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TESIS DOCTORAL

AVOCADO (*PERSEA AMERICANA*): COMPLEMENTARITY OF DIFFERENT OMICS TECHNOLOGIES FOR ITS METABOLIC CHARACTERIZATION

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AVOCADO (*PERSEA AMERICANA*): COMPLEMENTARITY OF DIFFERENT OMICS TECHNOLOGIES FOR ITS METABOLIC CHARACTERIZATION

Memoria presentada por Elena Hurtado Fernández
para optar al grado de Doctor Internacional en Química.

Granada, febrero de 2014

Elena Hurtado Fernández

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El trabajo que se presenta en esta tesis doctoral, con el título **"AVOCADO (*PERSEA AMERICANA*): COMPLEMENTARITY OF DIFFERENT OMICS TECHNOLOGIES FOR ITS METABOLIC CHARACTERIZATION",** ha sido realizado bajo mi dirección y la de la Prof. Dra. Dña. Alegría Carrasco Pancorbo en los laboratorios que el grupo FQM-297 tiene en el Departamento de Química Analítica y, parcialmente, en el Centro de Proteómica y Metabolómica del Centro Médico de la Universidad de Leiden (LUMC) en Holanda, y reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Elena Hurtado Fernández pueda optar al grado de Doctor Internacional en Química.

Y para que así conste, expido y firmo el presente certificado en Granada a de febrero de 2014:

Esta tesis doctoral ha sido realizada con la ayuda de una beca predoctoral del Programa de Contratos Predoctorales del Plan Propio de Investigación de la Universidad de Granada y también gracias a la financiación con cargo a fondos del grupo FQM-297 "Control analítico, Ambiental, Bioquímico y Alimentario" del Plan Andaluz de Investigación de la Junta de Andalucía, procedentes de diferentes contratos, proyectos y subvenciones de la Administración central y autonómica, plan propio de investigación de la UGR, así como de empresas interesadas en los resultados de la investigación.

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OBJECTIVES/ OBJETIVOS

OBJECTIVES

Avocado is a fruit increasingly valued by consumers, not only for its unique flavor and texture, but also for its reported healthy benefits. This fact together with adequate climatic conditions explains the rapid growing of its cultivation in coastal areas of Andalucia, situating Spain as one of the principal avocado producers and exporters.

The growing evidence of the health-beneficial properties of the avocado fruit is stimulating research on this area; however, it is absolutely necessary to know in depth the composition of what we eat to go further on the understanding of their physiological effects. Although the lipophilic fraction of avocado fruit had been extensively studied and some reports had been published describing the presence of proteins, sugars, vitamins, sterols, tannins, fatty acids or amino acids in this matrix, the overall composition of this tropical fruit, in terms of bioactive compounds and other phytochemicals, was still quite unknown when this Doctoral Thesis started.

The chemical diversity of avocado specific substances in combination with time dependent mechanisms –such as ripening– makes the analysis of its metabolome a challenging task; there is no single method that can give a complete qualitative and quantitative overview of its composition. This made us considered the combined use of targeted and exploratory analyses (both approaches involving the use of different analytical techniques) as very appropriate strategy for trying to characterize the avocado metabolome and its dynamic changes.

Therefore, the main goal of the current Doctoral Thesis is the metabolic characterization of avocado fruit by applying different metabolomics approaches and tools. In order to achieve this general purpose, we propose a series of partial aims that can assure the successful achievement of main one described above:

- ✓ Optimize different analytical methodologies by using CE, LC and GC as separation techniques coupled to diverse detection systems, particularly to MS. This goal involves the application of different electrophoretic approaches, the development of innovative LC methods, as well as the coupling of GC with novel ionization sources.
- ✓ Make a careful and highly standardized sample collection of avocado fruits, in such a way that gives us the chance to achieve useful and founded conclusions.
- ✓ Study the influence that processes such as ripening have on the evolution of metabolic profile of avocado fruits, as well as check the metabolic differences among diverse cultivar types (sometimes genetically related).
- ✓ Try to establish ripening markers that help identifying the optimum harvest time to collect the fruits.
- ✓ Apply the powerful developed methodologies to perform metabolomics studies that allow characterizing, both qualitative and quantitatively, an important number of metabolites present in avocado.
- ✓ Carry out the analytical validation of the methods to assure its appropriate analytical performance.
- ✓ Use different statistics and computational tools (chemometrics) to extract effectively and reliably the useful information from the generated datasets.
- ✓ Demonstrate the existing complementarity among different omics technologies and the importance of its combined use to achieve a holistic description of avocado metabolome.

OBJETIVOS

El aguacate es una fruta tropical cada vez más valorada por los consumidores, no sólo por su particular sabor y textura, sino por los posibles efectos beneficiosos para la salud de quien la consume. Esto, acompañado de unas condiciones climáticas muy favorables para su crecimiento, explica que su cultivo se haya extendido en ciertas zonas costeras de Andalucía, situando a España entre los principales países productores y exportadores de este alimento.

