtered through 0.2 µm Millipore (Bedford, MA, USA) membrane filter. Finally the extract was kept at -4°C until analysis.

Antioxidant capacity methods

1. ORAC assay

The antiradical activity against AAPH was estimated according to a slightly modified procedure reported by Prior *et al.* (2003). Fluorescent measurements were carried out on a spectrofluorimeter Polarstar Optima (BMG Labtechnologies) thermostated and a fluorescence filter with an excitation wavelength of 490 nm and an emission wavelength of 545 nm was used. The hydrophilic solution from the extracted sample was diluted (1/3500) as appropriate with 75 mM phosphate buffer solution (pH 7.4). The hydrophilic dilution and fluorescein (70 nM) solution were placed in each well of a black 96-well polystyrene microplate. Finally, AAPH solution (36 mM) was added rapidly using the multichannel micropipette, and fluorescence was recorded during 37 cycles, every 210 s, at 37°C. A blank (fluorescein + AAPH) using phosphate buffer (75 mM) instead of the sample solution and eight calibration solutions using Trolox (10, 20, 30, 40, 50, 60, 70, 80 µM) as antioxidant were also carried out in each assay.

The final ORACFL values were calculated by using a regression equation (y = 0.1577X+1.8121, r^2 = 0.9928) between Trolox concentration (Y) (µM) and net area under the FL decay curve (X).

Area under the curve (AUC) was calculated according to the equation:

$$AUC = 1 + F_1/F_0 + F_2/F_0 + \ldots + F_n/F_o$$

where F_0 is the initial fluorescence reading at 0 min, F_1 is the fluorescence intensity reading at time 3.5 min and F_n is the fluorescence intensity reading at 129.5 min (37 cycles of 210 s). Finally, the net AUC was calculated by subtracting the AUC of the blank sample from the AUC of each sample. ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay and final results were expressed in µmol equivalents of Trolox per g of sample.

2. ABTS$^{\bullet+}$ assay

The antioxidant capacity was estimated in terms of radical scavenging activity following the procedure described by Re *et al.* (1999) and Pulido *et al.* (2003). Absorbance was measured on a spectrophotometer Lambda 3B (Perkin Elmer). Briefly, ABTS$^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS$^{\bullet+}$ solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. After addition of 40 µL of hydrophilic solution (previously diluted 1/64 for *G. mangostana*), Trolox, or blank (phosphate buffer) to 4 mL of diluted ABTS$^{\bullet+}$ solution, an absorbance reading was taken at 20 min. Eight calibration solutions using Trolox (100-800 mM) as antioxidant were also carried out in each assay. The linear regression equation obtained was:
y=-1.8935X+1.1714, r^2=0.9918, between Trolox concentration (X) (mg/mL) and Absorbance (Y) (A_{Trolox}–A_{blank}). Results were expressed as µmol equivalents of Trolox per gram.

3. FRAP assay

The ferric reducing ability was estimated according to the procedure described by Pulido *et al.* (2000). The measure of absorbance was performed on a spectrophotometer Lambda 3B (Perkin Elmer) with thermostated bath.

The ferric reducing/antioxidant power (FRAP) reagent contained 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, plus 2.5 mL of 20 mM $FeCl_3.6H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6. Briefly, 900 µL of FRAP reagent, prepared fresh and warmed at 37 °C, was mixed with 90 µL of distilled water and 30 µL of hydrophilic solution (previously diluted 1/200), Trolox, or blank (acetate buffer).Temperature was maintained at 37 °C. an absorbance reading (595 nm) was taken at 30 min. Eight calibration solutions using Trolox (10-80 mM) as antioxidant were also carried out in each assay. The linear regression equation

obtained was: y = 6.2485X + 0.1865, r^2 = 0.9915, between Trolox concentration (X) (mg/mL) and Absorbance (Y) (A_{Trolox}–A_{blank}). Results were expressed as µmol equivalents of Trolox per gram.

The antioxidant capacity was also determined in a commercial product (dietary supplement), to compare it with *Garcinia* results, extracted and determined by the same procedure. The product is constituted according to labelling information by dehydrated vegetables (carrots, lemon, borage, tomato, alfalfa, red grape, cabbage, garlic, yeast) and propolis, and is recommended as an antioxidant and anti-aging supplement.

Each sample was extracted in triplicate and assayed in triplicate or quadruplicate.

CE-ESI-TOF-MS

CE experiments were performed using a P/ACE^{TM} System MDQ (Beckman Instruments, Fullerton, CA, USA). Fused-silica capillaries of 100 cm in length and 50 µm inner diameter (360 µm outer diameter) were used. After thorough optimization, a running buffer of 40 mM ammonium acetate at pH 9.5 was used. The separation voltage was set to 30 kV at the inlet of the capillary. Injection was performed hydrodynamically at 50 mbar during 15 s, corresponding to about 15 nL injected (0.9% of the capillary).

For CE-MS coupling, a coaxial sheath-liquid sprayer was used (Agilent Technologies). Isopropanol/water (60:40) with 0.1% (*v/v*) TEA was applied as sheath-liquid at a flow rate of 6 µL/min delivered by a 5 mL gas-tight syringe (Hamilton, Reno, NV, USA) using a syringe pump Cole-Parmer (Vernon Hill, IL, USA). The ESI-voltage of the TOF is applied at the end cap of the transfer capillary to the MS with the spray needle being grounded. An electrospray potential of + 4.1 kV was applied at the inlet of the MS (negative mode). A nebulizer gas (N_2) pressure of 0.3 bar was applied to assist the spraying. Dry gas temperature was set to 180ºC at a dry gas flow of 4 L/min.

Before first use, the bare capillaries were conditioned with 0.1 M sodium

hydroxide during 20 min followed by a water rinse for another 10 min. between runs the capillary was flushed with water and separation buffer for 5 min. At the end of the day the capillary was flushed with water for 10 min (all rinses during capillary conditioning were performed using N_2 at a pressure of 20 psi).

MS was performed using the micrOTOF^{TM} (Bruker Daltonik, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOF-MS). Transfer parameters were optimized by direct infusion experiments with Tuning Mix (Agilent Technologies) in the range of 50-800 *m/z*.

The trigger time was set to 50 µs, 49 µs for set transfer time and 1 µs pre-pulse storage time, corresponding to a mass range of 50-800 *m/z*. Spectra were acquired by summarizing 20,000 single spectra, defining the spectra rate to 1 Hz.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 3.3 (Bruker Daltonik GmbH), which provided a list of possible elemental formula by using the GenerateMolecularFormula^{TM} editor. The GenerateFormula^{TM} editor uses the sigmaFit^{TM} algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}) for increased confidence in the suggested molecular formula (Bruker Daltonics Technical Note #008, Molecular formula determination under automation).

During the development of the CE method external calibration was performed using sodium formate cluster by switching the sheath liquid to a solution containing 5 mM sodium hydroxide in the sheath liquid of 0.2 % formic acid in water:isopropanol 1:1 v/v at the end of the analysis. Using this method an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the MicroTOF, this external calibration provided accurate mass values (better at 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

RESULTS AND DISCUSSION

Measurement of antioxidant capacity

To evaluate the antioxidant capacities of foods, numerous *in vitro* methods have been developed and reviewed. However, there has not been a consensus as to the preferred method or methods. ORAC, ABTS (TEAC assays) and FRAP are among the more popular methods that have been used (Wu *et al*. 2004; USDA 2007). Reviews of some of the methods, advantages and disadvantages, have been fully discussed in several reviews (Frankel and Meyer 2000; Sánchez-Moreno 2002; Prior *et al.* 2005).

The ORAC assay is considered by some to be a preferable method as a standard tool to measure the antioxidant activity in the nutraceutical, pharmaceutical, and food industries because of its simplicity, biological relevance, mechanism HAT and the possibility to measure the lipophilic and hydrophilic antioxidant capacity (Prior *et al*. 2003). The $ABTS^{\bullet+}$ (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) assay has been popularized due to its ease of use and the FRAP assay previously was applied in plasma and in others types of products (Prior *et al*. 2005). In general, a product that has a high value for one measure of antioxidant capacity will also be high for another measure.
However, because antioxidant compounds with different chemical structures interact with different radical sources differently, the relation between any two antioxidant capacity methods will be quite low if considered across all products (USDA 2007).

Recently, Jiménez-Álvarez *et al*. (2008) assayed for the measurement of antioxidant capacity of food extracts methods that target different antioxidant mechanisms, that is, radical scavenging capacity (ORAC), reducing capacity (FRAP), and metal chelating properties (ICA) (iron (II) chelating activity) and Folin-Ciocalteu method to phenol total measure. ORAC and FRAP assays

provided a comprehensive, precise, and high-throughput assessment of antioxidant capacity in food extracts (Jiménez-Álvarez *et al*. 2008).

ORAC assay. *G. mangostana* extract (pulp and pericarp) showed a high radical scavenger capacity, 4088 μmol of TE/g to hydrophilic extract (**Table 1**), due to the high content of phenols (Haruenkit *et al.* 2007), which has been reported to correlate well with hydrogen-donating capacity (Proteggente *et al*. 2002; Prior *et al*. 2005). *Garcinia* extract exhibited an antioxidant capacity 19.6 times higher than a product (mixed vegetables) commercialized as a dietary supplement (200 μmol of TE/g). Patthamakanokporn *et al*. (2008) reported a value of 122.4 μmol of TE/g of dry matter from pulp of mangosteen. These authors applied the same determination method but used homogenized fresh pulp and acetone solvent to 50% to get the extracts. Interestingly, the peel and seed fractions of some fruits have higher antioxidant activity than the pulp fractions (Jayaprakasha *et al.* 2001).

The antioxidant capacity of ethanolic extract determined by Wu *et al*. (2004) (following the same method applied in this study) of 24 fresh fruit was between 2.97 μmol of TE/g to cantaloupe and 92.56 μmol of TE/g to cranberry; considering dry weight this would be 30.6 and 841.5, respectively. As can be observed the pericarp of mangosteen has a high contribution to antioxidant power of whole tropical fruit.

ABTS assay. The antioxidant capacity (AC) of *Garcinia* by this assay was 3591 μmol TE/g (**Table 1**). The peel of this fruit had recently been reported to have higher antioxidant activity than its pulp and seed (Li *et al*. 2006). Ethanolic extracts of mangosteen fruit peel showed high free radical-scavenging power, 3001 mmol of TE/g (Okonogi *et al*. 2007), approximately 900 times more than our results with whole fruit (pericarp and pulp). On the other hand, antioxidant capacity differences of mangosteen fruit extract can be observed depending on the solvent used from 10 to 50 μmol TE/g when acetone:water or water was

applied (Leontowicz *et al*. 2006). Antioxidant capacity of fresh mangosteen determined by Haruenkit (2007) was 20.16 μmol TE/g.

FRAP assay. Antioxidant capacity of *Garcinia* was 1222 μmol TE/g (**Table 1**). As for other methods the value is higher than obtained by Patthamakanokporn *et al.* (2008) for mangosteen edible part, 31.7 μmol TE/g dry weight. The commercial supplement dietary exhibited an antioxidant capacity 35 times less than *Garcinia* (34.4 μmol TE/g).

Higher values of antioxidant activity analysed by ORAC rather than by the FRAP method was found. It is likely that not all ORAC-active antioxidants in the studied fruits are reducing. However, Patthamakanokporn *et al.* (2008) analyzed seven tropical fruits and found than three of them showed less antioxidant capacity by ORAC than FRAP but not for mangosteen fruit. It is not surprising that a different antioxidant capacity among the studied fruit was shown by ORAC and ABTS compared to the FRAP method because in theory the measurement of each method is based on different mechanisms of reaction (Prior and Cao 1999). This confirms that the antioxidant capacity should be investigated by more than one method.

Table 1. Antioxidant capacity (µmol trolox equivalents/g)

Products	FRAP	ABTS	ORAC
Garcinia mangostana[b]	1222 ± 18*	3591 ± 133*	4088 ± 109*
Dietary supplement[a,c]	34 ± 11**	137 ± 7**	200 ± 7**

[a]Dehydrated vegetables labelling as supplement antioxidant and anti-aging
[b]n = 4
[c]n =7
*,**Statistical differences $p<0.05$

Identification of phenolic compounds in mangosteen

The proposed CE-ESI-TOF-MS method was applied for the identification of the phenolic compounds present in the *G. mangostana* extract. The formula, selected ion, *m/z* experimental and calculated, error (ppm and mDa), sigma value, tolerance, migration time and tentative compound proposed are summarized in Table 2.

The present method is able to detect several phenolic compounds previously observed in different studies. These tentative compounds are: (1) Garcinone E ($[M-H]^-_{exp.}$ 463.2126 *m/z*), (2) Garcinone D ($[M-H]^-_{exp.}$ 427.1762 *m/z*), (3) Mangostenone C ($[M-H]-_{exp.}$ 441.1568 *m/z*), (4) Mangostanol ($[M-H]^-_{exp.}$ 425.1612 *m/z*), (5) a-Mangostin, 1-Iso-mangostin, 3-Isomangostin, Cudraxanthone O, F, E, D, C, 8-Hydroxycudraxanthone G ($[M-H]^-_{exp.}$ 409.1667 *m/z*), (6) Garcinone C ($[M-H]^-_{exp.}$ 413.1621 *m/z*), (7) Epicatechin ($[M-H]^-_{exp.}$ 289.0717 *m/z*), (8) Mangostinone, 8-Desoxy-gartanin, Garcinone A ($[M-H]^-_{exp.}$ 379.1565 *m/z*), (9) Gartanin, γ-mangosteen, Mangoxanthone, Mangostenone D, Smeathxanthone A, Cudraxanthone P, M, L, J ($[M-H]^-_{exp.}$ 395.1513 *m/z*), (10) 1, 3, 5, 6-Tetrahydroxyxanthone ($[M-H]^-_{exp.}$ 259.0237 *m/z*).

As **Table 2** shows, several polar compounds can be found with the same molecular formula and that can be extracted with methanol and described in bibliography related to mangosteen.

As TOF-MS provides excellent mass accuracy over a wide dynamic range and allows measurements of the isotopic pattern, providing important additional information for the determination of the elemental composition. Therefore all detected compounds observed in **Table 2** exhibit good sigma values and mass accuracy (ppm and mDa) as indicated by the error values.

In comparison to the chromatographic methods, the proposed method is a good alternative for simultaneous characterization of phenolic compounds in *G. mangostana* as this technique provides fast and efficient separations and used reduced sample and solvent consumption. Also, the hyphenation of CE to MS combines the advantages of CE with the selectivity, sensitivity and mass accuracy inherent to TOF-MS.

Table 2. Well Known phenolic compounds determined by CE-ESI-TOF-MS in an extract of *Garcinia mangostana*

Formula	Selected ion	*m/z* experimental	*m/z* calculated	Error		Sigma value	Tolerance (ppm)	Migration time (min)	Compounds
				ppm	mDa				
$C_{28}H_{31}O_6$	$[M-H]^-$	436.2132	436.2126	1.4	-0.66	0.0119	5	11.8	Garcinone E (Suksamran *et al.* 2006)
$C_{24}H_{27}O_7$	$[M-H]^-$	427.1772	427.1762	2.3	-1.00	0.013	5	11.9	Garcinone D (Suksamran *et al.* 2006)
$C_{24}H_{25}O_8$	$[M-H]^-$	441.1568	441.1554	3.1	-1.36	0.0333	5	12	Mangostenone C (Suksamran *et al.* 2006)
$C_{24}H_{25}O_7$	$[M-H]^-$	425.1612	425.1600	1.7	-0.72	0.0553	5	12.1	Mangostanol (Suksamran *et al.* 2006)
$C_{24}H_{25}O_6$	$[M-H]^-$	409.1667	409.1656	2.7	-1.1	0.0145	5	12.2	α-Mangostin, 1-Isomangostin, 3-Isomangostin, Cudraxanthone O, F, E, D, C, 8-Hidroxycadraxanthone G (Ji *et al.* 2007; Jung *et al.* 2006)
$C_{23}H_{25}O_7$	$[M-H]^-$	413.1621	413.1605	3.9	-1.59	0.0043	5	12.3	Garcinone C (Suksamran *et al.* 2006)
$C_{15}H_{13}O_6$	$[M-H]^-$	289.0717	289.0717	0.0	-0.01	0.0146	5	12.4	Epicatechin (Suksamran *et al.* 2006)
$C_{23}H_{23}O_5$	$[M-H]^-$	379.1565	379.1550	3.9	-1.48	0.0282	5	12.5	Mangostinone, 8-desoxygartanin, Garcinone A (Ji *et al.* 2007; Jung *et al.* 2006)
$C_{23}H_{23}O_6$	$[M-H]^-$	395.1513	395.1550	3.5	-1.38	0.0216	5	12.6	Mangoxanthone, Mangostenone D, Smeathxanthone A, Cudraxanthone P, M, L, J (Suksamran *et al.* 2006, Nilar *et al.* 2005, Jung *et al.* 2006)
$C_{13}H_7O_6$	$[M-H]^-$	259.0237	259.0248	4.3	-1.1	0.0165	5	15.3	1,3,5,6-Tetrahidroxyxanthone (Nilar *et al.* 2005)

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CAPÍTULO 4

Influence of technological processes on phenolic compounds, organic acids, furanic derivatives, and antioxidant activity of whole-lemon powder

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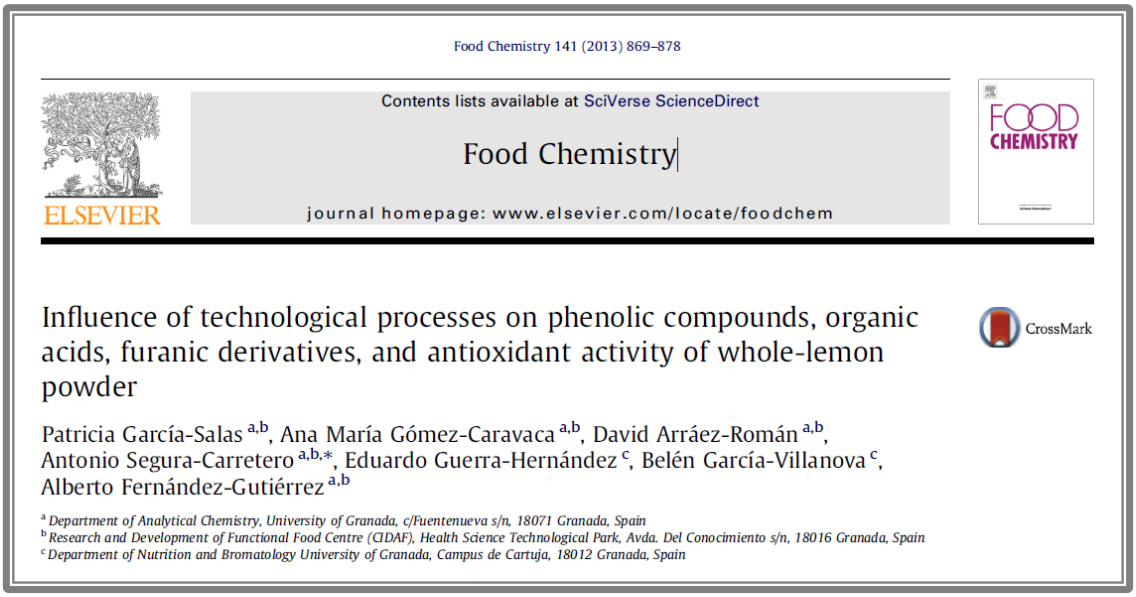

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Abstract

The healthy properties of citrus fruits have been attributed to ascorbic acid and phenolic compounds, mainly to flavonoids. Flavonoids are important phytonutrients because they have a wide range of biological effects that provide health-related properties. In this context, this study seeks to characterise the phenolic compounds in lemon and their stability in different drying processes (freeze-drying and vacuum-drying) and storage conditions (-18 and 50 ºC for 1 and 3 months). A powerful high-performance liquid chromatography coupled to DAD and electrospray-ionization time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) method has been applied for the separation, identification, and quantification of 19 phenolic compounds and 4 organic acids. To our knowledge, two hydroxycinnamic acids have been identified for the first time in lemon. Folin-Ciocalteu was applied to determine total phenolic compounds and TEAC, FRAP, and ORAC were applied to determine the antioxidant capacity of lemon. Total phenolic content significantly differed in the samples analysed, vacuum-dried lemon showing the highest phenolic content, followed by freeze-dried lemon and, finally, vacuum-dried lemon stored at 50 ºC for 1 and 3 months. The content in furanic compounds was determined to evaluate the heat damage in lemon and it was showed an increase with the thermal treatment because of the

triggering of Maillard reaction. As exception of ORAC, antioxidant-capacity assays were not correlated to phenolic content by HPLC due to the formation of antioxidant compounds during Maillard reaction.

Keywords: *Citrus limon* L., Lemon, Phenolic compound, HPLC–DAD–ESI-TOF-MS, Antioxidant activity, Maillard reaction.

1. Introduction

Lemon (*Citrus limon,* L.) is the third most important Citrus species after orange and mandarin, with a worldwide production over 4,200,000 tons in 2007. Spain is the third producer country in the world (González-Molina, Domínguez-Perles, Moreno, & García-Viguera, 2010), of which 50% of this production is exported, while 10% is used for fresh domestic consumption, and 40% for processing.

Commonly, fruits are dehydrated in order to be preserved, for which moisture must be removed in a way that has the least influence on the organoleptic qualities of the product (Davoodi, Vijayanand, Kulkarni, & Ramana, 2007). Indeed, the health-promoting capacity of fruits depends strictly on the processing of such foods (Hunter & Fletcher, 2002). The processed fruit quality is especially important, because high fruit consumption is encouraged throughout Europe for health considerations. Therefore, dehydrated fruit is under rapidly increasing demand both in the domestic and the international market and is being used largely for the preparation of nutraceutical products or convenience food. Fruit can be dehydrated by various methods and the quality of the product depends on different parameters (Mrkìc, Cocci, Rosa, & Sacchetti, 2006).

The rate of drying which usually lasts several days at intermediate temperature (50-80 ºC), affects the final quality of a dehydrated product. Drying processes can be quite different and may affect the sample depending on the time and dry temperature used, but generally the drying processes at high temperatures cause a reduction in the concentration of phenolic compounds (Gao, Wu, Wang, Xu, & Du, 2012; Leong & Oey, 2012). However, the use of vacuum systems allows the reduction of the applied temperature and/or dehydration time, improving the overall quality of foods.

Citrus species of various origins have been assessed for their phenolic constituents, essential oils (terpenoids), vitamins (vitamin C), minerals, dietary fibre, and carotenoids. The combination of these components appears to be

responsible for biological effects (Gorinstein *et al.*, 2004; Proteggente, Saija, De Pasquale, & Rice-Evans, 2003).

The health-promoting properties of *citrus* have been associated mainly with its antioxidant vitamin C and flavonoid contents (Ramful, Tarnus, Aruoma, Bourdon, & Bahorun, 2011). Recently, phenolic compounds in citrus fruits and co-products have received great attention for their health benefits in human nutrition (Abad- García *et al.*, 2012). For consumption, sensory attributes of fruits (colour, sweet taste, bitterness, and astringency) constitute decisive organoleptic and commercial properties.

The positive influence of this dietary component is attributed to antioxidant effects. It has been shown that phenolic compounds, and particularly flavonoids, bear antioxidant properties (Gorinstein *et al.*, 2004). Flavonoids in *citrus* fruits exhibit a wide range of promising biological properties including anti-atherogenic, anti-inflammatory, and antitumor activity, inhibition of blood clots, and strong antioxidant activity (Montanari, Chen, & Widmer, 1998). The phenolic composition of citrus fruits includes flavanones (major group), flavones, and flavonols. More than 60 individual flavonoids have been identified in *Citrus* sp. In addition, other phenolic compounds (phenolic acids, etc.) are also present in *Citrus* species (Abad-García *et al.*, 2012).

Citrus flavonoids are present in the glycoside or aglycone forms. Among the glycoside forms, two types of di-glycosides are common, i.e. neohesperidosides (rhamnosyl-α-1,2-glucose) and rutinosides (rhamnosyl-α-1,6-glucose). Flavanones are the most abundant *Citrus* flavanoids (e.g. 90% in lemon). The *Citrus* peel and seeds are also rich in phenolic compounds, such as phenolic acids and flavonoids. The main phenolic constituents of *citrus* peel are flavanone and flavone glycosides, although there are also numerous hydroxycinnamates, coumarins, psoralens, and polymethoxylated flavones (Peterson *et al.*, 2006; Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007).

Heat treatment of foods containing sugars and ascorbic acid may result in non-enzymatic browning or the Maillard reaction, which causes a change in the flavour, colour, and nutritional value of the foods. Furfural and 5-(hydroxymethyl)-furfural (HMF) are the main degradation products of the hydrolysis of hexoses and pentoses, respectively (Rufián-Henares, García-Villanova, & Guerra-Hernández, 2008).

The aim of the present work was to identify the phenolic compounds of lemon samples (dehydrated by freeze-dried and vacuum system, and storage under extreme conditions) by HPLC-ESI-TOF- MS and to evaluate their stability. The content in furanic compounds, i.e. (hydroxymethylfurfural (HMF) and furfural, was also determined to evaluate the heat damage.

2. Materials and methods

2.1. Samples

The lemons (*Citrus lemon*) studied, which were ecologically cultivated, were from Dispronusa S.L. company of ecological products (Granada, Spain). The fresh fruits were cleaned with tap water and the whole fresh fruits (pulp, seed and peel) were chopped and triturated using a Thermomix (Vorwerk España M.S.L).

A fraction of the triturated lemon sample was freeze-dried and kept at -18 C until used. Another fraction of the triturated lemon was dehydrated using a rotary vacuum evaporator in a bath at 50 ºC, after which the drying process was completed in an oven at 35 ºC for 48 h, and then kept at -18 ºC until used (L0 sample). This procedure was applied to simulate the process used in the vegetable-dehydration industry. Two fractions of L0 were stored under extreme conditions (50 ºC during for 1 and 3 months, L1 and L3, respectively) to study the stability of the compounds. This procedure was conducted to simulate the conditions of storage during summer months in the Mediterranean region.

2.2. Chemicals and reagents

The reagents used for HPLC-ESI-TOF-MS analyses were: HPLC grade acetic acid and acetonitrile purchased from, Sigma-Aldrich (St. Louis, MO, USA) and Lab-Scan (Gliwice, Sowinskiego, Poland), respectively, and methanol for dissolving the samples was purchased from Panreac (Barcelona, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). The reagents used to measure the antioxidant capacity, AAPH (2,20-azobis-2-methyl-propanimidamide, dihydrochloride), TPTZ (1,3,5-triphenyl-tetrazolium chloride), ABTS [2,20 -azinobis (3-ethyl- benzothiazoline-6-sulphonate)], trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylicacid), fluorescein, potassium persulphate, and ferric sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dehydrated sodium phosphate, trihydrated sodium acetate, sodium acetate, ferric chloride, hydrochloric acid, and acetic acid were obtained from Panreac (Barcelona, Spain).

The reagents used for the determination of furanic compounds were as follows: furfural (Merck, Darmstadt, Germany) and HMF (Sigma-Aldrich, St. Louis, MO, USA) were used to prepare the standard solutions; a clarified solution of 15% potassium ferrocyanide (w/v) (Carrez I) and 30% zinc acetate (w/v) (Carrez II) (Merck, Darmstadt, Germany); acetonitrile (J.T. Baker, Phillipsburg, NJ, USA) used for the mobile phase.

Double-deionised water with conductivity lower than 18.2 MX was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). Other unmarked reagents were of analytical grade.

2.3. Determination of phenolic compounds by HPLC-DAD-ESI-TOF-MS

Dried lemon (5 g) was placed in a test tube. Then, 25 mL methanol were added and the tube was shaken by vortex for 30 min, and centrifuged at 4500 rpm for 10 min. The supernatant was removed, evaporated, and reconstituted in 2 mL MeOH/water (80:20 v/v). Finally, the extract solution was diluted 1/10 and

filtered through a 0.22 µm micro-filter (Millipore, Bedford, MA, USA) before being injected for HPLC analysis.

The phenolic compounds from dried lemon extracts were separated on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of a vacuum degasser, an autosampler, and a binary pump equipped with a C18 Eclipse Plus analytical column (4.6 x 150 mm, 1.8 µm) from Agilent Technologies. The mobile phases used were water with acetic acid (0.5%) (mobile phase A) and acetonitrile (mobile phase B) and the solvent gradient changed according to the following conditions: 5% B to 30% B for 10 min; from 30% B to 33% B for 2 min; from 33% B to 38% B for 5 min; from 38% B to 50% B for 3 min; from 50% B to 95% B for 3 min; from 95% B to 5% B for 2 min; equilibration of the column for 10 min. The flow rate used was set at 0.80 mL/ min throughout the gradient. The effluent from the HPLC column was split using a T-type phase separator before being placed in the mass spectrometer. The flow which arrived to the ESI-TOF-MS detector was 0.2 mL/min. The column temperature was maintained at 25 ºC and the injection volume was 10 µL. The UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 210, 240, 280, and 330 nm.

The HPLC system was coupled to a microTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The analysis parameters were set using a negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-TOF source parameters were: capillary voltage +4.5 kV, drying gas temperature 190 ºC; drying gas flow 9 L/min and nebulizing gas pressure 2 bar. Indeed, the optimum values of transfer parameters were: capillary exit -120 V; skimmer 1 -40 V; hexapole 1 -23 V; RF hexapole 100 Vpp and skimmer 2, was set at -22.5 V.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulas by using the Generate Molecular Formula™ Editor. The latter uses a CHNO algorithm providing 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^{TM}) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm for most of the compounds.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium acetate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). With this method, an exact calibration curve was drawn based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$). Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass values (better 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

2.4. Antioxidant capacity assays

The extraction was conducted as follows: 5 mg of dried lemon was placed in a test tube. Then, 1 mL MeOH/water (80:20 v/v) was added and the sample was sonicated for 20 min and filtered through a 0.22 µm micro-filter (Millipore, Bedford, MA, USA).

The trolox equivalent antioxidant capacity (TEAC) assay, which measures the reduction of the ABTS radical cation by antioxidants, was based on the method described by Miller, Rice-Evans, Davies, Gopinathan, and Milner (1993). Oxygen Radical Absorbance Capacity assay (ORAC,) which uses fluorescein

(FL) as the fluorescent probe (ORACFL), was used (Ou, Hampsch-Woodill, & Prior, 2001). The final ORAC values were calculated using a regression equation between the trolox concentration and the next area of the FL decay curve (area under curve, AUC). The ferric reducing antioxidant power (FRAP) was performed according to Al-Duais, Mueller, Boehm, and Jetschke (2009). Ferrous sulphate solutions (0-300 µM) were used for calibration.

The instrument employed for the antioxidant capacity determinations was a FLOUstar Omega (BMG LABTEHC with Microplate Reader; Osfenburg, Germany), and 96-well polystyrene microplates were used.

2.5. Determination of furanic compounds

The extraction and determination was based on a procedure proposed by Guerra-Hernández, García-Villanova, and Montilla-Gómez (1992) with some modifications. Firstly, 0.4 g of sample, mixed with 6 mL of demineralised water, were shaken vigorously for 1 min and centrifuged at 9000 rpm for 10 min. The same procedure was followed twice again, adding 3 and 2 mL of demineralised water, respectively. The supernatants were combined and 0.5 mL each of Carrez I and II solutions were added and the resulting mixture was centrifuged at 9000 rpm for 10 min. The supernatant was brought to 10 mL with demineralised water. The aqueous extract was filtered through a 0.2 µm disk filter before injection. Both determinations (HMF and furfural) were performed in triplicate.

The HPLC system used in these analyses was equipped with a Varian Prostar model 230 pump, a Varian Prostar autosampler injector model 410, and a Varian Prostar model 325 ultraviolet detector. Samples were put into the column through an automatic injector equipped with a sample loop (50 µL). The mobile phase was filtered through 47 mm Nylon 0.45 µm paper filters (Waters, Milford, Massachusetts, USA), before being introduced into the chromatographic system. Separations were performed on a Nova pack 4 µm C18 (250 4.6 mm) Waters

column. The mobile phase was acetonitrile: water (5:95), flow rate 1 mL/min and a wavelength of 280 nm.

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

All chemical analyses were performed in triplicate, and the analytical data were used for statistical comparisons.

3. Results and discussion

3.1. Identification of phenolic compounds by HPLC-DAD-ESI-TOF-MS

Lemon extracts obtained by solid-liquid extraction were analysed using an HPLC coupled to DAD and ESI-TOF-MS (HPLC-DAD-ESI-TOF-MS) in negative ionization mode in order to identify the phenolic compounds. Peak identification was performed on basis of their relative retention time values, their UV-vis spectra and mass spectra obtained using TOF-MS together with information previously reported in the literature.

Fig. 1 a and b show, as a representative example, the UV spectrum at λ = 280 nm and base peak chromatogram (BPC) of the lemon extract L0, respectively. TOF-MS instrumentation provides excellent mass resolution and mass accuracy in combination with the true isotopic pattern, thus TOF-MS is the perfect choice for molecular formula determination using the SmartFormula^{TM} Editor. The HPLC-ESI-DAD-TOF-MS profiles of the extracts analysed showed several peaks from which 23 compounds corresponding to different polyphenols and organic acids could be identified, although most of the compounds tentatively identified were flavonoids.

These compounds are summarised in Table 1, including the retention time, molecular formula, experimental and calculated *m/z*, sigma values, and error. The information in Table 1 concern sample L0. The phenolic compounds and organic acids were identified taking as reference the sample L0 while the compounds were identified by the UV data and MS data provided by the software Data Analysis 3.4 (Bruker Daltonik, Bremen, Germany), which gives a list of possible elemental formulae by using the Generate Molecular Formula^{TM} Editor. The identification of phenolic compounds of samples L1, L3 and freeze-dried sample was accomplished by TargetAnalysis 1.2 from Bruker Daltonics platform, which reports the tools for the simultaneous identification of different compounds from LC-MS chromatograms using a previous database made with the retention time, molecular formula, and experimental *m/z* of the compounds identified on sample L0.

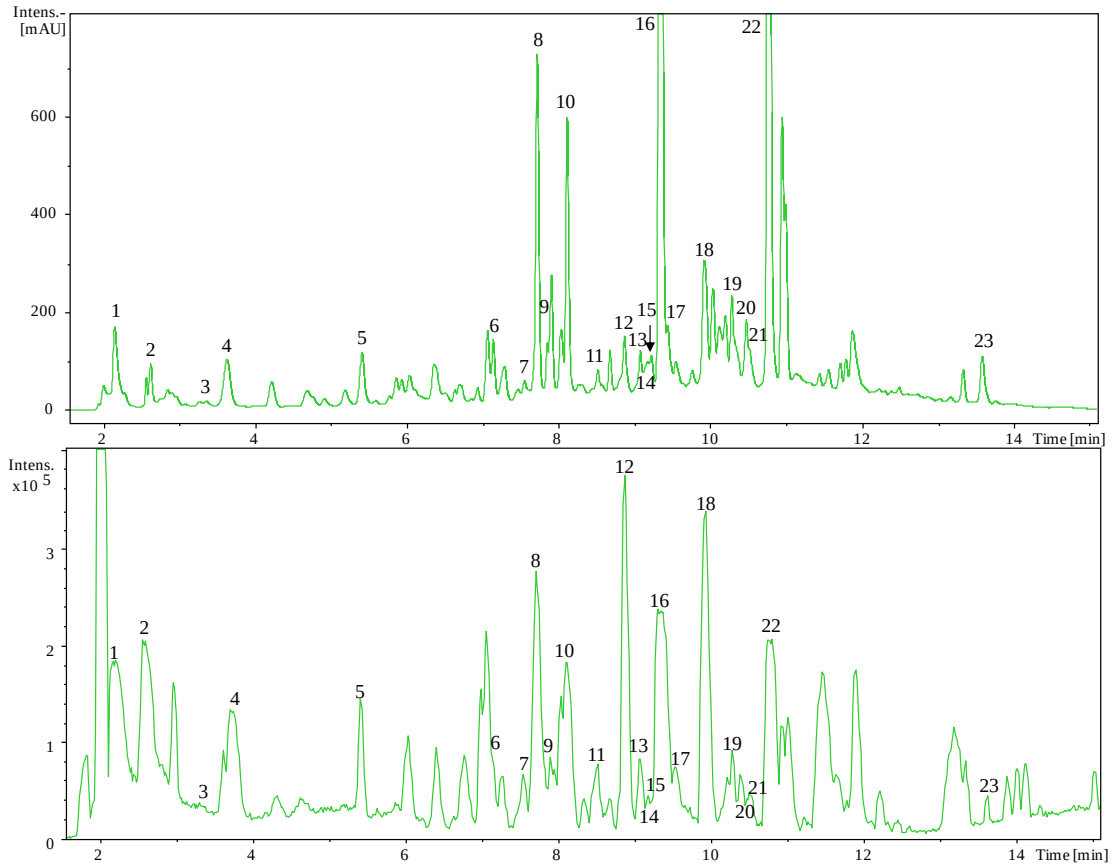

Fig. 1. Chromatograms of the phenolic fraction of lemon extract L0 drawn by UV–vis at λ = 280 nm (a) and by mass spectrometry (b). See Table 1 for identification of phenolic compounds.

The first family of compounds identified, according to their retention times, were organic acids (peaks 1-4, Table 1), citric acid, and their isomers I and II (peaks 1-3) with retention times 2.21, 2.61 and 3.62 min, respectively (Uckoo, Jayaprakasha, Nelson, & Patil, 2011), and homocitric acid with *m/z* 205.0348 (peak 4) at time 3.74 min.

Table 1. Phenolic compounds tentatively identified in whole-lemon samples.

Peak	RT [min]	*m/z* experimental	*m/z* calculated	Molecular Formula	Error (ppm)	mSigma	Compound Name
1	2.21	191.0208	191.0197	$C_6H_8O_7$	5.5	2.2	Citric acid
2	2.61	191.0202	191.0197	$C_6H_8O_7$	2.5	3.6	Citric acid (Isomer I)
3	3.62	191.0195	191.0197	$C_6H_8O_7$	1.4	2.5	Citric acid (Isomer II)
4	3.74	205.0348	205.0354	$C_7H_{10}O_7$	2.8	1.3	Homocitric acid
5	5.41	299.0763	299.0772	$C_{13}H_{16}O_8$	3.3	5.6	Hydroxybenzoic acid hexose
6	7.14	609.1456	609.1461	$C_{27}H_{30}O_{16}$	0.8	16.0	Quercetin-3-rutinoside (Rutin)
7	7.54	357.1184	357.1191	$C_{16}H_{22}O_9$	2.0	19.6	3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose
8	7.72	593.1518	593.1512	$C_{27}H_{30}O_{15}$	1.0	9.1	Apigenin 6,8-di-C-glucoside (Vicenin-2)
9	7.91	623.1622	623.1618	$C_{28}H_{32}O_{16}$	0.7	17.3	Chrysoeriol 6,8-di-C-glucoside (Stellarin-2)
10	8.12	623.1631	623.1618	$C_{28}H_{32}O_{16}$	2.2	11.5	Diosmetin 6,8-di-C-B-glucoside
11	8.52	447.0941	447.0933	$C_{21}H_{20}O_{11}$	1.8	7.1	Quercitrin
12	8.87	357.1209	357.1191	$C_{16}H_{22}O_9$	5.0	13.2	3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose (Isomer I)
13	9.08	563.1402	563.1406	$C_{26}H_{28}O_{14}$	0.8	12.2	Apigenin-6-C-pentosyl-8-C-hexoside
14	9.16	577.1567	577.1563	$C_{27}H_{30}O_{14}$	0.7	5.0	Apigenin-7-O-neohesperidoside (Rhoifolin)
15	9.23	563.1405	563.1406	$C_{26}H_{28}O_{14}$	0.2	14.9	Apigenin-6-C-pentosyl-8-C-hexoside (Isomer I)
16	9.33	595.1690	595.1668	$C_{27}H_{32}O_{15}$	3.6	4.4	Eriodictyol 7-O-rutinoside (Eriocitrin)
17	9.54	431.1001	431.0984	$C_{21}H_{20}O_{10}$	4.0	3.0	Apigenin-8-C-glucoside (Vitexin)
18	9.92	461.1116	461.1089	$C_{22}H_{22}O_{11}$	5.9	5.9	Diosmetin glucoside
19	10.28	579.1734	579.1719	$C_{27}H_{32}O_{14}$	2.6	7.6	Naringin
20	10.47	609.1840	609.1825	$C_{28}H_{34}O_{15}$	2.4	27.2	Hesperetin-7-O-rutinoside (Hesperidin)
21	10.52	607.1674	607.1668	$C_{28}H_{32}O_{15}$	1.0	15.1	Diosmin
22	10.76	609.1848	609.1825	$C_{28}H_{34}O_{15}$	3.9	2.8	Hesperetin-7-O-neohesperidoside (Neohesperidin)
23	13.57	287.0558	287.0561	$C_{15}H_{12}O_6$	1.0	5.6	Eryodictiol

Among the phenolic acids, 3 compounds were identified. Peak 5, with *m/z* 299.0772 was tentatively identified as hydroxybenzoic acid hexose according to the MS data and its fragmentation pattern, the neutral loss of a glycoside moiety (162 Da) and the fragment ion at *m/z* 137.0236, previously reported in other vegetables (Abu-Reidah *et al.*, 2012; Daayf, Ongena, Boulanger, El Hadrami, & Belanger, 2000). Peaks 7 and 12, 3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose and its isomer, belong to the hydroxycinnamic family of acids. They showed *m/z* 357.1184 and 357.1209, respectively, and a fragment ion was found at *m/z* 195.0654. These fragments have previously been reported in other vegetables (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010).

This is the first available record of these hydroxycinnamic acids having been detected in lemon extracts.

Most of the compounds tentatively identified in the lemon extracts belong to the flavonoid family (Table 1). Of these compounds, 10 were determined to be flavones. Peak 8 with *m/z* 593.1518 was identified as apigenin 6,8-di-C-glucoside (vicenin-2). This compound has previously been identified in lemon fruit juice (Abad-García *et al.*, 2012; Caristi, Bellocco, Gargiulli, Toscano, & Leuzzi, 2006; Gil-Izquierdo, Riquelme, Porras, & Ferreres, 2004) and citrus peels (Mandalari *et al.*, 2006). Peaks 9 and 10 were identified as chrysoeriol 6,8-di-C-glucoside or stellarin-2 (*m/z* 623.1631) and diosmetin 6,8-di-C-b-glucoside (*m/z* 623.1622), respectively, as previously reported in lemon juice (Abad-García *et al.*, 2012; Caristi *et al.*, 2006; Dugo *et al.*, 2005). Compounds 13 and 15 presented *m/z* 563.1402 and 563.1405, respectively, and fragments at *m/z* 443.1192 and 473.1290. This pattern, which has been found previously in other vegetables (Abu-Reidah *et al.*, 2012), is typical of a flavone-C- diglycoside. Therefore, these compounds were proposed as apigenin-6-C-pentosyl-8-C-hexoside and its isomer. Peak 14, with *m/z* 577.1567 was identified as apigenin 7-*O*- apigenin-7-*O*-neohesperidoside (rhoifolin). This compound has also been identified in lemon juice and other *citrus* juices by other authors (Abad-García *et al.*, 2012; Dugo *et al.*, 2005). Peak 18 with *m/z* 461.1116 was identified as diosmetin-glucoside, this

flavone having already been identified in lemon juice and *citrus* peel by other authors (Abad-García *et al*., 2012; González-Molina *et al.*, 2010; Mandalari *et al.*, 2006). Diosmin (peak 21) at *m/z* 607.1674 and retention time 10.32 min was identified, as previously found in hand-pressed lemon juice, commercial lemon juice, lemon peels, and the edible lemon portion (González-Molina *et al.*, 2010).

Flavanones are the most abundant *Citrus* flavonoids and they are not equally distributed in lemon fruit (González-Molina *et al.*, 2010). The flavanones identified were: peak 16 with *m/z* 595.169 and retention time 9.33 min; this data corresponds to eriodictyol 7-O-rutinoside (eriocitrin), as found previously in lemon juice, peels, seeds, and the edible portion (Abad-García *et al.*, 2012; González-Molina *et al.*, 2010) The compound with *m/z* 579.1734 (peak 19) and retention time 10.28 was identified as naringin according to Abad-García *et al.* (2007). Hesperidin and neohesperidin (peaks 20 and 22) with *m/z* 609.1840 and 609.1848, respectively, were found at retention times 10.468 and 10.765. These compounds have been reported in the literature (Abad-García *et al.*, 2012; González-Molina *et al.*, 2010). Eryodictiol was identified at a retention time of 13.575 min and *m/z* 287.0558 (peak 23).

The third group of flavonoids identified was flavonols (Table 1). Peak 6 with *m/z* 609.1456 and retention time 7.14 was tentatively identified as quercetin-3-rutinoside (rutin). This compound, a typical citrus flavonol, has been previously identified in *citrus* juices (Abad-García *et al.*, 2012) and other citrus fruits (Ramful *et al.*, 2011). Peak 11 at *m/z* 447.0933 was identified as quercitrin, as found previously in fruit juices (Abad-García *et al.*, 2007).

3.2. Quantification of phenolic compounds by HPLC-DAD-MS

Six calibration curves were prepared using the following standards: citric acid, sinapic acid, hydroxybenzoic acid, rutin, apigenin, and hesperetin, in the range of concentrations from the quantification limit (LOQ) to 500 µg/mL. Seven calibration points were used in each case, and the analyses were replicated five

times for each calibration point (n = 5). The different parameters of each are summarised in Table 2: sensitivity [relative standard deviations (RSDs) (%)], linearity (r^2), calibration range and correlation coefficient.

Table 2. Analytical parameters of the method proposed.

Analyte	RSD	LOD (µg/mL)	LOQ (µg/mL)	Calibration range (µg/mL)	Calibration equations	r^2
Citric acid	0.53	0.153	0.475	LOQ-500	y = 0.55518x - 0.0132	0.9991
Sinapic acid	0.25	0.068	0.224	LOQ-500	y = 16,746x - 6,7425	0.9999
Hydroxybenzoic acid	0.37	0.087	0.287	LOQ-500	y = 19,244x - 60,211	0.9989
Rutin	0.33	0.182	0.601	LOQ-500	y = 8,8262x - 3,5516	0.9999
Apigenin	0.48	0.096	0.317	LOQ-500	y = 29,042x - 119,97	0.9983
Hesperetin	0.15	0.101	0.333	LOQ-500	y = 33,56x - 34,061	0.9987

All calibration curves showed good linearity between different concentrations depending on the analytes studied. The calibration plots indicate good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.990 in all cases. The LOQ was determined as the signal-to noise ratio of 10:1, and the limit of detection (LOD) was determined as a signal-to-noise ratio of 3:1. The LOD was found to be within the range 0.068-0.182 µg/mL, while the LOQ was within 0.224-0.601 µg/mL. Intraday and interday precision was developed to evaluate the repeatability of HPLC-ESI-TOF-MS method. A methanol/water extract was injected (n = 6) on the same day (intraday precision) for 3 consecutive days (interday precision, n = 18). The RSDs of analysis time and peak area were determined.

The intraday repeatability (expressed as % RSDs) of the retention times was from 0.15% to 0.53%, whereas the interday repeatability was from 0.98% to 2.27%. The intraday repeatability (expressed as% RSDs) of the total peak area was 0.28%, whereas the interday repeatability was 1.62%.

The quantification was performed by using the previous calibration curves. The calibration curve of citric acid at k = 210 nm was used to quantify organic acids such as citric acid and their isomers and homocitric acid.

Hydroxybenzoic acid hexose was quantified with the calibration curve of hydroxybenzoic acid at k = 280 nm, while 3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose and its isomer were quantified using the calibration curve of sinapic acid at k = 280 nm. Rutin was quantified using the rutin calibration curve at k = 280 nm.

Apigenin calibration curve at λ = 280 nm was used to quantify flavones (apigenin 6,8-di-C-glucoside (vicenin-2), chrysoeriol 6,8-di-C-glucoside (stellarin-2), diosmetin 6,8-di-C-B-glucoside, quercitrin, apigenin-6-C-pentosyl-8-C-hexoside and its isomer, apigenin-7-O-neohesperidoside (rhoifolin), apigenin-8-C-glucoside (vitexin), diosmetin glucoside and diosmin) and hesperetin calibration curve at λ = 280 nm was used to quantify flavanones (eriodictyol 7-O-rutinoside (eriocitrin), naringin, hesperetin-7-O-rutinoside (hesperidin), hesperetin-7-O-neohesperidoside (neohesperidin), eryodictiol).

The methanolic extracts of the different lemon extracts were analysed by the HPLC-DAD-ESI-TOF-MS method, and the quantitative results are presented in Table 3.

It should be taken into account that the response of the standards can differ from the response of the analytes present in the lemon samples and, consequently, the quantification of these compounds is only an estimation of their actual concentrations.

Table 3. Concentration of organic acids and phenolic-compound extracts expressed as µg/g of lemon powder

Peak	Compound Name	L0	L1	L3	Freeze-dried
1	**Citric acid**	632.17^{a}	601.97^{b}	606.21^{b}	533.97^{c}
2	**Citric acid (Isomer I)**	208.76^{a}	138.85^{b}	143.15^{b}	112.47^{c}
3	**Citric acid (Isomer II)**	249.37^{a}	nd	nd	88.33^{b}
4	**Homocitric acid**	491.79^{b}	508.20^{a}	474.66^{c}	475.86^{c}
5	**Hydroxybenzoic acid hexose**	19.50^{a}	14.89^{b}	11.92^{c}	15.61^{b}
6	**Quercetin-3-rutinoside (Rutin)**	22.72^{a}	18.35^{b}	14.78^{c}	16.31^{b}
7	**3-(2-hydroxy-4-methoxyphenyl) propanoic acid hexose**	7.49^{a}	0.80^{b}	0.80^{b}	7.56^{a}
8	**Apigenin 6,8-di-C-glucoside (Vicenin-2)**	40.89^{a}	22.11^{c}	18.49^{d}	32.45^{b}
9	**Chrysoeriol 6,8-di-C-glucoside (Stellarin-2)**	20.39^{a}	15.07^{c}	12.39^{d}	17.44^{b}
10	**Diosmetin 6,8-di-C-B-glucoside**	35.09^{a}	22.49^{c}	17.47^{d}	24.79^{b}
11	**Quercitrin**	13.53^{a}	13.25^{a}	11.81^{b}	11.90^{b}
12	**3-(2-hydroxy-4-methoxyphenyl)-propanoic acid hexose (Isomer I)**	27.16^{a}	21.13^{c}	16.46^{d}	22.65^{b}
13	**Apigenin-6-c-pentosyl-8-c-hexoside**	14.90^{a}	13.74^{b}	11.81^{c}	13.58^{b}
14	**Apigenin-7-O-neohesperidoside (Rhoifolin)**	13.86^{a}	13.43^{a}	12.25^{b}	12.37^{b}
15	**Apigenin-6-c-pentosyl-8-c-hexoside (Isomer I)**	14.38^{c}	18.17^{b}	20.59^{a}	13.58^{d}
16	**Eriodictyol 7-O-rutinoside (Eriocitrin)**	122.89^{a}	106.01^{b}	86.47^{d}	103.85^{c}
17	**Apigenin-8-C-glucoside (Vitexin)**	15.94^{a}	13.79^{c}	12.28^{d}	14.82^{b}
18	**Diosmetin glucoside**	29.45^{a}	22.70^{b}	19.81^{c}	21.90^{b,c}
19	**Naringin**	12.44^{a}	8.949^{c}	8.46^{c}	9.84^{b}
20	**Hesperetin-7-O-rutinoside (Hesperidin)**	10.11^{b}	14.04^{a}	9.45^{b}	8.16^{c}
21	**Diosmin**	15.60^{a,b}	16.99^{a}	14.49^{b}	15.94^{a,b}
22	**Hesperetin-7-O-neohesperidoside (Neohesperidin)**	110.28^{a}	110.68^{a}	108.97^{a}	109.48^{a}
23	**Eryodictiol**	2.02^{c}	4.521^{a}	4.23^{b}	2.03^{c}
	Total	2130.82^{a}	1722.19^{b}	1637.05^{c}	1683.98^{b}

3.3. Effect of drying systems and storage conditions on phenolic content

Table 3 presents the concentration values of the different identified compounds in lemon samples (L0, L1, L3 and freeze-dried samples) and bar graphs in Fig. 2 represent the concentrations and standard deviations of L0, L1, L3, and freeze-dried samples.

Of the organic acids present in the samples (Fig. 2a), citric acid and its isomers behaved similarly; freeze-dried samples showed a content in those compounds that was significantly lower than in L0 samples, while they also decreased in L1 and L3. Homocitric acid was significantly higher in L1 than in L0, L3 and freeze-dried samples; indeed homocitric acid in freeze-dried lemon was significantly lower than in L0 lemon samples. Organic acids comprised the family with the highest concentration in methanol-water lemon extracts.

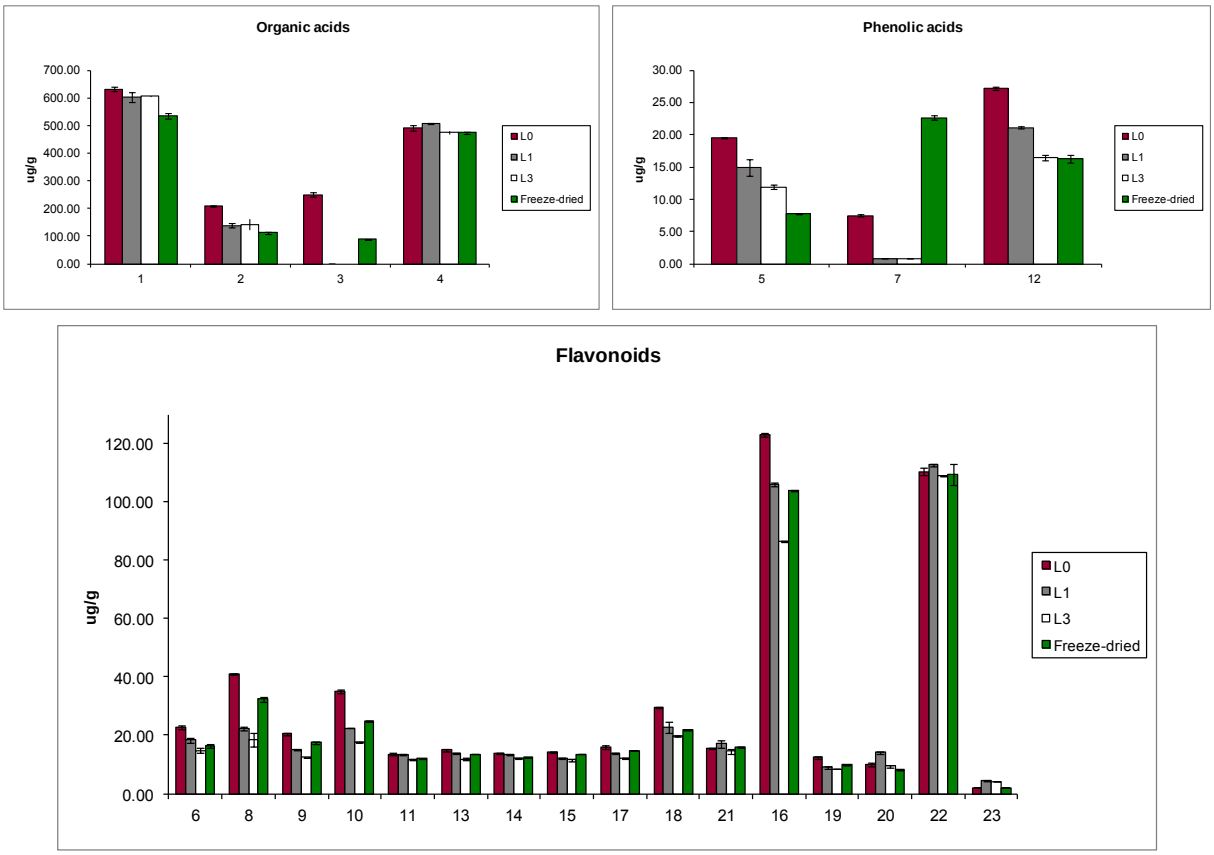

Fig. 2. Bar graphs of the concentrations of the different families of compounds contained in L0, L1, L3, and freeze-dried whole-lemon powder samples.

Among phenolic compounds, the highest concentration values corresponded (in descending order) to peaks 16 (flavanone), 22 (flavanone), 8 (flavone), and 10 (flavone). These peaks, all flavonoids, corresponded to the following tentatively identified compounds: eriocitrin, neohesperidin, vicenin-2, and diosmetin 6,8-di-C-B-glucoside, respectively. These results agree with the major phenolic compounds found previously in the literature (Gil-Izquierdo *et al.*, 2004; González-Molina, Moreno, & García- Viguera, 2008; Peterson *et al.*, 2006).

The phenolic acid content changed depending on the different storage systems used (Fig. 2b). Different phenolic acids did not change in the same manner, but it was found that the storage at 50 ºC lowered their content in lemon. The percentage of losses after 1 and 3 months at 50 ºC ranged from 4% to 100%. Compound 5 content was similar in L1 and freeze-dried samples, while compounds 7 and 12 significantly differed in those samples.

Concerning flavonoids (Fig. 2c), most behaved similarly; in most cases, samples dried only under vacuum and kept at low temperature (L0) presented the highest content in flavonoids, followed by freeze-dried samples that showed significant decreases compared to L0. Sample storage at high temperatures, L1 and L3, had a significantly lower content of flavonoids than the other samples, as expected because phenolic compounds are affected by temperature (Chang, Zuo, Chow, & Ho, 2006). The losses in flavonoids after 1 month at 50 ºC were between 2% and 46% and 7-55% for samples stored at 50 ºC for 3 months. Finally compound 23 was the only one that increased with the temperature, because temperature can affect phenolic compounds breaking the glycosidic bonds, giving rise to the aglycone molecules. This could have increased eryodictiol after the storage at high temperatures (Deng, West, Jensen, & Palu, 2009).

No studies have reported findings on flavonoid stability during the storage of whole-lemon powder. However, the studies made on fresh orange juice treated by thermal (low pasteurisation, high pressure/40 ºC) and non-thermal technologies (pulsed electric fields) and stored at 4 ºC for 0-40 days showed a

significant decrease of flavanones after 20 days (Plaza *et al.*, 2011). Besides storage at 18, 28, and 38 ºC for 2, 4, and 6 months of fresh orange juices affected vitamin C and free and conjugated hydroxycinnamic acids. A 6-month storage of orange juices at various temperatures resulted in an approximately 10-22% decline in total phenolic and vitamin C content. Small changes in flavanone content were observed, indicating high stability of these compounds upon storage (Klimczak, Malecka, Szlachta, & Gliszczynska-Swiglo, 2007).

In agreement with the literature, the phenolic content significantly differed in the samples analysed, diminishing in the following order: L0 > freeze-dried > L1 > L3.

3.4. Total phenolic content trend during storage

The values found in dried samples were 10.43 and 10.11 mg GAE (gallic acid equivalent)/g for freeze-dried and vacuum-dried lemon, respectively. The samples dried and stored 1 and 3 months at 50 ºC, L1 and L3, exhibited values of 9.88 and 11.60 mg GAE/g, respectively. No significant differences were detected between the two drying procedures or between dried (L0) samples stored at 50 ºC (L1 and L3) (Table 4).

Table 4. Antioxidant capacity (µmol TEs/g dried lemon) and total phenols (mg GAE/g of dried lemon).

Lemon samples	TEAC	FRAP	ORAC	Total Phenols by Folin-Ciocalteu
Freeze-dried	61.45 [a]	70.92 [a]	913 [a]	10.43 [a]
Vacuum Dried, t = 0 (L0)	40.15 [b]	67.07 [a]	1043 [a]	10.11 [a]
Stored[(a)] t = 1 month (L1)	38.16 [b]	66.37 [a]	914 [a]	9.88 [a]
Stored[(a)] t = 3 months (L3)	36.64 [b]	65.42 [a]	911 [a]	11.60 [a]

These results agree with those found in literature, taking into account that the samples were obtained from the whole lemon. In fact, reported values of 4.98-9.92 mg GAE/g in lemon peel after 24-26 h of oven-drying (Li, Smith, & Hossain, 2006) and 15.68-23.57 mg GAE/g as dry weight in peel of Mauritanian lyophilised lemon (Ramful, Bahorun, Bourdon, Tarnus, & Aruoma, 2010). The values reported for lemon juice were 17.5 mg GAE/g as dry weight and 5.1 mg GAE/g as dry weight for lemon raw peeled (USDA, Department of Agriculture, Agricultural Research Service, US., Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods, 2007, http://www.ars.usda.gov/nutrientdata). However, the total phenolic content did not behave in the same way as the phenolic content determined by HPLC-ESI-TOF-MS. This may have resulted from the overestimation by Folin-Ciocalteu because of interference from other agents present in food, such as carotenoids, amino acids, sugars, and vitamin C (Vinson, Su, Zubik, & Bose, 2001).

3.5. Antioxidant activity

For the evaluation of the antioxidant capacities of foods, numerous *in vitro* methods have been developed and reviewed. However, there is still no consensus for the preferred method. ORAC (oxygen radical absorbance capacity) and TEAC (trolox equivalent antioxidant capacity) were suggested to be used in the routine quality control and measurement of antioxidant capacity of food, dietary supplements, and other botanicals, at the First International Congress on Antioxidant methods, held in Orlando, Florida, USA in 2004 (Prior, Wu, & Schaich, 2005). Recently, Jiménez-Alvarez *et al.* (2008) assayed methods with different antioxidant mechanisms and held that ORAC and FRAP (ferric ion reducing antioxidant power) assays provided a comprehensive, precise and high-throughput assessment of antioxidant capacity in food extracts.

These three methods, with different mechanisms, have been applied to determine the antioxidant capacity of lemon: ORAC and TEAC based on a hydrogen-transfer mechanism (HAT) and FRAP based on single electron transfer (SET).

These methods measure the radical-scavenging capacity or the reducing ability, respectively. The results are summarised in Table 4.

ORAC assay: Lemon freeze-dried samples showed a value of 913 µmol TEs (trolox equivalents)/g lemon and the vacuum dried samples stored at 50 ºC for 1-3 months presented values of 1043 (L0), 914 (L1), and 911 (L3) µmol TEs/g lemon, respectively (Table 4). No statistical differences were detected between the two drying procedures or among dried (L0) and stored samples L1 and L3.

ORAC assay is considered a standard tool to measure the antioxidant activity in the nutraceutical, pharmaceutical, and food industries, because of its simplicity, biological relevance, and the possibility of measuring the lipophilic and hydrophilic antioxidant capacity (Prior *et al.*, 2005). The literature consulted has not reported information on antioxidant capacity in whole lemon (pulp, peel, and/or seeds) powder. The values reported for lemon juice and raw lemon without peel were 1225 and 1346 µmol TEs/ 100 g of fresh weight (FW), respectively (Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods-2007; U.S. Department of Agriculture, http://www.ars.usda.gov/nutrientdata). According to these results, whole-lemon powder reported around 10-fold more antioxidant activity than juice or peeled lemon, if 12% dry matter is considered.

***FRAP assay*:** In this assay the antioxidant capacity of fruit extracts was determined by the ability of the antioxidants in these extracts to reduce ferric to ferrous iron in FRAP reagent. The antioxidant capacities of the lemon-powder extracts tested were of 70.9 µmol of TEs/g for freeze-dried sample and 67.07, 66.37, and 65.42 µmol of TEs/g for lemon dried by vacuum (L0) and stored for 1 month (L1) and 3 months (L3) at 50 ºC, respectively (Table 4). A decrease in antioxidant capacity could be detected between freeze-dried and vacuum-dried samples, but no statistical differences were detected between the two drying procedures or among vacuum-dried (L0) and stored samples (L1 and L3).

TEAC assay: The results showed higher antioxidant capacity in freeze-dried lemon (63.25 µmol of TEs/g) than in vacuum-dried lemon (L0) (43.16 µmol of

TEs/g). The storage for 1 and 3 months reduced the antioxidant capacity to 41.72 and 40.11 lg TEs/g, respectively (Table 4). Significant differences were found between freeze-dried and vacuum-dried samples and between freeze-dried and stored samples at 50 ºC. Antioxidant activity detected in peel extracts was 13.3 and 11.5 µmol TEs/g of fresh weight (Ramful *et al.*, 2010) The average antioxidant capacity of fruits consumed in Spain is 10.2 µmol TEs/g as dry matter of the edible part (Saura-Calixto & Goni, 2006), much lower than the results found for the lemon powders studied.

The results indicate that ORAC and FRAP registered similar values while TEAC had a higher value for freeze-dried lemon than for L0, L1, or L3, in agreement with Jimenez-Alvarez *et al.* as stated above. However, the only antioxidant activity assay that was correlated to the phenolic content obtained by HPLC-ESI-TOF-MS was ORAC ($r = 0.9909$; $p < 0.05$). FRAP and TEAC were not correlated with the phenolic content by HPLC.

3.6. Indicators of non-enzymatic browning: hydroxymethylfurfural and furfural

During drying and storage, chemical changes take place, such as the non-enzymatic browning reactions, which are responsible of nutritional and sensorial quality changes. These reactions are favoured in systems with intermediate moisture content, temperatures over 50 ºC, pH 5-7 and long processing times, which are the main characteristics of the dehydration process (Maillard reaction) or high temperature and acid medium in the presence of sugar (caramelization); as well as from degradation of L-ascorbic acid (Clegg, 1964). Later, furan-type compounds, lactones, acids and 3-hydroxy-2-pyrone as degradation products of L-ascorbic acid were isolated and these compounds were identified as non-enzymatic browning products from citrus fruit concentrates and powders (Meydav, Saguy, & Kopelman, 1977). The effect of L-ascorbic acid degradation on browning can be accelerated by amino acids (Clegg, 1964).

Hydroxymethylfurfural (HMF) is a parameter normally used by the industry and researchers to evaluate heat damage in vegetable and fruits processed. Furfural is formed in processed food from ascorbic acid during thermal treatment or storage (Nagy & Dins- more, 1974) but also from the degradation of sugars (pentoses) or hydroxymethylfurfural (Yuan & Chen, 1998), and it is a useful indicator for assessing the extent of non-enzymatic browning (Guerrero, Ventura, & Bota, 1988). In 1974, it was observed that browning resulted from reducing sugar (fructose and glucose) in orange juice (Wolfrom, Kashimura, & Horton, 1974). In addition, HMF is formed from hexoses, such as fructose in acid medium from orange juice (Resnik & Chirife, 1979). The thermal treatment and storage of citric juices causes a gradual loss of sugars, and fructose is the potential source of HMF formation (Guerrero *et al.*, 1988). Since the accumulation of these compounds influences the quality loss of citrus products, furfural and/or HMF indexing has been recommended as a basis for quality control.

HMF was detected in freeze-dried lemon samples with values of 0.95 mg/kg and the dehydration at low pressure increased the value at 5.03 mg/kg. The values of furfural were 0.21 mg/kg for lemon freeze-dried and 0.29 mg/kg for samples dehydrated at low pressures (Table 5).

Table 5. HMF and furfural values (mg/kg) of dried and stored lemon samples

Furanic compounds	Freeze-dried	Vacuum Dried (L0) (t =0)	Stored[a] (L1) (t =1 month)	Stored[a] (L3) (t =3 months)
HMF	0.95	5.03	1717	1830
Furfural	0.21	0.29	68	105

[a] Storage temperature: 50 ºC

The values for both procedures were considerably lower than the HMF determined in orange and banana commercial powders previously analysed, 20.2 and 32.1 mg/kg, respectively (Fernández-Artigas, Guerra-Hernández, & García-

Villanova, 1999). These results also agree with data reported by Rufian-Henares *et al.* where 42 vegetables were treated in a similar way as L1 lemon samples, and HMF was determined to control the thermal damage and storage. HMF was not detected in most of the vegetables analysed. Only garlic, onion, and tomato showed measurable HMF concentrations in the different samples with values between 0.01 to 4.77 mg/kg for garlic and tomato samples, respectively (Rufián-Henares *et al.*, 2008). As expected, according to the browning of the lemon samples, storage under extreme conditions (1 and 3 months at 50 ºC) drastically raised the HMF and furfural values. 1 and 3 months of storage resulted in HMF values of 1717 and 1830 mg/kg, respectively, and 68 and 105 mg/kg for furfural, respectively. The acid pH, the sugar content, and the ascorbic acid content of lemon considerably intensified the non-enzymatic browning reactions.

It is well known that natural antioxidants in foods are lost during such processing as sterilization, pasteurisation, dehydration, storage, handling, and high-temperature cooking (Jonsson, 1991). Nevertheless, during the last two decades, it has been demonstrated that thermal treatment can induce the formation of new compounds with new antioxidant properties. In this sense, 2-furoylmethylamino acid, which is generated during the acid hydrolysis of the corresponding Amadori compounds, is the most reliable compound to detect the early stages of Maillard reactions in food products. This compound has been determined in orange juice (Del Castillo, Villamiel, Olano, & Corzo, 2000).

The experiment of determining the antioxidant capacity in lemon-powder storage showed no losses of antioxidant capacity by any of the methods. To date, the Maillard reaction products have lead to controversial findings. However, in the literature related to Maillard reaction products (MRPs), there are many works that have indicated that MRPs could act as antioxidants (Benjakul, Lertittikul, & Bauer, 2005; Osada & Shibamoto, 2006).

According to the literature, it is possible to explain the results found in the antioxidant capacity assays and their lack of correlation to the phenolic content

determined by HPLC-ESI-TOF-MS. The lack of correlation between phenolic content and antioxidant activity may be due to the increase in antioxidant Maillard reaction products, which, even after a decrease of phenolic compounds because of thermal treatments, maintain the antioxidant activity constant because of the new antioxidant Maillard reaction products.

4. Conclusions

Here, different treatments, storage temperature, and storage times were applied to lemon samples in order to evaluate the effects of these variables on the phenolic profile, the content in furanic derivatives, and the antioxidant activity of whole-lemon powders.

A powerful HPLC method coupled to DAD and TOF-MS has been used to study the polar fraction of whole lemon, and 23 compounds have been identified. It is important to highlight that, this is the first available report of two hydroxycinnamic acids identified in lemon. The analysis of the different samples showed that freeze-dried lemon powder had a phenolic content significantly lower than the low-vacuum-dried lemon (L0), which demonstrates that low temperature drying negatively influences the phenolic composition of lemon. Indeed, high-temperature storage for long time periods (L1 and L0) dramatically reduces the phenolic compounds in whole-lemon powder.

Moreover, the correlation between the phenolic compounds and the antioxidant activity, indicated that only the ORAC assay was correlated to the phenolic content. This finding could be explained by the results for the HMF and furfural compounds, which revealed an increase after the thermal and long-term storages. The increase in furanic derivatives indicates the Maillard reaction, which can promote the formation of antioxidant Maillard reaction products that influence the antioxidant activity assays. Therefore, the phenolic profile and the antioxidant activity may indicate the presence of Maillard reaction products and heat damage in processed vegetables and fruit.

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CAPÍTULO 5

Identification and quantification of phenolic compounds in diverse cultivars of eggplant grown in different seasons by high-performance liquid chromatography coupled to diode array detector and electrospray-quadrupole-time of flight-mass spectrometry

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Identification and quantification of phenolic compounds in diverse cultivars of eggplant grown in different seasons by high-performance liquid chromatography coupled to diode array detector and electrospray-quadrupole-time of flight-mass spectrometry

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Abstract

Eggplant (*Solanum melongena* L.) is an important vegetable for its richness in healthy components such as phenolic compounds. As environmental conditions and growing techniques may influence the phenolic content in plants, this study was focused on characterizing and quantifying of phenolic compounds, by high-performance liquid chromatography coupled to diode array detector and electrospray-quadrupole-time of flight-mass spec-trometry (HPLC-DAD-ESI-TOF-MS), in three eggplant cultivars grown in different seasons. A total of 25 compounds were identified and quantified by the optimized methodology. To our knowledge, of these compounds, 14 have been reported for the first time in eggplant, 9 of which have been found for the first time in Solanaceae family. Two of the major compounds were chlorogenic acid and delphinidin rutinoside in all eggplant cultivars studied; furthermore, most of the phenolic compounds showed significant differences according to the cultivar and the harvest season. Besides, phenolic compounds of two of the cultivars sharply decreased from spring to summer. These results showed that phenolic compounds concentration depends on the cultivar adaptation to abiotic variation. Indeed, it was seen that cultivar and harvest season have a marked influence on polyphenols in eggplant which could be due mainly to climatic factors such as the high increase of

temperatures in the summer season, in the region of Andalusia. This information may be important for the agricultural sector because it can help to know the suitable period for harvesting eggplants with high content in phenolic compounds.

Keywords: Solanum melongena L., Eggplant Cultivar, Phenolic compounds, Season, HPLC–DAD–ESI-Q-TOF-MS

1. Introduction

It has been well demonstrated that fruits and vegetables provide, in addition to other basic nutrients, bioactive substances with beneficial effects on human health (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010). In fact, the consumption of fruits and vegetables has been associated with lower incidence and lower mortality rates of cancer, and cardiovascular diseases in humans (Raigón, Prohens, Muñoz-Falcón, & Nuez, 2008). Besides, the human diet is fundamental in the protection against oxidative stress, because many important antioxidants cannot be synthesized by the human body, and must be derived from diet (Okmen *et al.*, 2009).

Vegetables belonging to the Solanaceae family, such as tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.), pepper (*Capsicum annuum* L.), and potato (*Solanum tuberosum* L.) are important for their richness in these healthy components, as previously described (Helmja, Vaher, Gorbatsova, & Kaljurand, 2007; Morales-Soto, Gómez-Caravaca, García-Salas, Segura-Carretero, & Fernández-Gutiérrez, 2013). In particular, eggplant fruit is one of the most widely consumed vegetables in the world, the most popular species being the common eggplant (*S. melongena*) (Sánchez-Mata, Yokoyama, Hong, & Prohens, 2010). *S. melongena*, a native of Southeast Asia, is also one of the most common vegetable crops in some Mediterranean countries (Concellón, Zaro, Chaves, & Vicente, 2012), although, there are many eggplant cultivars that vary in the color, size, and shape of the fruit (Luthria *et al.*, 2010).

Eggplant has been found to have strong antioxidant activity, this being attributed to its high levels of phenolic compounds. The main family of polyphenols found in the eggplant flesh are phenolic acids (chlorogenic acid, caffeic acid, p-coumaric acid), and anthocyanins such as nasunin and delphinidin conjugates in eggplant peels of pigmented cultivars (Azuma *et al.*, 2008; Helmja *et al.*, 2007). Apart from their free-radical scavenging activity, eggplant phenolics also have verified antitumoral and anticholeresterolemic properties (Raigón, Rodríguez-

Burruezo, & Prohens, 2010; Whitaker & Stommel, 2003). Several *in vivo* studies have also demonstrated that phenolic compounds extracted from eggplant fruit have a significant hypolipidemic action (Sudheesh, Presannakumar, Vijayakumar, & Vijayalakshmi, 1997); in addition, eggplant extracts have been found to inhibit protein-activated receptor-2 inflammation associated with atherosclerosis (Han *et al.*, 2003). In fact, the human-health benefits of hydroxycinnamoyl esters of quinic acid, collectively referred to as chlorogenic acids, are attributed to a broad range of biological activities, including free-radical scavenging and anti-inflammatory, antiviral, antimicrobial, antimutagenic, and anti-carcinogenic action (Ma, Dastmalchi, Whitaker, & Kennelly, 2011; Wu, Meyer, Whitaker, Litt, & Kennelly, 2012).

Moreover, different authors have documented that cultivar, environment, soil type, and growing and storage conditions can influence in the quantity and quality of phenolic phytochemicals present in vegetables (Singh *et al.*, 2009).

Several methods have been used to determine polyphenols in eggplant matrices. As reported in literature, high-performance liquid chromatography (HPLC), because of its high separation capacity and relative simplicity, has been the most frequently used technique for separating and quantifying phenolic compounds and anthocyanins in eggplant fruits and Solanum species (Luthria *et al.*, 2010; Singh *et al.*, 2009; Wu, Meyerb, Whitaker, Litt, & Kennelly, 2013). Although, capillary zone electrophoresis (CZE) has also been used to determine phenolic compounds in the skin of plants of the Solanaceae family (Helmja *et al.*, 2007).

In combination with HPLC, some studies have used NMR for structural elucidation (Azuma *et al.*, 2008; Ichiyanagi *et al.*, 2005; Ma *et al.*, 2011). However, the most common detection system used has been the diode-array detector (DAD) (Luthria *et al.*, 2010; Todaro *et al.*, 2009), while only a few research works have used mass spectrometry (Alarcón-Flores, Romero-González, Vidal, & Frenich, 2013; Ichiyanagi *et al.*, 2005; Singh *et al.*, 2009).

Therefore, the aim of our study was the comprehensive identification and quantification of phenolic compounds of three eggplant cultivars grown in Andalusia (Spain) and collected in different seasons, in order to study the evolution of phenolic compounds. Eggplant samples were analyzed by a high-performance liquid chromatography coupled to diode array detector and electrospray-quadrupole-time of flight-mass spectrometry (HPLC-DAD-ESI-TOF-MS) methodology to determine structures based on fragmentation patterns using the accurate mass measurements of the deprotonated molecules and product ions obtained with MS/MS experiments. To our knowledge, this is the first available report using a QTOF-MS detector to analyze phenolic compounds in eggplant samples.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetic acid and acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and methanol for dissolving the samples was 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 standard compounds were bought from different sources: delphinidin-3-O-rutinoside was from Extrasynthese (Lyon, France), and chlorogenic acid (3-caffeoylquinic acid) and rutin were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples

About 1-1.5 kg of three eggplant cultivars, purple-striped (PSE), long (LE), and round (RE) eggplant were collected in spring and summer during 2011 (the warmer seasons, because eggplants need warm temperatures to develop high-

quality products). These cultivars were selected for their commercial and nutritional properties, as well as for being representative of crops from Almería (south-eastern Spain). All samples were from plants grown under the same agricultural conditions. Fresh samples were milled, homogenized, stored at -25 °C and placed on a lyophilizer shelf (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK), which was pre-cooled to -50 °C for 1 h at 1 mbar. Afterwards, the samples were kept at -18 °C until used.

2.3. Extraction of the phenolic compounds of eggplant

A solid-liquid extraction system was used to extract the phenolic fraction. Briefly, 0.5 g of freeze-dried eggplant powder was dissolved in 15 mL of a solution of methanol/water/acetic acid (80:20:0.5 v/v). The mixture was placed in an ultrasonic bath for 15 min at room temperature and was then centrifuged (Heraeus Sepatech Labofuge 200, Thermo Fisher Scientific Inc., Waltham, MA) for 15 min at 1000 g; the supernatant was removed, and the extraction was repeated once more.

The supernatants were collected, evaporated, and reconstituted in 2 mL of methanol/water/acetic acid (80:20:0.5 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.

2.4. HPLC-DAD-QTOF-MS analyses

An Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a UV-vis detector was used for the chromatographic determination. A methodology previously optimized by Gómez-Caravaca, Segura-Carretero, Fernández-Gutiérrez, and Caboni (2011) was used and further modified to analyze the eggplant samples. The separation was done using an Agilent Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 μm) from Agilent Technologies, operating at a

flow rate of 0.8 mL/min throughout the gradient. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the following conditions: 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B, 12 min, 18% B, 14 min, 20% B, 15 min, 22% B, 16 min, 25% B, 18 min, 28% B, 19 min, 30% B, 20 min 31% B, 21.50 min, 32% B, 25 min, 100% B, 30 min, 100% B, 32 min, 5% B. The injection volume was 0.5 μL and UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, 330 and 520 nm.

The HPLC system was coupled to a microTOF-Q (Bruker Daltonik, Bremen, Germany) instrument, using an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The flow rate used in the HPLC method was too high to achieve stable electrospray ionization (ESI); therefore, a flow divisor 1:3 was used to reduce the flow delivered into the mass spectrometer to 0.2 mL/min. Parameters for analysis were set using negative and positive ion modes with spectra acquired over a mass range from *m/z* 50 to 1100. According to this, the optimum values of ESI-Q-TOF-MS/MS parameters in negative mode were: capillary voltage, 4000 V; drying gas temperature, 210 °C; drying gas flow, 8 L/min; nebulizing gas pressure, 2 bar; and end-plate offset, -500 V. And the optimum parameter values in the positive mode were: capillary voltage 4500 V; drying gas temperature 210 °C; drying gas flow 8 L/min; and nebulizing gas pressure 2 bar.

During the use of the HPLC method, an external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, 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 plotted based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$). Due to the compensation of temperature drift in the TOF, this external calibration provided

accurate mass values for a complete run without the need for a dual sprayer set-up for internal mass calibration.

The accurate mass data for the molecular ions were processed using the software Data Analysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as a 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.

To perform the quantification of phenolic compounds four calibration curves were prepared using the following standards: chlorogenic acid, rutin, delphinidin-3-O-rutinoside and 4-hydroxybenzoic acid, in the range of concentrations from the limit of quantification (LOQ) to 1000 μg/mL and 500 μg/mL for delphinidin-3-O-rutinoside. Ten calibration points were used for each of them except for anthocyanidin, where eight points were used, and the analyses were replicated three times for each calibration point (n = 3).

2.5. Statistical analysis

Factorial ANOVA univariate analysis was also used to determine the effects of the eggplant cultivars, harvest season, and interaction between these two factors as variables (Statistica 8.0 software, StatSoft, Tulsa, OK, USA).