Las evidencias que confirman los efectos saludables de esta fruta han provocado un aumento de las investigaciones biomédicas/nutricionales donde se evalúan sus propiedades. Sin embargo, un paso previo y absolutamente necesario para la realización de esos estudios es conocer en profundidad la composición de aquello que comemos, hecho que ayuda de manera considerable a comprender sus efectos fisiológicos. Si bien la fracción lipofílica del aguacate había sido estudiada bastante en detalle y se habían publicado diferentes artículos donde se ponía de manifiesto la presencia de proteínas, azúcares, vitaminas, esteroles, taninos, aminoácidos o ácidos grasos en esta matriz, su composición en lo referente a compuestos bioactivos y otros fitoquímicos era bastante desconocida cuando se comenzó la presente Tesis Doctoral.

La diversidad química de los metabolitos presentes en este fruto, junto con ciertos procesos (como el de maduración) que provocan que sus concentraciones varíen con el tiempo, hacen que la completa caracterización del metaboloma del aguacate sea una tarea difícilmente alcanzable. No existe ninguna metodología analítica que sea capaz de dar una visión completa del metaboloma de ningún sistema biológico. Esto nos hizo considerar que el uso combinado de aproximaciones *targeted* and *non-targeted* (empleando en ambos casos varias herramientas analíticas) podría representar una estrategia adecuada para intentar caracterizar el metaboloma del aguacate, así como sus cambios dinámicos.

Por tanto, el objetivo principal de esta Tesis Doctoral es llevar a cabo la caracterización metabólica del aguacate empleando diferentes aproximaciones y herramientas ómicas. Para la exitosa consecución del mismo, se han formulado una serie de objetivos parciales:

- ✓ Optimizar diferentes metodologías analíticas que empleen CE, LC y GC como técnicas separativas, acopladas a distintos sistemas de detección (prestando particular atención a MS). Este objetivo conlleva la aplicación de diferentes aproximaciones electroforéticas, el desarrollo de métodos innovadores para LC, así como el acoplamiento de GC y MS mediante fuentes de ionización novedosas.

- ✓ Seleccionar de la manera más cuidadosa y estandarizada posible las muestras de aguacate a estudiar, de modo que permitan alcanzar conclusiones relevantes y fundadas.

- ✓ Estudiar la influencia del proceso de maduración en la evolución del perfil metabólico del aguacate, así como examinar las diferencias metabólicas que existen entre distintas variedades de la muestra tropical bajo estudio (algunas de ellas relacionadas genéticamente).

- ✓ Identificar posibles compuestos "marcadores de maduración" que faciliten la decisión acerca de cuál es el momento óptimo de la campaña para recolectar los frutos.

- ✓ Emplear las metodologías puestas a punto para llevar a cabo estudios metabolómicos que permitan la caracterización, tanto cualitativa como cuantitativa, de un número importante de metabolitos presentes en el fruto del aguacate.

- ✓ Realizar una validación completa y rigurosa de los métodos desarrollados que asegure la obtención de resultados fiables.

- ✓ Emplear herramientas estadísticas y computacionales (Quimiometría) para extraer de modo efectivo y fiable toda la información relevante contenida en los datos analíticos generados.

- ✓ Demostrar la complementariedad existente entre las herramientas ómicas empleadas y poner de manifiesto la importancia de usar varias de ellas para analizar una misma muestra, si lo que se persigue es obtener una visión global de la composición de la misma.

SUMMARY/ RESUMEN

SUMMARY

This manuscript shows the results achieved during the realization of the Doctoral Thesis entitled "Avocado (*Persea americana*): Complementarity of different omics technologies for its metabolic characterization". The report has been divided in two main sections: the introduction and the experimental part, results and discussion.

In the **Introduction**, the first half presents a global vision of the matrix under study, from the origin and botany of the avocado fruit to its production and consumption throughout the world, paying particular attention to the composition of this tropical fruit –which has been related to several benefitial effects for the human health– and all the analytical platforms used to its characterization. In the second half, an overview of the omics sciences is shown, focusing on the relevant importance of metabolomics studies in current investigations in different fields, as well as on the different steps that define the usual workflow where different analytical strategies can be followed.

The second part of the manuscript, so-called **Experimental Part. Results and discussion**, is divided in three thematic sections related to the three analytical techniques applied for avocado characterization, each of which consists on several chapters and a general introduction. It is important to mention that chapters are not presented in chronological order and they are consecutively numbered, regardless of the section they belong to.

The first section deals with the application of CE as separation technique coupled to different detectors to identify and quantify several polar metabolites of avocado extracts. It is composed by three chapters, being the first one (Chapter 1) a bibliographic revision of all the CE methods published up to 2010 for the analysis of antioxidant phenolic compounds in food derived from plants. Plants are rich sources of this type of substances, which have been widely studied on over the last decades for its potential healthy benefits.