All chemical analyses were carried out in triplicate, and the analytical data were used for statistical comparisons.

3. Results and discussion

3.1. Identification of phenolic compounds in eggplant

Eggplant extracts obtained by solid-liquid extraction were analyzed using an HPLC coupled to DAD and ESI-QTOF-MS in negative and positive ionization modes in order to identify the phenolic compounds. Peak identification was based on their relative retention time values, their UV-vis spectra and mass spectra determined by the QTOF-MS, and information from the literature. Fig. 1 shows the UV chromatograms at λ = 280 and 520 nm of the phenolic fraction of an eggplant extract obtained under the optimum conditions of the method. A total of 25 phenolic compounds and derivatives were tentatively identified, as summarized in Table 1, including the retention time, molecular formula, experimental and calculated *m/z*, fragments, sigma value, and error. Also, Fig. 2 presents the MS/MS spectra of the compounds that, to our knowledge, have been identified for the first time in eggplant.

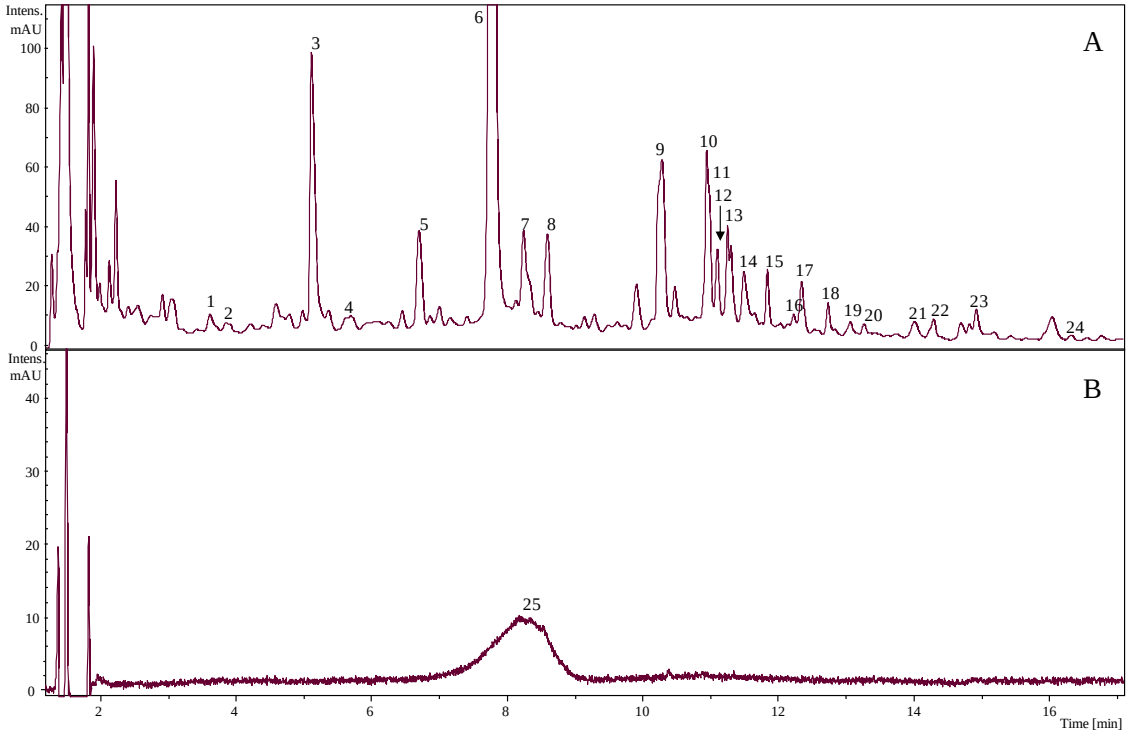

Fig. 1. Chromatograms of the phenolic fraction of eggplant extract PSE obtained by UV–vis at λ = 280 nm (A) and UV–vis at λ = 520 nm (B). See Table 1 for the identification of phenolic compounds

Of these compounds, 24 were studied in negative ionization. Most of the compounds identified were caffeoylquinic acid derivatives as follows: peak 5 with *m/z* 515.1422 and retention time 6.72 min was presumably assigned to dicaffeoylquinic acid according with the MS data and fragmentation pattern at *m/z* 353 corresponding to the loss of the caffeoyl quinic acid fragment and *m/z* 191 due to the loss of quinic acid moiety (Clifford, Johnston, Knight, & Kuhnert, 2003). The presence of this compound in eggplant has been reported by other authors (Whitaker & Stommel, 2003; Wu *et al.*, 2013), and has also been found in other vegetables of the Solanum genus family (Shakya & Navarre, 2006).

Three caffeoylquinic acid isomers with retention times of 7.76, 8.23, and 8.58 min (peaks 6, 7, and 8) were identified and designated as 3-caffeoylquinic acid (chlorogenic acid; *m/z* 353.0883), 5-caffeoylquinic acid (*m/z* 353.0876) and 4-caffeoylquinic (*m/z* 353.0885) acid, respectively (Clifford, Knight, & Kuhnert, 2005). These compounds have been identified previously in different eggplant

cultivars (Singh *et al.*, 2009; Whitaker & Stommel, 2003). Peak 6 was compared with 3-caffeoylquinic acid standard used to plot the calibration curve and it presents the same retention time, molecular formula, and fragmentation pattern, with a representative fragment at *m/z* 191 resulting from the loss of quinic acid and another fragment at *m/z* 179 as reported by Clifford *et al.* (2005). Peak 7 presented a strong fragment at *m/z* 173 and other minor fragments at *m/z* 191 and 179, and thus it was tentatively identified as 4-caffeoylquinic acid. The last caffeoylquinic acid isomer, peak 8, showed a strong fragment at *m/z* 191 and a very weak ion at *m/z* 179, and therefore it was tentatively identified as 5-caffeoylquinic acid. This identification was based on Clifford *et al.* (2005).

Peak 11 with *m/z* 705.1690, was detected at a retention time 10.99 min. Another five isomers were found at retention times 11.22 min (peak 13, *m/z* 705.1688), 12.33 min (peak 17, *m/z* 705.1669), 12.71 min (peak 18, *m/z* 705.1676), 14.27 min (peak 22, *m/z* 705.1665), 14.90 min (peak 23, *m/z* 705.1666). The MS/MS spectra of all of these showed two fragments at *m/z* 513 and 191 derived from the loss of a quinic acid moiety (Fig. 2A), and thus were tentatively identified as caffeoylquinic dehydrodimers that could have resulted from oxidative coupling. The presence of these compounds has previously been reported in other vegetables (Guyot, Bernillon, Poupard, & Renard, 2008, 2009).

Table 1. Phenolic compounds tentatively identified in eggplant samples (n.f.: non-fragmented).

Peak	Compound	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula	MS/MS	UV (nm)	Error (ppm)	mSigma
				Negative mode					
1	(Dihydrocoumaroyl glucoside) amide	3.61	326.1239	326.1245	$C_{15}H_{21}NO_7$	164	229	1.8	4.5
2	(Dihydrocoumaroyl glucoside) amide (isomer 2)	3.84	326.1239	326.1245	$C_{15}H_{20}NO_7$	164	229	1.9	29.4
3	N-Caffeoylputrescine	5.36	249.1243	249.1245	$C_{13}H_{18}N_2O_3$	250/135	229-292-320	0.7	2.7
4	Homovanillic acid hexose	5.60	343.1035	343.1035	$C_{15}H_{20}O_9$	181	229	0.1	11.5
5	Dicaffeoylquinic acid	6.72	515.1422	515.1195	$C_{25}H_{24}O_{12}$	353/323/191	229-283	40.4	16.8
6	Chlorogenic acid (3-caffeoylquinic acid)	7.76	353.0883	353.0878	$C_{16}H_{18}O_9$	191	236-295-326	1.3	3.4
7	5-Caffeoylquinic acid	8.23	353.0876	353.0878	$C_{16}H_{18}O_9$	191	229-282-320	0.6	36.5
8	4-Caffeoylquinic acid	8.58	353.0885	353.0878	$C_{16}H_{18}O_{11}$	191	229-293-325	2.1	5.5
9	Bis(dihydrocaffeoyl)spermidine	10.25	472.2475	472.2453	$C_{25}H_{35}N_3O_6$	350/308	229-284-322	4.7	12.5
10	Caffeoyl(dihydrocaffeoyl)spermidine	10.92	470.2305	470.2297	$C_{25}H_{33}N_3O_6$	308	230-288-321	1.8	28.1
11	Caffeoylquinic dehydrodimer (isomer 1)	10.99	705.1690	705.1672	$C_{32}H_{34}O_{18}$	513/191	230-288-324	2.4	16.9
12	Caffeoylshikimic acid	11.11	335.0778	335.0772	$C_{16}H_{16}O_8$	179	231-292-325	1.6	8.0
13	Caffeoylquinic dehydrodimer (isomer 2)	11.22	705.1688	705.1672	$C_{32}H_{34}O_{18}$	513/191	231-288-325	2.4	16.9
14	Feruoylquinic acid	11.49	367.1031	367.1035	$C_{17}H_{20}O_9$	191	233-291-325	1.4	6.2
15	N,N-Dicaffeoylspermidine	11.83	468.2158	468.2140	$C_{25}H_{31}N_3O_6$	332	234-291-320	3.7	36.1
16	Quercetin 3-O-gentiobioside	12.21	625.1431	625.1410	$C_{27}H_{29}O_{17}$	n.f.	234-267-334	3.3	20.5
17	Caffeoylquinic dehydrodimer (isomer 3)	12.33	705.1669	705.1672	$C_{32}H_{34}O_{18}$	513/191	235-288-322	0.5	30.0
18	Caffeoylquinic dehydrodimer (isomer 4)	12.71	705.1676	705.1672	$C_{32}H_{34}O_{18}$	513/191	236-288-321	0.5	24.3
19	Kaempferol dihexoside (isomer 1)	13.06	609.1481	609.1461	$C_{27}H_{30}O_{16}$	285	236-264-324	3.2	29.3
20	Kaempferol dihexoside (isomer 2)	13.26	609.1524	609.1461	$C_{27}H_{30}O_{16}$	285	237-265-321	10.3	8.1
21	Tris(dihydrocaffeoyl) spermine	13.98	693.3531	693.3505	$C_{37}H_{50}N_4O_9$	350	237-285	3.7	31
22	Caffeoylquinic dehydrodimer (isomer 5)	14.27	705.1665	705.1672	$C_{32}H_{34}O_{18}$	513/191	238-287-322	1.0	18.1
23	Caffeoylquinic dehydrodimer (isomer 6)	14.90	705.1666	705.1672	$C_{32}H_{34}O_{18}$	513/191	238-285-323	0.9	17.3
24	Kaempferol-3-O-rutinoside	16.29	593.1537	593.1512	$C_{27}H_{30}O_{15}$	285	238	4.2	28.5
				Positive mode					
25	Delphinidin rutinoside	8.33	611.1639	611.1607	$C_{27}H_{30}O_{16}$	303	520	5.3	8.8

Compound 12 at 11.11 min and *m/z* 335.0778 showed different fragments in the MS/MS spectrum at *m/z* 179, 161 [179-H_2O], and 135 (Fig. 2B), which are typical fragments of caffeic acid derivatives. The deprotonated molecular ion at *m/z* 335 indicated that this compound had one H_2O molecule less than caffeoylquinic acid; consequently, it was reasonable to assume that compound 12 was an ester of caffeic acid and dehydrated quinic acid (shikimic acid), and so it was designated as caffeoylshikimic acid. This compound has previously been identified in other vegetable matrices such as plum (Fang, Yu, & Prior, 2002).
At a retention time 11.49 and *m/z* 367.1031, the compound feruoylquinic acid (peak 14) was identified. A common fragment with *m/z* 191, which corresponds to the loss of quinic acid, was also found. The presence of this compound in *S. melongena* and other Solanaceae vegetables has been reported elsewhere (Gómez-Romero *et al.*, 2010; Shakya & Navarre, 2006; Wu *et al.*, 2013).

A benzoic acid was also found, at a retention time of 5.60 min and *m/z* 343.1035 (peak 4) and it was tentatively identified as homovanillic acid hexose. This compound presented a fragment at *m/z* 181 corresponding to homovanillic acid after the loss of the hexose molecule (Fig. 2C). This compound has previously been found in tomato (Gómez-Romero *et al.*, 2010) and pepper (Morales-Soto *et al.*, 2013), which are also Solanaceae vegetables.

Flavonoids comprised another family of phenolic compounds found to be present in eggplant. Quercetin 3-O-gentiobioside was tentatively identified at 12.21 min (peak 16), *m/z* 625.1410 and fragment at *m/z* 301. This compound had been identified previously in *S. melongena* and other Solanum species (Wu *et al.*, 2013). Peaks 19 and 20 at *m/z* 609 and retention times 13.06 and 13.26 were tentatively identified as dihexoside derivatives of kaempferol due to the fragments found at *m/z* 285, indicating the loss of a kaempferol moiety (Fig. 2D). Furthermore, different authors confirm the presence of kaempferol and kaempferol derivatives in eggplant and other vegetables of Solanaceae family (Calderón-Montano, Burgos-Morón, Pérez-Guerrero, & López-Lázaro, 2011; Ferreres, Taveira, Pereira, Valentao, & Andrade, 2010; Miean & Mohamed,

2001). Another flavonoid, peak 24, at retention time 16.29 min and *m/z* 593.1512 was proposed as kaempferol-3-O-rutinoside, with MS/MS fragmentation generating the kaempferol aglycone at *m/z* 285 [M-H-rutinose] (Fig. 2E). This compound was previously reported in other Solanaceae vegetables, i.e. potato and tomato (Gómez-Romero *et al.*, 2010; Shakya & Navarre, 2006).

Furthermore, a total of seven hydroxycinnamic acid amide conjugates were tentatively identified, using their UV spectra and mass data. In addition, it has been reported that amide conjugates of hydroxycinnamic acids are common in species belonging to Solanaceae (Whitaker & Stommel, 2003). Also, (dihydrocoumaroyl glucoside) amide (isomer 1) (peak 1) and isomer 2 (peak 2) at retention times 3.61 min and 3.85 min, respectively, were tentatively identified. Both, isomer 1 and isomer 2 with *m/z* 326.1239 presented a fragment with *m/z* 164 corresponding to dihydrocoumaroyl amide moiety (Fig. 2F).

Peak 3 at a retention time 5.36 min, *m/z* 249.1243 and fragments at *m/z* 250 and 135, was designated as N-caffeoylputrescine. This compound has previously been described in eggplant samples (Singh *et al.*, 2009; Wu *et al.*, 2013) as well as in potato samples (Shakya & Navarre, 2006). Peak 9 (*m/z* 472.2475 and retention time 10.25 min), was recognized as bis-(dihydrocaffeoyl)-spermidine (Wu *et al.*, 2013). This peak showed fragments at *m/z* 308 corresponding to the loss of one of the dihydrocaffeoyl groups and *m/z* 350. Peak 20 (*m/z* 693.3531 and retention time 13.982 min) was identified as tris-(dihydrocaffeoyl)-spermine. This compound presents a fragment at *m/z* 350 (Fig. 2G) as previously reported in potato (Shakya & Navarre, 2006). At 11.83 min and *m/z* 468.2158, N,N-dicaffeoylspermidine (peak 15) was identified; this compound has been reported in eggplant pulp by other authors (Singh *et al.*, 2009; Whitaker & Stommel, 2003). The last caffeic acid derivate tentatively identified was caffeoyl-(dihydrocaffeoyl)-spermidine (peak 10) at a retention time of 10.92 min and *m/z* 470.2305; the fragment found at *m/z* 308 explains the loss of dihydrocaffeoyl molecule, this compound has been reported previously in *S. melongena* species (Wu *et al.*, 2013).

In positive mode (Singh & Saldana, 2011), an anthocyanidin was found. This compound corresponded to peak 25 at 8.33 min and *m/z* 611.1639; when its MS and UV data together with its retention time were compared with those of its standard as well as with previously published data, this compound was proposed as delphinidin rutinoside, which has previously been reported in this species of eggplant by different authors (Azuma *et al.*, 2008; Wu & Prior, 2005).

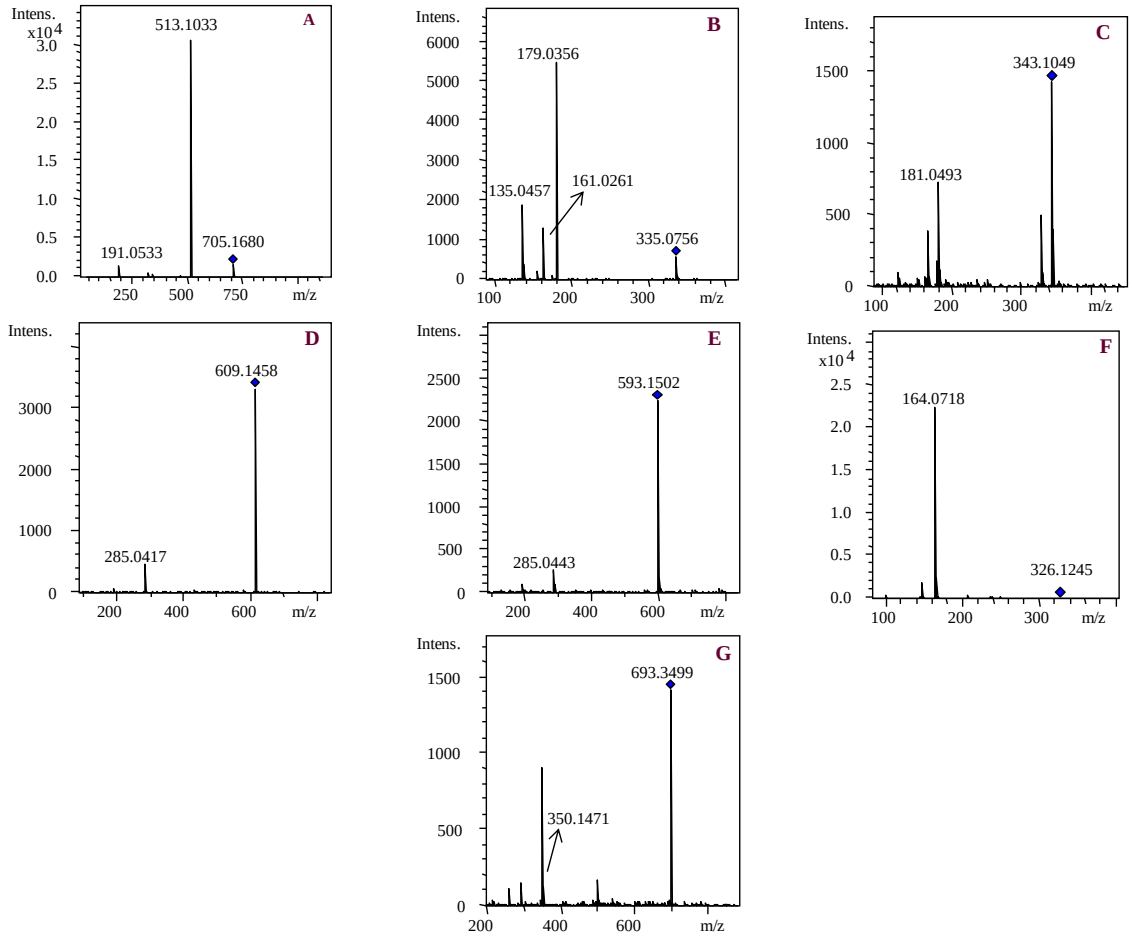

Fig. 2. MS/MS spectra of phenolic compounds tentatively identified for the first time in eggplant. A, caffeoylquinic dehydrodimer; B, caffeoylshikimic acid; C, homovanillic acid hexose; D, kaempferol dihexoside; E, kaempferol-3-O-rutinoside; F (dihydrocoumaroyl glucoside) amide; G, tris(dihydrocaffeoyl) spermine.

3.2. Quantification of phenolic compounds of eggplant

The different parameters for each calibration curve have been summarized in Table 2: sensitivity [relative standard deviations (RSDs) (%)], linearity (r^2), calibration range, and correlation coefficient.

All calibration curves showed good linearity among different concentrations, depending on the analytes studied. The calibration plots indicate good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.999 in all cases. The LOQ was determined as the signal-to-noise ratio of 10:1, and the limit of detection (LOD) was determined as a signal-to-noise ratio of 3:1. The LOD was found to be within the range 0.007-0.051 μg/mL, while the LOQ was within 0.024-0.168 μg/mL.

Intraday and interday precision was developed to evaluate the repeatability of HPLC-ESI-QTOF-MS method. A methanol-water extract was injected (n = 3) on the same day (intraday precision) for 3 consecutive days (interday precision, n = 9). The RSDs of the retention time and peak area were determined.

The repeatability (expressed as % RSDs) of the intraday retention times was from 0.06 to 0.86%, whereas the interday repeatability was 2.77%. The intraday repeatability (expressed as% RSDs) of the total peak area was from 1.02 to 2.29%, while, the interday repeatability was 4.94%.

The quantification was performed by using the previous calibration curves. The calibration curve of chlorogenic acid at λ = 280 nm was used to quantify hydroxycinnamic acids such as caffeic acid and coumaric acid derivatives.

Homovanillic acid hexose was quantified with the calibration curve of 4-hydroxybenzoic acid at λ = 280 nm, flavonoids were quantified using the calibration curve of rutin at λ = 280 nm while the anthocyanidin was quantified with delphinidin-3-O-rutinoside.

Table 2. Analytical parameters of the method proposed.

Analyte	RSD	LOD (µg/mL)	LOQ (µg/mL)	Calibration range (µg/mL)	Calibration equations	r^2
Chlorogenic acid	1.140	0.018	0.060	LOQ-1000	y = 12.59x – 13.29	0.999
Rutin	1.583	0.051	0.168	LOQ-1000	y = 4.51x + 5.18	0.999
Delphinidin-3-O-rutinoside	2.058	0.007	0.024	LOQ-500	y = 31.87x + 99.72	0.999
4-hydroxybenzoic acid	1.189	0.030	0.101	LOQ-1000	y = 7.50x + 7.60	0.999

The methanolic extracts of the different eggplant extracts were analyzed by the HPLC-DAD-ESI-QTOF-MS method and the quantitative results are presented in Table 3.

It should be taken into account that the response of the standards can differ from the response of the analytes present in the eggplant samples, and consequently, the quantification of these compounds is only an estimation of their actual concentrations.

Table 3. Phenolic compounds, expressed in mg/g, found in different eggplant cultivar according to the harvest season sampled.

	COMPOUNDS	SPRING						SUMMER					
		PSE	RSD	LE	RSD	RE	RSD	PSE	RSD	LE	RSD	RE	RSD
1	(Dihydrocoumaroyl glucoside) amide (isomer 1)	25.7-28.4	7.0	69.9-87.6	15.9	20.9-25.1	13.0	19.5-21.9	8.2	18.1-27.0	19.6	23.0-24.0	2.9
2	(Dihydrocoumaroyl glucoside) amide (isomer 2)	17.2-19.7	9.5	133.4-181.0	21.4	22.3-24.0	5.2	12.8-12.9	0.7	25.5-33.4	16.6	22.8-25.1	6.9
3	N-Caffeoylputrescine	142.1-143.0	0.5	1622.6-1630.4	0.3	35.7-38.7	5.6	116.7-201.0	37.5	237.9-271.4	22.3	38.0-43.0	8.7
4	Homovanillic acid hexose	20.9-29.6	24.3	99.2-164.6	35.1	29.9-30.4	1.0	22.5-27.6	14.5	21.8-28.2	13.8	35.0-35.3	0.6
5	Dicaffeoylquinic acid	26.5-69.7	63.5	n.d.	n.d.	59.2-62.5	3.8	24.5-26.9	6.8	n.d.	n.d.	58.9-66.7	8.8
6	Chlorogenic acid (3-caffeoylquinic acid)	926.5-997.8	5.2	3058.4-4178.0	21.9	314.5-331	3.6	862.1-870.7	0.7	887.9-1075.8	11.2	316.4-334.5	3.9
7	5-Caffeoylquinic acid	71.2-82.4	10.3	169.7-672.9	84.5	19.1-20.8	6.0	59.5-61.6	2.5	52.1-65.7	11.6	22.4-23.6	3.7
8	4-Caffeoylquinic acid	68.7-75.8	7.0	345.4-501.7	26.1	38.7-40.0	2.5	53.6-56.5	3.7	98.4-115.9	8.2	38.9-45.5	10.9
9	Bis(dihydrocaffeoyl)spermidine	40.1-66.3	34.9	301.4-328.2	6.0	43.5-44.2	1.1	47.5-48.4	1.1	104.9-211.8	33.2	42.5-44.3	2.9
10	Caffeoyl(dihydrocaffeoyl)spermidine	75.63-86.4	9.4	402.7-540.8	20.7	21.7-22.6	2.7	66.3-70.7	4.6	113.8-126.1	5.2	22.0-23.9	5.8
11	Caffeoylquinic dehydrodimer (isomer 1)	29.8-42.0	24.0	117.2-173.0	27.2	n.d.	n.d.	15.5-25.7	34.7	22.2-42.8	33.5	n.d.	n.d.
12	Caffeoylshikimic acid	41.0-45.1	6.8	n.d.	n.d.	n.d.	n.d.	26.8-31.8	12.1	n.d.	n.d.	n.d.	n.d.
13	Caffeoylquinic dehydrodimer (isomer 2)	41.0-46.5	9.0	n.d.	n.d.	29.3-33.3	8.9	32.1-32.8	1.5	n.d.	n.d.	31.8-33.6	3.9
14	Feruoylquinic acid	44.5-46.9	3.7	167.1-168.2	0.5	42.0-42.3	0.5	27.9-29.6	4.3	39.3-42.0	3.4	41.3-42.8	2.4

	COMPOUNDS	SPRING						SUMMER					
		PSE	RSD	LE	RSD	RE	RSD	PSE	RSD	LE	RSD	RE	RSD
15	N,N-Dicaffeoylspermidine	31.9-33.4	3.2	288.6-311.2	5.3	22.8-23.1	0.9	26.3-29.6	8.5	36.9-61.1	24.6	21.8-23.0	3.5
16	Quercetin 3-O-gentiobioside	39.0-43.9	8.4	250.5-381.6	29.3	17.0-19.1	8.3	24.4-29.9	14.5	33.9-51.2	20.7	14.4-18.8	18.8
17	Caffeoylquinic dehydrodimer (isomer 3)	37.6-38.4	1.4	n.d.	n.d.	32.3-33.1	1.8	15.9-28.5	40.1	n.d.	n.d.	28.3-33.2	11.3
18	Caffeoylquinic dehydrodimer (isomer 4)	22.9-24.6	5.0	74.6-107.7	25.7	19.6-20.2	2.5	11.2-19.2	37.3	16.1-23.9	20.2	18.9-20.6	6.2
19	Kaempferol dihexoside (isomer 1)	33.8-36.5	5.4	75.5-163.1	51.9	30.4-32.1	3.8	9.1-14.7	33.3	25.9-41.6	26.8	28.6-36.7	17.6
20	Kaempferol dihexoside (isomer 2)	22.7-28.1	15.1	86.1-122.3	24.5	30.0-34.4	9.6	11.3-20.0	39.4	29.1-34.9	9.9	30.7-33.1	5.2
21	Tris(dihydrocaffeoyl) spermine	30.7-31.2	1.1	80.8-159.4	46.3	n.d.	n.d.	11.7-14.1	13.3	14.3-32.6	41.3	n.d.	n.d.
22	Caffeoylquinic dehydrodimer (isomer 5)	17.6-18.1	2.0	n.d.	n.d.	15.1	n.d.	10.4-12.5	13.3	n.d.	n.d.	16.2-19.1	11.7
23	Caffeoylquinic dehydrodimer (isomer 6)	26.5-28.2	4.2	74.8-88.1	11.6	17.0-18.2	4.5	12.1-18.5	29.7	18.2-25.9	19.8	19.1-21.3	7.8
24	Kaempferol-3-O-rutinoside	20.8-21.4	2.1	122.8-171.6	23.4	17.6-18.4	2.8	9.0-19.7	52.6	23.0-29.8	13.3	16.1-18.7	10.8
25	Delphinidin rutinoside	48.1-59.7	15.2	1861.8-2102.0	8.6	12.8-13.6	4.5	40.5-132.7	67.9	289-432.6	20.5	17.2-20.9	13.8
	Total	1964.9-2080.7	4.0	9955.1-11680.9	11.3	908.3-925.3	1.3	1778.6-1787.4	4.5	2280.5-2678.0	8.5	925.8-966.0	3.0

3.3. Effect of the harvest season on phenolic compounds of different eggplant cultivars

Several studies show that differences in soil, climatic conditions, growing season, postharvest storage, and other factors can affect the amount of bioactive compounds and nutrients of vegetables (Queiroz, Lopes, Fialho, & Valente-Mesquita, 2011). In addition, phenolic compounds present in these kinds of samples are especially sensitive to various biotic and abiotic stresses, such as air pollutants, extreme temperatures (including freezing), drought, high light intensity, salinity, and mechanical damage (Bartwal, Mall, Lohani, Guru, & Arora, 2013; Brandt, Giannini, & Lercari, 1995; Dixon & Paiva, 1995). In the present study, the main variables are the cultivars and the harvest season of eggplant; therefore, the influence of the harvest season has been evaluated, as there is evidence of the change of phenolic compounds because of these variables in other vegetables (Mirdehghan & Rahemi, 2007; Raffo, La Malfa, Fogliano, Malani, & Quaglia, 2006).

Table 3 shows the ranges of the phenolic compound content of the different cultivars analyzed according to the harvest season. PSE proved to be the cultivar where a higher number of compounds could be identified and quantified; however, LE registered the highest quantity of phenolic compounds.

The concentration of hydroxycinnamic acids found in the eggplant cultivars PSE and LE decreased with rising temperatures due to the summer season, while RE showed no significant differences. This effect was more evident in the LE cultivar, where all hydroxycinnamic acids decreased by more than 70%. The greatest loss was for 5-caffeoylquinic acid (86.0%) and the changes in the other compounds of this family were similar: 73.7% for chlorogenic acid, 74.6% for 4-caffeoylquinic acid, 78.4% for caffeoylquinic dehydrodimer (isomer 1), 75.6% for feruloylquinic acid, and 78.5 and 74.1% for caffeoylquinic dehydrodimer isomers 4 and 6, respectively. Compounds 5, 12, 13, 16, and 21 were not detected in this eggplant cultivar (LE).

In PSE, the concentration of hydroxycinnamic acids from spring to summer declined to a lesser extent than in LE. Dicaffeoylquinic acid, 5-caffeoylquinic acid, and 4-caffeoylquinic acid concentrations decreased 46.6, 21.2, and 23.8%, respectively; however, chlorogenic acid was only 10% lower in summer than in spring. Moreover, caffeoylquinic dehydrodimer (isomer 1) reduced its concentration 42.6%, as occurs with the other isomers of caffeoylquinic dehydrodimer, for which the loss rate ranged 25.8-44.1%. Caffeoyshikimic and feruloylquinic acids decreased 32 and 37.1%, respectively.

The third cultivar, RE, showed a different behavior from the other two, resulting in no significant differences or in small changes in the amount of most of the hydroxycinnamic acids found from spring to summer. The highest gains were for caffeoylquinic dehydrodimer isomers 5 and 6 (17.2 and 14.5%, respectively) and 5-caffeoylquinic acid (15.1%). In this cultivar, isomer 1 of caffeoylquinic dehydrodimer and caffeoyshikimic acid were not found.

Samples collected in summer also showed lower concentrations of flavonoids than those harvested in spring. LE was the cultivar that showed the greatest degradation. Quercetin 3,4′-O-diglucoside and kaempferol-3-O-rutinoside were the two flavonoids that showed the highest loss, 86.7%, and 82.3%, respectively. Finally, isomers 1 and 2 of kaempferol dihexoside and its isomer diminished 73.4 and 79.9%, respectively.

The loss of flavonoids in the PSE cultivar during the summer season was lower. Kaempferol dihexoside (isomer 1) registered the sharpest decrease (66.0%), while reductions of 34.6% in quercetin 3-O-gentiobioside, 38.3% in kaempferol dihexoside (isomer 2) and 32.2% in kaempferol-3-O-rutinoside were found.

In the case of RE, flavonoids did not register significant differences with the seasons.

Trends for amide conjugates of hydroxycinnamic acids resembled those of hydroxycinnamic acids. The most considerable loss was found in LE, which had

a degradation rate of 70.9-83.1% with the exception of bis-(dihydrocaffeoyl)-spermidine, which presented a reduction of 47.1% (Table 3).

For the cultivar PSE, the highest losses in hydroxycinnamic acid amide conjugates occurred for tris-(dihydrocaffeoyl)-spermine (58.2%), (dihydrocoumaroyl glucoside) amide (isomer 2) (30.2%) and, (dihydrocoumaroyl glucoside) amide (isomer 1) (23.5%). The other compounds decreased at the following rates: 9.7% (bis-(dihydrocaffeoyl)-spermidine), 14.3% (N,N-dicaffeoylspermidine) and 15.4% (caffeoyl-(dihydrocaffeoyl)-spermidine). As exception, N-caffeoylputrescine slightly increased (11.5%).

The behavior of the amide conjugates of hydroxycinnamic acids in the RE cultivar was also similar to hydroxycinnamic acids, with small gains in concentration from spring to summer season. The biggest increase was registered for N-caffeoylputrescine (8.8%), followed by (dihydrocoumaroyl glucoside) amide (isomer 2) (3.9%), caffeoyl-(dihydrocaffeoyl)-spermidine increase (3.7%) and (dihydrocoumaroyl glucoside) amide (isomer 1) (2.1%). Minimal losses were recorded for (bis-(dihydrocaffeoyl)-spermidine) (1.0%) and N,N-dicaffeoylspermidine (2.4%). Tris(dihydrocaffeoyl) spermine was not detected in this cultivar.

The only benzoic acid identified (homovanillic acid) varied differently according to the eggplant cultivar. In PSE, values remained unchanged from spring to summer; in LE the concentration fell by 81.5%; and, in RE the concentration rose by 16.4%. Delphinidin rutinoside values climbed 44.5 and 44.2% in PSE and RE, respectively, but plunged 82.15% in LE.

As mentioned above, the phenolic compound content declined in two of the three eggplant cultivars studied (PSE and LE), this loss being most pronounced in LE. The variation in temperature and UV radiation, before and/or during the harvest in the different seasons, might explain the differences found (Brandt *et al.*, 1995; Raffo *et al.*, 2006). The third cultivar, RE, presented no differences between samples collected in spring and summer. Polyphenolic profiles are to some extent

correlated to variations in gene expression profiles. Therefore, the control of the gene expression plays an essential role in the polyphenolic biosynthetic pathway. Although, the range of induction or repression of gene expression in other vegetables of Solanum genus has been showed to be highly cultivar-dependent. This can explain the behavior of RE cultivar (André *et al.*, 2009).

The explanation of the varying trends in the eggplant cultivars may be due to the different effects of the seasons, while the concentrations in the phenolic compounds could depend on the cultivar adaptation to abiotic variation and, therefore, all cultivars would not be affected in the same way (Aires *et al.*, 2011). The seasonal variations of phenolic compounds in tomato (Solanaceae) have been reported by Raffo *et al.* (2006), reporting a decrease in the concentrations of hydroxycinnamic acids such as chlorogenic acid (3.12 mg/100 g on July vs. 5.44 mg/100 g on April), coumaric acid (0.36 mg/100 g on July vs. 0.68 mg/100 g on April), and ferulic acid (0.09 mg/100 g on July vs. 0.16 mg/100 g on April) in summer months (June-July) vs. spring months (March-April) (Raffo *et al.*, 2006). A similar effect was also observed in fruit such as apple, which lost total polyphenols and proanthocyanidins at high temperatures (Queiroz *et al.*, 2011). Furthermore, Aires *et al.* indicated high levels of total phenolic contents in *Brassica* plants in the summer-winter period rather than spring-summer period (Aires *et al.*, 2011) and the same effect occurred in inedible plants such as Calluna vulgaris (Jalal, Read, & Haslam, 1982).

Thus, the behavior of phenolic compounds in eggplant could be explained by the fact that, in vegetables, phenolic compounds may act as defense mechanisms, i.e. at high temperatures (Queiroz, Ribeiro da Silva, Mendes Lopes, Fialho, & Valente-Mesquita, 2011) and may also act as a precursor for the synthesis of polyphenolic barriers (Dixon & Paiva, 1995), or may simply degrade with increasing temperatures (Khanal, Howard, & Prior, 2010; Queiroz *et al.*, 2011). Nevertheless, phenolic compounds can also be synthesized in response to pathogen attacks or injury (Raffo *et al.*, 2006), which could explain the small increases in the concentrations of some compounds found in RE.

A factorial ANOVA univariate analysis of variance was performed to determine the effects of the eggplant cultivars, harvest season, and interaction between these two factors as variables (Table 4).

Table 4. Factorial ANOVA (Univariate Results). Column V, significant effect of the eggplant cultivar; Column S, significant effect of season; Column V*S, significant effect of cultivar and season (** $p < 0.01$; * $p<0.05$; NS not significant)

	Compounds	V	S	V x S
1	(Dihydrocoumaroyl glucoside) amide (isomer 1)	**	**	**
2	(Dihydrocoumaroyl glucoside) amide (isomer 2)	**	**	**
3	N-Caffeoylputrescine	**	**	**
4	Homovanillic acid hexose	**	**	**
5	Dicaffeoylquinic acid	**	NS	NS
6	Chlorogenic acid (3-caffeoylquinic acid)	**	**	**
7	5-Caffeoylquinic acid	*	NS	NS
8	4-Caffeoylquinic acid	**	**	**
9	Bis(dihydrocaffeoyl)spermidine	**	**	**
10	Caffeoyl(dihydrocaffeoyl)spermidine	**	**	**
11	Caffeoylquinic dehydrodimer (isomer 1)	**	**	**
12	Caffeoylshikimic acid	**	*	**
13	Caffeoylquinic dehydrodimer (isomer 2)	**	NS	NS
14	Feruoylquinic acid	**	**	**
15	N,N-Dicaffeoylspermidine	**	**	**
16	Quercetin 3-O-gentiobioside	**	**	**
17	Caffeoylquinic dehydrodimer (isomer 3)	**	**	**
18	caffeoylquinic dehydrodimer (isomer 4)	**	**	**
19	Kaempferol dihexoside (isomer 1)	**	**	**
20	Kaempferol dihexoside (isomer 2)	**	**	**
21	Tris(dihydrocaffeoyl) spermine	**	**	**
22	Caffeoylquinic dehydrodimer (isomer 5)	**	NS	**
23	Caffeoylquinic dehydrodimer (isomer 6)	**	**	**
24	Kaempferol-3-O-rutinoside	**	**	**
25	Delphinidin rutinoside	**	**	**
	Total	**	**	*

According to the p-level, differences in all phenolic compounds identified due to the eggplant cultivar were observed. Furthermore, the influence of the harvest time proved important for all compounds except for dicaffeoylquinic acid, 5-caffeoylquinic acid and caffeoylquinic dehydrodimer isomers 1 and 4, which did not show significant differences in this respect.

In most cases, changes in phenolic concentrations were associated with the combined effects of the cultivar and season.

4. Conclusions

In summary, a total of 25 phenolic compounds were identified in different eggplant cultivars. It is important to highlight, that to our knowledge, this is the first study available to report 14 compounds in eggplant, 9 of which have been found for the first time in Solanaceae.

Our results showed that cultivar and harvest season have a marked influence on the content of bioactive secondary metabolites as polyphenols in eggplant. The variations in polyphenol content according to the cultivar and season could be due mainly to climatic factors (abiotic effect) and, particularly, to the high increase of temperatures in the summer season, in the region of Andalusia, which is more pronounced than in other regions. Therefore, our results confirm that the high temperatures of summer in the Mediterranean region may provoke a significantly negative effect on the phenolic compound content in eggplant.

This information may be important for the agricultural sector because it can help to know the suitable period and ideal climatic conditions for harvesting eggplants with high content in phenolic compounds.

Appendix A. Supplementary data

Table 5. Eggplant cultivars, harvest date and location.

Cultivar	Reference	Harvest date	Origin
Purpel-Striped Eggplant	**PSE-1**	Apr.2011	Andalusia, Almeria (San Agustin)
	PSE-2	May.2011	
	PSE-3	Jul. 2011	
	PSE-4	Aug. 2011	
Long Eggplant	**LE-1**	Mar. 2011	Andalusia, Almeria (San Agustin)
	LE-2	Apr.2011	
	LE-3	Jul. 2011	
	LE-4	Aug. 2011	
	LE-5	Sep.2011	
Round Eggplant	**RE-1**	Mar. 2011	Andalusia, Almeria (Las Norias)
	RE-2	Aug. 2011	
	RE-3	Sep.2011	

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CAPÍTULO 6

Identification and quantification of phenolic and other polar compounds in the edible part of Annona cherimola and its by-products by HPLC-DAD-ESI-Q-TOF-MS

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Abstract

Annona cherimola fruit is native to inter-Andean valleys from Peru and Ecuador. In the Mediterranean region, the main producer of cherimoya is the coast of Granada-Malaga (Spain), also called "Costa Tropical", where the two most important cultivars found are 'Fino de Jete' and 'Campa'.

Cherimoya, like most fruits, is an important source of bioactive compounds, such as phenolic compounds. Therefore, the aim of this study was the tentative identification and quantification of phenolic and organic acids in pulp, peel and seed of two cherimoya cultivars ('Fino de Jete' and 'Campa') using HPLC-DAD-ESI-QTOF-MS.

By using the proposed method, 21 phenolic and organic acids were detected in the edible portion of cherimoya, 37 in peel and 22 in seed. Procyanidins were the main phenolic compound family identified in the pulp and peel of the two cultivars, whereas in cherimoya seeds higher quantities of organic acids and flavonoids were detected. Most of the compounds were identified for the first time in cherimoya.

According to these results, cherimoya pulp and its by-products are a natural source of procyanidins and other phenolic and polar compounds. In particular, cherimoya peel, with a higher concentration of phenolic and polar compounds in

comparison with pulp and seed, represents an interesting food by-product that could be used as an ingredient in the functional food and/or pharmaceutical industry.

Keywords: *Annona cherimola*, by-products, phenolic compounds, HPLC-DAD-ESI-Q-TOF-MS

1. Introduction

Nowadays, the idea that a healthy diet can prevent the development of some diseases has been supported by several studies (Liu, 2013). In addition, research works have shown the high relationship between the consumption of fruits in prevention of various chronic health problems (Loizzo *et al.*, 2012; Monteiro Egydio, Catarina, Segal Floh, & Alves Cursino dos Santos, 2013). Not only fruit pulp, but also peels and seeds from dietary plants and traditional medicinal herbs, play an important role in health because of their nutritional, antioxidant properties and wealth due to bioactive compounds (Huang, Cai, Corke, & Sun, 2010). Particularly, exotic fruits are an important source of these compounds in the human diet. Furthermore, consumption of exotic fruits is increasing around the world due to the high recognition of their nutritional value and beneficial properties (Loizzo *et al.*, 2012).

As a result of the interest in the efficacy and use of naturally derived antioxidants, the great potential of tropical fruit pulps and their by-products is the focus of current research. In fact, the isolation of specific phytochemicals for application in nutraceutical supplements, dietary additives, functional food and pharmaceutical products are among the main objectives of industry (Huang *et al.*, 2010; Ribeiro da Silva *et al.*, 2014). The revalorization of food by-products consists of 5 stages that allow the recovery of the compounds of interest: macroscopic pretreatment, separation of macro- and micro-molecules, extraction, isolation and purification, and product formation (Galanakis, 2012).

One tropical fruit that has become an important crop due to its characteristics is the cherimoya (*Annona cherimola* Mill.) (Albuquerque, Santos, Sanches-Silva, Oliveira, Bento, & Costa, 2014). The cultivation of cherimoya is native to inter-Andean valleys of Peru and Ecuador, and in the last few decades it has become endemic in several subtropical areas of the world (Brito, Rodríguez, Samaniego, Jaramillo, & Vaillant, 2008).

The main cherimoya producer in the Mediterranean basin is the coast of Granada-Malaga (Spain), the so-called "Costa Tropical". The two major cultivars grown in "Costa Tropical" are 'Fino de Jete' and 'Campa'. 'Fino de Jete'is also the most widespread cultivar in the world (Alique & Oliveira, 1994; Brito *et al.*, 2008).

Cherimoya fruit contains bioactive compounds with antioxidant properties that can contribute to the prevention of diseases associated with oxidative stress such as cancer, atherosclerosis and neurodegenerative diseases (Loizzo *et al.*, 2012; Monteiro Egydio *et al.*, 2013). Prior studies have reported its application in traditional medicine, in particular for the treatment of digestive disorders and skin disease (Loizzo *et al.*, 2012). According to the literature, seeds and stems of *Annona cherimola* have diverse compounds that have demonstrated anti-neoplastic activity against human cancer cell lines (Quispe, Callacondo Riva, Rojas-Camayo, Zavala Curzo, Posso Rivera, & Vaisberg Wolach, 2009). In particular, most of these compounds related to therapeutic properties in plant-food are polyphenols, which have important physiological functions in plants and are one of the most extensive groups of phytochemicals. Therefore, interest in the determination of polyphenolic composition of tropical and subtropical fruits is growing, and studies focused on the characterisation and quantification of phenolic compounds in plant by-products for their use in the manufacture of functional foods are becoming important (Jiménez, Gruschwitz, Schweiggert, Carle, & Esquivel, 2014; Rinaldo, Mbéguié-A-Mbéguié, & Fils-Lycaon, 2010).

It is also interesting to know the presence and concentration of polar compounds as organic acids, since these compounds have influence in organoleptic and sensorial properties. Besides, organic acids could be used as chemical markers of ripeness, bacterial activity or storage conditions because these compounds present good stability during processing and storage (Sandín-España, Mateo-Miranda, López-Goti, De Cal, Alonso-Prados, 2016).

Currently, there are few studies on phenolic compounds and organic acids of cherimoya. Most of them are focused on the determination of total phenolic and total flavonoid composition and antioxidant activity by spectrometric techniques (Gupta-Elera, Garrett, Martínez, Robison, & O'Neill, 2011; Loizzo *et al.*, 2012). Albuquerque *et al.* (2014) also reported total phenolics and total flavonoids in cherimoya pulp and its by-products by spectrometric techniques. Phenolic constituents of cherimoya pulp, particularly catechins, have also been analysed by RP-HPLC-DAD-IT-MS (Barreca *et al.*, 2011). Furthermore, different studies have been conducted on the determination of amino acids, organic acids and general composition of cherimoya (Alique & Oliveira, 1994; Monteiro Egydio *et al.*, 2013).

Due to the few available research studies about the characterization of bioactive compounds in *Annona cherimola*, and particularly in its byproducts, the aim of the present work was to comprehensive and tentative identify and, for the first time, quantify phenolic compounds and organic acids in the edible part and its by-products of two cherimoya cultivars grown in Andalusia (Spain). The variation of these compounds among the different parts of the fruit was also studied. Cherimoya samples were analyzed by a HPLC-diode array detector (DAD)-electrospray ionisation (ESI)-quadrupole-time of flight (QTOF)-mass spectrometry (MS). To our knowledge, this is the first available report using a QTOF-MS detector to analyze the complete phenolic profile of pulp, peel, and seed of cherimoya. Indeed, this is the first report about the quantification of the complete profile of phenolic compounds and organic acids in pulp, peel, and seed of cherimoya. Therefore, this study presents cherimoya by-products potential sources of bioactive compounds with applications in the food, pharmaceutical, and cosmetic industries.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade acetic acid and acetonitrile were purchased from Fisher Scientific (Leicestershire, UK), and methanol for dissolving the samples was purchased from Panreac (Barcelona, Spain). Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Double-deionised water with conductivity lower than 18.2 MΩ was obtained with a Milli-Q system from Millipore (Bedford, MA, USA). The standard compounds, (+)-catechin, rutin, and 4-hydroxybenzoic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Samples

About 10 kg of each cherimoya cultivar, 'Fino de Jete' and 'Campa', were collected in Almuñécar (Granada, Spain) in February 2014. These cultivars were selected because they are the two major cultivars commercialised in Andalucia (Spain). The samples were from plants grown under the same agricultural conditions. Pulp, peel and seed of fresh samples were manually separated and dried in a lyophiliser (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK). Afterwards, dried samples were milled and kept at -18 ºC until use.

2.3. Extraction of the polar fraction of cherimoya samples

A solid-liquid extraction was used to extract the polar fraction. Briefly, 0.5 grams of freeze-dried sample powder were 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 it was centrifuged for 15 min at 1000 g; the supernatant was removed, and the extraction was repeated once 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 μm (Millipore, Bedford, MA, USA) and stored at -18 ºC until the analyses.

2.4. HPLC-DAD-QTOF-MS analyses

Cherimoya extracts obtained by solid-liquid extraction were analysed using an HPLC coupled to DAD and ESI-QTOF-MS in negative ionisation mode in order to identify the phenolic compounds. An Agilent 1200-LC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, binary pump, and DAD was used for the chromatographic determination. A methodology previously optimised and validated by García-Salas *et al.* (García-Salas, Gómez-Caravaca, Morales-Soto, Segura-Carretero & Fernández-Gutiérrez, 2014) was used to analyse the cherimoya samples. The separation was done using an Agilent Poroshell 120 EC-C18 column (4.6 x 100 mm, 2.7 μm) from Agilent Technologies, operating at a flow rate of 0.8 mL/min and 25ºC, throughout the gradient. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the following conditions: 0 min, 5% B; 2 min, 7% B; 4 min, 9% B; 7 min, 12% B; 8 min, 15% B; 9 min, 16% B; 12 min, 18% B; 14 min, 20% B; 15 min, 22% B; 16 min, 25% B; 18 min, 28% B; 19 min, 30% B; 20 min 31% B; 21.50 min, 32% B; 25 min, 100% B; 30 min, 100% B; 32 min, 5% B. The injection volume was 0.5 μL and UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, 330 and 520 nm.

The HPLC system was coupled to a microTOF-Q (Bruker Daltonik, Bremen, Germany) instrument, using an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The flow rate used in the LC method was too high to achieve stable electrospray ionisation (ESI); therefore, a flow divisor 1:3 was used to reduce the flow delivered into the mass spectrometer. Parameters for analysis were set using negative and positive ion modes with spectra acquired over a mass range from *m/z* 50 to 1200. According to this, the optimum values of

ESI-Q-TOF-MS/MS parameters in negative mode were: capillary voltage, 4000 V; drying gas temperature, 210 ºC; drying gas flow, 8 L/min; nebulizing gas pressure, 2 bar; and end-plate offset, -500 V. The optimum parameter values in the positive mode were: capillary voltage, 4500 V; drying gas temperature, 210 ºC; drying gas flow, 8 L/min; and nebulising gas pressure, 2 bar.

During the use of the HPLC method, an external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, 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 plotted based on numerous cluster masses, each differing by 68 Da ($NaCHO_2$). Due to the compensation of temperature drift in the TOF, this external calibration provided accurate mass values for a complete run without the need for a dual sprayer set-up for internal mass calibration.

The accurate mass data for the molecular ions were processed using the software Data Analysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula^{TM} editor. The editor uses a CHNO algorithm, which provides standard functionalities such as a 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.

2.5. 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

Peak identification was based on their relative retention time values, their UV-Vis spectra, their mass spectra determined by QTOF-MS, and information from the literature (Tables 1, 2 and 3). Fig. 1 and Fig. 2 show the base peak chromatograms (BPC) of the polar fraction of cherimoya 'Fino de Jete' and 'Campa' extracts obtained under the optimum conditions of the method. Peaks numbering is independent in pulp, peel and seed.

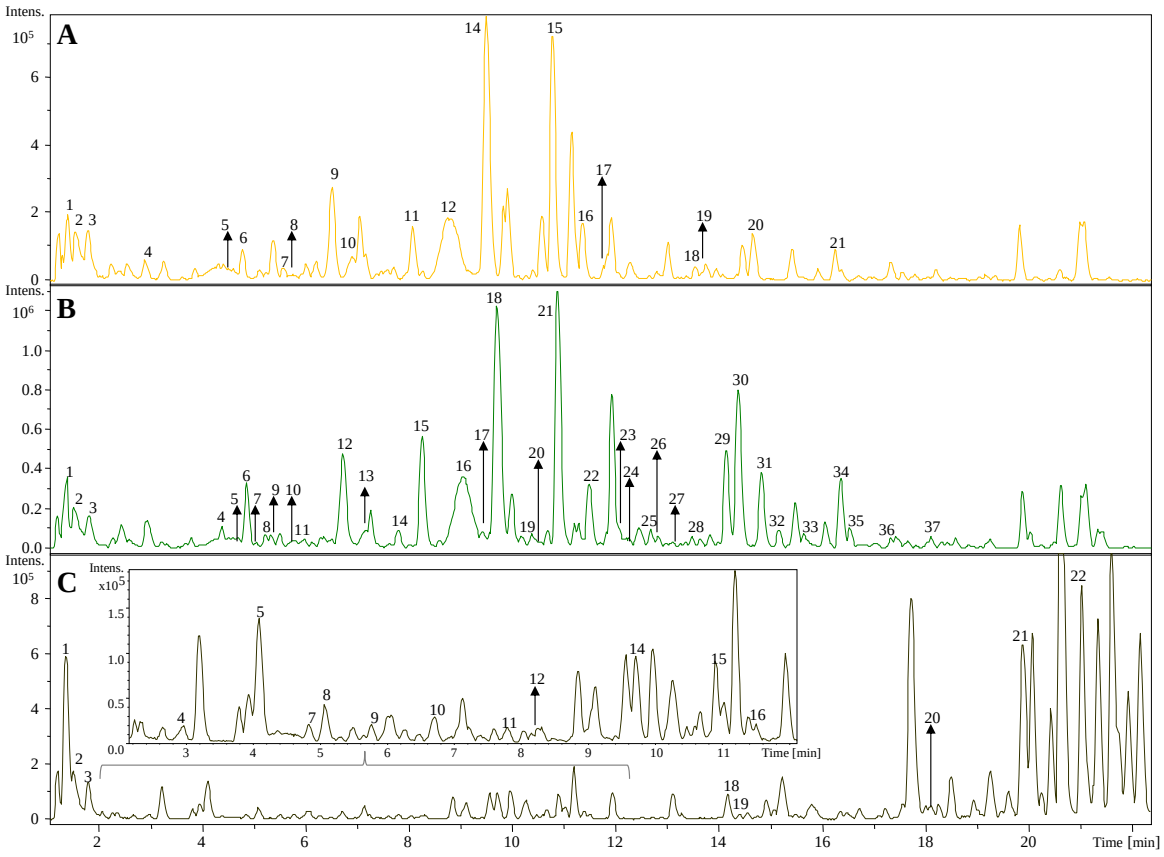

Fig. 1. Base peak chromatograms of the phenolic fraction of 'Fino de Jete' pulp and by-product, A: pulp, B: peel, C: seeds. See Tables 1, 2 and 3 for the identification of phenolic compounds.

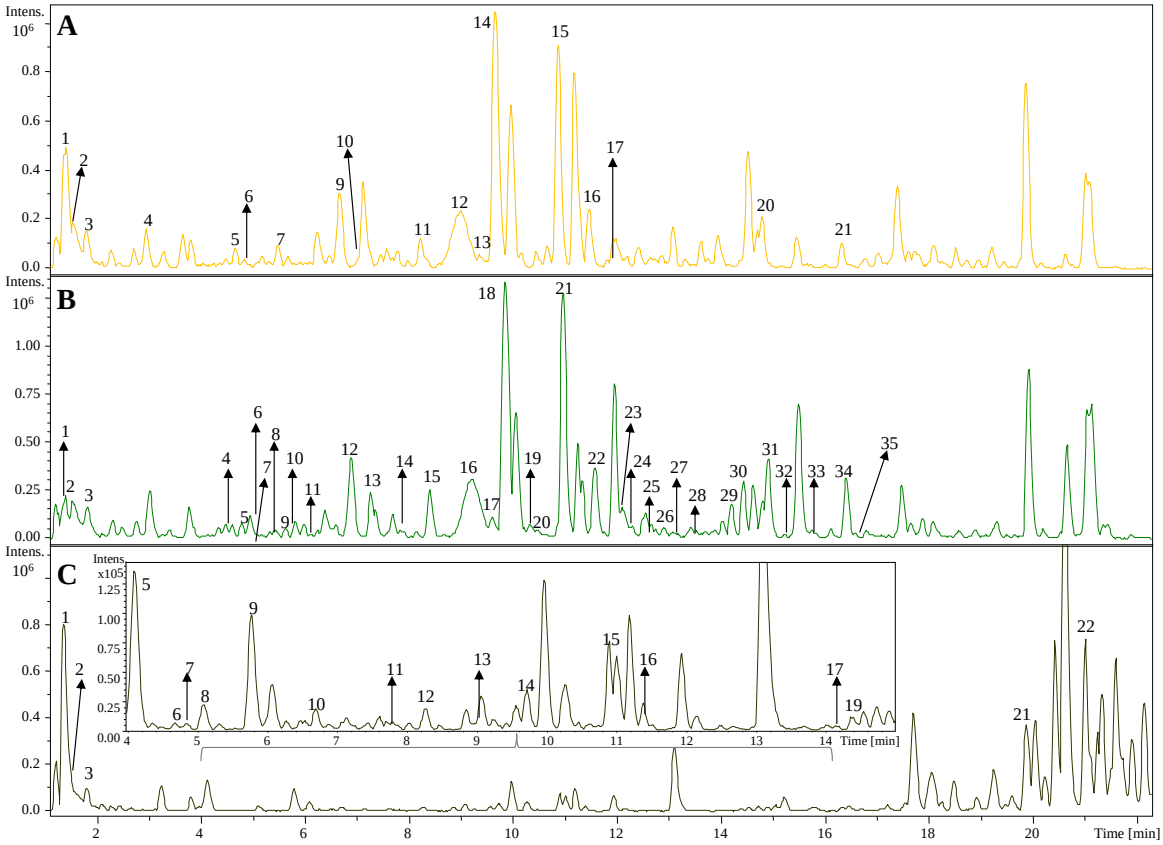

Fig. 2. Base peak chromatograms of the phenolic fraction of 'Campa' pulp and by-product, A: pulp, B: peel, C: seeds. See Tables 1, 2 and 3 for the identification of phenolic compounds.

3.1.1. Identification of phenolic and other polar compounds in cherimoya pulp

A total of 21 phenolic and other polar compounds were tentatively identified in pulp of both cultivars analysed, as summarised in Table 1, including the retention time, molecular formula, experimental and calculated *m/z*, fragments, sigma value, error and UV-Vis maximum absorbance. The most important family of phenolics present in cherimoya pulp is flavan-3-ols, in fact, most compounds were (epi)catechin and derivatives (procyanidins) as follows: peak 11 with *m/z* 289.0725, at retention time 8.06 min was identified as catechin. The fragmentation pattern showed fragments at *m/z* 245, 205, and 179. The fragment at *m/z* 205 corresponded to the loss of the flavonoid A-ring, whereas the one at *m/z* 179 was due to the loss of the B-ring. At retention time 10.77 min and *m/z* 289.0732, epicatechin (peak 15) was found. The presence of these compounds in

Annona cherimola pulp has previously been reported (Barreca *et al.*, 2011). Differentiation between isomers was based on retention time; it has been reported that in similar conditions catechin eluted before epicatechin (Tomás-Barberán & Espín, 2001; Bocalandro *et al.*, 2012).

Procyanidin dimer type A at *m/z* 575.1197 was only found in 'Fino de jete' pulp at retention time 5.74 min (peak 8). In MS^2, the ions were at *m/z* 423, 449, 289 corresponding to $[(epi)catechin\text{-}H]^-$, and 285 corresponding to $[(epi)catechin\text{-}2H_2\text{-}H]^-$. The last two fragments are generated by the splitting of the interflavanoid bonds. This fragmentation pattern is typical of the A-type proanthocyanidin dimer. This compound has previously been reported for other tropical fruits (Cádiz-Gurrea, Lozano-Sánchez, Contreras-Gámez, Legeai-Mallet, Fernández-Arroyo, & Segura-Carretero, 2014; Lin, Sun, Chen, Monagas, & Harnly, 2014).

B-type procyanidins were the most abundant compounds identified in pulp of both cultivars. The chemical structure of these compounds was based on the presence of (epi)catechin units, which are linked by a single bond. Compounds 9, 10, 12, 14, 20 which *m/z* was 577 were tentatively identified as procyanidin dimer type B and its isomers. The result of MS^2 spectra showed ions at *m/z* 425 [retro-Diels-Alder reaction (RDA)] and *m/z* 289 corresponding to an (epi)catechin unit. The fragment at *m/z* 407 resulted from water elimination of *m/z* 425. These compounds have previously been identified in pulp of cherimoya fruit, and the fragmentation pattern was in agreement with previous studies (Barreca *et al.*, 2011; Cádiz-Gurrea *et al.*, 2014; Lin *et al.*, 2014; Spinola, Pinto, & Castilho, 2015).

Other B-type procyanidins found were procyanidin trimer type B and its isomers. Compound 13 was tentatively designated as procyanidin trimer type B isomer 1, it was detected only in 'Campa' cultivar at retention time 9.33 min and *m/z* 865.1904. Procyanidin trimer type B isomer 2 with *m/z* 865.2035 was also identified in both pulp extracts at retention time 11.36 min and numbered as

compound 16. The fragmentation pattern produced by MS^2 analysis was composed of ions at *m/z* 713 [retro-Diels-Alder reaction (RDA)], 577 and 289 indicating the loss of the dimer and monomer of (epi)catechin. Procyanidin trimer type B has been reported in cherimoya previously with the same fragmentation pattern by Barreca *et al.*, (2011).

Compound 17, at retention time 11.94 min and experimental *m/z* 1153.2629 was identified as procyanidin tetramer type B isomer 1 due to MS^2 ions at *m/z* 289, 577 and 865. These fragments are characteristics of (epi)catechin monomer and polymers. Isomers 2 (peak 18) and 3 (peak 19) were identified only in 'Fino de Jete' cultivar at *m/z* 1153.2621 and 1153.2628 with retention times at 13.48 and 13.73 min, respectively. This type of procyanidin has been reported for strawberry and avocado pulp (Simirgiotis & Schmeda-Hirschmann, 2010; Simirgiotis, Schmeda-Hirschmann, Borquez, & Kennelly, 2013; Wang, Bostic, & Gu, 2010).

Among phenolic acids tentatively described in cherimoya pulp were 3-(2-hydroxyphenyl)-propanoic acid hexose (compound 4, at retention time 2.93 and *m/z* 327.1084). The MS^2 spectrum showed a fragment at *m/z* 165 corresponding to the loss of hexose molecule $[M\text{-}H\text{-}hexoside]^-$. This compound has previously been found in tomato (Gómez-Romero, Segura-Carretero, & Fernández-Gutiérrez, 2010).

Peak 5 at retention time 4.58 and *m/z* 315.1071 showed a fragmentation pattern with two major fragments, one at *m/z* 153 and the other at *m/z* 123. This evidence suggested that the compound could be identified as hydroxytyrosol hexoside as reported by Savarese and co-workers (Savarese, De Marco, & Sacchi, 2007) in olive fruits. An isomer of hydroxytyrosol hexoside (peak 6), with the same experimental *m/z*, was found at retention time of 4.77 min.

Table 1. Phenolic and other polar compounds identified in cherimoya pulp samples

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
1	Quinic acid	x	x	1.37	191.0580	191.0561	$C_7H_{11}O_6$	1.4	13	228/264	111
2	Citric acid (isomer 1)	x	x	1.54	191.0215	191.0197	$C_6H_7O_7$	8.5	2.7	229/267	111
3	Citric acid (isomer 2)	x	x	1.78	191.0214	191.0197	$C_6H_7O_8$	7.1	3.7	264	111
4	3-(2-hydroxyphenyl)-propanoic acid hexose	x	x	2.93	327.1084	327.1085	$C_{15}H_{19}O_8$	0.4	4.7	228/278	117/145/165
5	Hydroxytyrosol hexoside (isomer 1)	x	x	4.58	315.1071	315.1085	$C_{14}H_{19}O_8$	4.6	26.3	228/277	132/153
6	Hydroxytyrosol hexoside (isomer 2)	x	x	4.77	315.1071	315.1085	$C_{14}H_{19}O_8$	4.5	2.1	228/277	153
7	Luteolin-glucopyranoside	x	x	5.55	447.1523	447.1508	$C_{19}H_{27}O_{12}$	3.7	22.1	228/278	153/285/161
8	Procyanidin dimer type A	x		5.74	575.1197	575.1195	$C_{30}H_{23}O_{12}$	0.4	14.0	228/278	423/449/289/285
9	Procyanidin dimer type B (isomer 1)	x	x	6.51	577.1379	577.1351	$C_{30}H_{25}O_{12}$	4.8	5.5	230/279	289
10	Procyanidin dimer type B (isomer 2)	x	x	6.87	577.1380	577.1351	$C_{30}H_{25}O_{13}$	4.9	7.1	230/280	289
11	Catechin	x	x	8.06	289.0725	289.0718	$C_{15}H_{13}O_6$	2.6	0.5	231/279	179/245/205
12	Procyanidin dimer type B (isomer 3)	x	x	8.73	577.1372	577.1351	$C_{30}H_{25}O_{12}$	3.6	4.6	231/279	289
13	Procyanidin trimer type B (isomer 1)		x	9.33	865.1904	865.1985	$C_{45}H_{37}O_{18}$	5.3	5.6	231/279	289/577
14	Procyanidin dimer type B (isomer 4)	x	x	9.48	577.1372	577.1351	$C_{30}H_{25}O_{12}$	3.6	4.6	231/279	289
15	Epicatechin	x	x	10.77	289.0732	289.0718	$C_{15}H_{13}O_6$	5.1	6.0	231/279	179/245/205

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
16	Procyanidin trimer type B (isomer 2)	**x**	**x**	11.36	865.2035	865.1985	$C_{45}H_{37}O_{18}$	5.7	3.7	231/279	289/575/577/713
17	Procyanidin tetramer type B (isomer 1)	**x**	**x**	11.94	1153.2629	1153.2692	$C_{60}H_{49}O_{24}$	11.4	48.8	230/279	289
18	Procyanidin tetramer type B (isomer 2)	**x**		13.48	1153.2621	1153.2692	$C_{60}H_{49}O_{24}$	2.0	10.6	231/279	289/577
19	Procyanidin tetramer type B (isomer 3)	**x**		13.73	1153.2628	1153.2692	$C_{60}H_{49}O_{24}$	1.8	52.9	231/279	289/577
20	Procyanidin dimer type B (isomer 5)	**x**	**x**	14.63	577.1348	577.1351	$C_{30}H_{25}O_{12}$	0.7	6.9	237/280	289
21	Secoisolariciresinol beta-D-hexoside	**x**	**x**	16.22	523.2177	523.2185	$C_{26}H_{35}O_{11}$	1.6	5.1	238/279	347/361

x: identified in the cultivar.

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Compound 21 was found at 16.22 min with *m/z* 523.2177 and was tentatively identified as secoisolariciresinol beta-D-hexoside, due to the fragmentation profile that showed ions at *m/z* 347 and 361 [M-H-162] due to the loss of a hexose moiety. This fragmentation pattern and the molecular formula provided by the software ($C_{26}H_{35}O_{11}$) are in agreement with data previously reported for pomegranate (Mena *et al.*, 2012).

At retention time 5.55 min (peak 7) a flavonoid was tentatively identified as luteolin-glucopyranoside with experimental *m/z* 447.1523. It presented MS^2 (*m/z* 285) fragments indicating the loss of 162 uma (dehydrated glucose moiety). This compound had previously been found in another tropical fruit, as *Passiflora tripartita* fruit, with the same fragmentation pattern (Simirgiotis *et al.*, 2013).

Three organic acids were also detected in 'Fino de Jete' and 'Campa' pulp. Peak 1 was assigned as quinic acid with experimental *m/z* 191.0580 and retention time at 1.37 min. Quinic acid has previously been identified in cherimoya (Spinola *et al.*, 2015). Citric acid was also identified at retention time 1.54 min and experimental *m/z* 191.0215 (peak 2). Isomer 2 of this compound was detected at 1.78 min with experimental *m/z* 191.0214 (peak 3). Citric acid is typical of many fruits, and it has been reported in cherimoya by other authors (Manríquez, Muñoz-Robredo, Gudenschwager, Robledo, & Defilippi, 2014; Spinola *et al.*, 2015). Literature and mass spectrometry data obtained (Table 1) for the first time by QTOF analyzer allowed the identification of peaks 2 and 3 as citric acid isomers.

In this study, procyanidin dimer type A, procyanidin tetramer type B (isomers 1, 2, 3), 3-(2-hydroxyphenyl)-propanoic acid hexose, hydroxytyrosol hexoside (isomers 1 and 2), secoisolariciresinol beta-D-hexoside and luteolin-glucopyranoside were identified in cherimoya pulp for the first time.

3.1.2. Identification of phenolic and other polar compounds in cherimoya peel

Phenolic and other polar compounds tentatively identified in 'Fino de Jete' and 'Campa' peel are reported in Table 2. A total of 37 compounds were tentatively identified, and peel composition of both cultivars was qualitatively similar.

As in cherimoya pulp, catechin, epicatechin and procyanidins were the most abundant phenolic compounds, in terms of number of compounds, present in cherimoya peel. Catechin (peak 15) and epicatechin (peak 21) were found at 8.25 min and 10.86 min, respectively, with *m/z* 289.0717 in both compounds and fragments at *m/z* 245 and 205 (Barreca *et al.*, 2011). At retention time 5.96 min procyanidin dimer type A was detected in both cultivars at *m/z* 575.1211 (peak 11). Nevertheless, procyanidin dimer type A isomer was only found in 'Fino de Jete' cherimoya peel (peak 36). The fragmentation pattern typical of the A-type proanthocyanidin dimer was obtained by MS^2 analysis. It showed fragments at *m/z* 423 (RDA, Retro Diels Alder product), 289 and 285. According to the literature these compounds have been found in peel of *Annona squamosa* and other tropical fruits (Huang *et al.*, 2010; Ramírez, Zambrano, Sepulveda, & Simirgiotis, 2014).

Table 2. Phenolic and other polar compounds identified in cherimoya peel samples

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
1	Quinic acid	x	x	1.37	191.0580	191.0561	$C_7H_{11}O_6$	9.9	19.5	226/265	111
2	Citric acid (isomer 1)	x	x	1.51	191.0215	191.0197	$C_6H_7O_7$	9.3	1.30	227/270	111
3	Citric acid (isomer 2)	x	x	1.79	191.0219	191.0197	$C_6H_7O_8$	11.5	6.0	243/285	111
4	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 1)	x	x	4.37	373.1120	373.1140	$C_{16}H_{21}O_{10}$	5.4	5.0	225/277/193	193
5	Hydroxytyrosol hexoside (isomer 1)	x	x	4.67	315.1057	315.1085	$C_{14}H_{19}O_8$	9.1	7.0	226	153
6	Hydroxytyrosol hexoside (isomer 2)	x	x	4.85	315.1074	315.1085	$C_{14}H_{19}O_8$	3.7	7.3	227/278	153
7	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 2)	x	x	5.04	373.1106	373.1140	$C_{16}H_{21}O_{10}$	9.1	15.6	225/277/193	193
8	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 3)	x	x	5.20	373.1128	373.1140	$C_{16}H_{21}O_{10}$	3.4	5.3	225/277/193	193
9	Tyrosol hexoside pentoside	x	x	5.32	431.1532	431.1559	$C_{19}H_{27}O_{11}$	6.3	1.5	226/277	137
10	Vanillic acid hexoside	x	x	5.81	329.0862	329.0831	$C_{14}H_{17}O_9$	4.9	1.5	226/271	167
11	Procyanidin dimer type A (isomer 1)	x	x	5.96	575.1211	575.1195	$C_{30}H_{23}O_{12}$	2.72	65.5	227/277	449/423/289/285
12	Procyanidin dimer type B (isomer 1)	x	x	6.71	577.1338	577.1351	$C_{30}H_{25}O_{12}$	2.4	1.6	227/279	425/407/289
13	Procyanidin dimer type B (isomer 2)	x	x	7.14	577.1338	577.1351	$C_{30}H_{25}O_{12}$	2.4	9.4	227/280	425/407/290
14	Sibiricose A3	x	x	7.78	461.1275	461.1301	$C_{19}H_{25}O_{13}$	5.5	6.3	227/279	138/153/163

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
15	Catechin	x	x	8.25	289.0717	289.0718	$C_{15}H_{13}O_6$	0.3	1.4	229/278	245/205
16	Procyanidin dimer type B (isomer 3)	x	x	9.05	577.1333	577.1351	$C_{30}H_{25}O_{12}$	3.3	3.0	227/278	425/407/290
17	Procyanidin trimer type B (isomer 1)	x	x	9.40	865.1946	865.1985	$C_{45}H_{37}O_{18}$	4.9	15.1	227/279	289/577
18	Procyanidin dimer type B (isomer 4)	x	x	9.68	577.1337	577.1351	$C_{30}H_{25}O_{12}$	2.4	2.1	232/278	425/407/290
19	Procyanidin tetramer type B (isomer 1)	x	x	10.17	1153.2646	1153.2692	$C_{60}H_{49}O_{24}$	2.2	7.2	228.5	865/575/287
20	Procyanidin tetramer type B (isomer 2)	x	x	10.35	1153.2629	1153.2692	$C_{60}H_{49}O_{24}$	0.7	7.3	227/279	865/575/287
21	Epicatechin	x	x	10.86	289.0717	289.0718	$C_{15}H_{13}O_6$	0.1	2.2	232/278	245/205
22	Procyanidin trimer type B (isomer 2)	x	x	11.47	865.1958	865.1985	$C_{45}H_{37}O_{18}$	3.2	16.6	232/279	577/425/407/289
23	Procyanidin tetramer type B (isomer 3)	x	x	12.20	1153.2629	1153.2692	$C_{60}H_{49}O_{24}$	1.43	33.6	227/279	865/575/287
24	Procyanidin trimer type B (isomer 3)	x	x	12.28	865.1955	865.1985	$C_{45}H_{37}O_{18}$	3.5	19.4	229/279	577/425/407/289
25	Calabricoside A (isomer 1)	x	x	12.66	741.1884	741.1884	$C_{32}H_{37}O_{20}$	5.9	6.5	228/276	300
26	Calabricoside A (isomer 2)	x	x	12.81	741.1882	741.1884	$C_{32}H_{37}O_{20}$	0.2	18.0	228/277	300
27	Procyanidin tetramer type B (isomer 4)	x	x	12.96	1153.2629	1153.2692	$C_{60}H_{49}O_{24}$	0.67	36.0	227/279	865/575/287
28	Lariciresinol-glucopyranoside	x	x	13.46	521.2010	521.2028	$C_{26}H_{33}O_{11}$	2.5	18.1	229/279	151/289/387/360
29	Rutin (isomer 1)	x	x	14.13	609.1470	609.1461	$C_{27}H_{29}O_{16}$	1.5	3.5	229/268/356	300
30	Rutin (isomer 2)	x	x	14.35	609.1470	609.1461	$C_{27}H_{29}O_{16}$	1.5	8.1	229/265/355	300
31	Procyanidin dimer type B (isomer 5)	x	x	14.80	577.1347	577.1351	$C_{30}H_{25}O_{12}$	0.8	4.4	229/279	425/407/290
32	Quercetin hexoside	x	x	15.14	463.0879	463.0882	$C_{21}H_{19}O_{12}$	0.6	5.5	230/277	300
33	Catechin diglucopyranoside (isomer 1)	x	x	15.62	593.1498	593.1512	$C_{27}H_{29}O_{15}$	6.4	13.5	231/270	285

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
34	Secoisolariciresinol β-D- hexoside	x	x	16.34	523.2181	523.2185	$C_{26}H_{35}O_{11}$	0.7	6.1	230/280	361/165
35	Catechin diglucopyranoside (isomer 2)	x	x	16.52	593.1508	593.1512	$C_{27}H_{29}O_{15}$	0.7	3.6	231/270	285
36	Procyanidin dimer type A (isomer 2)	x		17.13	575.1170	575.1195	$C_{30}H_{23}O_{12}$	4.4	12.0	231/278	449/423/289/285
37	Hesperidine	x		18.08	609.1821	609.1825	$C_{28}H_{33}O_{15}$	0.7	9.0	232/278	301

x: identified in the cultivar.

As in cherimoya pulp, some procyanidins type B were identified in both cultivars. Procyanidin dimer type B and its isomers were tentatively identified at experimental *m/z* between 577.1347 and 577.1333. The retention times for each compound were: isomer 1 at 6.71 min, isomer 2 at 7.14 min, isomer 3 at 9.05 min, isomer 4 at 9.68 min and isomer 5 at 14.80 min. These compounds correspond to peaks 12, 13, 16, 18 and 31, respectively. The fragmentation pattern was similar to that obtained in the pulp and these compounds had previously been reported in peel of other *Annonaceae* fruits and in pulp of *Annona cherimolla* (Barreca *et al.*, 2011; Giomaro *et al.*, 2014; Huang *et al.*, 2010; Ramírez *et al.*, 2014). Thus, data indicates that these compounds are procyanidin dimers type B. Peak 17 was tentatively assigned as procyanidin trimer type B isomer 1. It was detected at retention time 9.40 min and *m/z* 865.1946. Procyanidin trimer type B isomer 2 (peak 22) was found at retention time 11.47 min and *m/z* 865.1958, whereas at 12.28 min (peak 24) it was identified the isomer 3 with *m/z* 865.1955. In the MS^2 spectra ions were seen at *m/z* 577 and 289, indicating the loss of (epi)catechin dimer and monomer. The fragment at *m/z* 425 is typical of a retro-Diels-Alder reaction. The presence of procyanidin trimer type B has previously been published in cherimoya pulp (Barreca *et al.*, 2011) and in other peel fruits (Giomaro *et al.*, 2014; Tomás-Barberán & Espín, 2001).

The last type B procyanidin identified in all peel extracts was procyanidin tetramer type B and its isomers. Peak 19 presented *m/z* 1153.2646 and retention time of 10.17 min. It was identified as procyanidin tetramer type B isomer 1. Isomer 2 (peak 20) was found at 10.35 min and *m/z* 1153.2629. At 12.20 (peak 23) and 12.96 min (peak 27) procyanidin tetramer type B isomers 3 and 4 were detected. A fragmentation pattern with ions was observed at *m/z* 289, 577 and 865, the typical losses of procyanidin tetramers. This procyanidin type has been reported before in apple peel (Giomaro *et al.*, 2014).

Another catechin derivative identified was peak 33 at retention time 15.26 min and experimental *m/z* 593.1512. One of the major ions produced in MS^2 was at

m/z 285, corresponding to the loss of (epi)catechin monomer, and this fragment revealed two consecutive losses of glucose moieties. So this compound was tentatively assigned as catechin diglucopyranoside isomer 1. Isomer 2 was detected at retention time 16.52 min (peak 35). This fragmentation pattern and data found in literature for other tropical fruits such as *Theobroma cacao* (Cádiz-Gurrea *et al.*, 2014) indicates that these compounds are catechin diglucopyranoside isomers.

As in cherimoya pulp, at retention time 4.67 min and *m/z* 315.1057 (peak 5) and at retention time 4.85 min with *m/z* 315.1074 (peak 6) hydroxytyrosol hexoside isomer 1 and isomer 2 in cherimoya peel were identified. The MS^2 analysis showed a fragment at *m/z* 153 that corresponded to the loss of glucose moiety (Savarese *et al.*, 2007).

Tyrosol hexoside pentoside (peak 9) was found with *m/z* 431.1532 and at retention time 5.32 min. The MS^2 spectrum showed a fragment with *m/z* 137 corresponding to the loss of tyrosol moiety. This compound has previously been described in persimmon leaves (Varughese, Rahaman, Kim, Cho, & Moon, 2009). This previous data, the fragmentation pattern, exact mass, molecular formula, error, mSigma and UV-Vis data (Table 2) identify this compound as tyrosol hexoside pentoside.

Peak 10 was tentatively identified as vanillic acid hexoside. The experimental *m/z* obtained was 329.0862 at a retention time 5.81 min and presented a fragment at *m/z* 167 due to the loss of a vanillic acid moiety. Vanillic acid hexoside and the same fragmentation pattern have previously been reported by other authors in melon (Rodríguez-Perez, Quirantes-Piné, Fernández-Gutiérrez, & Segura-Carretero, 2013) and pomegranate (Fischer, Carle, & Kammerer, 2011).

Peak 14 eluted at retention time 7.78 min with experimental *m/z* 461.1275. The MS^2 spectrum showed fragments at *m/z* 153 and 163 (hexose moiety) and 138 (hydroxybenzoic acid moiety). The molecular formula obtained was $C_{19}H_{26}O_{13}$. Thus, the name tentatively proposed was sibiricose A3. The same fragmentation

pattern has previously been reported in other vegetable studies (Abu-Reidah, Arráez-Román, Lozano-Sánchez, Segura-Carretero & Fernández-Gutiérrez, 2013).

Two isomers of calabricoside A were detected at 12.66 (peak 25) and 12.81 min (peak 26) and experimental *m/z* 741.1884 and 741.1882, respectively. As occurs in quercetin derivative compounds in the fragmentation profile, a major fragment was found at *m/z* 300 in accordance with the loss of a quercetin moiety. The molecular formula ($C_{32}H_{37}O_{20}$) also coincides with the one of calabricoside A. This compound with a similar mass profile has previously been identified in plants with medicinal uses from genus Silphium (Williams 2007).