The second chapter (Chapter 2) includes the optimization of an effective and sensitive CE-UV method for the analysis of different phenolic acids in 13 avocado varieties at two different ripening degrees (just harvested and ready for consumption). All the variables involved in CE separation and UV detection were exhaustively optimized; the method was fully validated and successfully applied to the different avocado samples afterwards. It was possible to carry out the

identification of a considerable number of phenolic acids that ranged from 8 to 14 depending on the variety, which were present in variable concentrations. In general, compounds such as *p*-coumaric, benzoic and protocatechuic acids were the most abundants in ripe avocado extracts. Principal component analysis was applied to highlight the differences between varieties and ripening degrees, standing out the fact that hydroxycinnamic acids, in particular *p*-coumaric acid (which concentration increases during ripening) was the variable with the highest influence in the data variability. Hydroxybenzoic acids composition seemed to be more cultivar-related.

The last chapter of this section (Chapter 3) is related to the development of a CZE method with two different MS detection conditions (*MRM* and *Full Scan*) to evaluate, both qualitative and quantitatively, changes in the metabolic profile of avocado fruits, paying attention to 10 specific metabolites that belong to different chemical categories. 18 avocado samples were used for this study; which came from the same avocado variety, but they were collected at different moments of the harvest time. Both MS approaches were compared in terms of analytical parameters and also in terms of the quantitative results achieved, showing a good agreement. The concentration of 8 of the 10 analyzed compounds presented a diminution as the fruit ripened, whereas the other 2 compounds (ferulic acid and again *p*-coumaric acid) showed the oppsite trend.

Section II is principally about the usefulness of LC and MS for metabolic profiling of avocado fruits, and it embraces three chapters. The first one is Chapter 4 and it describes a simple method for LC-DAD-ESI-TOF MS to performe the characterization of three different avocado varieties in terms of phenolic compounds, including both phenolic acids and flavonoids. By using the developed method, we were able to identify unequivocally 17 compounds and other 25 metabolites were tentatively identified taking into account the accuracy and isotopic information provided by TOF MS.

Chapter 5 makes reference to the development and optimization of an UHPLC-UV/ESI-TOF MS method for the comprehensive profiling of the metabolites found in the methanolic extracts of 13 different varieties of avocado at two different ripening degrees. The application of this analytical platform allowed achieving, for the first time, the tentative identity of around 200 metabolites from different chemical families. It was possible to assure with certainty the identity of 60 metabolites, 20 of which could be quantified in terms of their own pure standards.

Finally, Chapter 6 encloses a very interesting study about the metabolic evolution of the samples from 4 avocado varieties that have been collected at different moments during the harvest season. A powerful LC-DAD/FL/ESI-IT MS has been optimized and applied to the analysis of the samples mentioned, being possible to observe clearly the evolution of five compounds such as *p*-coumaric acid, chlorogenic acid, pantothenic acid, epicatechin and abscisic acid.

The third and final section is formed by 5 chapters where GC-MS has been applied to study the avocado metabolome. First of all, Chapter 7 shows the optimization of a methodology where GC is used as separation technique and it is coupled to two detectors (FID and APCI-TOF MS) working in parallel. This method was applied to determine quantitative changes in the metabolic profile of *Persea americana*, quantifying analytes that belong to several chemical families (organic acids, phytohormones, vitamins, flavonoids and phenolic acids) in 13 varieties of avocado at two specific ripening stages. The method was fully validated and the analytical parameters of both detectors were compared, showing both of them acceptable analytical features. 27 compounds were quantified by GC-APCI-MS, whilst GC-FID allowed the proper quantification of 7 analytes. In general, the concentration of flavonoids and organic acids tended to decrease with the ripening process, observing an opposite tendency for hydroxycinnamic acid derivatives and vitamin B_5. After the statistical analysis, quinic and *p*-coumaric acids, epicatechin and quercetin ended up being some of the most influential compounds explaining the classification of the samples under study.

The subsequent chapter (Chapter 8) summarizes the development of a database for GC-FID/APCI-TOF MS. It is not a very common analytical technique used to perform metabolomics studies, which is promoted in a large extent by the the lack of spectral databases to carry out a "simple" identification of the compounds. The database was built including those metabolites that can be potentially found both in food matrices and biofluids. Up to now, the databse contains information about retention indices, as well as MS and MS/MS spectra of around 150 compounds (it is already being updated). This work was developed during a stay in the Centre for Proteomics and Mabolomics of the Leiden University Medical Centre (LUMC) in Leiden, The Netherlands.

Chapter 9 probably describes the first application where GC-APCI-MS is applied to carry out a metabolic profiling approach for determining all the metabolites subceptibles to be analyzed with the proposed method in avocado samples. This unusual coupling for GC was compared with the traditional GC platform, which makes use of EI as ionization source. The comparison was made considering the MS signals observed in both ionization sources. By using these techniques it was possible to identify a total of 100 metabolites in 3 avocado varieties. This work also allowed proving the usefulness and applicability of the new spectral library for the identification of an important number of compounds.