Several flavonoids were also found in cherimoya peel. Peaks 4, 7 and 8 were detected and tentatively identified as 3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one isomers. The retention times and experimental *m/z* were as follows: isomer 1, retention time 4.37 min and *m/z* 373.112; isomer 2, at 5.04 min and *m/z* 373.1106; finally, isomer 3 at 5.20 min and *m/z* 373.1128. The daughter ion obtained at *m/z* 193 was in agreement with the fragmentation pattern previously reported for the same compound (Liimatainen, Karonen, Sinkkonen, Helander, & Salminen, 2012).

At retention time 14.13 min and *m/z* 609.1470 eluted peak 29 with spectrum of MS^2 showed a majority fragment at *m/z* 300, corresponding to the loss of a quercetin moiety. The molecular formula was $C_{27}H_{29}O_{16}$. Similar fragmentation patterns were found in other peels of tropical fruits as mango and longan (Dorta, González, Gloria Lobo, Sánchez-Moreno, & de Ancos, 2014; Huang *et al.*, 2010) and also in the edible portion of cherimoya (Spinola *et al.*, 2015). Because of that, this compound was identified as rutin. Another isomer of rutin was found at retention time 14.35 min (peak 30).

Quercetin hexoside (peak 32) was identified at 15.14 min and experimental *m/z* 463.0879. The fragmentation profile showed the even ion at *m/z* 301 and the odd ion at *m/z* 300, which corresponded to quercetin aglycone. This compound has

been reported in *Annonaceae* species from Brazil (Santos & Salatino, 2000) and in *Annona cherimola* pulp (Spinola *et al.*, 2015).

Peak 37 with *m/z* 609.1821 was identified only in 'Fino de Jete' cultivar at retention time 18.08 min. MS^2 spectrum showed a major ion at *m/z* 301; thus, it was assigned as hesperidine. Hesperidine has been detected in orange peel (Khan, Nawaz, Ahmad, Afzal, Malik, & Saleem, 2010).

Two types of phenylpropanoids were identified in both cherimoya peel cultivars. At retention time 13.46 min the compound 28 with *m/z* 521.2010 was identified. This compound was proposed as lariciresinol-glucopyranoside due to the fragment obtained at *m/z* 360, characteristic of the lariciresinol moiety. Lariciresinol-glucopyranoside was reported in Brazilian *Lippia* species previously (Funari, Eugster, Martel, Carrupt, Wolfender, & Silva, 2012). Peak 34 was proposed as secoisolariciresinol beta-D-hexoside. The retention time and *m/z* values were 16.34 min and 523.2181, respectively. The fragmentation pattern with daughter ions at *m/z* 361 and 347 was according to the literature. This compound has previously been reported in other fruits such as pomegranate (Mena *et al.*, 2012).

Several organic acids were also detected in peel extracts. Peak 1 was assigned as quinic acid with experimental *m/z* 191.0580 at retention time 1.37 min. Citric acid isomer 1 and 2 were detected (peak 2 and 3) at retention times 1.54 min and 1.78 min, respectively. Citric acid was reported in quince peel extract (B. M. Silva, Andrade, Valentao, Ferreres, Seabra, & Ferreira, 2004). These organic acids have also been found in cherimoya pulp (Spinola *et al.*, 2015).

To our knowledge, this is the first time that procyanidin dimer type A (isomers 1 and 2), procyanidin tetramer type B (isomers 1, 2, 3 and 4), catechin diglucopyranoside (isomers 1 and 2), hydroxytyrosol hexoside (isomers 1 and 2), tyrosol hexoside pentoside, vanillic acid hexoside, sibiricose A3, calabricoside A (isomers 1 and 2), 3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomers 1, 2 and 3), hesperidine, lariciresinol-

glucopyranoside and secoisolariciresinol beta-D-hexoside have been identified in cherimoya fruit. In addition, all compounds identified in peel are reported for the first time because it is the first time that an analysis of phenolic compounds in *Annona cherimola* peel has been done.

3.1.3. Identification of phenolic and other polar compounds in cherimoya seeds

A total of 22 phenolic and other polar compounds were tentatively identified in the seeds of cherimoya cultivars (Table 3).

Some flavan-3-ols were also present in cherimoya seed. However, fewer procyanidins were found in this matrix. At retention time 8.25 min and *m/z* 289.0713 catechin (peak 12) was identified. Epicatechin (peak 15) was detected at 10.87 min. The same fragmentation pattern obtained in pulp and peel analysis was also found in seed. Besides in cherimoya (Spinola *et al.*, 2015), catechin and epicatechin have previously been reported in other fruit seeds (Huang *et al.*, 2010).

Peak 10 and 14 were identified in both cultivars at retention times 6.71 min (*m/z* 577.1332) and 9.70 min (*m/z* 577.1276). Isomer 3 of this compound was detected only in 'Campa' cultivar at 8.91 min (peak 13). The fragmentation pattern showed ions at *m/z* 425 [retro-Diels-Alder reaction (RDA)] and *m/z* 289 corresponding to (epi)catechin moiety. The same fragmentation pattern using an ion trap mass spectrometer has previously been reported in the edible portion of cherimoya (Spinola *et al.*, 2015). Thus, they were identified as procyanidin dimer type B isomer 1 and isomer 2.

Peak 16 at retention time 11.49 min and *m/z* 865.1963 was proposed as procyanidin trimer type B. The fragments found in the MS^2 spectra of seed extracts were similar to the fragments observed in pulp and peel. This fragmentation pattern has also been reported in cherimoya pulp (Barreca *et al.*, 2011; Huang *et al.*, 2010).

The presence of hydroxybenzoic acid hexoside (peak 4) was confirmed by their MS^2 data. The *m/z* value was 299.0772 and retention time 2.98 min. The presence of this compound and its mass spectra has previously been published regarding pomegranate fruit (Mena *et al.*, 2012). This compound was only found in 'Fino de Jete' seed samples.

Vanillic acid hexoside isomer 1 (peak 5, retention time 4.10 min) and isomer 2 (peak 9, retention time 5.77 min) were identified at *m/z* 329.0867 and 329.0870, respectively. The fragmentation pattern was the same found in peel samples. This compound has previously been reported in other seed of fruits and in some plant seeds (Nyam, Tan, Lai, Long, & Man, 2009).

Peak 6, at retention time 4.67 and *m/z* 315.1069 with characteristic fragment ions of hydroxytyrosol at *m/z* 153 and 123 was assigned as hydroxytyrosol hexoside isomer 1. Identification of this compound was based on the fragmentation pattern described in the literature for olives (Savarese *et al.*, 2007). Isomer 2 of hydroxytyrosol hexoside (peak 7) was found at retention time 4.84 min in both cherimoya seeds. However, isomer 1 was only detected in 'Campa' seeds.

Peak 8 was identified at *m/z* 359.0983, retention time 5.07 min and $C_{15}H_{19}O_{10}$ as molecular formula. The MS^2 spectra showed a fragment at *m/z* 197 corresponding to the syringic acid moiety and the loss of a glucopyranoside moiety. Similar fragmentation pattern have previously been detected in bark (Liimatainen *et al.*, 2012). Therefore, this compound could be identified as syringic acid glucopyranoside.

Peak 11 was detected at retention time 7.80 min with experimental *m/z* 461.1273. The fragmentation pattern obtained for this compound was the same as that in peel. So this phenolic compound was identified as sibiricose A3 (Abu-Reidah *et al.*, 2013).

Table 3. Phenolic and other polar compounds identified in cherimoya seeds samples.

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
1	**Quinic acid**	x	x	1.37	191.0580	191.0561	$C_7H_{11}O_6$	6.2	7.6	226/265	111
2	**Citric acid (isomer 1)**	x	x	1.49	191.0210	191.0197	$C_6H_7O_7$	2.8	3.1	228/267	111
3	**Citric acid (isomer 2)**	x	x	1.78	191.0212	191.0197	$C_6H_7O_8$	1.0	2.7	228/267	111
4	**Hydroxybenzoic acid hexoside**	x		2.98	299.0772	299.0754	$C_{13}H_{15}O_{18}$	6.3	1.4	225/251	137/179
5	**Vanilic acid hexoside (isomer 1)**	x	x	4.10	329.0867	329.0878	$C_{14}H_{17}O_9$	3.3	10.6	225/255/293	167
6	**Hydroxytyrosol hexoside (isomer 1)**		x	4.67	315.1069	315.1085	$C_{14}H_{19}O_8$	5.1	64.4	225	153
7	**Hydroxytyrosol hexoside (isomer 2)**	x	x	4.84	315.1089	315.1085	$C_{14}H_{19}O_8$	1.0	12.2	228/277	153
8	**Syringic acid glucopyranoside**	x	x	5.07	359.0983	359.0984	$C_{15}H_{19}O_{10}$	0.1	3.8	226/263	197
9	**Vanilic acid hexoside (isomer 2)**	x	x	5.77	329.0870	329.0878	$C_{14}H_{17}O_9$	2.4	8.7	226	323
10	**Procyanidin dimer type B (isomer 1)**	x	x	6.71	577.1332	577.1351	$C_{30}H_{25}O_{12}$	3.4	10.3	226/273	425/407/289
11	**Sibiricose A3**	x	x	7.80	461.1273	461.12742	$C_{19}H_{25}O_{13}$	6.0	23.5	226	138/153
12	**Catechin**	x	x	8.25	289.0713	289.0718	$C_{15}H_{13}O_6$	1.6	3.8	226/276	245
13	**Procyanidin dimer type B (isomer 2)**		x	8.91	577.1276	577.1351	$C_{30}H_{25}O_{12}$	13.0	12.8	227	425/407/289
14	**Procyanidin dimer type B (isomer 3)**	x	x	9.70	577.1336	577.1351	$C_{30}H_{25}O_{12}$	2.7	3.7	227/279	425/407/289
15	**Epicatechin**	x	x	10.87	289.0707	289.0718	$C_{15}H_{13}O_6$	3.8	9.5	228/278	245
16	**Procyanidin trimer type B**	x	x	11.49	865.1963	865.1985	$C_{45}H_{37}O_{18}$	2.6	33.6	232/279	289/577/407

Peak	Tentative identity	'Fino de Jete'	'Campa'	RT (min)	*m/z* experimental	*m/z* calculated	Molecular formula $[M-H]^-$	Error (ppm)	mSigma	UV-Vis (nm)	MS^2
17	**Rutin (isomer 1)**		**x**	14.12	609.1405	609.1461	$C_{27}H_{29}O_{16}$	9.3	68.4	235/320	301
18	**(Neo)eriocitrín**	**x**		14.15	595.1648	595.1668	$C_{27}H_{31}O_{15}$	3.5	4.1	232/282	287/459
19	**Rutin (isomer 2)**	**x**	**x**	14.38	609.1442	609.1461	$C_{27}H_{29}O_{16}$	3.1	3.7	236/350	301
20	**Hesperidine**	**x**		18.10	609.1805	609.1825	$C_{28}H_{33}O_{15}$	3.3	27.3	235/278	301
21	**(Neo)poncirin**	**x**	**x**	19.87	593.1924	593.1876	$C_{28}H_{33}O_{14}$	8.1	33.4	255	285/269/226
22	**Miconioside A**	**x**	**x**	21.01	607.2073	607.2136	$C_{29}H_{35}O_{14}$	6.6	32.8	230/255	444/283

x: identified in the cultivar.

Rutin isomer 1 (peak 17) was identified at retention time 14.12 min and *m/z* 609.1405. The ion found in MS^2 analysis corresponded to quercetin aglycone (*m/z* 301) after the loss of a hexoside moiety. Rutin isomer 2 (peak 19) was found at 14.38 min. This compound has previously been reported in the edible portion of cherimoya (Spinola *et al.*, 2015).

Compound 18 eluted at retention time 14.15 min with *m/z* 595.1648, and it was only detected in 'Fino de Jete' seeds. This compound was tentatively identified as eriocitrin/neoeriocitrin because the fragmentation pattern showed an ion at *m/z* 287 [M-H-308], a typical fragment of rutinosides. Another fragment was also found at *m/z* 459 previously reported in neoeriocitrin (Spinola *et al.*, 2015). (Neo)eriocitrin has previously been reported in citrus fruit seeds (Bocco, Cuvelier, Richard, & Berset, 1998).

Hesperidine (peak 20) was only found in 'Fino de Jete' seeds (retention time 18.10 min and *m/z* 609.1805). It was identified according to the molecular formula ($C_{28}H_{34}O_{15}$) and the fragment obtained at *m/z* 301, characteristic of this compound. This compound with a similar fragmentation pattern has previously been detected in seed of citrus fruits (Bocco *et al.*, 1998).

At retention time 19.87 min and *m/z* 593.1924, peak 21 was detected. This compound was tentatively assigned as poncirin/neoponcirin due to the ions obtained by MS^2 analysis that were in agreement with the fragmentation pattern reported by Spinola *et al.* for other fruits (Spinola *et al.*, 2015).

Peak 22 was identified at retention time 21.01 min and *m/z* 607.2073. Two fragments were found at *m/z* 444 and 283, that showed the consecutively loss of two hexose moieties. This fragmentation pattern agrees with the compound previously described in *Miconia trailii* as miconioside A (Zhang *et al.*, 2003). The molecular formula obtained ($C_{29}H_{36}O_{14}$) also matched with the one described in *Miconia traili.*

As in the other parts of cherimoya, some organic acids were also detected in seed extracts. Peak 1 was assigned as quinic acid with experimental *m/z* 191.0580 at

retention time 1.37 min. Citric acid isomer 1 (peak 2) at retention times 1.49 min and isomer 2 (peak 3) at retention time 1.78 min were identified. These compounds were confirmed by the fragments found by MS^2 analysis. This data was in agreement with previous reports in cherimoya (Spinola *et al.*, 2015).

As far as we are concerned, hydroxybenzoic acid glycoside, vanillic acid hexoside (isomers 1 and 2), hydroxytyrosol hexoside (isomers 1 and 2), syringic acid glucopyranoside, sibiricose A3, (neo)eriocitrín, hesperidine, (neo)poncirin and miconioside A have been identified in cherimoya fruit for the first time. In addition, as in the peel, it is the first time that an analysis of phenolic compounds in *Annona cherimola* seeds has been done, and all compounds identified in seeds are reported for the first time.

3.2. Quantification of phenolic and other polar compounds content

Three calibration curves were prepared using the following standards: 4-hydroxybenzoic acid, rutin, and catechin, in the range of concentrations from the limit of quantification (LOQ) to 1000 μg/mL. Eight calibration points were used for each of them and the analyses were replicated three times for each calibration point (n = 3).

The calibration plots indicate good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.99 in all cases. The LOQ was determined as the signal-to-noise ratio of 10:1, and the limit of detection (LOD) was determined as a signal-to-noise ratio of 3:1. The LOD was 0.09 μg/mL for rutin, 2.79 μg/mL for 4-hydroxybenzoic acid and 0.138 for catechin. LOQ was 0.30 μg/mL for rutin, 9.30 μg/mL for 4-hydroxybenzoic acid and 0.451 for catechin.

The quantification was performed by using the previous calibration curves. The flavan-3-ols family was quantified with the calibration curve of catechin, flavonoids, and derivatives compounds were quantified using the calibration

curve of rutin, whereas organic acids and phenolic acids were quantified with 4-hydroxybenzoic acid.

Table 4 shows the content of phenolic compounds in the different parts of the two cherimoya cultivars analysed. It should be taken into account that the response of the standards can differ from the response of the analytes present in the cherimoya samples, and consequently, the quantification of these compounds is only an estimation of their actual concentrations.

Table 4. Phenolic and other polar content (mg/100g dry weight) in the different parts of 'Fino de Jete'and 'Campa' cherimoya cultivars.

Fruit part	Tentative identity	'Fino de Jete'	'Campa'
PULP			
	Quinic acid	38.06±2.79^{a}	47.77±0.94^{a}
	Citric acid (isomer 1)	201.42±10.22^{b}	282.70±14.12^{a}
	Citric acid (isomer 2)	192.90±10.38^{a}	199.57±9.60^{a}
	3-(2-Hydroxyphenyl)propanoic acid hexose	60.61±2.20^{a}	20.67±1.05^{b}
	Hydroxytyrosol hexoside (isomer 1)	27.50±2.39^{b}	59.94±10.72^{a}
	Hydroxytyrosol hexoside (isomer 2)	82.00±6.43^{a}	17.66±1.17^{b}
	Luteolin-glucopyranoside	< LOQ	< LOQ
	Procyanidin dimer type A	0.17±0.01	nd
	Procyanidin dimer type B (isomer 1)	3.30±0.12^{a}	3.42±0.12^{a}
	Procyanidin dimer type B (isomer 2)	0.95±0.06^{a}	0.66±0.05^{b}
	Catechin	1.32±0.05^{a}	0.93±0.03^{b}
	Procyanidin dimer type B (isomer 3)	7.00±0.34^{b}	7.75±0.31^{a}
	Procyanidin trimer type B (isomer 1)	nd	3.32±0.07
	Procyanidin dimer type B (isomer 4)	14.45±0.61^{b}	18.75±0.59^{a}
	Epicatechin	11.92±0.16^{b}	14.24±0.67^{a}
	Procyanidin trimer type B (isomer 2)	5.48±0.19^{b}	7.63±0.14^{a}
	Procyanidin tetramer type B (isomer 1)	2.26±0.06^{b}	2.87±0.11^{a}
	Procyanidin tetramer type B (isomer 2)	1.29±0.09	nd
	Procyanidin tetramer type B (isomer 3)	1.51±0.10	nd
	Procyanidin dimer type B (isomer 5)	1.91±0.12^{b}	2.36±0.16^{a}

Fruit part	Tentative identity	'Fino de Jete'	'Campa'
	Secoisolariciresinol beta-D- hexoside	88.96±6.00[b]	94.59±1.35[a]
	Total	743.02±36.59[b]	784.82±14.67[a]
PEEL			
	Quinic acid	304.28±41.37[a]	217.94±3.30[b]
	Citric acid (isomer 1)	292.95±14.37[a]	295.63±7.70[a]
	Citric acid (isomer 2)	225.22±10.00[a]	229.60±9.62[a]
	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 1)	1.16±0.03[a]	0.11±0.03[b]
	Hydroxytyrosol hexoside (isomer 1)	73.79±34.59[a]	63.83±3.21[a]
	Hydroxytyrosol hexoside (isomer 2)	348.84±24.38[a]	122.24±2.38[b]
	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 2)	< LOQ	< LOQ
	3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one (isomer 3)	< LOQ	< LOQ
	Tyrosol hexoside-pentoside	67.61±1.71[a]	41.16±1.34[b]
	Vanillic acid hexoside	38.13±0.24[a]	8.12±0.43[b]
	Procyanidin dimer type A (isomer 1)	0.40±0.03[a]	0.17±0.01[b]
	Procyanidin dimer type B (isomer 1)	7.21±1.09[a]	5.23±0.12[b]
	Procyanidin dimer type B (isomer 2)	1.93±0.71[a]	0.71±0.03[b]
	Sibiricose A3	106.49±11.86[a]	13.51±0.33[b]
	Catechin	5.40±0.83[a]	2.10±0.07[b]
	Procyanidin dimer type B (isomer 3)	16.47±4.28[a]	10.62±0.25[b]
	Procyanidin trimer type B (isomer 1)	5.75±0.97[a]	5.03±0.29[a]
	Procyanidin dimer type B (isomer 4)	25.98±2.32[a]	25.52±0.62[a]
	Procyanidin tetramer type B (isomer 1)	0.54±0.03[a]	0.31±0.01[b]
	Procyanidin tetramer type B (isomer 2)	1.45±0.25[a]	1.58±0.10[a]
	Epicatechin	25.78±2.39[a]	22.70±0.62[b]
	Procyanidin trimer type B (isomer 2)	12.92±1.66[a]	12.02±0.37[a]
	Procyanidin tetramer type B (isomer 3)	4.30±0.74[a]	4.18±0.10[a]
	Procyanidin trimer type B (isomer 3)	2.52±0.46[a]	2.47±0.10[a]
	Calabricoside A (isomer 1)	0.37±0.16	< LOQ
	Calabricoside A (isomer 2)	< LOQ	< LOQ
	Procyanidin tetramer type B (isomer 4)	1.11±0.23[a]	1.03±0.05[a]
	Lariciresinol-glucopyranoside	42.41±4.94[a]	34.24±0.83[b]

Fruit part	Tentative identity	'Fino de Jete'	'Campa'
	Rutin (isomer 1)	13.63±1.35[a]	3.04±0.14[b]
	Rutin (isomer 2)	26.77±3.83[a]	6.60±0.26[b]
	Procyanidin dimer type B (isomer 5)	5.50±0.83[a]	5.36±0.25[a]
	Quercetin hexoside	1.07±0.57	< LOQ
	Catechin diglucopyranoside (isomer 1)	0.36±0.01	< LOQ
	Secoisolariciresinol β-D-hexoside	375.04±3.85[a]	339.66±6.93[b]
	Catechin diglucopyranoside (isomer 2)	0.52±0.02[a]	0.15±0.01[b]
	Procyanidin dimer type A (isomer 2)	0.28±0.09	n.d
	Hesperidine	< LOQ	n.d
	Total	2035.37±38.88[a]	1474.85±10.43[b]
SEED			
	Quinic acid	73.80±3.70[a]	51.85±1.62[b]
	Citric acid (isomer 1)	243.67±12.29[a]	169.27±3.94[b]
	Citric acid (isomer 2)	152.22±5.87[a]	107.26±1.91[b]
	Hydroxybenzoic acid hexoside	16.27±4.11	nd
	Vanilic acid hexoside (isomer 1)	157.01±6.20[a]	156.74±6.23[a]
	Hydroxytyrosol hexoside	nd	< LOQ
	Hydroxytyrosol hexoside	14.74±2.23	< LOQ
	Syringic acid glucopyranoside	36.52±1.61[a]	16.75±1.20[b]
	Vanilic acid hexoside (isomer 2)	13.20±1.21[b]	101.29±4.09[a]
	Procyanidin dimer type B (isomer 1)	0.38±0.06[a]	0.11±0.01[b]
	Sibiricose A3	9.98±0.49	< LOQ
	Catechin	0.15±0.01[a]	0.14±0.01[a]
	Procyanidin dimer type B (isomer 2)	nd	0.23±0.01
	Procyanidin dimer type B (isomer 3)	1.62±0.23[a]	0.52±0.05[b]
	Epicatechin	1.30±0.13[a]	0.93±0.02[b]
	Procyanidin trimer type B	0.61±0.08[a]	0.17±0.02[b]
	Rutin (isomer 1)	nd	< LOQ
	(Neo)eriocitrín	0.29±0.02	nd
	Rutin (isomer 2)	< LOQ	< LOQ
	Hesperidine	< LOQ	nd
	(Neo)poncirin	18.14±1.59[a]	10.76±1.13[b]
	Miconioside A	19.67±1.52[a]	18.63±1.60[a]
	Total	758.42±17.14[a]	634.49±18.84[b]

3.2.1. Quantification of phenolic and other polar compounds in cherimoya pulp

The total amount of phenolic and other polar compounds in 'Campa' cultivar pulp was 784.82 mg/100g, whereas in 'Fino de Jete' it was 743.02 mg/100g d.w. The concentrations found in this fruit were higher than the quantity reported in pulp of other tropical fruit as pineapple or cajú which was found 48.69 and 378.36 mg/100g of total phenolic compounds respectively. It is reported that taperebá has 712.72 mg/100g of total phenolic compounds, this amount was similar to the cherimoya pulp (Bataglion, da Silva, Eberlin & Koolen, 2015)

The content of catechin, epicatechin and procyanidins was similar in both cultivars. Regarding the total content, in Campa pulp, flavan-3-ols were 7.83%, whereas in 'Fino de Jete' they were 6.94%. Catechin was quantified in a range of 0.93-1.32 mg/100 g in both cultivars. However, epicatechin concentration was higher, varying between 11.92 and 14.24 mg/100 g.

The procyanidin present in the lowest concentration was procyanidin dimer type A, which was only detected in 'Fino de Jete' cultivar with 0.17 mg/100 g. The total concentration of the five isomers of procyanidin dimer type B was higher in 'Campa' than in 'Fino de Jete'. The percentage of this compound and its isomers regarding to the total content was 3.72 and 4.20 % in 'Fino de Jete' and 'Campa', respectively.

Procyanidin trimer type B isomer 1 was only found in 'Campa' pulp in a quantity of 3.32 mg/100 g. Procyanidin trimer type B isomer 2 content was higher in 'Campa' than in 'Fino de Jete' pulp, 7.63 mg/100 g versus 5.48 mg/100 g. Procyanidin tetramer type B isomer 1 content was slightly higher in 'Campa' pulp than in 'Fino de Jete', 2.87 and 2.26 mg/100 g, respectively. Isomers 2 and 3 of this compound were detected only in 'Fino de Jete' pulp.

The content of 3-(2-hydroxyphenyl)-propanoic acid hexose was higher in 'Fino de Jete' than in 'Campa' pulp, 60.61 mg/100g versus 20.61 mg/100g.

The hydroxytyrosol hexoside quantity was also different in the two cherimoya pulps analysed. Hydroxytyrosol hexoside isomer 2 concentration was higher in 'Fino de Jete', whereas 'Campa' pulp presented a major content of isomer 1. The total quantity of the two isomers of this compound was greater in 'Fino de Jete', where the concentration found was 109.50 mg/100 g. In 'Campa' the total content of hydroxytyrosol hexoside was 74.61 mg/100 g.

Another compound found in pulp with a high content and similar in two cultivars was secoisolariciresinol beta-D-hexoside that ranged from 88.96 to 94.59 mg/100 g. Luteolin-glucopyranoside was lower than LOQ.

The major compound found in both cultivars was citric acid (isomer 1 and 2). The citric acid percentage was 53.07% in 'Fino de Jete' whereas in 'Campa' pulp it was 61.45%, regarding to the total content. Quinic acid was quantified in a range from 38.06 to 47.77 mg/100 g.

3.2.2. Quantification of phenolic and other polar compounds in cherimoya peel

The total content of compounds found in peel was much higher than in the other parts of the fruit. In 'Fino de Jete' peel the total content of phenolic and other polar compounds was 2035.37 mg/100 g. However, the total concentration found in 'Campa' was lower, 1474.85 mg/100 g. The content of total phenolic compound in cherimoya peel was greater than the content reported in avocado 'Hass' peel which concentration was 699.1 mg/100g (Kosinska, Karamac, Estrella, Hernández, Bartolomé, & Dykes, 2012).

Regarding the total compounds content, the quantity of catechin, epicatechin and derivative compounds was 4.77% and 8.03% for 'Fino de Jete' and 'Campa' peel, respectively. The amount of catechin ranged between 2.10 and 5.75 mg/100 g, being greater in 'Fino de Jete' peel. Epicatechin concentration was also higher in 'Fino de Jete' (25.78 mg/100 g) than in 'Campa' (22.70 mg/100 g). The total content of catechin diglucopyranoside isomer 1 and 2 was found in a range from

0.15 to 0.88 mg/100 g. The lowest concentration was found for ‘Campa’ cherimoya peel.

The content of procyanidin dimer type A isomer 1 varied from 0.40 to 0.17 mg/100 g and isomer 2 was only quantified in ‘Fino de Jete’ peel (0.28 mg/100g). As in the pulp, five isomers of procyanidin dimer type B were quantified. The percentage estimated of all isomers of this compound, regarding the total content, was 2.66% in ‘Fino de Jete’ and 3.22 % in ‘Campa’ peel. The contents of procyanidin trimers type B were similar in both peel cultivars, and they were 19.52 and 21.19 mg/100 g in ‘Campa’ and ‘Fino de Jete’, respectively. Four procyanidin tetramer type B isomers were quantified having also similar concentrations in the two peel cultivars, 7.10 to 7.40 mg/100g in ‘Campa’ and ‘Fino de Jete’, respectively.

A great amount of hydroxytyrosol hexoside was quantified. The concentration of isomers 1 and 2 was 186.07 and 422.63 mg/100 g for ‘Campa’ and ‘Fino de Jete’, respectively. Tyrosol hexoside pentoside content was 67.61 and 41.16 mg/100 g, being also higher in ‘Fino de Jete’ than in ‘Campa’. Vanillic acid content was 8.12 and 38.13 mg/100 g and it was also higher in ‘Fino de Jete’ than in ‘Campa’ peel. The same happened with sibiricose A3, 13.51 and 106.49 mg/100 g for ‘Campa’ and ‘Fino de Jete’, respectively. Calabricoside A isomer 1 was quantified only in ‘Fino de Jete’ cherimoya peel, the content was 0.37 mg/100 g. However, calabricoside A isomer 2 concentration was lower than LOQ. The concentration for the compound 3-beta-glucopyranosyloxy-2-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one isomer 1 was 0.11 and 1.16 mg/100 g, being slightly higher in ‘Fino de Jete’ than in ‘Campa’ peel. Isomers 2 and 3 contents were below the LOQ.

Two isomers of rutin were quantified and their content was 9.64 and 40.40 mg/100 g for ‘Campa’ and ‘Fino de Jete’, respectively. The content of this compound was greater in ‘Fino de Jete’ than in ‘Campa’ samples. Quercetin

hexoside was quantified only in ‘Fino de Jete’ peel with a concentration of 1.07 mg/100 g. Hesperidine content was less than LOQ.

The phenylpropanoids detected were quantified in a high amount. The concentration of secoisolariciresinol beta-D-hexoside was 339.66 and 375.04 mg/100 g in ‘Campa’ and ‘Fino de Jete’, respectively, whereas lariciresinol glucopiranose content was 34.24 and 42.41 mg/100g ‘Campa’ and ‘Fino de Jete’, respectively. Citric acid content was 25.46% in ‘Fino de Jete’ and 35.61 % in ‘Campa’ of the total compounds quantified. Quinic acid content was lower in ‘Campa’ (217.94 mg/100g) than in ‘Fino de Jete’ (304.28 mg/100g).

3.2.3. Quantification of phenolic and other polar compounds in cherimoya seeds

As opposed to pulp, in ‘Fino de Jete’ seeds the total concentration of phenolic and other polar compounds was higher than in ‘Campa’ cultivar, being observed at 758.42 and 634.49 mg/100 g, respectively, these concentrations were higher than in avocado ‘Hass’ seeds which content was 394.9 mg/100g(Kosinska, Karamac, Estrella, Hernández, Bartolomé, & Dykes, 2012).

The content of catechin, epicatechin and derivative compounds in cherimoya seeds was much lower than in the other parts of the fruit. Regarding the total compounds quantified, the total percentage of these compounds in ‘Fino de Jete’ was 0.54 %, the double than in ‘Campa’ seeds with 0.33%.

Catechin concentration was similar in both cultivars varying only from 0.14 to 0.15 mg/100 g. Epicatechin was 0.93 and 1.30 mg/100 g. The amount of these two compounds was higher in ‘Fino de Jete’ than in ‘Campa’ seeds. The total quantity of procyanidin dimer type B isomers was 0.86 and 2.00 mg/100 g. This amount was lower in ‘Campa’ than in ‘Fino de Jete’ cherimoya seed. Procyanidin trimer type B was 0.17 and 0.61 mg/100 g, also being higher in ‘Fino de Jete’ seeds.

Vanillic acid was present in a high concentration. The content of isomer 1 of this acid was similar in both peel cultivars, around 157 mg/100 g. However, the concentration of vanillic acid isomer 2 was higher in 'Campa' than in 'Fino de Jete' seeds, and it was 13.20 and 101.29 mg/100 g. Sibiricose A3 and (neo)eriocitrin were quantified only in 'Fino de Jete' cultivar, whose content were 9.98 and 0.29, respectively. The concentrations obtained of these compounds in 'Campa' seeds were lower than LOQ. Rutin and hesperidin concentrations were also below the LOQ. The (Neo)poncirin amount was 10.76 and 18.14 mg/100 g, being lower in 'Campa' than in 'Fino de Jete' samples. Miconioside A was found in the same quantity in both seed cultivars, around 19 mg/100 g.

Finally, as in the pulp, the compounds found with major concentrations in both seed samples were citric acid isomer 1 and 2, and their percentage were 43.58 and 52.20% of the total quantified compounds in 'Campa' and 'Fino de Jete', respectively. Quinic acid concentration was also higher in in 'Fino de Jete' (73.80 mg/100 g) than in 'Campa' seeds (51.85 mg/100 g).

3.3. Comparison between cherimoya pulp and its by-products

Out of the three parts of the fruit, the peel had the largest amount of phenolic and other polar compounds. In particular, the total content of these compounds in 'Fino de Jete' peel was two times more than in the other fruit parts, and higher than in 'Campa' peel. However, the amount of these compounds in pulp and seeds was analogous, also between cultivars. It was noted that the lowest quantity of polyphenols was found in 'Campa'seeds.

Procyanidins content was higher in cherimoya pulp, and in seeds the content of total procyanidins was quite low. The quantity of epicatechin was greater than catechin content, particularly in pulp and peel. The greatest amount of epicatechin was found in 'Fino de Jete' cultivar, with the exception of the pulp. The contents of procyanidin dimer type B and procyanidin trimer type B, were

higher in ‘Fino de Jete’ than in ‘Campa’ cultivar in peel and seed. The part of cherimoya with the highest concentration of these flavan-3-ols was the peel followed by pulp and seed. Procyanidin tetramer type B was found only in pulp and peel of cherimoya. The content was similar in both peel cultivars, whereas in pulp, this content was higher in ‘Fino de Jete’ than in ‘Campa’.

Regarding the other phenolic compounds, cherimoya peel was also the part with the greatest content, and pulp contained the lowest quantity. The presence of hydroxytyrosol hexose is remarkable, especially in pulp and peel, being more concentrated in ‘Fino de Jete’ than in ‘Campa’ peel.

Rutin was found in peel and seeds but only quantified in peel. Sibiricose A3 was also present in peel and seeds, and a much greater quantity in ‘Fino de Jete’ peel was observed. Secoisolariciresinol beta-D- hexoside was found only in pulp and in peel. Its content was two times higher in peel than in pulp in both cultivars.

In seeds an important content of vanillic acid glycoside, particularly isomer 1, was found. The concentration of isomer 2 was especially low in ‘Fino de Jete’ cherimoya. Hydroxybenzoic acid glycoside, syringic acid glucopyranoside, (neo)poncirin and miconioside A were found only in seeds. The quantity of these compounds was higher in ‘Campa’ cultivar.

The content of citric acid was similar in the three parts of the fruit, whereas quinic acid content was found to be much higher in peel. Between cultivars, few differences were observed concerning the quantity of these organic acids.

4. Conclusions

To our knowledge, this is the first study available that provides an exhaustive identification and quantification in pulp, peel and seed of two different cultivars of *Annona cherimola*. A total of 21 phenolic and other polar compounds have been tentatively identified in cherimoya pulp, 37 in peel and 22 in seeds. The most important family of polyphenols present in cherimoya pulp and peel was

flavan-3-ols. Of all compounds detected, 9 compounds in pulp, 37 in peel and 22 in seeds have been identified for the first time in cherimoya fruit. As far as we know, there are no previous studies about single phenolic or other polar compounds in this fruit of *Annona* genus.

In summary, quantification results show that cherimoya peel is the richest part of the fruit in phenolic compounds, followed by seeds and pulp. Cherimoya 'Fino de Jete' presented the highest content of polyphenols in peel and seeds, whereas in pulp the amount was greater in 'Campa' cultivar.

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CAPÍTULO 7

Determination of lipid composition of the two principal cherimoya cultivars grown in Andalusian Region

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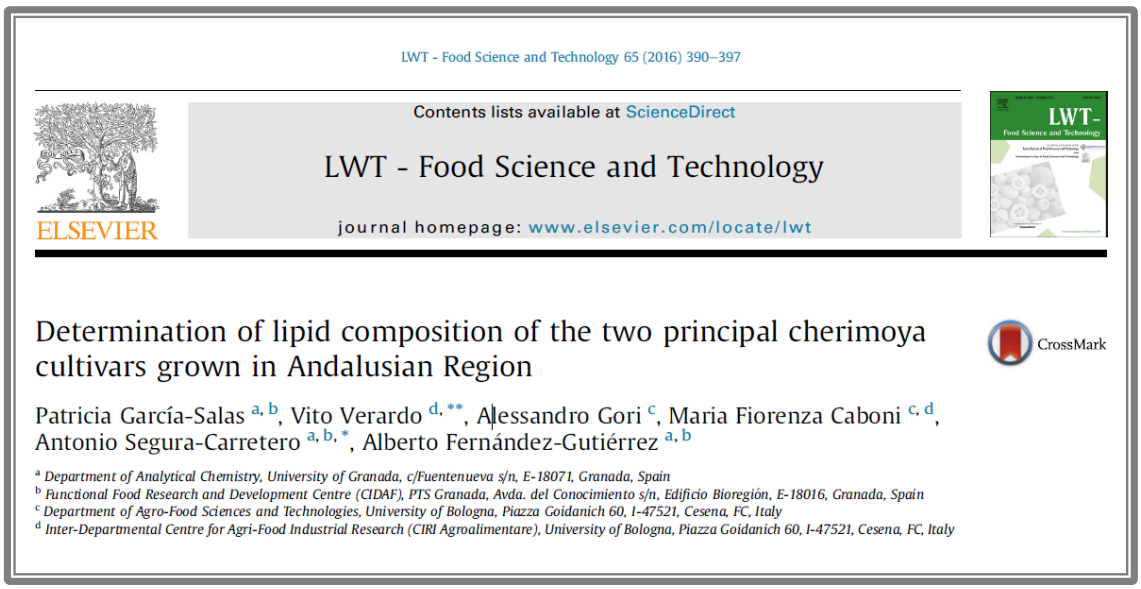

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Abstract

Cherimoya (*Annona cherimola*) is a tropical fruit, native to inter-Andean valleys from Peru and Ecuador. The main cherimoya growing in the Mediterranean basin is the coast of GranadaeMalaga (Spain), the so called “Costa Tropical”.

Recently, the number of studies related to economic exploitation of seeds and other by-products proceeding from new oleaginous vegetable sources has increased. Therefore, the aim of this work was to characterize the lipid fraction of the edible portion of the cherimoya and its by-products.

Different fatty acids were identified in pulp, seed, and skin. The major fatty acids were palmitic, oleic, linoleic, and α-linolenic. On the other hand, α-tocopherol was identified in seeds and peel. Moreover, three different phospholipids were identified. Finally, β-sitosterol was the principle sterol in all samples and, to our knowledge, γ-sitosterol was identified only in pulp and peel of cherimoya for the first time.

Keywords: Cherimoya Fatty acids Tocopherols Phospholipids Phytosterols

1. Introduction

Cherimoya (*Annona cherimola* Mill.) is one of the tropical fruit that has become an important crop due to its excellent sensorial properties and because it is a source of bioactive compounds.

The main cherimoya growing in the Mediterranean basin is the coast of Granada-Malaga (Spain), the so-called "Costa Tropical". Spain is considered one of the major producers worldwide. Two different cultivars are resident in "Costa Tropical", 'Fino de Jete', which is the widest spread in the world, and 'Campa' (Alique & Oliveira, 1994; Barreca *et al.*, 2011).

The high potential of tropical fruit pulps and their by-products in human nutrition has led food research to investigate the isolation of specific phytochemicals for application in nutraceutical supplements, dietary additives, and new foods and pharmaceutical products (Ribeiro da Silva *et al.*, 2014).

Special attention has been focused by food researchers on the lipid fraction in fruits and vegetables due to the presence of antioxidant compounds that play a natural preventive role in cardiovascular disease and several degenerative illness (Ayala-Zavala *et al.*, 2011; Fadavi, Barzegar, & Azizi, 2006; Melgarejo & Artes, 2000). In fact, lipophilic characterization at fruits and by products has opened a promising field of research in the cosmetic and food industries for their potential uses as nutritional supplements in functional foods (Villaverde *et al.*, 2013). Plant seed oil is one of the most interesting essential oils due to its properties; nevertheless there are relatively reports few studies in the literature about the lipid composition of fruit seeds such as prickly pear, apple, and some *Annona* species (Bada, Leon-Camacho, Copovi, & Alonso, 2014; Monteiro & Alves, 2011; Ramadan & Morsel, 2003). It has been found that fruit peel lipids can be a source of essential fatty acids, sterols, and lipid soluble antioxidants (Fiorentino *et al.*, 2009; Ramadan *et al.*, 2003). Previous studies about lipid composition of cherimoya fruit have been developed. Gutierrez *et al.* reported a general study about lipids fraction of the fruit mesocarp studying only the fatty acids content

(Gutiérrez, Sola, & Vargas, 2005). Cordeiro and coworkers studied the fatty acids, sterols, and δ-tocopherol content also in pulp samples (Cordeiro, Sousa, Freitas, & Gouveia, 2013). Recently, Albuquerque *et al.* reported the vitamin E content in some cherimoya cultivars and its by-products (Albuquerque *et al.*, in press).

There are few research studies about the determination of lipid compounds in *Annona cherimola* and its by-products. For this reason, the aim of this work was to determine the lipid composition (FA, sterols, tocopherols, and phospholipids) of cherimoya pulp and by-products, from two different cultivars from the Granada coast (Spain).

2. Materials and methods

2.1. Samples

Two different cherimoya cultivars, 'Fino de Jete' and 'Campa', were collected in February 2012. These cultivars were selected because they are the most commercialized in Andalucia (Spain).

The cultivars were grown under the same agronomic and environmental conditions in the same experimental field located in Motril (Spain) (36º44'43''N 3º 31'14''O). Trees of each cultivar (10 trees) is planted at 7 × 7 m spacing.

The fruits were collected at commercial maturation. A total of 30 fruits from 6 individual trees for each cultivar were taken. The pulp, seeds, and peel of fresh samples were manually separated and freeze dried by a lyophilizer (Christ Alpha 1-2 LD Freeze dryer, Shropshire, UK). Afterwards, the dried samples were milled and kept at -18 ºC until use.

2.2. Chemicals

All solvents and reagents were purchased from Merck (Darmstadt, Germany). The following standards were supplied by Sigma Aldrich (Saint Louis, MO, USA): L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC), L-α-phosphatidylinositol (PI), α-, δ-tocopherol and dihydrocholesterol. The GLC-463 mix for FA was from Nu-Check (Elysian, MN, USA).

2.3 Determination of oil content

To determine the total oil content, cherimoya pulp, seeds, and peel were extracted with diethyl ether in a Soxtech instrument, and the remaining solvent was removed by vacuum evaporation. Each extraction was carried out three times for each sample.

2.4. Lipid extraction for analysis

The pulp, seeds, and peel oils were extracted using the procedure described by Boselli, Velazco, Caboni, and Lercker (2001) using a chloroform/methanol solution. The lipid extract was stored at -18 ºC until it was analysed.

2.5. Total fatty acid composition analysis

The FA composition of cherimoya samples was determined from extracted oil as FAMEs by capillary gas chromatography analysis as reported by Verardo, Gomez-Caravaca, Gori, Losi, and Caboni (2013) with minor modifications as follows: the sample volume injection was 0.2 mL with a split ratio set at 1:400.

2.6. Tocopherol analysis

Approximately 50 mg of oil from the pulp, seed or peel, was dissolved in 500 mL of n-hexane. α-, γ-, and δ-tocopherol were determined by HPLC (Agilent 1200 series, Agilent Technologies, Palo Alto, CA, USA) equipped with a fluorescence

detector (Agilent Technologies, Palo Alto, CA, USA) under the conditions as reported by Gomez-Caravaca, Verardo, and Caboni (2010). A calibration curve was constructed with α-tocopherol standard solution (r^2 0.9999) and it was used for quantification.

2.7. Phytosterol and triterpenic alcohol analysis

To determine the phytosterols and triterpenic alcohols, 100 mL of dihydrocholesterol (c = 2.0 mg/mL) was added to 200 mg of each oil sample and saponification was conducted at room temperature (Iafelice, Verardo, Marconi, & Caboni, 2009). The unsaponifiable matter was sylilated according to Sweeley, Bentley, Makita, and Wells (1963) and it was analysed using a GC/MS (GCMS-QP2010 Plus, Shimadzu, Tokyo, Japan) with the chromatographic conditions reported by Cardenia, Rodriguez-Estrada, Baldacci, Savioli, and Lercker (2012).

Compound identification was achieved by comparing chromatographics peaks and peak mass spectra with peaks in a standard mixture and with GC-MS data reported by Pelillo, Iafelice, Marconi, and Caboni (2003). Quantification of identified phytosterol and triterpenic alcohol compounds was performed in relation to dihydrocholesterol used as an internal standard.

2.8. Phospholipid determination

To determine the phospholipids in the pulp and by-products of cherimoya fruit, approximately 100 mg of oil were weighed and dissolved in 1 mL of 88/12 (v/v) chloroform/methanol and used for HPLC analysis.

Quantitation of the phospholipid classes was performed using HPLC-ELSD. The chromatographic method used by Verardo *et al.* (2013) to separate the polar lipids was carried out.

2.9. Determination of phospholipid fatty acids

HPLC-MS analyses were carried out on the same extract analyzed by HPLC-ELSD. A liquid chromatography apparatus HP 1100 Series from Agilent Technologies, including a degasser, a binary pump delivery system and an automatic liquid sampler was used and coupled with a mass spectrometer (mod. G1946A) from Agilent Technologies operated in negative mode using an electrospray ionization-source (ESI). The chromatographic and mass spectrometry conditions were the same as those used by Verardo *et al*. (2013).

2.10. Statistical analysis

All chemical analyses were carried out in triplicate, and the analytical data were used for statistical comparisons. Tukey's honest significant difference multiple comparison (one-way ANOVA), $p < 0.05$ level, was evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA)

3. Result and discussion

3.1. Oil content and fatty acids composition analysis

Fat extraction was carried out by soxtech instrument, to evaluate the oil content. However, the lipid composition was determined in the Folch extracts, because it is the best method for the recovery of polar lipids.

The oil content of the cherimoya pulp varied from 0.89 to 1.13 % between 'Campa' and 'Fino de Jete' cultivars. These data are similar to those for the pulp lipid contents of other fruits such as jatoba (*Hymenaea stigonocarpa*) (Dias, Luzia, & Jorge, 2013). In cherimoya by-products, the percentage of oil was slightly high in peel, ranging from 1.41 to 2.26 %, and, as expected, greater in the seeds than in the other parts of the fruit, ranging between 15.12 and 24.20% in both cultivars. In cherimoya seeds the oil content was higher than in other fruit seeds such as *Opuntia ficus-indica* seeds (Matthäus & Özcan, 2011), and of the

same order of magnitude if compared with different cultivars of apple seeds (Bada *et al.*, 2014).

The content of each FA found in different parts of the 'Fino de Jete' and 'Campa' cultivars of cherimoya are shown in Table 1: nine saturated, five mono-unsaturated, and five poly-unsaturated FAs were identified and quantified.

Seventeen different FAs were detected in peel and pulp, and the qualitative composition of both samples was similar. According to the literature, the major FAs were palmitic acid (C16:0), linoleic acid (C18:2n6), and α-linolenic acid (C18:3n3) (Gutiérrez *et al.*, 2005). A similar composition was previously reported in mango (Pierson *et al.*, 2014; Vilela *et al.*, 2013), and banana fruit (Vilela *et al.*, 2014).

Briefly, the main compound in the peel and pulp of 'Campa' and 'Fino de Jete' was C18:2n6, ranging from 24.85 to 35.72 % and 29.30-31.02 % in the peel and pulp of the two varieties, respectively, and being higher in 'Fino de Jete'. These data are in accordance with Gutiérrez *et al.* (2005).

The second major FA was C18:3n3, which was particularly high in 'Fino de Jete' pulp. It comprised 25.56-28.43 % and 29.91-30.99 % of the peel and pulp of 'Campa' and 'Fino de Jete' varieties respectively. These values were particularly higher compared to those quantified in cherimoya fruit mesocarp by Gutiérrez *et al.* (2005), who reported a range from 15.35 to 23.37 % of C18:3n3. These differences can be due by the different agronomic practices or season of harvesting.

Table 1. Oil and FA content (g/100 g of FAME) in peel, pulp and seeds of 'Fino de Jete' and 'Campa' cultivars of cherimoya fruit.

	Cherimoya 'Fino de jete'			Cherimoya 'Campa'		
	Peel	**Pulp**	**Seed**	**Peel**	**Pulp**	**Seed**
Oil Content	1.41	1.13	15.12	2.26	0.89	24.20
C12:0	0.29±0.03^{c}	0.17±0.01^{b}	0.02±0.00^{a}	0.40±0.07^{d}	0.19±0.01^{b}	0.02±0.00^{a}
C14:0	0.77±0.01^{b}	0.85±0.01^{c}	0.05±0.00^{a}	0.96±0.02^{d}	0.79±0.00^{b}	0.04±0.00^{a}
C15:0	0.33±0.01^{b}	0.34±0.01^{b}	0.03±0.00^{a}	0.41±0.01^{c}	0.32±0.01^{b}	0.02±0.00^{a}
C16:0	22.73±0.08^{c}	26.56±0.05^{d}	15.67±0.01^{b}	26.87±0.14^{e}	27.00±0.04^{e}	12.90±0.01^{a}
***cis*-C16:1**	0.62±0.01^{d}	0.70±0.00^{e}	0.14±0.01^{b}	0.47±0.02^{c}	0.74±0.01^{f}	0.10±0.00^{a}
C17:0	0.91±0.02^{c}	0.94±0.02^{c,d}	0.23±0.00^{b}	0.97±0.02^{d}	0.94±0.01^{c,d}	0.12±0.01^{a}
***cis*-C17:1**	n.d.	n.d.	0.09±0.00^{b}	n.d.	n.d.	0.04±0.00^{a}
C18:0	2.14±0.06^{a}	2.71±0.04^{b}	4.41±0.01^{d}	4.71±0.09^{f}	2.83±0.01^{b}	4.23±0.00^{c}
***cis*-9-C18:1**	3.32±0.03^{b}	1.97±0.00^{a}	32.48±0.03^{e}	10.21±0.03^{d}	4.34±0.02^{c}	44.93±0.01^{f}
***cis*-11-C18:1**	1.74±0.02^{b}	1.28±0.01^{a}	n.d.	1.35±0.09^{a}	1.27±0.03^{a}	n.d.
C18:2n-6	35.72±0.14^{e}	31.02±0.02^{c}	43.72±0.02^{f}	24.85±0.10^{a}	29.30±0.04^{b}	35.20±0.02^{d}
C18:3n-6	n.d.	n.d.	0.120±0.005^{b}	n.d.	n.d.	0.073±0.009^{a}
C18:3n-3	28.43±0.02^{d}	30.99±0.02^{f}	2.02±0.00^{b}	25.46±0.10^{c}	29.91±0.04^{e}	1.25±0.01^{a}
C20:0	0.28±0.02^{a}	0.34±0.02^{b}	0.49±0.00^{d}	0.43±0.01^{c}	0.33±0.00^{b}	0.47±0.00^{d}
C20:1	0.18±0.01^{c}	0.04±0.00^{a}	0.21±0.00^{d}	0.15±0.00^{b}	0.05±0.01^{a}	0.27±0.01^{e}
C20:2	0.14±0.01^{c}	0.07±0.00^{b}	0.02±0.00^{a}	0.13±0.03^{c}	0.07±0.01^{b}	0.01±0.00^{a}
C22:0	0.87±0.03^{d}	0.81±0.01^{b,c}	0.16±0.00^{a}	0.83±0.02^{c,d}	0.77±0.03^{b}	0.16±0.00^{a}
C22:2	0.50±0.02^{c}	0.40±0.01^{b}	0.03±0.00^{a}	0.42±0.01^{b}	0.39±0.01^{b}	0.02±0.00^{a}
C24:0	1.03±0.05^{c}	0.78±0.01^{b}	0.14±0.00^{a}	0.99±0.01^{c}	0.77±0.02^{b}	0.13±0.00^{a}
SFA	29.35	33.49	21.18	36.56	33.94	18.10
MUFA	5.87	4.04	32.92	12.18	6.40	45.34
PUFA	64.79	62.81	45.90	51.26	59.67	36.56
UFA	70.65	66.85	78.82	63.44	66.07	81.90
SFA/UFA	0.42	0.50	0.27	0.58	0.51	0.22
n-3	28.43	30.99	2.02	25.46	29.91	1.25
n-6	35.72	31.02	43.84	24.85	29.30	35.28

n.d.: non detected

The different lower case letters in the same line indicate significantly different values ($p<0.05$)

C18:3n3 was followed by C16:0, ranging from 22.73 to 26.87 % and 26.56-27.00 % in the peel and pulp of 'Fino de Jete' and 'Campa', respectively, similar to what was reported in earlier investigations (Gutiérrez *et al.*, 2005). 'Campa' cultivar peel and pulp also showed high concentrations of oleic acid (*cis*-9-C18:1), 10.21% and 4.34%, respectively. Moreover, the presence of the cis isomer of vaccenic acid (*cis*-11-C18:1) was detected, accounting for about 1e2 %

of total FAs. The stearic acid (C18:0) content ranged from 2.14 to 4.71 % and 2.71-2.83 % in the peel and pulp of the 'Fino de Jete' and 'Campa' varieties, respectively.

Cherimoya seeds also showed 18 different FAs, of which the major fatty acid compounds were C18:2n6 and *cis*-9-C18:1. The content of linoleic acid was higher in 'Fino de Jete' than'Campa' seeds. Oleic and linolenic acids amounts were lower in 'Fino de Jete' than 'Campa' (35.20-43.72 % and 32.48-44.93 % in linoleic and oleic acid, respectively). These data are in agreement with those previously reported in seeds of some *Annona* species (Monteiro, dos Santos, & Yara, 2011) and of the same order of magnitude of those reported by Gornas, Seglina, Lacis, and Pugajeva (2014) for cold pressed Japanese quince seed oil. Although the concentrations of these FAs were higher than in avocado and berry seed oils (Dulf, 2012; Rodrigues & Meirelles, 2008). The C18:2n6 content was also greater than in *Pistacia lentiscus* edible oil (Mezni *et al.*, 2014). Other minor FA compounds contained in 'Campa' and 'Fino de Jete' seeds were C16:0 (12.90-15.67 %), C18:0 (4.23-4.41 %), and mainly C18:3n3 (1.25-2.02 %). The content of C16:0 and C18:3 fatty acids were lower in the seed than in the peel and pulp; instead, higher amounts of C18:0 were detected in the seeds compared to the other parts of the fruit. Gamma-linolenic (C18:3n6) and heptadecenoic acid (*cis*-C17:1) were found only in cherimoya seeds ranging from 0.073 to 0.120 % and 0.04-0.09 % in 'Campa' and 'Fino de Jete', respectively.

The other FAs found in cherimoya peel, pulp, and seeds were lauric (C12:0), miristic (C14:0), pentadecanoic (C15:0), palmitoleic (*cis*-C16:1), margaric (C17:0), arachidic (C20:0), gadoleic (C20:1), eicosadienoic (C20:2), behenic (C22:0), docosadienoic (C22:2), and lignoceric acid (C24:0). Some of these have also been reported in seeds of *Annona* species, avocado and berries (Dulf, 2012; Monteiro *et al.*, 2011; Rodrigues & Meirelles, 2008). Although the amounts of these FAs in the seeds of both cultivars were not statistically different, their contents in peel and pulp samples were low.

In particular, total saturated FAs (SFAs) in pulp showed similar levels in both cherimoya varieties (about 33% of total FAMEs). Regarding the peel, some differences were noted between the two cultivars; indeed, the 'Campa' sample demonstrated a higher content of SFAs than 'Fino de Jete'. The SFA content in seeds of the two cultivars is lower (21.2-18.1 %) compared to pulp and peel, and similar behavior can be observed in berry oil (Dulf, 2012). It was noted that the content of unsaturated FAs was high in all three parts of the fruit, with seeds containing the highest amounts of UFA. Peel and pulp showed the highest values of polyunsaturated acids (PUFA), while for monounsaturated acids (MUFA) the levels were less than 6.5 % except in 'Campa' peel where it was 12.18%. The content of unsaturated FA in the seeds was balanced, ranging from 32.92 to 45.34 % and 36.56-45.90 % of MUFA and PUFA, respectively. The amount of unsaturated FAs in the seeds was similar to that reported by Monteiro *et al.* (2011) in seeds of *Annona* species.

It should also be noted that in the 'Fino de Jete' cultivar, omega-6 FAs were higher than in the 'Campa' cultivar, mostly in the seeds. The content of omega-3 FAs comprised between 25.46 and 28.43 % in peel, while the n-3 composition of the seeds was much lower. In any case, cherimoya seed oil reported appreciable amounts of oleic acid; its content, ranging between 32 and 45% of total FAMEs, is comparable with that of other unconventional seed oils (Gornas *et al.*, 2014; Habib, Kamal, Ibrahim, & Al Dhaheri, 2013).

3.2. Tocopherol determination

The content of tocopherols in the studied *Annona cherimola* cultivars is reported in Table 2. Tocopherols were detected only in peel and seeds. Only α-tocopherol was detected in cherimoya peel.

Table 2. Tocopherols content (μg/g) of oil in peel, pulp and seeds of 'Fino de Jete' and 'Campa' cultivars of cherimoya fruit.

Tocopherols	Cherimoya 'Fino de Jete'			Cherimoya 'Campa'		
	Peel	Pulp	Seed	Peel	Pulp	Seed
α-Tocopherol	18.6±4.0^{a}	n.d.	26.7±0.8^{b}	50.5±12.9^{c}	n.d.	17.4±1.7^{a}
δ-Tocopherol	n.d.	n.d.	96.3±1.3^{a}	n.d.	n.d.	216.3±7.3^{b}
Total Tocopherols	18.6±4.1^{a}	n.d.	122.9±2.1^{b}	50.5±12.9^{c}	n.d.	233.7±5.6^{c}

n.d.: non detected

The different lower case letters in the same line indicate significantly different values ($p<0.05$).

In addiction their contents were significantly different in each cultivar studied. The 'Campa' peel showed more than 50 mg/g of oil of α-tocopherol, although the amount of α-tocopherol in 'Fino de Jete' fruit was 18.6 mg/g of oil. This compound has also been reported by Albuquerque *et al.* (in press) in other cherimoya cultivars.

It has been reported that α-tocopherol contributes up to the 80% of the total tocopherol found in prickly pear peel (Ramadan *et al.*, 2003) and it has also been detected in other fruit peels such as kiwi (Fiorentino *et al.*, 2009), banana (Villaverde *et al.*, 2013) and mango (Li *et al.*, 2014). In 'Fino de Jete' seeds this compound was quantified at 26.7 mg/g, while 'Campa' cherimoya seeds showed 17.4 mg/g of oil.

These values were higher than results reported by Luzia and Jorge (2012) for *Annona muricata* and *Annona squamosa*. At the same time, the α-tocopherol values in the analyzed samples were in the same order of magnitude as those reported by Mariod, Elkheir, Ahmed, and Matthaeus (2010) for *Annona squamosa* and by Luzia and Jorge (2013) for *Annona crossiflora* (Luzia & Jorge, 2012; Luzia & Jorge, 2013; Mariod *et al.*, 2010).

Two tocopherol compounds were detected in cherimoya seed oil. A high content of δ-tocopherol was found. It was particularly high in 'Campa', 216.3 mg/g of oil compared to 96.9 mg/g in 'Fino de Jete' cherimoya. A similar concentration of δ-tocopherol has previously been reported in apple seeds (Gornas *et al.*, 2014). In prickly pear seed oil the level of δ-tocopherol ranged between 30.9 mg/g and 500.0 mg/g (Matthaus & Ozcan, 2011). The lowest amount of δ-tocopherol was noted in *Annona crossiflora* (Luzia *et al.*, 2013) and *Annona muricata* (Luzia *et al.*, 2012).

The seeds of 'Fino de Jete' and 'Campa' cultivars also contained α-tocopherol and statistical differences were observed between the seeds of the two varieties.

3.3. Phytosterol and triterpenic alcohol analysis

Phytosterol values presented in the literature are expressed in mg/Kg, mg/100g of dry weight or lipid fraction (Vilela *et al.*, 2014; Villaverde *et al.*, 2013); in this study the data are indicated in mg/ g of oil.

A total of eight compounds were identified (Table 3). The sterol composition was different between the different parts of the fruit (seed, peel, and pulp).

Table 3. Phytosterols and triterpenic alcohols content (mg/g of oil) in peel, pulp and seeds of 'Fino de jete'and 'Campa'cultivars of cherimoya fruit. The different lower case letters in the same line indicate significantly different values (p<0.05).

Sterols	Cherimoya 'Fino de jete'			Cherimoya 'Campa'		
	Peel	Pulp	Seed	Peel	Pulp	Seed
Ergosterol	1.5±0.2^{b}	3.0±0.3^{c}	n.d.	5.6±0.4^{d}	0.8±0.1^{a}	n.d.
Campesterol	6.6±0.8^{b,c}	7.5±0.8^{c}	1.1±0.0^{a}	0.4±0.3^{a}	5.7±0.4^{b}	0.6±0.0^{a}
Stigmasterol	4.4±0.6^{b}	4.8±0.5^{b,c}	1.2±0.0^{a}	3.8±2.7^{c,d}	6.5±0.4^{d}	0.8±0.0^{a}
Stigmastanol	n.d.	n.d.	0.3±0.0^{a}	n.d.	n.d.	0.5±0.0^{b}
Gamma-sitosterol	6.2±0.6^{b}	14.2±1.8^{c}	n.d.	0.2±0.2^{a}	5.2±0.5^{b}	n.d.
Beta-sitosterol	20.4±2.4^{b}	18.0±2.7^{b}	5.0±0.1^{a}	21.6±0.9^{b}	19.3±1.3^{b}	2.7±0.2^{a}
Lanosterol	1.5±0.2^{c}	2.4±0.2^{d}	0.1±0.0^{a}	0.6±0.4^{b}	1.4±0.1^{c}	0.1±0.0^{a}
24-methylencycloartanol	n.d.	n.d.	0.1±0.0^{a}	n.d.	n.d.	0.2±0.0^{b}

n.d.: non detected

Briefly, peel and pulp contained ergosterol, campesterol, stigmasterol, β-sitosterol, lanosterol, and an unknown compound that eluted before the β-sitosterol. This compound reported a first fragment at 414 Da corresponding to the loss of a TMS group and representing $[M\text{-}TMS]^{+}$. Other important fragments were at 396 Da (corresponding to $[M\text{-}90]^{+}$), 381 Da (corresponding to $[M\text{-}90\text{-}15]^{+}$), 275 Da (corresponding to $[M\text{-}TMSOH\text{-}C9H13]^{+}$), 255 Da (corresponding to $[M\text{-}SC\text{-}90]^{+}$), 213 Da (corresponding to $[M\text{-}SC\text{-}D\text{-}90]^{+}$), and finally one at 129 Da derived from the loss of the C1-C3 moiety from ring A, favored by the β-double bond. This fragmentation pattern was reported by Pelillo *et al.* (2003) for sitosterol. Because of that, due to its fragmentation pattern and comparing its mass spectrum with spectra present in NIST library (Fig. 1), this compound was tentatively identified as gamma-sitosterol.

Cherimoya seeds reported the presence of campesterol, stigmasterol, stigmastanol, β-sitosterol, lanosterol, and 24-methylencycloartanol.

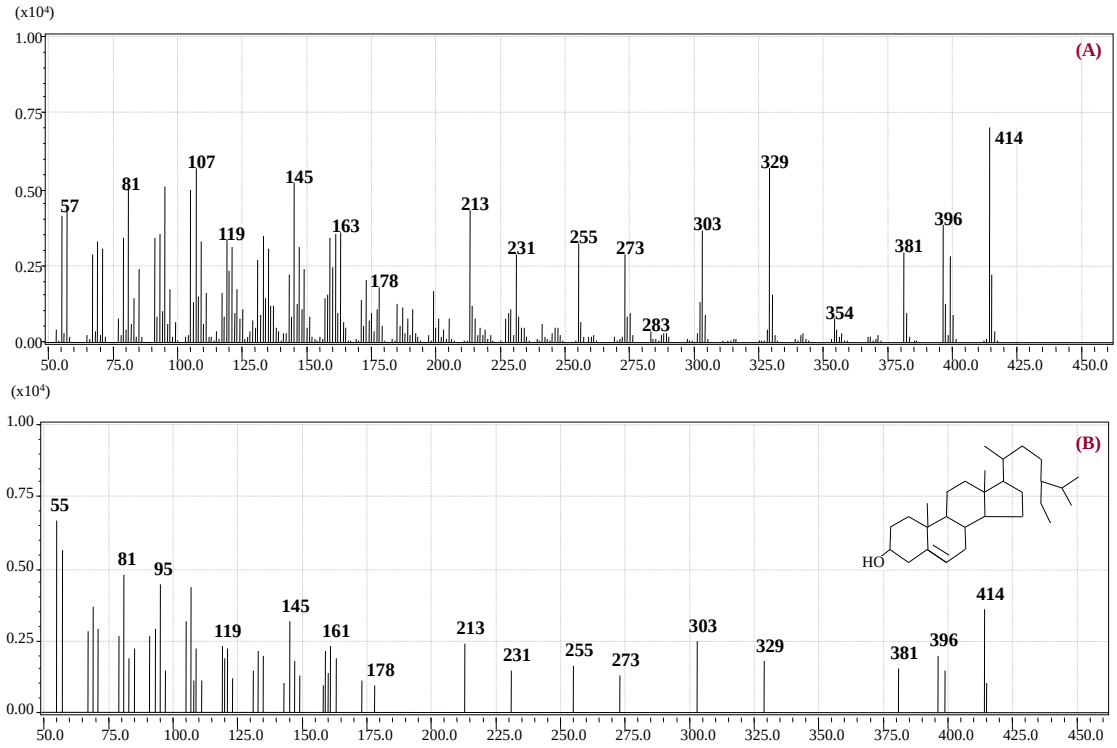

Fig. 1. Comparison of MS spectra of g-sitosterol obtained in samples (A) and g-sitosterol obtained by NIST Library (B).

The content of phytosterols and triterpenic alcohols are summarized in Table 3

β-sitosterol was the most characteristic sterol in peel and pulp. No statistical differences were noticed between the two cultivars. 'Fino de Jete' peel contained a concentration of 20.4 mg/g of β-sitosterol in the extracted oil, while in 'Campa' peel it accounted for 21.6 mg/g β-sitosterol was also identified previously in peel from banana (Villaverde *et al.*, 2013), and a similar concentration has been reported in prickly pear peel (Ramadan el al., 2003). The β-sitosterol content in pulp of both cultivars was similar, resulted in 18.0 mg/g and 19.3 mg/g for 'Fino de Jete' and 'Campa' respectively.

The amounts of β-sitosterol in the different pulps were of the same order of magnitude as the content of the same sterol in pulp from different cultivars of banana (Vilela *et al.*, 2014). Higher concentrations were found in mango pulp (Vilela *et al.*, 2013).

Other important phytosterols present in cherimoya peel and pulp and particularly in 'Fino de Jete' cherimoya, were γ-sitosterol and campesterol. For these compounds, the richest part of the fruit was the pulp and significant differences were observed between the different samples. The concentration of γ-sitosterol was 14.2 mg/g for 'Fino de Jete' pulp and 6.2 mg/g for 'Fino de Jete' peel. The cherimoya 'Campa' presented 5.2 mg/g and 0.2 mg/g of γ-sitosterol in the pulp and peel, respectively. These differences should be investigated in the future to evaluate if this compound can be used as a potential marker to distinguish between cherimoya cultivars.

Statistical differences were noticed with regards to the campesterol content in peel and pulp; in fact, 'Fino de Jete' peel and pulp showed the highest content compared to 'Campa' peel and pulp, respectively. The campesterol content of 'Campa' peel was similar to that reported by Ramadan *et al.* (2003) in prickly pear. 'Campa' pulp showed 5.7 mg/g of campesterol, a similar concentration similar to that in the 'Eilon' cultivar of banana pulp (Vilela *et al.*, 2014). The amount of campesterol in 'Fino de Jete' was 7.5 mg/g in pulp and 6.6 mg/g in peel. A similar concentrations of campesterol was previously reported by Vilela *et al.* (2013) in different cultivars of mango pulp. The data about campesterol content in banana pulp was lower than in 'Fino de Jete' pulp (Vilela *et al.*, 2014). However, Villaverde *et al.* (2013) found a similar content of campesterol in banana peel.

Stigmasterol was also identified, showing a statistically higher concentrations in 'Campa' pulp and peel compared to the pulp and peel, respectively, of 'Fino de Jete' cultivar. The stigmasterol pulp content was in the range of values obtained earlier in pulp from several cultivars of mango and banana (Vilela *et al.*, 2013, 2014). Stigmasterol in 'Fino de Jete' and 'Campa' peel was 4.4-3.8 mg/g, respectively. These levels were lower than those provided by Villaverde *et al.* (2013) in banana peel; however, cherimoya peel had a stigmasterol concentration higher than that in peel of *Opuntia focus indica* (Ramadan *et al.*, 2003).

In ‘Campa’ peel a major quantity of ergosterol was found 5.6 mg/g, while ‘Fino de Jete’ peel presented less amount, 1.5 mg/g. The ergosterol content in ‘Fino de Jete’ pulp was 3.0 mg/g, while it was 0.8 mg/g in ‘Campa’ pulp. Ergosterol has also been detected in prickly pear peel but in only small quantities (Ramadan *et al.*, 2003).

Lanosterol in the cultivars ranged from 1.4 to 2.4 mg/g in pulp and 0.6-1.5 mg/g in peel. The amount of lanosterol obtained in peel was similar to the content found in prickly pear peel (Ramadan *et al.*, 2003).

The sterol content in the lipid fraction of the seeds was lower than in other parts of the fruit. Moreover, according to the statistical analysis, important differences between the cultivars were not observed for any compounds, except for stigmastanol and 24-methylencycloartanol.

β-sitosterol was the most important sterol in the seeds, amounting to 5.0 mg/g in oil from ‘Fino de Jete’ and 2.7 mg/g in ‘Campa’ cherimoya seeds. A similar β-sitosterol content was previously reported in hazelnut oil (Ciemniewska-Zytkiewicz *et al.*, 2015). This compound has also been detected in oil from *Annona crassiflora* and apple seeds but in minor concentrations (Bada *et al.*, 2014; Luzia *et al.*, 2013). Campesterol and stigmasterol were quantified in ‘Fino de Jete’ at concentrations of 1.1 mg/g and 1.2 mg/ g respectively. However, ‘Campa’ presented 0.6 mg/g of campesterol and 0.8 mg/g of stigmasterol. Campesterol and stigmasterol have been found previously in fruit of the genus *Annona* (Luzia *et al.*, 2013). Lanosterol was also identified in *Annona cherimola* seeds, its content ranged between 0.06 and 0.1 mg/g 24-methylencycloartanol was one of the minor compound in the range of 0.1-0.2 mg/g.

3.4. Phospholipids composition

The phospholipids (PL) composition is shown in Table 4.

Three phospholipids, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC) were detected in the different *Annona cherimola* samples. The results denoted significant differences in the PL content between the cultivars.

Table 4. Phospholipids content (mg/g) of oil in peel, pulp and seeds of 'Fino de jete'and 'Campa'cultivars of cherimoya fruit.

Phospholipids	Cherimoya 'Fino de jete'			Cherimoya 'Campa'		
	Peel	Pulp	Seed	Peel	Pulp	Seed
PE	57.3±0.0^{e}	94.9±0.6^{f}	7.4±0.2^{b}	33.5±2.0^{d}	13.7±0.2^{c}	1.6±0.1^{a}
PI	37.7±0.6^{f}	17.6±0.3^{d}	4.2±0.0^{b}	24.5±0.7^{e}	10.6±0.0^{c}	1.0±0.0^{a}
PC	47.4±0.4^{e}	27.9±2.0^{c}	10.2±0.4^{b}	42.6±0.6^{d}	11.5±0.0^{b}	2.0±0.0^{a}

With regard to peel and pulp, the lipid fraction of the 'Fino de Jete' cultivar was richer in phospholipids than the 'Campa' cultivar. In particular, PE accounted for 94.8 mg/g and 57.2 mg/g in pulp and peel oil, respectively. 'Campa' peel and pulp oil contained PE at 33.5 mg/g and 13.7 mg/g, respectively. PE has been previously characterized in the edible portion of fig fruit (Pande & Akoh, 2010) and in prickly pear peel (Ramadan *et al.*, 2003).

Another abundant phospholipid was PC; the highest amount of PC was found in 'Fino de Jete' peel at a level of 47.4 mg/g of oil, and in 'Campa' peel at 42.6 mg/g. PC has been identified in other fruit peelsin smaller amounts (Ramadan *et al.*, 2003). PC in pulp comprised between 11.5 and 27.9 mg/g in both cultivars. Pande *et al.* (2010) has reported the presence of this compound in several fruits.

PI counted for more than 37 mg/g in 'Fino de Jete' peel, and more than 24 mg/g in 'Campa' peel, while its level in the pulp of two cultivars did not exceeded 18

mg/g. This phospholipid has been previously identified in prickly pear peel, in small quantities (Ramadan *et al.*, 2003).

As reported for the peel and pulp, the phospholipid content in the lipid extract from the seeds was less in the 'Campa' cultivar compared to 'Fino de Jete'. PC was the principal phospholipid in both samples, showing 10.2 mg/g in 'Fino de Jete' cherimoya. A similar result was reported in an earlier investigation by Ciemniewska-Zytkiewicz *et al.* (2015) in hazelnuts. The PC content in 'Campa' seeds was less than in the 'Fino de Jete' seeds, accounting for 2.0 mg/g of oil. The literature reports that PC has also been identified in loquat, mayhaw, and pawpaw seeds (Pande *et al.*, 2010). PE was quantified in 'Fino de Jete' seeds, accounting at a level of 7.4 mg/g, although in 'Campa' amounted to 1.6 mg/g. This compound has been found previously in other fruits' seeds and nuts (Ciemniewska-Zytkiewicz *et al.*, 2015; Pande *et al.*, 2010).

The concentration range for PI was 4.2-1.0 mg/g in 'Fino de Jete' and 'Campa' cultivar, respectively. The result obtained for the 'Campa' cultivar was similar to those reported in the literature for hazelnut oil (Pande *et al.*, 2010).

Table 5 reports the HPLC/ESI-MS data on phospholipid fatty acids present in the samples.

Table 5. Molecular species of phospholipids content (% of total area) in peel, pulp and seeds of 'Fino de jete'and 'Campa'cultivars of cherimoya fruit.

Selected Ion	*m/z*	Tentative identification	Cherimoya 'Fino de jete'			Cherimoya 'Campa'		
			Peel	Pulp	Seed	Peel	Pulp	Seed
PE[M-H]$^-$	712.5	C16:0/C18:3	n.d.	1.86	n.d.	n.d.	0.28	n.d.
	714.5	C16:0/C18:2; C16:0/C18:1	39.63	43.97	25.68	40.78	46.39	13.72
	716.5	C16:0/C18:1	4.16	3.36	13.94	2.76	3.29	23.60
	736.5	C18:2/C18:3	15.10	15.87	1.97	16.71	12.66	1.04
	738.5	C18:2/C18:2; C18:1/C18:3	30.26	26.42	21.77	28.69	22.81	12.22
	740.5	C18:1/C18:2; C18:0/C18:3	7.89	5.49	26.37	6.46	8.54	27.42
	742.5	C18:1/C18:1	1.48	2.20	8.29	3.52	4.77	15.87
	744.5	C18:0/C18:1	0.77	0.82	1.98	0.87	1.26	6.13
	766.3	C20:2/C18:2	0.71	n.d.	n.d.	0.20	n.d.	n.d.
PI[M-H]$^-$	831.5	C16:0/C18:3	42.76	46.01	24.07	18.67	51.99	12.84
	833.5	C16:0/C18:2	28.01	40.72	12.36	37.11	39.39	30.47
	835.5	C16:0/C18:1	27.34	12.52	46.07	41.15	8.25	31.85
	837.5	C16:0/C18:0	n.d.	n.d.	15.35	n.d.	n.d.	24.25
	857.5	C18:2/C18:2; C18:1/C18:3	1.59	0.75	0.30	3.07	0.37	0.05
	859.8	C18:1/C18:2; C18:1/C18:3	0.30	n.d.	0.76	n.d.	n.d.	0.28
	861.5	C18:0/C18:2; C18:1/C18:1	n.d.	n.d.	0.76	n.d.	n.d.	0.21
	863.5	C18:0/C18:1; C16:0/C20:1	n.d.	n.d.	0.33	n.d.	n.d.	0.04
PC[M-H]$^-$	802.5	C16:0/C18:2	51.39	63.18	34.94	64.98	30.95	23.89
	804.5	C16:0/C18:1	48.61	32.82	26.20	35.02	56.91	33.03
	806.5	C16:0/C18:0	n.d.	4.00	38.86	n.d.	12.14	43.08

The ESI-MS spectrum of PE in negative ion mode revealed eight different molecular species for peel and pulp and seven species for seeds. In cherimoya peel and pulp the main molecular species at *m/ z* 714, 738 and 736, accounted for more than 85% of the total PE molecular species. However, in seed samples the most relevant molecular species were at *m/z* 714, 740, 738 and 716. The percentage obtained for these species was higher than 80%. The most

representative fatty acids in these species were C16:0, C18:0, C18:1, C18:2, and C18:3.

Eight molecular species were detected in negative ion mode for PI in *Annona cherimola* seeds, with those at *m*/*z* 835, 831, 833, and 837 representing more than 95% of the total PI species. The molecular species detected in the other parts of the fruit included five in 'Fino de Jete' and four in'Campa', with the principal *m*/*z* at 831 and 833. C16:0, C18:0, C18:1, C18:2, and C18:3 were the most representative fatty acids in this phospholipid class.

The ESI-MS spectrum of PC in negative ion mode revealed three molecular ion clusters at *m*/*z* 802, 804, and 806 in pulp and seeds. In pulp from the 'Fino de Jete' cultivar the main specie was at 802, nevertheless in the 'Campa' sample it was at 804, the percentages being 63.2 and 56.9% respectively. In peel only 802 and 804 were found. The fatty acids C16:0, C18:0, C18:1, and C18:2 were predominant.

The majority fatty acids of the phospholipids are in accordance with those reported by Gutiérrez *et al.* (2005) in cherimoya mesocarp. The molecular species founded in cherimoya pulp and seeds were also detected by Pacetti, Boselli, Lucci, and Frega (2007) in pulp and seeds of avocado samples.

4. Conclusions

This work presented, for the first time, an extensive characterization of the lipid composition in cherimoya fruit. The results obtained confirmed that the content of lipid compounds (i.e. fatty acids, sterols, tocopherols, and phospholipids) depend on the cultivar. Within the same cultivar, it also varied in different parts of the fruit. Appreciable differences were detected in pulp, peel, and seed fatty acid composition; the seed contained *cis*-9-C18:1, C18:2n-6, and C16:0 as principal fatty acids respectively. Peel and pulp contained a higher content of C18:2n-6 fatty acid. Regarding tocopherol composition, α-tocopherol and d-tocopherol were identified only in seeds; moreover α-tocopherol was also

detected in skin. No tocopherols were quantified in pulp. Differences in sterol composition were noted in seeds, pulp, and peel of cherimoya fruit. These preliminary results suggest that g-sitosterol could be used as a marker for the 'Fino de Jete' cultivar, due to the high content found in this variety; however, further investigations are needed.

Finally, three different phospholipids, PE, PC, and PI, were identified and their molecular species were studied by HPLC-ESI-MS.

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CAPÍTULO 8

Pomegranate seeds as a source of nutraceutical oil naturally rich in bioactive lipid

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Abstract

Pomegranate seed oil is constituted primarily of the rare conjugate linolenic acid (punicic acid), whose activities against inflammation and metabolic syndrome have been confirmed in various *in vivo* experiments. Because of this, the lipid composition of pomegranate seeds, representing one of the principal by-products of the pomegranate juice industry, was analyzed. After juice production, the lipolytic enzymes present in the pomegranate seeds were inactivated by thermal treatment. Total lipid content of the pomegranate seeds varied between 7.9 and 16 %. The pomegranate seed oil contained large amounts of conjugated linolenic acids (CLnA), such as punicic acid, in the range of 74-85 % of the total fatty acid content. Other major fatty acids were oleic, linoleic and palmitic acid, respectively. Pomegranate seeds also showed high contents of tocopherols; one of them, γ-tocopherol, was 87.9-95.0 % of the total tocopherols. Moreover, two tocotrienols were identified for the first time in pomegranate seed oil. The major phytosterols analyzed were campesterol, stigmasterol, sitosterol, Δ5-avenasterol and citrostadienol. Furthermore, some triterpene compounds such as cycloartenol, 24-methylene-cycloartenol and betulinol and squalene were quantified. Three different phospholipids were detected;

phosphatidylethanolamine was the principle compound, accounting for 56-86 % of total phospholipids.

Briefly, these data promote pomegranate seeds as rich sources of bioactive lipids with potential beneficial physiological activities and encourage their use in human nutrition.