To close this section, in Chapter 10 and 11 it is expected to highlitgh the utility of chemometrics tools for metabolomics studies. Different multivariate analysis were applied to treat a dataset achieved by using GC-APCI-TOF MS (chapter 10) and GC-EI-Q MS (chapter 11), and to obtain valuable information about the metabolic evolution of 13 avocado varieties at two ripening stages.

Both platforms have provided very useful information and they have allowed knowing the identity of some of the most influential compounds on the classification of the samples under study (some of which have been common in both chapters): mannoheptulose, aspartic acid and linoleic acid, among other metabolites.

Finally, there is a separated section/chapter where the existing complementarity among the different omics technologies used in this thesis is detailed. It is well known that plant and food matrices consist of a wide variety of compounds from different chemical families which are found in very different abundancies. This complexity of the metabolome makes extremely difficult to understand the complete metabolic profile of a plant or food when only a single omics technology is applied, being necessary to use different analytical approaches that provide complementary information.

RESUMEN

En esta memoria se presentan los resultados obtenidos durante la realización de la Tesis Doctoral titulada "Aguacate (*Persea americana*): Complementariedad de distintas tecnologías ómicas para su caracterización metabólica." La memoria se ha dividido en dos secciones principales: introducción y parte experimental, resultados y discusión.

En la **Introducción**, la primera parte recoge una visión general de la matriz objeto de estudio, desde el origen y estudio botánico del aguacate hasta su producción y consumo a nivel mundial, aunque se presta especial atención a la composición de esta fruta tropical –la cual ha sido relacionada con distintos efectos beneficiosos para la salud humana– y a todas las plataformas analíticas que se han utilizado para su caracterización. En segundo lugar, se muestra un resumen de las distintas ciencias ómicas, centrándonos en la importancia de los estudios metabolómicos en las investigaciones que se llevan a cabo actualmente en distintos ámbitos, así como en las distintas etapas que componen el flujo de trabajo habitual y las diferentes estrategias analíticas que se pueden seguir.

La segunda parte de la memoria hace referencia a la **Parte Experimental. Resultados y discusión** y está dividida en tres secciones temáticas, relacionadas con las metodologías analíticas puestas a punto para la caracterización del aguacate, cada una de las cuales consta de varios capítulos y una breve introducción. Es importante mencionar que los capítulos recogidos en este bloque de la tesis no siguen un orden cronológico y están numerados de manera consecutiva independientemente de la sección en la que se encuentren.

La primera sección versa sobre la aplicación de CE como técnica separativa acoplada a diferentes detectores para identificar y cuantificar varios metabolitos polares en extractos de aguacate. Está formada por tres capítulos, siendo el primero de ellos (Capítulo 1) una revisión bibliográfica que incluye todos aquellos métodos de CE publicados hasta 2010 para el análisis de compuestos fenólicos antioxidantes en alimentos derivados de plantas. Las plantas son importantes fuentes de este tipo de sustancias, las cuales han sido ampliamente estudiadas en los últimos años por sus potenciales efectos saludables.

El segundo (Capítulo 2) incluye la optimización de un método CE-UV sensible y eficaz para el análisis de diversos ácidos fenólicos en 13 variedades de aguacate en dos estados de maduración,

recién cogidos del árbol y listos para ser consumidos. Todas las variables involucradas tanto en la separación electroforética como en la detección UV fueron optimizadas exhaustivamente. El método fue completamente validado y aplicado con éxito al análisis de las muestras de aguacate. Fue posible llevar a cabo la identificación de un número considerable de ácidos fenólicos, que osciló entre 8 y 14 compuestos dependiendo de la variedad, los cuales se encontraron en concentraciones variables. En general, compuestos como los ácidos *p*-cumárico, benzoico y protocatecuico resultaron ser los más abundantes en los extractos de aguacate maduro. Se realizó el análisis de componentes principales para evaluar las diferencias existentes entre las distintas variedades y los estados de maduración, destacando el hecho de que la oscilación en la concentración de los distintos ácidos hidroxicinámicos —en particular el ácido *p*-cumárico (cuya concentración aumenta con la maduración)— fue el factor con la mayor influencia en la variabilidad de los datos. La influencia de los ácidos hidroxibenzóicos parece estar más relacionada con la variación entre los cultivos.

El último capítulo de esta sección (Capítulo 3) está relacionado con el desarrollo de un método para CZE acoplado a MS utilizando dos aproximaciones diferentes (*MRM* y *Full Scan*) para evaluar, tanto cualitativa como cuantitativamente, los cambios en el perfil metabólico de frutos de aguacate, prestando particular atención a 10 metabolitos específicos que pertencen a diferentes familias químicas. Para este estudio se emplearon 18 muestras de aguacate que procedían de una misma variedad de aguacate, pero habían sido recogidas en distintos momentos de la campaña de recolección. Ambas aproximaciones de MS se compararon en base a sus parámetros analíticos y también en base a los resultados cuantitativos obtenidos con cada una de ellas, mostrando buena concordancia. La concentración de 8 de los 10 metabolitos analizados disminuyó con la maduración, mientras que los otros dos compuestos (ácido ferúlico y de nuevo el ácido *p*-cumárico) presentaron un comportamiento opuesto.

La sección II está dedicada principalmente a la utilidad de LC y MS para el estudio del perfil metabólico de los frutos de aguacate. En ella es posible encontrar 3 capítulos. El primero es el Capítulo 4 y describe un método LC-DAD-ESI-TOF MS para llevar a cabo la caracterización de 3 variedades distintas de aguacate en cuanto a compuestos fenólicos se refiere, incluyendo tanto ácidos fenólicos como flavonoides. Haciendo uso del método desarrollado fuimos capaces de identificar 17 compuestos de forma inequívoca, mientras que otros 25 metabolitos se identificaron tentativamente teniendo en cuenta la exactitud y la información isotópica proporcionada por el espectrómetro de masas TOF.

El Capítulo 5 hace referencia al desarrollo y optimización de un método para UHPLC-UV/ESI-TOF MS para realizar un análisis completo del perfil de metabolitos encontrado en los extractos metanólicos de 13 variedades de aguacate en dos estados de maduración distintos. La aplicación

de esta técnica analítica permitió conocer por primera vez la identidad tentativa de unos 200 metabolitos de distintas familias químicas. Fue posible asegurar con certeza la identidad de 60 de los compuestos, 20 de los cuales se pudieron cuantificar en base a su estándar comercial.
Finalmente, el Capítulo 6 engloba un estudio muy interesante sobre la evolución metabólica de muestras pertenecientes a 4 variedades de aguacate que han sido recogidas en distintos puntos del periodo de cosecha. Se ha puesto a punto un potente método LC-DAD/FL/ESI-IT MS y se ha utilizado para el análisis de las muestras antes mencionadas, siendo posible observar la clara evolución de 5 compuestos como el ácido *p*-cumárico, ácido clorogénico, ácido pantoténico, epicatequina y ácido abscísico.

La tercera y última de las secciones está compuesta por 4 capítulos en los que GC-MS ha sido utilizada para el estudio del metaboloma del aguacate. En primer lugar, el Capítulo 7 muestra la optimización de una metodología donde GC es usada como técnica de separación y se acopla a dos detectores (FID y APCI-TOF MS) que trabajan en paralelo. Este método se aplicó a la determinación cuantitativa de los cambios producidos en el perfil metabólico de *Persea americana*, cuantificando analitos provenientes de varias familias químicas (ácidos orgánicos, fitohormonas, vitaminas, flavonoides y ácidos fenólicos) en 13 variedades de aguacate en dos estados de maduración específicos. Dicho método fue también completamente validado y se compararon los parámetros analíticos de los dos detectores, manifestando ambos características analíticas más que aceptables. Una vez establecidos los parámetros analíticos del método, se logró la cuantificación de 27 compuestos mediante GC-APCI-MS, y sin embargo GC-FID sólo permitió la correcta cuantificación de 7 analitos. En general, la concentración de flavonoides y ácidos orgánicos tendió a disminuir con el proceso de maduración, observándose una tendencia opuesta para los derivados del ácido hidroxicinámico y la vitamina B_5. Tras los análisis estadísticos, los ácidos quínico y *p*-cumárico, la epicatequina y la quercetina resultaron ser algunos de los metabolitos más influyentes en la clasificación de las muestras bajo estudio.
El siguiente capítulo (Capítulo 8) resume los pasos seguidos en la creación de una base de datos para GC-FID/APCI-TOF MS. Se trata de una técnica poco habitual en estudios metabolómicos, hecho propiciado en gran medida a la ausencia de bibliotecas espectrales que faciliten la identificación de compuestos. La base de datos se construyó incluyendo aquellos metabolitos que pueden ser encontrados con facilidad en matrices alimentarias y en fluidos biológicos. Hasta la fecha, dicha biblioteca contiene información sobre índices de retención, así como espectros de MS y MS/MS, de aproximadamente 150 compuestos (está siendo actualizada). Este trabajo se desarrolló durante una estancia en el Centro de Proteómica y Metabolómica del Centro Médico de la Universidad de Leiden (LUMC) en Leiden, Holanda.

El Capítulo 9 describe la primera aplicación en la que GC-APCI-MS es empleada para llevar a cabo el estudio del perfil metabólico para el análisis de todos los metabolitos, susceptibles de ser analizados con el método propuesto, en muestras de aguacate. Este acoplamiento, poco habitual para GC, se comparó con otra más habitual en los laboratorios, como es GC-EI-Q MS, que hace uso de EI como fuente de ionización y Q como analizador de masas. La comparación se hizo en base a las señales de MS obtenidas con ambos sistemas de ionización. Utilizando dichas técnicas fue posible identificar 100 metabolitos en 3 variedades de aguacate. Este trabajo también permitió poner de manifiesto la utilidad y aplicabilidad de la nueva biblioteca espectral para la identificación de un importante número de compuestos.