Keywords: pomegranate seed, lipid composition, punicic acid, sterols, phospholipids, tocopherols

1. Introduction

The pomegranate is native to the Himalayas, from northern India to Iran, but has been cultivated and naturalized since ancient times over the entire Mediterranean region. It is popularly consumed as a fresh fruit, beverages, food products (jams and jellies), and extracts wherein they are used as botanical ingredients in herbal medicines and dietary supplements (Goula & Adamopoulos, 2012; Jurenka, 2008). Pomegranate seeds are the by-products obtained during some pomegranate processing. The oil contained in pomegranate seeds (PSO) consists of 65-80 % conjugated fatty acids, the most important of which is 9-cis, 11-trans, 13-cis, octadecatrienoic acid, so-called punicic acid (Abbasi, Rezaei, & Rashidi, 2008; Eikani, Golmohammad, & Homami, 2012). Punicic acid is a conjugated linolenic acid (CLnA). Yamasaki et al. (2006) reported that dietary CLnA modulated lipid metabolism and promoted Ig production by mouse splenocytes. In fact, several authors (Al-Muammar & Khan, 2012; McFarlin, Strohacker, & Kueht, 2009; Vroegrijk et al., 2011) explored the effects of PSO on obesity; its supplementation in mice resulted in decreases in total weight gain, leptin and insulin, and an increase in plasma adiponectin concentration. Moreover, dietary PSO rich in 9-cis, 11-trans, 13-cis-CLnA alleviate hepatic triacylglycerol accumulation in obese hyperlipidemic rats (Arao *et al.*, 2004).

Anti-inflammatory properties of PSO were also noticed by Coursodon-Boyiddle *et al.* (2012); they demonstrated that the administration of PSO protects against necrotizing enterocolitis in a neonatal rat model. This protective effect is associated with improved intestinal epithelial homeostasis and a strong anti-inflammatory effect of PSO on the developing intestinal mucosa. Moreover, the anti-inflammatory properties of PSO were confirmed by Boussetta *et al.* (2009), who investigated the regulation of neutrophil activation by PSO in inflammatory diseases. Their data showed that punicic acid exerts a potent anti-inflammatory effect, inhibiting TNFa-induced priming of NADPH oxidase by targeting the p38MAPKinase/Ser345-p47phox-axis and myeloperoxidase (MPO) release. Because of this, they suggested PSO as a novel alternative therapeutic strategy in

inflammatory diseases such as inflammatory bowel diseases. In addition, other authors (Spilmont *et al.*, 2013) demonstrated that PSO consumption (5 % of the diet) significantly improved bone mineral density and prevented trabecular microarchitecture impairment in ovariectomized (OVX) mice C57BL/6 J.

Finally, Gasmi and Sanderson (2010) investigated the growth inhibitory, antiandrogenic, and pro-apoptotic effects of some compounds found in the pomegranate, among which was punicic acid, in androgen-dependent LNCaP human prostate cancer cells; the results obtained con- firmed its potent growth inhibitory activities in androgen-dependent LNCaP cells, which appeared to be mediated by both antiandrogenic and pro-apoptotic mechanisms.

Due to its nutritional and medicinal properties, in recent years PSO has also been investigated as a functional ingredient in the food industry (Çam, Erdogan, Aslan, & Dinç, 2013; Jurenka, 2008; Mohagheghi, Rezaei, Labbafi, & Ebrahimzadeh Mousavi, 2011); particular attention has been given to its fatty acid composition; however, the literature is lacking in information about the other lipidic components present in PSO. Because of that, the aim of this work was to determine the lipidic composition (fatty acids, sterols, tocopherols and phospholipids) of the oil in seed by-products from different pomegranate cultivars recovered after juice production.

2. Materials and methods

2.1. Samples

Seeds were obtained from fruits several the pomegranate varieties after juice production. Wonderful1 and Wonderful, Akko and Hershkovitz were imported from Israel, Mollar(1), Mollar(2) and Valenciana were from Spain, Hijaz was from Turkey, Shiraz was from Iran, and G(1) and G(2) were imported from Tunisia; all of these samples were collected during the second and third week of September. Dente di Cavallo was provided by the Department of Scienze Agro

Ambientali e Territoriali of the University of Bari and collected in the second week of October. Fruits of other clones were harvested from non-commercial trees and identified with experimental nicknames: Ecotipo(1) and Ecotipo(2), harvested near Naples (approx 40° 40′N, 14° 45′E); Ravenna, Radisa and Veneti, harvested near Ravenna (44° 25′N, 12° 12′E). Clone fruits were collected in the second week of September.

The seeds obtained after juice production were wet seeds that contained aril peel (the moisture ranged between 58 and 76 %). Because of that, to dry the seeds, to eliminate the peel and inactivate lipolytic enzymes, the seeds were roasted in a hot air oven at 155 °C for 20 min as reported by Ruge, Changzhong, and Zaigui (2012). The seeds were stored at -18 °C until use.

2.2. Chemicals

All solvents and reagents were purchased from Merck (Darmstadt, Germany). The following standards were supplied by Sigma-Aldrich (Saint Louis, MO, USA): L-α-phosphatidylethanolamine (PE), L-α- phosphatidylcholine (PC), L-α-phosphatidylinositol (PI), α, γ, δ- tocopherol, dihydrocholesterol, and punicic acid. GLC-463 mix for fatty acids was from Nu-Check (Elysian, MN, USA).

2.3. Lipid extraction

PSO was extracted using the procedure described by Boselli, Velazco, Caboni, and Lercker (2001). Approximately 20 g of sample was homogenized with 100 mL of a chloroform/methanol solution (1/1, v/v) in a glass bottle with a screw cap. The bottle was kept at 60 °C for 20 min before adding an additional 100 mL of chloroform. After 3 min of homogenization, the contents of the mixture were filtered through filter paper. The filtrate was then mixed thoroughly with 70 mL of 1 M KCl solution and left overnight at 4 °C to allow phase separation. The organic phase was collected and dried in a rotary evaporator at 40 °C, dissolved

in 5 mL n-hexane/isopropanol solution (4/1, v/v) and stored at -18 °C until it was analyzed.

2.4. Total fatty acid composition analysis

The fatty acid composition of PSO samples was determined from the oil as fatty acid methyl esters (FAMEs) by capillary gas chromatography analysis after alkaline treatment as described by Christie (1982).

Fatty acids were transmethylated and the FAMEs were analyzed by capillary gas chromatography (CGC) using a fused silica capillary column BPX70 (10 m × 0.1 mm i.d., 0.2 μm f.t.) from SGE Analytical Science (VIC, Australia). The column was fitted on a GC-2010 Plus gas chromatograph from Shimadzu (Shimadzu Corp., Tokyo, Japan).

The chromatographic conditions were the same as reported by Verardo, Gómez-Caravaca, Gori, Losi, and Caboni (2013) with some modifications as follows: the sample volume injection was 0.2 μL with a split ratio set at 1:400.

2.5. Tocol analysis

Approximately 50 mg of PSO was dissolved in 1 mL of n-hexane. The solution was filtered through a 0.45 μm nylon filter. The tocopherols were determined by HPLC (Agilent 1200 series, Palo Alto, CA, USA) equipped with a fluorimeter detector (Agilent, Palo Alto, CA, USA). The excitation wavelength was 290 nm and the emission wavelength was 325 nm. The column used was a Luna Hilic Phenomenex column (250 mm × 4.6 mm i.d., 5 μm particle size) under isocratic conditions as reported by Gómez-Caravaca, Verardo, and Caboni (2010).A calibration curve was constructed with α-tocopherol standard solution and used for quantification.

2.6. Phytosterol and triterpene analysis

To determine phytosterols and terpene compounds, 200 µL of dihydrocholesterol (c = 2.0 mg/mL) was added to 200 mg of PSO and saponification was conducted at room temperature (Iafelice, Verardo, Marconi, & Caboni, 2009). After saponification, the organic fraction was washed with 10 mL diethyl ether/water (1/1 v/v), and the unsaponifiable matter was further extracted in the sequence: two times with 10 mL diethyl ether, washed two times with 10 mL 0.5 N aqueous KOH and again two times with 10 mL distilled water. The diethyl ether solvent was removed under vacuum and the unsaponifiable matter was used for the phytosterol and terpene determination.

The unsaponifiable matter was silylated according to Sweeley, Bentley, Makita, and Wells (1963) and analyzed using a GC/MS (GCMS-QP2010 Plus, Shimadzu, Tokyo, Japan) with a Restek RTX-5 (10 m × 0.1 mm, i.d. 0.1 µm film thickness; Bellafonte, PA, USA) under the chromatographic conditions reported by Cardenia, Rodriguez-Estrada, Baldacci, Savioli, and Lercker (2012).

Data were filed and processed by the software GCMS solution version 2.50 SU1 from Shimadzu. Phytosterol identification was achieved by comparing peak mass spectra with peaks in a standard mixture and with GC-MS data reported by Pelillo, Iafelice, Marconi, and Caboni (2003). Quantification of identified phytosterols and terpene compounds was performed in relation to dihydrocholesterol used as internal standard.

2.7. Phospholipid determination

To determine the phospholipids in PSO, approximately 100 mg of PSO was weighed and dissolved in 1.5 mL of 88/12 (v/v) chloroform/methanol and used for the HPLC analysis.

The quantitation of the phospholipid classes was performed using HPLC-ELSD. The chromatographic method used by Verardo *et al.* (2013) to separate polar

lipids was carried out. Phospholipid separation was performed on an Agilent liquid chromatography HP 1200 Series (Agilent Technologies, Palo Alto, CA, USA). The detector was an evaporative light scattering detector (ELSD; PL-ELS1000, Polymer Laboratories, Church Stretton, Shropshire, UK). The control of the HPLC system was accomplished by the software Agilent ChemStation (Agilent Technologies, Santa Clara, CA, USA) while chromatogram registration and data processing were assessed by ClarityLite (version 2.4.0.190, DataApex, Praha, The Czech Republic). The separation was achieved using a silica column, 150 mm × 3 mm with a 3 μm particle diameter (Phenomenex, Torrance, CA, USA). The calibration curves were prepared separately for each phospholipid identified.

2.8. Determination of phospholipid fatty acids

About 20 mg of PSO was dried under nitrogen, dissolved in 0.5 mL of chloroform and loaded on a 20 × 20 cm Silica gel 60 TLC plate (Merck KGaA, Darmstadt, Germany). The mobile phase was 100 mL of a n-hexane/diethyl ether mixture, 3/2 (v/v). The phospholipid band was visualized under UV light (254 nm) by spraying the TLC plate with a 0.02 % (m/v) ethanolic solution of 2,7-dichlorofluorescein (sodium salt) and then scraped off and collected. Phospholipids were extracted three times with chloroform (3 × 1 mL). The organic extracts were pooled, dried under nitrogen and phospholipid fatty acids determined as reported for total fatty acids.

2.9. Statistical analysis

Tukey's honest significant difference multiple comparison (one-way ANOVA), $p < 0.05$ level, was evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Oil content and fatty acid composition

The oil content in pomegranate seeds varied from 7.6 to 16.2 % (Table 1). These data are in agreement with results obtained by Fadavi, Barzegar, and Hossein Azizi (2006). In particular, Akko showed the highest oil content; seeds from Valenciana, Wonderful and Hershkovitz contained about 13 % oil; in Hijaz, Radisa, and G(1) the seed oil content was determined to be about 11 %; and the oil content in Ravenna, Veneti, Mollar(1), Mollar(2), Wonderful1, G(2) and Ecotipo(1) was about 10 %, B while the other cultivars all reported oil contents less than 10 %.

The fatty acid compositions of the seventeen pomegranate seed oils are shown in Table 1. No qualitative differences in fatty acid composition were observed among the extracted oil samples.

According to the literature (Jing *et al.*, 2012) total saturated fatty acids (SFA) ranged between 4.9 and 7.0 % of total fatty acids, mono-unsaturated fatty acids (MUFA) were in the range of 5.7-9.8 % and polyunsaturated fatty acids (PUFA) were the principal fatty acid class, comprising between 84.0 and 89.3 % of total fatty acids.

Akko, Wonderful1, G(2) and Ecotipo(2) reported the highest saturated/unsaturated ratios (SFA/UFA), similar to those reported by Fadavi et al. (2006) and Melgarejo and Artés (2000), while Akko, Shiraz, Wonderful1, G(2) and Ecotipo(1) showed the highest MUFA/PUFA ratios. These data underline that these cultivars have the lower degree of unsaturation.

According to the literature (Fadavi *et al.*, 2006; Garima & Akoh, 2009) the major fatty acid is punicic acid (C18:3 c9, t11, c13), in the range of 72.4-84.1 % of total fatty acids. Radisa, Hershkovitz, Valenciana, Ravenna, Veneti, Hijaz, Dente di Cavallo, Mollar(1), Mollar(2), Wonderful and G(1) samples all contained punicic

acid contents higher than 80 %. This content is very important because punicic acid is the principal CLnA contained in pomegranate seeds that promotes the potential biological and health effects of PSO (Özgül-Yücel, 2005). The second and third most common fatty acids in pomegranate oils were linoleic (C18:2 n-6) and oleic (C18:1 c9) acid, which were in the ranges 4.1-11.3 and 3.6-7.1 %, respectively.

Table 1. Oil and fatty acid content (g/100 g of oil) in the analyzed pomegranate seed oils. The different lower case letters in the same line indicate significantly different values ($p < 0.05$)

	Akko	Radisa	Hershkovitz	Valenciana	Ravenna	Veneti	Hijaz	Shiraz	Dente di Cavallo	Mollar (1)	Mollar (2)	Wonderful 1	Wonderful	G (1)	G (2)	Ecotipo (1)	Ecotipo (2)
Oil content	16.21^{a}	10.84^{c}	12.88^{b}	13.08^{b}	9.75^{d}	9.91^{d}	11.0^{c}	9.17^{d}	7.56^{e}	10.1^{c}	9.82^{d}	10.08^{c}	12.96^{b}	11.8^{c}	10.2^{d}	10.02^{d}	7.88 ^{e}
Myristic acid	0.03^{a}	0.03^{a}	0.02^{b}	0.03^{a}	0.02^{b}	0.03^{a}	0.02^{b}	0.02^{b}	0.02 ^{b}	0.03^{a}	0.03^{a}	0.03^{a}	0.02^{b}	0.02^{b}	0.03^{a}	0.02^{b}	0.03^{a}
Palmitic acid	3.52^{b}	2.81^{d}	2.68^{d}	2.89^{d}	2.98^{d}	3.06^{c}	2.72^{d}	3.18^{c}	3.19^{c}	3.62^{b}	3.33^{c}	3.87^{b}	2.53^{d}	3.02^{c}	3.75^{b}	3.47^{b}	4.28^{a}
Palmitoleic acid	0.03^{b}	0.04^{a}	0.03^{b}	0.02^{c}	0.02^{c}	0.02^{c}	0.03^{b}	0.02^{c}	0.02^{c}	0.02^{c}	0.02^{c}	0.03^{b}	0.02^{c}	0.02^{c}	0.02^{c}	0.04^{a}	0.04^{a}
Margaric acid	0.06^{b}	0.05^{b}	0.05^{b}	0.05^{b}	0.06^{b}	0.06^{b}	0.05^{b}	0.06^{b}	0.07^{a}	0.06^{b}	0.05^{b}	0.06^{b}	0.05^{b}	0.06^{b}	0.07^{a}	0.06^{b}	0.08^{a}
Stearic acid	1.74^{c}	1.92^{a}	1.51^{d}	1.44^{d}	1.82^{b}	1.87^{b}	1.57^{d}	1.83^{b}	1.82^{b}	1.63^{c}	1.60^{c}	1.53^{d}	1.70^{c}	1.54^{d}	1.65^{c}	1.71^{c}	1.78^{b}
Oleic acid	6.63^{b}	4.62^{d}	4.88^{d}	3.63^{e}	4.23^{e}	3.81^{e}	4.40^{e}	6.17^{b}	4.76^{d}	4.17^{e}	3.74^{e}	7.12^{a}	4.09^{e}	3.94^{e}	5.82^{c}	6.82^{b}	4.82^{d}
cis-Vaccenic acid	0.56^{a,b}	0.36^{d}	0.46^{c}	0.57^{a,b}	0.39^{d}	0.43^{c}	0.52^{b}	0.49^{c}	0.47^{c}	0.63^{a}	0.55^{b}	0.50^{b}	0.38^{d}	0.59^{a}	0.67^{a}	0.46^{c}	0.65^{a}
Linoleic acid	7.37^{c}	5.90^{e}	4.71^{f}	4.11^{f}	5.91^{e}	5.49^{e}	4.85^{f}	6.35^{d}	6.49^{d}	6.24^{d}	5.21^{e}	11.32^{a}	4.39^{f}	4.66^{f}	7.28^{c}	9.02^{b}	9.30^{b}
Linolelaidic acid	0.05^{a}	0.03^{c}	0.03^{c}	0.03^{c}	0.02^{c}	0.03^{c}	0.04^{b}	0.03^{c}	0.03^{c}	0.03^{c}	0.03^{c}	0.03^{c}	0.03^{c}	0.04^{b}	0.05^{a}	0.03^{c}	0.03^{c}
Linolenic acid	0.11^{d}	0.17^{c}	0.05^{e}	0.09^{d}	0.09^{d}	0.10^{d}	0.08^{d,e}	0.10^{d}	0.14^{c}	0.18^{c}	0.16^{c}	0.38^{a}	0.07^{e}	0.06^{e}	0.11^{d}	0.16^{c}	0.22^{b}
Arachidic acid	0.55^{a}	0.44^{b}	0.44^{b}	0.38^{b}	0.48^{a,b}	0.41^{b}	0.52^{a}	0.41^{b}	0.44^{b}	0.46^{b}	0.42^{b}	0.50^{a}	0.41^{b}	0.37^{b}	0.53^{a}	0.44^{b}	0.53^{a}
Gondoic acid	1.07^{a}	0.60^{c}	0.74^{b}	0.44^{d}	0.56^{c}	0.44^{d}	0.94^{a}	0.76^{b}	0.58^{c}	0.49^{d}	0.44^{d}	0.55^{c}	0.61^{c}	0.56^{c}	0.62^{c}	0.53^{c}	0.41^{d}
Behenic acid	0.16^{b}	0.13^{c}	0.10^{c}	0.14^{c}	0.16^{b}	0.16^{b}	0.14^{c}	0.13^{c}	0.17^{b}	0.20^{a}	0.18^{b}	0.19^{b}	0.11^{c}	0.12^{c}	0.22^{a}	0.17^{b}	0.25^{a}
Punicic acid	75.41^{f}	80.92^{c}	82.34^{b}	84.11^{a}	81.30^{c}	82.42^{b}	81.70^{c}	78.69^{e}	80.00^{d}	80.25^{d}	82.33^{b}	72.42^{g}	84.09^{a}	82.86^{b}	77.13^{e}	75.00^{f}	75.46^{f}
Eicosapentaenoic acid	0.71^{a}	0.61^{b}	0.60^{b}	0.68^{a}	0.73^{a}	0.56^{b}	0.69^{a}	0.59^{b}	0.67^{a}	0.62^{b}	0.59^{b}	0.51^{c}	0.47^{c}	0.70^{a}	0.61^{b}	0.63^{b}	0.67^{a}
Erucic acid	1.54^{a}	1.17^{b}	0.99^{b}	1.09^{b}	0.99^{b}	0.87^{c}	1.14^{b}	0.89^{c}	0.87^{c}	1.02^{b}	0.98^{b}	0.71^{d}	0.76^{d}	1.07^{b}	1.03^{b}	1.15^{b}	1.16^{b}
Docosadienoic acid	0.37^{b}	0.14^{d}	0.29^{c}	0.24^{c}	0.19^{d}	0.19^{d}	0.49^{a}	0.21^{c}	0.20^{d}	0.29^{c}	0.27^{c}	0.17^{d}	0.24^{c}	0.33^{b}	0.33^{b}	0.18^{d}	0.21^{d}
Lignoceric acid	0.10^{a}	0.06^{c}	0.08^{b}	0.06^{c}	0.04^{d}	0.05^{c,d}	0.10^{a}	0.05^{c,d}	0.06^{c}	0.06^{c}	0.06^{c}	0.07^{b}	0.05^{c,d}	0.07^{b}	0.08^{b}	0.10^{a}	0.08^{b}
SFA	6.16^{b}	5.45^{b,c}	4.88^{c}	4.99^{c}	5.56^{b,c}	5.64^{b,c}	5.12^{c}	5.69^{b}	5.78^{b}	6.05^{b}	5.67^{b}	6.25^{b}	4.86^{c}	5.19^{c}	6.32^{b}	5.98^{b}	7.02^{a}
MUFA	9.83^{a}	6.78^{c}	7.09^{c}	5.75^{d}	6.19^{d}	5.57^{d}	7.03^{c}	8.33^{b}	6.70^{c}	6.34^{d}	5.73^{d}	8.92^{a}	5.86^{d}	6.17^{d}	8.17^{b}	8.99^{a}	7.08^{c}
PUFA	84.01^{d}	87.77^{b}	88.04^{a}	89.26^{a}	88.25^{a}	88.79^{a}	87.84^{b}	85.98^{c}	87.52^{b}	87.61^{b}	88.60^{a}	84.83^{c}	89.28^{a}	88.64^{a}	85.51^{c}	85.02^{c}	85.90^{c}
MUFA/PUFA	0.12^{a}	0.08^{b}	0.08^{b}	0.06^{c}	0.07^{c}	0.06^{c}	0.08^{b}	0.10^{a}	0.08^{b}	0.07^{c}	0.06^{c}	0.11^{a}	0.07^{c}	0.07^{c}	0.10^{a}	0.11^{a}	0.08^{b}
SFA/UFA	0.07^{a}	0.06^{b}	0.05^{c}	0.05^{c}	0.06^{b}	0.06^{b}	0.05^{c}	0.06^{b}	0.06^{b}	0.06^{b}	0.06^{b}	0.07^{a}	0.05^{c}	0.05^{c}	0.07^{a}	0.06^{b}	0.08^{a}

The fatty acid linoleic n6 was the highest in the Wonderful1 sample, which contained more than 11 %; higher amounts were also revealed in Akko, Shiraz, Dente di Cavallo, Mollar(1), G(2), Ecotipo(1) and Ecotipo(2), which showed more than 6 % of this fatty acid. According to Garima and Akoh (2009), and Jing et al. (2012), oleic acid was the most prevalent monounsaturated fatty acid. The oleic fatty acid content was higher than 5 % in the Akko, Shiraz, Wonderful1, G(2) and Ecotipo(1) samples. Moreover, erucic and gondoic fatty acids were also identified in the range of 0.7-1.5 and 0.4-1.1 %, respectively.

3.2. Tocol composition

Total tocol content ranged between 678.3 and 2627.4 μg/g of oil (Table 2). The tocopherol values determined in the PSO samples were variable among the different cultivars, but overall they were higher than the values recorded for nut oils (Miraliakbari & Shahidi, 2008).

The α-, γ- and δ-tocopherols were identified by co-elution with commercial standards; according to Garima and Akoh (2009), β- tocopherol was not detected.

The γ-tocopherol was observed in higher amounts in PSO, in the range of 616-2400 μg/g of oil in G(1) and Ecotipo(2) samples, respectively. Similar results were reported by Habibnia, Ghavami, Ansaripour, and Vosough (2012), while other authors reported either higher (Elfalleh *et al.*, 2011; Garima & Akoh, 2009) or lower (Caligiani, Bonzanini, Palla, Cirlini, & Bruni, 2010; Jing *et al.*, 2012) contents of γ-tocopherol. These data are very interesting because γ-tocopherol is more resistant to oxidation compared to other tocols and showed the highest antioxidant activity in model systems (Maguire, O'Sullivan, Galvin, O'Connor, & O'Brien, 2004). The amounts of γ-tocopherol in PSO were higher than that in walnut, which was the only nut that contained γ-tocopherol in higher concentrations than α-tocopherol (Maguire *et al.*, 2004). The γ-tocopherol PSO content was from two to eight times higher than in walnut oil and similar to or

higher than that the maize, soybean and *Cucurbita pepo* L. (Gemrot, Barouh, Vieu, Pioch, & Montet, 2006), representing 88-94.5 % of the total tocols in PSO. The α-tocopherol was the second most prevalent tocol, in the range of 25.4-169.0 μg/g of oil in G(1) and Ecotipo(2) samples, respectively. Its content was 3.3-8.4 % of total tocol content. Similar amounts were reported by Caligiani *et al.* (2010) for a Wonderful sample; however, other authors showed higher contents of α-tocopherol in PSO (Elfalleh *et al.*, 2011; Habibnia *et al.*, 2012; Jing *et al.*, 2012). This discrepancy may be due to different factors such as the genotypes, growing conditions, or harvesting period. Moreover, the other authors analyzed fresh seeds; on the contrary, this survey was carried out after a thermal treatment that was necessary to inactivate enzymes that can affect the lipidic and phenolic fraction. This heating process could have influenced the α-tocopherol content due its low oxidation stability.

The δ-tocopherol was also determined in PSO samples. It ranged between 0.5 and 2.2 % of total tocols. The highest amount was detected in the Radisa sample (35.6 μg/g of oil) and the lowest was shown by Wonderful PSO (7.8 μg/g of oil). As with α-tocopherol, the δ- tocopherol content was also lower than that reported by other authors in other pomegranate genotypes (Caligiani *et al.*, 2010; Elfalleh *et al.*, 2011; Garima & Akoh, 2009; Habibnia *et al.*, 2012; Jing *et al.*, 2012).

Table 2. Tocols content (µg/g of oil) in the analyzed pomegranate seed oils. The different lower case letters in the same column indicate significantly different values ($p < 0.05$)

Pomegranate cultivars	α-tocopherol	α-tocotrienol	γ-tocopherol	β-tocotrienol	δ-tocopherol	Total
Akko	$57.6 \pm 0.2^{f,g}$	20.8 ± 0.4^{b}	1714.0 ± 5.7^{b}	$15.1 \pm 0.2^{g,h}$	$15.3 \pm 0.3^{d-f}$	1822.7 ± 4.7^{b}
Radisa	$56.9 \pm 2.5^{f-h}$	$17.7 \pm 1.3^{b,c}$	$1447.4 \pm 42.3^{c,d}$	$33.9 \pm 1.1^{b-d}$	35.6 ± 0.8^{a}	$1591.5 \pm 48.0^{c,d}$
Hershkovitz	65.6 ± 1.9^{f}	$14.7 \pm 0.5^{c-e}$	1182.2 ± 15.5^{f}	24.6 ± 0.3^{f}	$19.1 \pm 1.2^{c,d}$	1306.3 ± 18.9^{h}
Valenciana	$51.8 \pm 0.2^{g,h}$	19.3 ± 0.1^{b}	$1337.8 \pm 26.9^{d,e}$	$33.5 \pm 0.1^{c-e}$	$16.2 \pm 0.0^{c-e}$	$1458.6 \pm 27.0^{d-g}$
Ravenna	$59.1 \pm 1.8^{f,g}$	$9.4 \pm 0.9^{f-h}$	1732.5 ± 30.0^{b}	$23.3 \pm 2.2^{f,g}$	$9.5 \pm 1.0^{g,h}$	1833.8 ± 35.9^{b}
Veneti	90.6 ± 2.2^{d}	27.6 ± 0.1^{a}	1507.9 ± 20.1^{c}	52.1 ± 0.9^{a}	20.1 ± 0.2^{c}	$1698.3 \pm 21.1^{b,c}$
Hijaz	104.9 ± 0.7^{c}	$9.1 \pm 0.5^{f-h}$	$1263.6 \pm 1.9^{e,f}$	$22.9 \pm 1.8^{f,g}$	$14.6 \pm 1.0^{d-f}$	$1415.2 \pm 5.9^{e-h}$
Shiraz	114.9 ± 0.6^{b}	$13.0 \pm 0.2^{d-f}$	$1394.5 \pm 19.8^{c-e}$	$25.1 \pm 0.7^{e,f}$	$12.9 \pm 0.2^{e-g}$	$1560.3 \pm 21.4^{c-e}$
Dente di Cavallo	122.5 ± 3.0^{b}	$8.8 \pm 0.4^{f-h}$	$1293.8 \pm 4.0^{e,f}$	$25.8 \pm 1.7^{d-f}$	$11.3 \pm 0.6^{f-h}$	$1462.2 \pm 1.7^{d-g}$
Mollar (1)	48.2 ± 1.0^{h}	$16.5 \pm 1.0^{b-d}$	1176.3 ± 54.2^{f}	$25.3 \pm 1.8^{d-f}$	$15.1 \pm 1.0^{d-f}$	1281.4 ± 49.5^{h}
Mollar (2)	$58.4 \pm 0.5^{f,g}$	$12.7 \pm 1.1^{d-f}$	$1268.5 \pm 3.3^{e,f}$	$36.4 \pm 2.5^{b,c}$	$13.7 \pm 2.4^{e-g}$	$1389.6 \pm 2.2^{f-h}$
Wonderful1	$97.6 \pm 0.7^{c,d}$	7.3 ± 0.1^{h}	1155.2 ± 50.7^{f}	42.3 ± 3.8^{b}	$11.4 \pm 1.0^{f-h}$	$1313.7 \pm 55.1^{g-h}$
Wonderful	75.4 ± 3.1^{e}	$8.4 \pm 1.4^{g,h}$	$1382.1 \pm 17.8^{c-e}$	24.6 ± 0.7^{f}	7.8 ± 0.9^{h}	$1498.3 \pm 23.9^{d-f}$
G1	25.4 ± 1.2^{i}	$9.7 \pm 0.3^{f-h}$	616.5 ± 21.6^{g}	12.5 ± 3.5^{h}	$14.3 \pm 0.6^{e,f}$	678.3 ± 27.2^{i}
G2	$58.8 \pm 3.7^{f,g}$	$11.7 \pm 2.5^{e-g}$	1487.1 ± 4.9^{c}	$26.2 \pm 3.7^{d-f}$	$12.9 \pm 2.5^{e-g}$	$1596.7 \pm 17.4^{c,d}$
Ecotipo (1)	64.7 ± 5.7^{f}	$18.5 \pm 1.4^{b,c}$	1162.6 ± 50.6^{f}	$42.0 \pm 4.0^{b,c}$	25.7 ± 2.3^{b}	$1313.5 \pm 64.0^{g-h}$
Ecotipo (2)	169.0 ± 1.3^{a}	$18.7 \pm 1.9^{b,c}$	2400.8 ± 83.9^{a}	$26.0 \pm 1.1^{d-f}$	$12.8 \pm 1.1^{e-g}$	2627.4 ± 86.7^{a}

Finally, two tocotrienols were determined. To our knowledge, α and β-tocotrienol were determined in PSO samples for the first time. To identify these tocols, the retention times of α and β-tocotrienol in PSO samples were compared with their retention times in a barley extract obtained by hot saponification according to Panfili, Fratianni, and Irano (2003); in addition a PSO samples was spiked with the same barley extract. The total tocotrienol content was between 1.7 and 4.8 % of total tocols. The α-tocotrienol was detected in the range of 7.3-27.6 μg/g of oil and represented 0.5-1.4 % of total tocols. The β-tocotrienol was 0.8-3.2 % of total tocols, with amounts ranging between 12.5 and 52.1 μg/g. The contents of α and β-tocotrienol determined in the PSO samples were higher than those reported for berry and currant seed oils; in fact, α-tocotrienol was from 10 to 30 times higher than blueberry, blackcurrant and redcurrant seed oils, and β-tocotrienol in PSO was 2-11 times higher than in blackberry, raspberry, blackcurrant and redcurrant seed oils (Bada, León-Camacho, Copovi, & Alonso, 2014).

3.3. Phytosterol and triterpene composition

Phytosterol and triterpene compounds were determined in the same analysis without previous isolation. GC-MS analysis permitted the identification of four 4-desmethylsterols and a 4-α-methylsterol (Fig. 1).

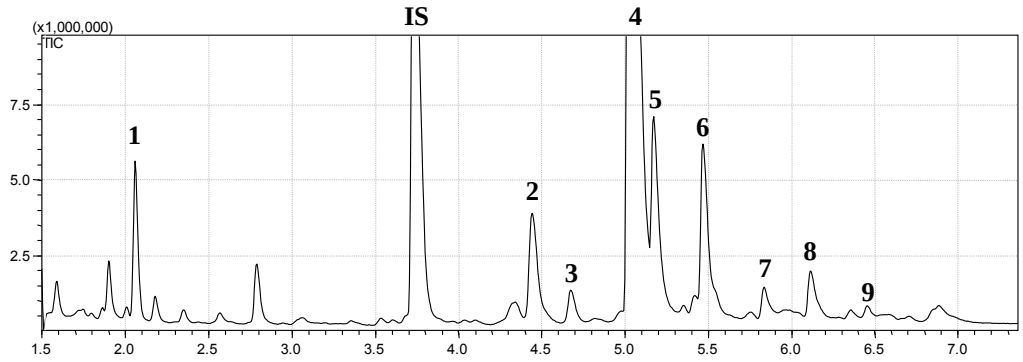

Fig. 1. GC-MS chromatogram of unsaponifiable fraction of pomegranate seed oil, 1, Squalene; 2, Campesterol; 3, Stigmasterol; 4, Sitosterol; 5, d5-avenasterol;6, Cycloartenol; 7, 24-methylencicloartenol; 8, Citrostadienol; 9, Betulinol

A compound at 4.45 min reported an M^+ at 472 *m/z* and two base peaks at 343 *m/z* (corresponding to a loss of 129 amu) and 129 *m/z*; moreover, two major fragments at 382 *m/z* ($[M-90]^+$), obtained by the loss of trimethylsilyl hydroxide (TMSOH), and 367 *m/z* ($[M-15-90]^+$), derived from the combined loss of TMSOH and a methyl group, were also produced during the ionization. According to Pelillo *et al.* (2003) and a comparison to the NIST library, this compound was identified as campesterol. The compound at 4.68 min showed an M^+ at 484 *m/z*, a principal fragment at 129 *m/z* and another fragment at 394 *m/z* ($[M-90]^+$). According to Pelillo *et al.* (2003) and an NIST library comparison, this fragmentation pattern was assigned to stigmasterol. The peak at 5.10 min reported an M^+ at 486 *m/z*; the base peak was at 129 *m/z* and two major fragments corresponding to $[M-129]^+$ (357 m/z) and $[M-90]^+$ (396 *m/z*) and a minor fragment at 381 *m/z* ($[M-15-90]^+$) were also described; as reported by Pelillo *et al.* (2003), this compound corresponds to sitosterol. Another major peak at 5.18 min with 484 *m/z* (M^+) and a base peak at 386 *m/z* ($[M-98]^+$) and a fragment ion at 129 *m/z* was identified as Δ5-avenasterol.

The peak at 6.15 min showed an M^+ at 498 *m/z* and a base peak at 357 *m/z*; moreover, three minor fragments were reported at 483, 408 and 393 *m/z*, corresponding to $[M-15]^+$, $[M-90]^+$ and $[M-15-90]^+$, respectively. According to Pelillo *et al.* (2003), this compound was identified as citrostadienol.

The total and single content of these phytosterols in the PSO samples are reported in Table 3. Total sterol content varied between 7.5 and 16.4 mg/g of oil. These data are in agreement with the results reported by Caligiani *et al.* (2010).

Table 3. Phytosterols content (mg/g of oil) in the analyzed pomegranate seed oils. The different lower case letters in the same column indicate significantly different values ($p < 0.05$)

Pomegranate cultivars	Campesterol	Stigmasterol	Sitosterol	Δ^5-avenasterol	Citrostadienol	Total
Akko	0.75 ± 0.02^{d}	$0.28 \pm 0.02^{d,e}$	6.21 ± 0.08^{g}	0.93 ± 0.08^{i}	$0.56 \pm 0.04^{b,c}$	$8.73 \pm 0.04^{f,g}$
Radisa	0.81 ± 0.03^{d}	0.21 ± 0.03^{e}	$7.66 \pm 0.44^{d-f}$	$1.41 \pm 0.07^{f-h}$	$0.36 \pm 0.03^{e,f}$	$10.45 \pm 0.55^{c,d}$
Hershkovitz	0.72 ± 0.03^{d}	$0.27 \pm 0.01^{d,e}$	6.40 ± 0.15^{g}	$1.13 \pm 0.04^{h,i}$	$0.46 \pm 0.04^{c,d}$	$8.98 \pm 0.27^{e-g}$
Valenciana	$0.71 \pm 0.07^{d,e}$	$0.24 \pm 0.04^{d,e}$	6.41 ± 0.01^{g}	$1.76 \pm 0.18^{c-f}$	$0.36 \pm 0.03^{e,f}$	$9.47 \pm 0.09^{c-f}$
Ravenna	0.50 ± 0.01^{f}	$0.22 \pm 0.01^{d,e}$	$6.68 \pm 0.11^{f,g}$	$1.61 \pm 0.25^{e-g}$	$0.34 \pm 0.03^{e,f}$	$9.36 \pm 0.09^{d-f}$
Veneti	0.82 ± 0.01^{d}	$0.23 \pm 0.01^{d,e}$	$6.82 \pm 0.01^{f,g}$	$1.95 \pm 0.05^{b-e}$	$0.41 \pm 0.01^{d,e}$	$10.23 \pm 0.08^{c-e}$
Hijaz	1.03 ± 0.02^{c}	$0.39 \pm 0.01^{b,c}$	$7.87 \pm 0.19^{c-e}$	$2.10 \pm 0.10^{a-c}$	0.76 ± 0.03^{a}	12.15 ± 0.16^{b}
Shiraz	$0.56 \pm 0.02^{e,f}$	0.20 ± 0.00^{e}	5.17 ± 0.12^{h}	$1.50 \pm 0.03^{f-h}$	$0.42 \pm 0.00^{d,e}$	$7.86 \pm 0.14^{g,h}$
Dente di Cavallo	$1.23 \pm 0.04^{a,b}$	$0.30 \pm 0.01^{c,d}$	$8.67 \pm 0.13^{b,c}$	2.38 ± 0.09^{a}	0.63 ± 0.04^{b}	13.22 ± 0.03^{b}
Mollar (1)	0.75 ± 0.06^{d}	$0.24 \pm 0.02^{d,e}$	$7.14 \pm 0.19^{e-g}$	$2.08 \pm 0.08^{a-d}$	$0.42 \pm 0.02^{d,e}$	10.63 ± 0.17^{c}
Mollar (2)	0.54 ± 0.03^{f}	$0.22 \pm 0.01^{d,e}$	5.13 ± 0.14^{h}	$1.29 \pm 0.04^{g-i}$	0.28 ± 0.01^{f}	7.46 ± 0.07^{h}
Wonderful1	$1.20 \pm 0.00^{a,b}$	0.57 ± 0.02^{a}	11.42 ± 0.39^{a}	2.42 ± 0.05^{a}	0.80 ± 0.01^{a}	16.42 ± 0.42^{a}
Wonderful	$1.23 \pm 0.05^{a,b}$	0.43 ± 0.04^{b}	$8.32 \pm 0.31^{c,d}$	$1.68 \pm 0.02^{d-g}$	$0.56 \pm 0.02^{b,c}$	12.22 ± 0.24^{b}
G (1)	1.35 ± 0.05^{a}	0.45 ± 0.04^{b}	10.44 ± 0.56^{a}	$2.15 \pm 0.10^{a-c}$	0.82 ± 0.02^{a}	15.21 ± 0.77^{a}
G (2)	0.85 ± 0.04^{d}	$0.27 \pm 0.01^{d,e}$	$6.89 \pm 0.08^{e-g}$	$2.18 \pm 0.13^{a,b}$	$0.34 \pm 0.01^{e,f}$	$10.53 \pm 0.00^{c,d}$
Ecotipo (1)	$1.23 \pm 0.04^{a,b}$	$0.39 \pm 0.04^{b,c}$	9.40 ± 0.19^{b}	$1.45 \pm 0.13^{f-h}$	0.26 ± 0.03^{f}	12.73 ± 0.43^{b}
Ecotipo (2)	$1.15 \pm 0.07^{b,c}$	$0.27 \pm 0.02^{d,e}$	$8.63 \pm 0.26^{b-d}$	$1.60 \pm 0.09^{e-g}$	$0.36 \pm 0.01^{e,f}$	12.00 ± 0.42^{b}

As reported in the literature (Caligiani *et al.*, 2010; Garima & Akoh, 2009), sitosterol was the first phytosterol present in PSO samples (Table 3). It was 65-74 % of total sterols; Shiraz reported the lowest content (5.1 mg/g of oil), whereas Wonderful1 showed the highest content (11.4 mg/g of oil). The sitosterol content reported for the PSO samples was comparable to the content detected in wheat germ oil (Caligiani *et al.*, 2010) and was from 2.5 to 12.3 times higher than the content of the same phytosterol in some nut oils (Maguire *et al.*, 2004) and 2-32 times higher than in other vegetable oils (Segura-Carretero *et al.*, 2008). The high sitosterol content in the PSO samples encourages the use of pomegranate seed oil as a nutraceutical for the modulation of cholesterol absorption.

The Δ5-avenasterol and campesterol were the second and third most prevalent phytosterols, representing 10.7-20.7 and 5.4-10.0 % of total phytosterols, respectively. Other minor phytosterols were citrostadienol and stigmasterol, representing 2.1-6.4 and 2.0-3.6 %, respectively.

Analysis by GC-MS also permitted the identification of three triterpenic compounds and squalene.