Para cerrar esta sección, en los Capítulos 10 y 11 se pretende destacar la utilidad de herramientas quimiométricas en estudios metabolómicos. Se emplearon distintos análisis multivariantes para tratar un conjunto de datos obtenido mediante GC-APCI-TOF MS (capítulo 10) y GC-EI-Q MS (capítulo 11) y para obtener información de interés sobre la evolución metabólica de 13 variedades de aguacate que se encontraban en dos estados de maduración diferentes. Ambas plataformas han resultado ser muy útiles y han permitido conocer la identidad de algunos de los compuestos más influyentes en la clasificación de las muestras bajo estudio (algunos de los cuales han sido comunes en ambos trabajos): manoheptulosa, ácido aspártico y ácido linoleico, entre otros metabolitos.

Finalmente, hay un capítulo independiente (Capítulo 12) donde se discute la complementariedad existente entre las distintas tecnologías ómicas. Es bien conocido que las matrices vegetales o alimentarias están constituidas por una amplísima variedad de sustancias que provienen de familias químicas muy diversas y que están presentes en cantidades muy diferentes. Esta complejidad del metaboloma hace extremadamente difícil llegar a conocer completamente el perfil metabólico de una planta o un alimento, siendo necesario hacer uso de distintas aproximaciones analíticas que nos proporcionen información complementaria.

INTRODUCTION

1. Avocado

1.1. Botany and origin

Avocado is an evergreen tree, despite the fact that the leaves present a surprisingly short longevity, which is no longer than 12 months. It is characterized by a rapid growth in height and spread, reaching heights up to 20 m, its roots are shallow and have poor water uptake and hydraulic conductance. Although the trees produce high amounts of flowers, usually less than 0.1% of these flowers set fruits. The flowering and fruit set could be influenced by three different climacteric factors: (i) the occurrence of frost during the winter; (ii) low mean temperatures; and (iii) the occurrence of extreme high temperatures during fruit set [1]. Avocado tree belongs to the Lauraceae family –typical from the tropical or subtropical climates– and the *Persea* genus, which is divided in three different subgenera that enclose more than 150 species:

1) *Persea* → Only 2 species, *P. americana* and *P. schiedeana*
2) *Eriodaphne* → About 70 species, such as *P. caerulea*, *P. indica* and *P. lingue* among others.
3) *Machilus* → Around 80 species, such as *P. japonica*, *P. kobu*, etc.

The most studied and the most important member of the *Persea* genus is *P. americana*, whose fruit is the commercial avocado. Avocado is the most common English name but it is also known as alligator pear and butter pear. Spanish-speaking people normally call it aguacate, cura or cuprandra, although in Chile, Peru and Ecuador it is called by its Inca name, palta. The name *avocado* is derived from the Aztec Nahuatl language word, *ahuacatl*, menaing "testicle". This name refers to the shape of the fruit, which was considered by the Aztecs as the fertility fruit.

Avocado is native from Central America and Mexico, where it has been a staple dietary component for at least 9000 years [2]. Within *Persea americana* it is possible to differentiate three different ecological races: Mexican, Guatemalan and West Indian (or Antillean). Each race presents typical characteristics in terms of leaves, fruits, flowering period, etc., which have been summarized in the following table (***Table 1***) [3]:

Table 1. Comparison of the three different horticultural races of avocado fruit.

	TRAIT	RACE		
		Guatemalan (G)	***Mexican (M)***	***West indian (WI)***
TREE	Climate	Subtropical	Semi-tropical	Tropical
	Cold tolerance	Intermediate	Most	Least
	Salinity tolerance	Intermediate	Least	Most
	Leaf anise	Absent	Present	Absent
	Young leaf colour	Green with red tinge	Green	Pale yellow
	Mature leaf colour	Dark green	Dark green	Pale green
FRUIT	Bloom to fruit maturity	10–18 months	5–7 months	6–8 months
	Size	Small–large	Tiny–medium	Medium–very large
	Shape	Mostly round	Mostly elongate	Variable
	Colour	Green	Often dark	Green or reddish
	Skin thickness	Thick	Very thin	Medium
	Skin surface	Rough	Waxy bloom	Shiny
	Skin peelability	Rigid	Membranous	Leathery
	Seed size	Small	Large	Variable
	Seed cavity	Tight	Loose	Variable
	Seed surface	Smooth	Smooth	Rough
	Oil content	High	Highest	Low
	Pulp flavour	Rich	Anise-like, rich	Sweeter, milder

There are no sterility barriers among the three races or among any taxa classified under *P. americana*. Hence, hybridization readily occurs wherever trees of differente races are growing in proximity. For this reason, most commercial avocado cultivars are interracial hybrids, developed from chance seedlings, with different degrees of hybridization [4, 5]. Avocado cultivars present very different characteristics among them, for instance in size, shape and colour, as can be appreciated in the next figure (***Figure 1***).

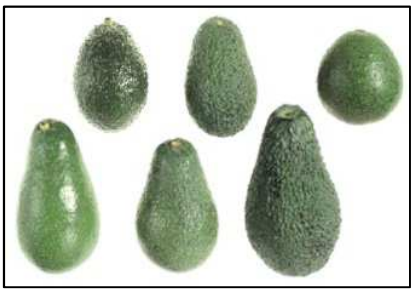

Figure 1. Example of different avocado cultivars.
From left to right, Top: Gwen, Hass, Reed. Bottom: Ettinger, Fuerte, Pinkerton.

Usually, the cultivars grown in tropical climates are not the same to those of the subtropical areas. Some of the principal avocado cultivars commercially available are enclosed in ***Table 2***.

Table 2. Some of the most relevant avocado cultivars grown in tropical and subtropical climates.

SUBTROPICAL CLIMATE		TROPICAL CLIMATE	
Cultivar	***Race***	***Cultivar***	***Race***
Bacon	MxG	Blair	G
Colin V 33	M	Booth 7	GxWI
Edranol	G	Booth 8	GxWI
Ettinger	M	Choquette	GxWI
Fuerte	MxG	Hickson	G
Gwen	GxM	Lisa	MxWI
Hass	G/GxM	Loretta	GxWI
Lamb Hass	GxM	Lula	GxWI
Pinkerton	GxM	Monroe	GxWI
Reed	G	Pollock	WI
Ryan	MxG	Simmonds	WI
Shepard	M	Taylor	G
Sir Prize	M	Tonnage	G
Zutano	MxG	Waldin	WI

Botanically, avocado (*Persea americana*) is a berry that consists of a large central seed and pericarp, which is the sum of the skin (exocarp), the edible portion (mesocarp) and the inner layer surrounding the seed (endocarp) (see ***Figure 2***) [6].

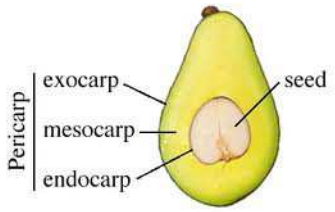

Figure 2. Different parts that conform the avocado fruit.

Avocado is a climacteric fruit that reach the physiological or the harvest maturity on the tree, which means that fruits are picked green and hard. However, the ripening process occurs after the harvest, reaching the optimun moment for consumption approximately after 10 days at room temperature. Knowing the adequate date to harvest avocado fruits is difficult, because there are no external changes on the fruits that indicate

that moment [7]. For this reason, parameters such as minimum content of oil and dry matter in the flesh have to be controlled to determine the optimum picking time [8].

1.2. Nutritional composition and related healthy benefits

The first information about the nutritional composition of avocado fruits dates back to 1922. Data about the macro- and micronutrients found in avocado have been compiled in different tables of food composition, although nutrient content of the edible portion of the fruit (pulp or mesocarp) highly varies depending, among others factors, on the variety, ripening degree and cultivation conditions. ***Figure 3*** shows the nutritional composition of 100 g of avocado fruit according to the USDA National Nutrient Database for Standard Reference [9].

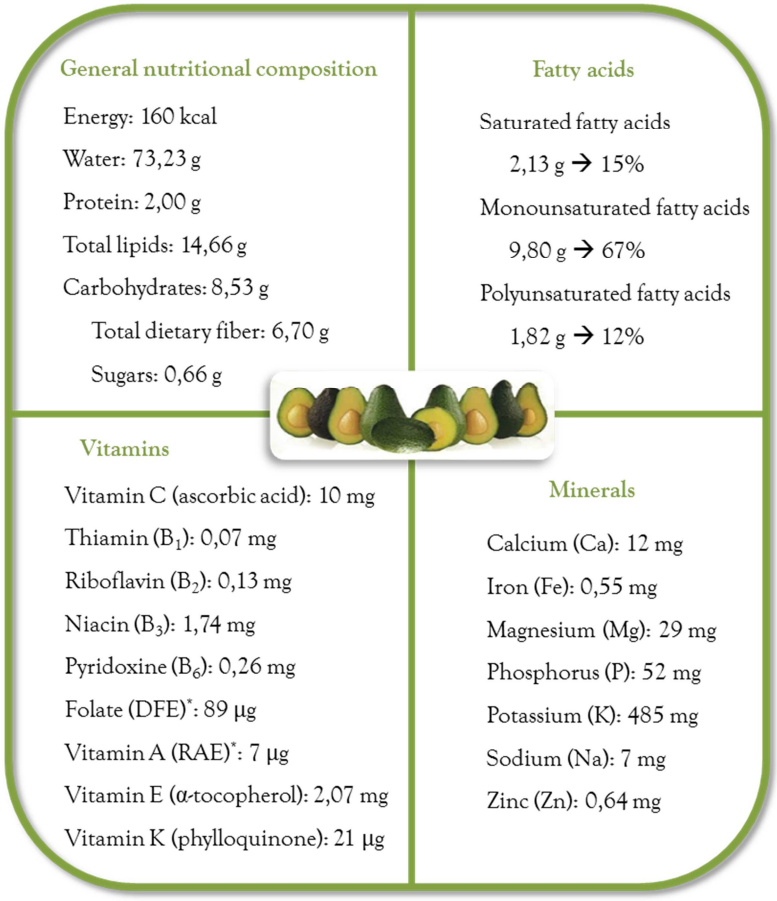

Figure 3. Nutritional content of 100 g of avocado fruit.
*RAE=Retinol Activity Equivalents; DFE=Dietary Folate Equivalents