Squalene eluted at 2.05 min. A major peak at 5.50 min showed an M^+ at 498 *m/z* and two major peaks at 393 and 365 *m/z*; according to Sakouhi *et al.* (2009) this peak was assigned to cycloartenol. Another peak at 5.85 min reported an M^+ at 586 *m/z* and three fragments at 496 ($[M-90]^+$), 481 ($[M-105]^+$) and 398 *m/z*; according to the NIST library and Sakouhi et al. (2009), this compound was identified as 24-methylene-cycloartenol. Finally, a peak at 6.45 min was identified as betulinol; to our knowledge this is the first time that this compound has been identified in PSO.

Squalene content ranged between 0.6 and 3.2 mg/g of oil (Table 4). The content described in PSO samples was 3 to 34 times higher than in nut oils (Maguire *et al.*, 2004). This is an additional nutritional value because, in humans, dietary squalene is absorbed and benefits to human health have been described; because

of this, squalene extraction and food-related applications are being investigated (Naziri & Tsimidou, 2013).

Total triterpene compounds were in the range of 0.99-3.13 mg/g of oil (Table 4). Cycloartenol, which was synthesized from squalene according to Caligiani *et al.* (2010), represents the first triterpene compound detected in PSO samples, ranging between 0.8 and 2.4 mg/g of oil. The second terpene compound was 24-methylene-cycloartenol, representing 9.7-35.9 % of total triterpene compounds with a content varying between 0.16 (in the Shiraz sample) and 0.81 mg/g of oil (in the Wonderful sample).

Betulinol was the third triterpene compound that was detected, in variable amounts (0.02-0.12 mg/g of oil) and ranging between 1.3 and 5.8 % of the total triterpenes.

Table 4. Squalene and triterpene compounds content (mg/g of oil) in the analyzed pomegranate seed oils. The different lower case letters in the same column indicate significantly different values ($p < 0.05$)

Pomegranate cultivars	Squalene	Cycloartenol	24-methylene-cycloartenol	Betulinol	Total triterpene compounds
Akko	$1.07 \pm 0.08^{e,f}$	1.19 ± 0.03^{b}	0.31 ± 0.03^{c}	0.03 ± 0.00^{f}	$1.53 \pm 0.06^{c,d}$
Radisa	$0.70 \pm 0.04^{h,i}$	1.09 ± 0.05^{b}	$0.20 \pm 0.02^{d,e}$	$0.07 \pm 0.01^{c,d}$	$1.36 \pm 0.06^{d,e}$
Hershkovitz	1.60 ± 0.06^{c}	1.13 ± 0.02^{b}	$0.26 \pm 0.01^{c-e}$	$0.03 \pm 0.00^{e,f}$	1.42 ± 0.02^{d}
Valenciana	$1.25 \pm 0.05^{d,e}$	1.29 ± 0.10^{b}	$0.27 \pm 0.00^{c,d}$	$0.03 \pm 0.01^{e,f}$	$1.59 \pm 0.12^{c,d}$
Ravenna	$0.84 \pm 0.04^{f-i}$	0.96 ± 0.08^{b}	$0.25 \pm 0.03^{c-e}$	$0.07 \pm 0.01^{c,d}$	$1.29 \pm 0.03^{d,e}$
Veneti	$0.99 \pm 0.07^{e-g}$	1.03 ± 0.04^{b}	$0.25 \pm 0.03^{c-e}$	$0.06 \pm 0.01^{d,e}$	$1.34 \pm 0.08^{d,e}$
Hijaz	2.13 ± 0.11^{b}	2.41 ± 0.10^{a}	$0.27 \pm 0.03^{c,d}$	0.12 ± 0.00^{a}	$2.80 \pm 0.13^{a,b}$
Shiraz	0.60 ± 0.01^{i}	0.81 ± 0.02^{b}	0.16 ± 0.01^{e}	$0.03 \pm 0.00^{e,f}$	1.00 ± 0.03^{e}
Dente di Cavallo	$1.07 \pm 0.00^{e,f}$	1.28 ± 0.02^{b}	0.50 ± 0.01^{b}	$0.10 \pm 0.02^{a-c}$	1.88 ± 0.02^{c}
Mollar (1)	$1.41 \pm 0.12^{c,d}$	1.30 ± 0.26^{b}	0.30 ± 0.02^{c}	0.02 ± 0.00^{f}	$1.63 \pm 0.24^{c,d}$
Mollar (2)	$1.03 \pm 0.02^{e,f}$	0.79 ± 0.02^{b}	$0.18 \pm 0.01^{d,e}$	0.02 ± 0.00^{f}	0.99 ± 0.02^{e}
Wonderful1	3.18 ± 0.14^{a}	2.02 ± 0.01^{a}	0.46 ± 0.02^{b}	$0.11 \pm 0.01^{a,b}$	2.58 ± 0.01^{b}
Wonderful	$0.91 \pm 0.03^{f-h}$	2.23 ± 0.16^{a}	0.81 ± 0.02^{a}	$0.08 \pm 0.01^{b-d}$	3.13 ± 0.14^{a}
G (1)	2.40 ± 0.05^{b}	2.42 ± 0.05^{a}	0.44 ± 0.05^{b}	$0.04 \pm 0.00^{e,f}$	$2.90 \pm 0.00^{a,b}$
G (2)	$1.07 \pm 0.09^{e,f}$	1.26 ± 0.02^{b}	0.17 ± 0.02^{e}	$0.03 \pm 0.01^{e,f}$	1.46 ± 0.01^{d}
Ecotipo (1)	$0.93 \pm 0.02^{f-h}$	1.19 ± 0.11^{b}	$0.23 \pm 0.01^{c-e}$	0.02 ± 0.00^{f}	1.44 ± 0.12^{d}
Ecotipo (2)	$0.72 \pm 0.02^{g-i}$	0.80 ± 0.08^{b}	0.46 ± 0.05^{b}	0.02 ± 0.00^{f}	$1.28 \pm 0.13^{d,e}$

3.4. Phospholipid composition

Phospholipid compounds in PSO have been poorly studied; moreover, the literature includes different determination methods such as two-dimensional TLC (Isamukhamedov & Akramov, 1982), spectrophotometric determination (Habibnia *et al*., 2012) and HPLC (Garima & Akoh, 2009).

HPLC-ELSD was used to determine the phospholipid classes, each being identified using a respective standard. Phospholipids represented 0.4-2.3 % of the total lipids. As reported in Table 5, the total content of phospholipids ranged between 3.6 and 23.1 mg/g of the oil, in agreement with the data reported by other authors (Garima & Akoh, 2009).

The principal phospholipid was represented by PE, which was 59.1-86.1 % of the total phospholipids. Veneti oil had the lowest content (2.1 mg/g of oil), whereas Wonderful1 presented the highest amount with 16.2 mg/g of oil.

The second phospholipid was PC, ranging between 0.7 and 4.2 mg/g of oil and representing 7.2-29.7 % of the total phospholipid. Moreover, PI was also identified and quantified in PSO in amounts ranging from 0.5 to 2.4 mg/g of oil; its presence in pomegranate has only been reported before by Isamukhamedov and Akramov (1982), who identified the PI by TLC.

Table 5. Phospholipid content (mg/g of oil) in the analyzed pomegranate seed oils. The different lower case letters in the same column indicate significantly different values ($p < 0.05$)

Pomegranate cultivars	PE	PI	PC	Total
Akko	3.58 ± 0.03^{i}	$0.70 \pm 0.01^{h-l}$	1.09 ± 0.09^{e}	5.37 ± 0.13^{i}
Radisa	5.07 ± 0.16^{h}	0.48 ± 0.01^{l}	0.78 ± 0.01^{e}	$6.33 \pm 0.19^{h,i}$
Hershkovitz	$6.25 \pm 0.09^{f,g}$	$0.77 \pm 0.01^{h-l}$	$2.33 \pm 0.02^{c,d}$	$9.35 \pm 0.08^{d,e}$
Valenciana	$6.53 \pm 0.32^{e,f}$	$1.21 \pm 0.03^{f,g}$	0.67 ± 0.04^{g}	$8.41 \pm 0.39^{e-g}$
Ravenna	12.56 ± 0.51^{b}	2.41 ± 0.03^{a}	4.23 ± 0.03^{a}	19.20 ± 0.45^{b}
Veneti	2.15 ± 0.01^{l}	$0.60 \pm 0.02^{h-l}$	0.88 ± 0.05^{e}	3.64 ± 0.06^{l}
Hijaz	$5.29 \pm 0.35^{g,h}$	$0.85 \pm 0.04^{h,i}$	$2.60 \pm 0.01^{b,c}$	$8.73 \pm 0.37^{e-g}$
Shiraz	$6.15 \pm 0.28^{f-h}$	$1.71 \pm 0.04^{c,d}$	3.07 ± 0.06^{b}	10.92 ± 0.39^{c}
Dente di Cavallo	8.12 ± 0.06^{d}	2.05 ± 0.07^{b}	1.90 ± 0.05^{d}	12.07 ± 0.06^{c}
Mollar (1)	$6.19 \pm 0.18^{f,g}$	$1.63 \pm 0.04^{c-e}$	0.74 ± 0.04^{e}	$8.55 \pm 0.18^{e-g}$
Mollar (2)	$6.36 \pm 0.09^{e-g}$	$1.35 \pm 0.07^{d-f}$	1.22 ± 0.04^{e}	$8.92 \pm 0.05^{e-g}$
Wonderful 1	16.25 ± 0.53^{a}	2.68 ± 0.14^{a}	4.13 ± 0.34^{a}	23.07 ± 1.01^{a}
Wonderful	$5.94 \pm 0.03^{f-h}$	$0.78 \pm 0.02^{h-l}$	$2.32 \pm 0.01^{c,d}$	$9.03 \pm 0.04^{d-f}$
G1	$5.49 \pm 0.44^{f-h}$	$0.97 \pm 0.05^{g,h}$	1.01 ± 0.08^{e}	$7.47 \pm 0.57^{g,h}$
G2	$7.55 \pm 0.05^{d,e}$	$1.76 \pm 0.05^{b,c}$	1.22 ± 0.02^{e}	$10.54 \pm 0.12^{c,d}$
Ecotipo (1)	$5.50 \pm 0.33^{f-h}$	$1.38 \pm 0.05^{e,f}$	0.90 ± 0.08^{e}	$7.77 \pm 0.46^{f-h}$
Ecotipo (2)	10.21 ± 0.21^{c}	$0.80 \pm 0.02^{h,i}$	0.85 ± 0.03^{e}	11.86 ± 0.26^{c}

The phospholipid fraction was isolated from the total lipids by TLC separation and was used to determine the composition of phospholipid fatty acids.

The phospholipid fatty acid content is shown in Table 6. All samples showed a high degree of unsaturation. More specifically, polyunsaturated fatty acids (PUFA) were the most representative unsaturated fatty acids except for the G(2) sample, while monounsaturated fatty acids (MUFA) were a minor component except in the Wonderful1 sample. Saturated fatty acids (SFAs) were in the range of 17.9-44.8 % of total phospholipid fatty acid.

Punicic acid (C18:3 c9, t11, c13) was the principal phospholipid fatty acid except in the Dente di Cavallo, Wonderful1 and G(2) samples; Hejaz reported the highest content with 62.4 % of the total phospholipid fatty acids.

The other principal fatty acids were palmitic, stearic and oleic acid, which were 10.0-22.7, 6.1-21.4 and 8.6-32.1 % of the total phospholipid fatty acids, respectively.

Several differences were underlined when the fatty acid compositions of the total lipids and phospholipids were compared. The SFA/UFA ratio was higher ($p < 0.05$) in phospholipid fatty acids, thus confirming a higher saturation level of phospholipid compared to total lipids.
Similarly, the monounsaturated/polyunsaturated ratio (MUFA/PUFA) was higher in phospholipids than in total lipids because the phospholipids were characterized by a lower ($p<0.05$) content of PUFA and a higher ($p< 0.05$) content of MUFA than the total lipids.

4. Conclusions

Briefly, the seed by-products obtained after pomegranate juice production were used to obtain the PSO. Before extracting the oil, the lipolytic enzymes in the seeds were inactivated by thermal treatment. The oil was then extracted and analyzed. As expected, the pomegranate oil was a rich source of punicic acid, which represented more than 70 % of the total fatty acids. In addition, pomegranate seed oil was a good source of γ-tocopherol (from 616 to 2400 μg/g of oil in G(1) and Ecotipo(2), respectively), which was higher than in other seed oils such as nut oil. Furthermore, to our knowledge, α and β-tocotrienol were determined in the PSO samples for the first time and they represent the 1.7-4.8 % of total tocols. With respect to phytosterol and triterpene compounds, sitosterol was determined to be present at high levels (65-74 % of total sterols), comparable with wheat germ oil, and higher than in other vegetable oils (including olive oil). Betulinol was also described for the first time in PSO.

Finally, phospholipids were also determined and they were 0.4-2.3 % of the total lipids.

In summary, PSO is a unique nutritional source of CLnA, tocopherol, sterol and squalene. The results obtained encourage the use of pomegranate seeds for the production of pomegranate oil or the formulation of nutraceuticals for human nutrition.

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CONCLUSIONES

1. Se ha realizado un trabajo de búsqueda bibliográfica donde se recogen los sistemas de extracción utilizados, en los últimos años, para el análisis de compuestos fenólicos en frutas y verduras. El sistema de extracción de compuestos fenólicos más utilizado en muestras vegetales es la extracción sólido-líquido, al ser un método económico que sólo requiere el uso de disolventes; sin embargo, los tiempos de extracción son largos y esto podría influir negativamente en la composición fenólica. Una técnica alternativa para la extracción de muestras líquidas es la extracción en fase sólida. Otras técnicas como extracción con fluidos supercríticos, extracción con fluidos presurizados, extracción asistida por microondas y extracción asistida por ultrasonidos, están reemplazando a las previamente mencionadas, ya que se reduce considerablemente el uso de disolventes y se acelera el proceso de extracción.

2. Se ha evaluado la capacidad antioxidante de la porción comestible de 15 frutas y 29 verduras que se producen en Andalucía mediante tres métodos diferentes: TEAC, FRAP y ORAC. Los resultados obtenidos muestran que limón, granada, uva y aguacate son las frutas que presentan la mayor capacidad antioxidante. Por otro lado, pimiento, cebolla, ajo, espárrago y berenjena son las verduras con una capacidad antioxidante más elevada. De forma general, se observa que en los casos en los que la porción comestible consiste solamente en la pulpa, la capacidad antioxidante es más baja que en las muestras que incluyen piel y semillas.

3. Debido a que la toma de muestra de los distintos vegetales ha sido realizada en diferentes periodos del año, el estudio ofrece información general y relevante acerca de la capacidad antioxidante de las frutas y verduras más consumidas en el mercado europeo, concluyéndose que algunos de estos alimentos pueden poseer importantes propiedades antioxidantes. Por otro lado, los resultados obtenidos podrían utilizarse como ampliación de la información proporcionada en las tablas de composición de alimentos.

4. Se ha llevado a cabo el estudio de la capacidad antioxidante de un extracto de *Garcinia mangostana* L., compuesto de pericarpio, pulpa y semillas, mediante los métodos TEAC, FRAP y ORAC. Los resultados obtenidos fueron comparados con la capacidad antioxidante de un suplemento alimenticio obtenido a partir de una mezcla de vegetales deshidratados, observándose que el extracto objeto de estudio presenta una capacidad antioxidante bastante superior. Los datos aportados por los métodos TEAC, FRAP y ORAC para el extracto de *Garcinia mangostana* L. muestran una capacidad antioxidante de 1222, 3591 y 4088 µmol equivalentes de Trolox/g respectivamente, mientras que el extracto de la mezcla de vegetales posee una capacidad antioxidante aproximadamente 30 veces menor.

5. Se ha desarrollado un método para la caracterización de los compuestos fenólicos en un extracto de *Garcinia mangostana* L. mediante electroforesis capilar acoplada a espectrometría de masas con analizador de tiempo de vuelo. El método propuesto ha permitido la identificación de 10 compuestos pertenecientes a la familia de las xantonas y los flavanoles. En comparación con otros métodos cromatográficos, el método propuesto es una buena alternativa para la caracterización simultánea de compuestos fenólicos en *Garcinia mangostana* L. debido a que la técnica proporciona una separación rápida, eficiente y se reduce el volumen de muestra y el consumo de disolventes. Así mismo, la combinación de la electroforesis capilar con la espectrometría de masas de alta resolución ofrece una alta sensibilidad, selectividad y precisión en los valores de masas.

6. Se ha evaluado el efecto del proceso de deshidratación en muestras de limón entero, así como de la temperatura y el tiempo de almacenamiento, sobre la composición fenólica, la capacidad antioxidante y el contenido de compuestos furánicos. Se pudo concluir que no existen diferencias significativas en el contenido de compuestos fenólicos totales y la capacidad antioxidante determinada mediante ORAC y FRAP, de las muestras estudiadas. Sin embargo,

mediante el método TEAC se observó que las muestras liofilizadas presentaban un valor más alto de capacidad antioxidante en comparación con las muestras deshidratadas, lo que podría ser debido al efecto de la temperatura. Por otra parte, las concentraciones de furfural e hidroximetilfurfural son más bajas en las muestras de limón liofilizadas y aumentan en función de la temperatura y el tiempo de almacenamiento. Estos datos ponen de manifiesto que se ha producido la reacción de Maillard y la producción de compuestos derivados de esta reacción pueden influir en la capacidad antioxidante.

7. Se ha desarrollado un método de cromatografía líquida de alta resolución acoplada a un detector de UV-Vis y espectrometría de masas con analizador de tiempo de vuelo que ha permitido la detección de 23 compuestos fenólicos y ácidos orgánicos, en las muestras de limón analizadas. La mayoría de los compuestos identificados pertenecen a la familia de los flavonoides, concretamente, flavonas, flavanonas y flavonoles. Por otro lado, se han identificado tres ácidos hidroxicinámicos que no estaban previamente documentados en este cítrico, éstos son el ácido hidroxibenzoico hexósido y dos isómeros del ácido hidroxi-metoxifenil-propanóico hexósido. Por otro lado, los datos de cuantificación indican que los compuestos que se encuentran en mayor cantidad en limón fueron eriocitrina, neohesperidina, vicenina-2 y diosmetina diglucósido. Finalmente, las muestras de limón deshidratadas a tiempo cero y liofilizadas presentan una mayor cantidad de compuestos fenólicos que las muestras que han sido almacenadas a altas temperaturas, esto demuestra la influencia negativa de la temperatura en la composición fenólica.

8. Se ha puesto a punto un método mediante cromatografía líquida de alta resolución acoplada a un detector de UV-Vis y espectrometría de masas con analizador de cuadrupolo-tiempo de vuelo para la identificación y cuantificación de los compuestos fenólicos de tres variedades diferentes de berenjena recolectadas en diferentes estaciones del año. El método propuesto ha permitido

la caracterización de 25 compuestos fenólicos, en 32 minutos. De estos compuestos, 14 han sido descritos por primera vez en berenjena en este trabajo y 9 de ellos descritos por primera vez en la familia Solenaceae.

9. Los resultados de cuantificación de las diferentes muestras de berenjena revelan que la variedad que posee una mayor cantidad total de compuestos fenólicos es berenjena larga, seguida de la berenjena púrpura rallada y la berenjena redonda. Por otra parte, el contenido fenólico de las mismas se ve influenciado por el periodo de recolección. Así, la composición fenólica de la berenjena puede estar negativamente afectada por factores climáticos, especialmente por las altas temperaturas propias del verano en la región del mediterráneo. Este hecho, se observa principalmente en la variedad de berenjena larga cuyo rango de concentración total de polifenoles es de 9955 a 11681mg/g en primavera, mientras que en verano se redujo de 2281 a 2678 mg/g.

10. Se ha llevado a cabo la identificación y cuantificación de los compuestos fenólicos y otros compuestos polares en dos variedades diferentes de chirimoya cultivadas en la "Costa tropical" de Granada, así como en sus subproductos, piel y hueso. Éste es el primer estudio disponible hasta la fecha en el que se realiza un análisis exhaustivo de la composición fenólica de la fruta completa, usando cromatografía líquida de alta resolución acoplada a un detector de UV-Vis y espectrometría de masas con analizador de cuadrupolo-tiempo de vuelo. Mediante la metodología propuesta se han identificado un total de 21 compuestos fenólicos y otros compuestos polares en la pulpa, de los cuales 9 no estaban descritos previamente en esta matriz. En la piel y el hueso de la fruta han sido caracterizados 37 y 22 compuestos respectivamente, estos resultados son relevantes ya que no existen estudios previos del contenido fenólico en estos subproductos. Por otra parte, la familia más importante de polifenoles presentes en la pulpa y la piel de la chirimoya son las proantocianidinas.

11. Los resultados cuantitativos muestran que la piel de la chirimoya es la parte de la fruta más rica en compuestos fenólicos, seguida por el hueso y la pulpa. En cuanto a las diferencias entre variedades, la chirimoya 'Fino de Jete' presentó mayor contenido de polifenoles en la piel y el hueso, mientras que la variedad 'Campa' presenta un contenido más alto en pulpa. En general, el contenido en catequina y proantocianidinas con respecto al contenido total de compuestos fenólicos, es mayor en la variedad 'Campa', presentando, 8.03 y 7.83% en la piel y la pulpa respectivamente frente al 4.77 y 6.94 % encontrado en la variedad 'Fino de Jete'.

12. Se ha determinado la composición de la fracción lipídica (ácidos grasos, fosfolípidos, esteroles y tocoferoles) en las dos variedades de chirimoya anteriormente mencionadas. Es la primera vez que se presenta un trabajo sobre la caracterización exhaustiva de la composición lipídica en la chirimoya y sus subproductos. Para ello, se ha desarrollado un método Fast-GC-FID que ha permitido el análisis y la identificación, en 6 minutos, de 19 ácidos grasos. En cuanto al contenido total de ácidos grasos insaturados, cabe destacar que éste es mayor en el hueso de la fruta, siendo su concentración total de 78.82% en la variedad 'Fino de Jete' y 81.90% en 'Campa'. Los principales ácidos grasos insaturados detectados en el hueso han sido el ácido oleico y linoleico, mientras que el ácido linoleico y α-linolénico han sido los mayoritarios en la pulpa y la piel, encontrándose concentraciones muy similares en ambas partes.

13. Los tocoferoles solamente han podido ser cuantificados en la piel y el hueso. En el hueso se han detectado α y δ-tocoferol, siendo la concentración total de los mismos mayor en la variedad 'Campa' con 233.7 µg/g. En la piel solamente se detectó α-tocoferol, siendo su contenido de 50.5 µg/g, también mayor cantidad en la chirimoya 'Campa'. Los resultados obtenidos en la composición de esteroles muestran diferencias entre las tres partes de la fruta y entre las distintas variedades analizadas. Cabe destacar el elevado contenido en γ-sitosterol,

especialmente en la pulpa de la variedad 'Fino de Jete', cuya concentración fue de 6.2 mg/g, este compuesto podría ser usado como marcador de la misma. Por último, se han detectado tres fosfolípidos, fosfatidiletanolamina, fosfatidilcolina y fosfatidilinositol, cuyas concentraciones son mayores en la variedad 'Fino de Jete'. En conclusión, los resultados obtenidos confirman que el contenido de compuestos lipídicos depende de la variedad y de la parte de la fruta estudiada.

14. Se ha determinado la composición de la fracción lipídica (ácidos grasos, fosfolípidos, esteroles y tocoferoles) del aceite extraído de las semillas de 17 variedades diferentes de granada. Los resultados revelaron que las semillas de granada son una fuente rica en ácido punícico, el cual representó más del 70% del total de ácidos grasos. En cuanto al contenido en tocoferoles, las semillas de granada contienen una cantidad importante de γ-tocoferol, siendo ésta mayor que en otros aceites extraídos de semillas. Los compuestos α y β-tocotrienol fueron determinados por primera vez en muestras de aceite de semillas de granada. En lo que respecta a los fitosteroles y compuestos triterpénicos, el sitosterol fue el compuesto mayoritario con una abundancia del 65 al 74 % del total y el betulinol fue encontrado por primera vez en este tipo de muestras. Por último, el fosfolípido mayoritario fue la fosfatidiletanolamina en todas las variedades de granada analizadas. Los resultados obtenidos ponen de manifiesto que las semillas de granada son una fuente importante de aceite rico en compuestos bioactivos que puede ser utilizado para la formulación de nutracéuticos y/o alimentos funcionales.

CONCLUSIONI

1. È stata condotta una ricerca bibliografica con lo scopo di riunire i sistemI di estrazione utilizzati negli ultimi anni per l'analisi dei composti fenolici in frutta e verdurre. Il sistema di estrazione dei composti fenolici più utilizzato su campioni vegetali, è sicuramente quella solido-liquido che, sebbene sia un metodo economico che richiede solo el'impiego di solventi, necesita di lunghi tempi di estrazione che potrebbero influire negativamente sulla composizione fenolica. Una tecnica alternativa per l'estrazione su matrici liquide è la estrazione in fase solida. Altre tecniche, come l'estrazione con fluidi supercritici, estrazione con fluidi pressurizzati, estrazione assistita con microonde ed estrazione assistita con ultrasuoni, stanno sostituendo le precedenti menzionate, riducendo considerevolmente l'uso di solventi ed acelerando il proceso di estrazione.

2. È stata valutata la capacità antiossidante della parte commestibile di 15 frutti e 29 verdure coltivate in Andalusia attraverso tre metodi differenti: TEAC, FRAP e ORAC. I risultati ottenuti evidenziano che il limone, il melograno, l'uva e l'avocado, sono i frutti che presentano la maggiore capacità antiossidante. Mentre il peperone, la cipolla, l'aglio, l'asparago e la melanzana, sono tra le verdure con capacità antiossidante più elevata. In linea generale, si osserva che nei casi in cui la porzione commestibile consista solamente nella polpa, la capacità antiossidante risulta più bassa rispetto a campioni che includono pelle e semi.

3. Poichè il campionamento dei diversi gruppi di vegetali è stato realizzato in differenti periodi dell'anno, questo studio offre una informazione generale e rilevante sulla capacità antiossidante della frutta e della verdura più consumata nel mercato europeo, con la conclusione che alcuni di questi alimenti possono possedere proprietà antiossidanti. Inoltre, i risultati ottenuti potrebbero essere utilizzati come estensione delle informazioni sulla capacità antiossidante fornite nelle tabelle di composizione di questi alimenti.

4. È stato condotto uno studio sulla capacità antiossidante di un estratto di *Garcinia mangostana* L., costituito da pericarpo, polpa e semi, attraverso i metodi TEAC, FRAP y ORAC. I risultati ottenuti sono stati confrontati con la capacità antiossidante di un supplemento alimentare ottenuto da una miscela di vegetali disidratati, ed è emerso che l'estratto oggetto di indagine presenta una capacità antiossidante decisamente superiore. I dati ottenuti attraverso i metodi TEAC, FRAP y ORAC per l'estratto di *Garcinia mangostana* L. hanno mostrato una capacità antiossidante di 1222, 3591 e 4088 µmol equivalenti di Trolox/g rispettivamente, mentre l'estratto della miscela di vegetali disidratati hanno mostrato una capacità antiossidante di circa 30 volte inferiore.

5. È stato messo a punto un metodo per la caratterizzazione dei composti fenolici in un'estratto di *Garcinia mangostana* L. attraverso elettroforesi capillare accoppiata a spettrometria di massa con analizzatore del tempo di volo. Il metodo proposto ha permesso l'identificazione di 10 composti appartenenti alla familia degli xantoni e flavonoli. Se confrontato con altri metodi cromatografici, il metodo proposto rappresenta una buona alternativa per la caratterizzazione simultanea di composti fenolici nella *Garcinia mangostana* L., dovuto al fatto che la tecnica fornisce una separazione rapida ed efficiente, la riduzione del volume del campione ed il consumo di solventi. Inoltre la combinazione dell'elettroforesi capillare con la spettrometria di massa ad alta risoluzione offre un'alta sensibilità, selettività e precisione nei valori di massa.

6. È stato valutato l'effetto del proceso di disidratazione in campioni di limoni interi, come la temperatura ed il tempo di stoccaggio, sulla composizione fenolica, la capacità antiossidante ed il contenuto di composti furanici. Si è potuto concludere che non esistono differenze significative nel contenuto di composti fenolici totali e la capacità antiossidante determinata attraverso ORAC e FRAP tra i campioni studiati. Tuttavia, attraverso il metodo TEAC, è stato osservato che i campioni liofilizzati presentavano un valore più alto in capacità

antiossidante, se confrontati con i campioni disidratati, e questo potrebbe essere dovuto all'effetto della temperatura. Peraltro la concentrazione di furfurolo ed idrossimetilfurfurolo sono più bassi nei campioni di limoni liofilizzati ed aumentano in funzione della temperatura e del tempo di stoccaggio. Questi dati rivelano che si è prodotta la reazione di Maillard e la produzione di composti derivati da questa reazione possono influenzare la capacità antiossidante.

7. È stato messo a punto un metodo di cromatografía liquida ad alta risoluzione accoppiata ad un rivelatore UV-Vis e spettrometria di massa con analizzatore del tempo di volo, che ha permesso la determinazione di 23 composti fenolici ed acidi inorganici in campioni di limone. La maggior parte dei composti identificati appartengono alla familia dei flavonoidi, nello specifico, flavoni, flavanoni, e flavonoli. Inoltre sono stati identificati tre nuovi acidi idrossicinnamici di cui non esisteva documentazione su quasto agrume, e che sono l'acido idrossibenzoico e due isomeri dell'acido idrossi-metossifenil-propanoico esaossido. Inoltre i dati di quantificazione indicano che i composti che si trovano in maggior quantità nel limone sono stati: eriocitrina, neohesperidina, vicenina-2 e diosmetina diglucoside. Infine, i campioni di limone disidratati a tempo zero e liofilizzati, presentano una maggior quantità di composti fenolici rispetto ai campioni che sono stati stoccati ad alte temperatura. Questo dimostra l'influeza negativa della temperatura nella composizione fenolica.

8. È stato messo a punto un metodo per la identificazione e quantificazione dei composti fenolici di 3 differenti varietà di melanzane raccolte in diverse epoche dell'anno, attraverso cromatografia liquida ad alta risoluzione accoppiata ad un rivelatore UV-Vis e spettrometria di massa con analizzatore quadrupolo-tempo di volo. Il metodo proposto ha permesso la caratterizzazione, in 32 minuti, di 25 composti fenolici. Tra questi, 14 sono stati descritti nella melanzana per la prima volta in questo lavoro, e 9 per la prima volta nella famiglia Solenacae.

9. I risultati della quantificazione dei differenti campioni di melanzana rivelano che la varietà che possiede una maggiore quantità totale di composti fenolici è la melanzana lunga, seguita dalla melanzana viola e la melanzana rotonda. Inoltre il contenuto fenolico è risultato essere influenzato dall'epoca di raccolta. Di conseguenza la composizione fenolica della melanzana può essere influenzata negativamente da fattori climatici, in particolare dalle alte temperature estive nell'area mediterranea. Questo effetto si osserva soprattutto nella varietà di melanzana lunga, dove la concentrazione totale di polifenoli varia da 9955 a 11681 mg/g in primavera, mentre in estate la variazione si riduce da 2281 a 2678 mg/g.

10. È stata condotta l'identificazione e la quantificazione dei composti fenolici ed altri composti polari in due differenti varietà di chirimoya, coltivate nella "Costa Tropical" della provincia di Granada (Spagna), così come i suoi sottoprodotti, pelle ed osso. Ad oggi rappresenta il primo studio disponibile nel quale si realizza un analisi esaustiva della composizione fenolica della totalità del frutto completo, utilizzando cromatografia liquida ad alta risoluzione accoppiata ad un rivelatore UV-Vis e spettrometria di massa con analizzatore quadrupolo-tempo di volo. Attraverso il metodo proposto sono stati identificati nella polpa un totale di 21 composti fenolici ed altri composti polari, 9 dei quali non sono mai stati descritti in questa matrice. Nella pelle e nel nocciolo del frutto sono stati caratterizzati 37 e 22 composti fenolici rispettivamente e, data la mancanza di precedenti studi sul contenuto fenolico in questi sottoprodotti, questi risultati sono senza dubbio rilevanti. In aggiunta, la famiglia più importante di polifenoli presenti nella polpa e nella buccia della chirimoya sono le proantocianidine.

11. I risultati quantitativi evidenziano che la buccia della chirimoya è la parte del frutto più ricca in composti fenolici, seguita dal nocciolo e dalla polpa. Per quanto riguarda la differenza tra varietà, la chirimoya 'Fino de Jete' presentò il contenuto più elevato di polifenoli nella buccia e nel nocciolo, mentre la varietà 'Campa' nella polpa. In generale, il contenuto in catechina e proantocianidine sul

totale dei composti fenolici, è maggiore nella varietà 'Campa', presentando un contenuto di 8.03 e 7.83% nella buccia e polpa rispettivamente, a fronte del 4.77 e 6.94 % riscontrato nella varietà 'Fino de Jete'.

12. È stata determinata la composizione della frazione lipidica (acidi grassi, fosfolipidi, steroli, e tocoferoli) in due varietà differenti di chirimoya menzionate precedentemente. Questa è la prima volta che viene presentato uno studio esaustivo della composizione lipidica nella chirimoya ed i suoi sottoprodotti. Per questo è stato messo a punto un método attraverso Fast-GC-FID che ha permesso l'analisi e la identificazione in 6 minuti di 19 acidi grassi. Per quanto riguarda il contenuto totale di acidi grassi insaturi, si rileva che questo è maggiore nell'osso del frutto, presentando una concentrazione totale del 78.82% e 81.90% nella varietà 'Fino de Jete' e nella varietà 'Campa' rispettivamente. I principali acidi grassi insaturi determinati nel nocciolo sono stati l'acido oleico e l'acido linoleico, mentre nella polpa e nella buccia l'acido oleico e l'acido α-linolenico, riscontrando concentrazioni molto simili in entrambe le parti.

13. È stato possibile quantificare i tocoferoli solamente nella buccia e nel nocciolo. Nell'osso sono stati determinati l' α y δ-tocoferolo, la cui concentrazione totale è risultata essere maggiore nella varietà 'Campa' con 233.7 µg/g. Nella buccia della varietà 'Campa' è stato rilevato solamente l' α-tocoferolo, con un contenuto di 50.5 µg/g, più elevato rispetto alla varietà 'Fino de Jete'. I risultati ottenuti nella composizione degli steroli evidenziano differenze tra le tre parti del frutto ed all'interno delle stesse varietà analizzate. Si rileva inoltre l'elevato contenuto in γ-sitosterolo, specialmente nella polpa della varietà 'Fino de Jete', il quale, con una concentrazione 6.2 mg/g, potrebbe essere utilizzato come marcatore di questa varietà. Infine, sono stati determinati tre fosfolipidi, fosfatidiletanolamina, fosfatidilcolina e fosfatidilinositolo, la cui concentrazione è risultata essere maggiore nella varietà 'Fino de Jete'. In

conclusione, i risultati ottenuti confermano che il contenuto dei composti lipidici dipendono sia dalla varietà che dalla parte del frutto studiato.

14. È stata determinata la composizione della frazione lipidica (acidi grassi, fosfolipidi, steroli e tocoferoli) dell'olio estratto dai semi di 17 differenti varietà di melograno. I risultati hanno evidenziato che i semi di melograno sono una ricca fonte di acido punico, rappresentando più del 70% della totalità degli acidi grassi. Per quanto riguarda il contenuto in tocoferoli, i semi di melograno contengono una quantità significativa di γ-tocoferolo, che resulta essere maggiore rispetto ad altri ali estratti da semi. I composti α y β-tocotrienolo sono stati determinati per la prima volta in campioni di olio di melograno. Per quanto riguarda i fitosteroli ed i composti triterpenici, il sitosterolo è stato il composto maggioritario con un'abbondanza dal 65 al 74% del totale, ed il betulinolo è stato riscontrato per la prima volta in questo tipo di campioni. Infine, il fosfolipide principale caratterizzato è stata la fosfatidiletanolamina in tutte le varietà di melograni analizzati. I risultati ottenuti evidenziano che i semi di melograno rappresentano una fonte importante di olio rico in composti bioattivi, che può essere utilizzato per la formulazione di nutraceutici e/o alimenti funzionali.

ANEXOS

ANEXO 1. Índice de tablas

ANEXO 2. Índice de figuras

ANEXO 3. Abreviaturas

AAPH: 2,2'-azobis-(2-aminopropano)-dihidrocloruro

ABTS: ácido 2,2'-azinobis (3-etilbenzotiazolín)-6-sulfónico

AD: detector amperométrico (Amperometric Detector)

ADN: ácido desoxirribonucleico

ALA: ácido α-linolénico

APCI: ionización química a presión atmosférica (Atmospheric Pressure Chemical Ionization)

APPI: fotoionización a presión atmosférica (Atomospheric Pressure Photoionization)

AUC: área bajo la curva (Area Under Curve)

BSA: bis-(trimetilsilil)-acetamida

BSTFA: N-O-bis-(trimetilsilil)-trifluoroacetamida

CE: electroforesis capilar (Capillary electrophoresis)

CI: ionización química (Chemical ionization)

CZE: electroforesis capilar en zona (capillary Zone electrophoresis)

DAD: detector de batería de diodos (Diode Array Detector)

DPPH: 2,2-difenil-1-picrilhidracilo

EI: impacto electrónico (Electron Impact)

EIC: cromatograma de ión extraido (Extract Ion Chromatogram)

EIE: electroferograma de ión extraido (Extract Ion Electropherogram)

ELSD: detector evaporativo de dispersión de luz (Evaporating Light Scattering Detector)

EOF: flujo electrosmótico (Electroosmotic Flow)

ESI: ionización por electrospray (Electrospray Ionization)

FAMEs: ésteres metílicos de ácidos grasos (Fatty Acid Methyl Esters)

Fast-GC: cromatografía de gases "fast" (Fast Gas Chromatography)

FCR: reactivo de Folin-Ciocalteu (Folin-Ciocalteu Reactive)

FID: detector de ionización de llama (Flame Ionization Detector)

FRAP: determinación de la capacidad del ion férrico (Ferric Reducing Antioxidant Power)

FT-ICR: transformada de fourier-resonancia de ión ciclotrónica (Fourier Transform Ion Cyclotron Resonance)

GC: cromatografía de gases (Gas Chromatography)

GLC: cromatografía gas-líquido (Gas-Liquid Chromatography)

HAT: transferencia de átomos de hidrógeno (Hydrogen Atom Transfer)

HPLC: cromatografía líquida de alta resolución (High Performance Liquid Chromatography)

HTLC: cromatografía líquida de alta temperatura (Ultra High Temperature Liquid Cromatography)

IT: trampa de iones (Ion Trap)

LC: cromatografía líquida (Liquid Chromatography)

LC×LC: cromatografía líquida de dos dimensiones

LDL: Lipoproteínas de baja densidad (Light Density Lipoprotein)

LLE: extracción líquido-líquido (Liquid-Liquid Extraction)

m/z: relación carga/masa

MAE: extracción asistida por microondas (Microwave Assisted Extraction)

MALDI: desorción/ionización láser asistida por matriz (Matrix-Assisted Laser Desorption/Ionization)

MS/MS: espectrometría de masas en tándem (Tandem Mass Spectrometry)

MS: espectrometría de masas (Mass Spectrometry)

MSTFA: N-metil-N-(trimetilsilil)-trifluoroacetamida

MUFA: ácido graso monoinsaturado

ORAC: determinación de la capacidad de absorción de radicales peroxilo (Oxygen Radical Absorbance Capacity)

PLE: extracción con fluidos presurizados (Pressurised Liquid Extraction)

PUFA: ácido graso poliinsaturado

Q: analizador de masas cuadrupolo (Quadrupole)

QQQ: triple cuadrupolo

QTOF: analizador de masas cuadrupolo-tiempo de vuelo (Quadrupole-Time of Flight)

RMN: Resonancia magnética nuclear

ROS: Especies reactivas de oxígeno (Reactive Oxygen Species)

SET: transferencia de un electrón desapareado (Single Electron Transfer)

SFE: extracción con fluidos supercríticos (Supercritical Fluid Extraction)

SLE: extracción sólido-líquido (Solid-Liquid Extraction)

SPE: extracción en fase sólida (Solid Phase Extraction)

SPME: microextracción en fase sólida (Solid Phase Microextraction)

TBDMS: N-(tert-butildimetilsilil)-N-metiltrifluoroacetamida

TEAC: determinación de la capacidad antioxidante en equivalentes de trolox (Trolox Equivalent Antioxidant Capacity)

TIC: cromatograma de iones totales (Total Ion Chromatogram)

TMCS: trimetilclorosilano

TMS: trimetilsililéter

TMS: trimetilsililo

TOF: analizador de masas de tiempo de vuelo (Time of Flight)

TPTZ: 4,6-tripiridil-s-triazina

t_R: tiempo de retención

TRAP: determinación del potencial antioxidante total (Total Radical Trapping Power)

Trolox: Ácido 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxílico

UHPLC: cromatografía líquida de ultra-alta presión (Ultra High Performance Liquid Cromatography)

UV: ultavioleta

UV-Vis: Ultravioleta-Visible