As it can be seen, one of the main components of avocado is the fat, and because of this characteristic is not surprising that it is also known as "butter fruit". In general, the oil content of avocado increases with the ripening procees [7]. Monounsaturated fatty

acids are the predominant ones, standing out oleic acid as the main representative. Other important fatty acids of avocado fruit, although less abundant, are linoleic (polyunsaturated) and palmitic (saturated) acids. The abundance of these substances, together with the fact that some of the principal healthy benefits of avocado have been attributed to its high monounsaturated fatty acid content; make lipids one of the most studied families of compounds in avocado [10]. Paying attention to the rest of constituents, the avocado protein level is higher than for other fruits, reaching values about 2%, whereas the majority of the fruits present a content of proteins around to the 1% [3]. Avocado is also a very important source of vitamins –especially vitamins E and C–, pigments (anthocyanins, clorophylls and carotenoids) [11, 12], sterols [13], phenolic compounds [14], and seven-carbon sugars and its related alcohols (D-mannoheptulose and perseitol), which are not very common sugars [15]. Trying to give an overview of all the substances that can be found in avocado fruit, the following table (***Table 3***) summarizes the families of compounds identified in this matrix since 1969.

Table 3. Applications where avocado fruit was the matrix under study including the determined metabolites, used techniques, part of the fruit considered and year of publication.

Metabolites	Technique	Avocado part	Year	Reference
Aliphatic compounds	IR NMR MS	Pulp	1969	[16]
Carotenoids	TLC MS	Pulp, peel, leaves	1973	[11]
Fatty acids Vitamins Minerals	AOAC methods	Pulp	1975	[17]
Triglycerides Fatty acids	HPLC-RI GC-FID	Pulp	1987	[18]
Phenolic acids	HPLC-UV/Vis	Pulp	1987	[19]
Lipids	GC-MS NMR	Pulp	1991	[20]
Triglycerides	HPLC-LSD	Oil	1992	[21]
Volatiles	GC-MS	Pulp	1998	[22]
Sugars	HPLC-RI	Pulp	1999	[23]
Flavanols	HPLC-UV	Pulp	2000	[24]
Antifungal compounds	HPLC-MS HPLC-UV-RI NMR IR	Idioblast cells of mesocarp	2000	[25]

Table 3 (continued)

Metabolites	Technique	Avocado part	Year	Reference
Fatty acids derivatives	NMR HR-FAB-MS HR-EI-MS IR HPLC GC	Pulp	2001	[26]
Sugars	HPLC-RI NIR	Honey	2002	[27]
Carbohydrates	HPLC-RI	Pulp	2002	[28]
Sterols	GC-MS	Pulp	2003	[29]
Fatty acids Volatiles	GC-FID GC-MS	Oil	2003	[30]
Flavanols	HPLC	Pulp	2004	[31]
Fatty acids	GC-FID	Pulp	2004	[7]
Minerals	ICP-OES	Honey	2004 2005	[32] [33]
Pigments	HPLC-PDA-FL	Oil, pulp, peel	2006	[12]
Vitamin E	HPLC-FL	Pulp	2006	[34]
Phenolic compounds Volatiles Fatty acids	HPLC-DAD GC-FID-MS GC-FID	Oil	2007	[35]
Fatty acids Glycolipids and phospholipids	GC-FID HPLC-MS-MS	Pulp	2007	[36]
Fatty acids Sugars	GC-FID HPLC-ELSD	Pulp	2008	[37]
Lipids Fatty acids	TLC GC-FID	Pulp	2008	[38]
Water content	NIR	Pulp	2009	[39]
Fatty acids Carbohydrates	GC-FID HPLC-ELSD	Pulp	2009	[40]
Fatty acids Carotenoids Tocopherols	GC-FID HPLC-UV LC-MS	Pulp	2009	[41]
Fatty acids Sterols	GC-MS	Oil, pulp	2009	[13]
Fatty acids	GC NMR	Oil	2009	[42]
Aminoacids	HPLC-UV/Vis	Oil	2010	[43]
Phenolic compounds	UV/Vis HPLC-UV	Pulp	2010	[14]
Fatty acids Sugars	GC-FID HPLC-ELSD	Pulp	2010	[15]
Phenolic compounds	HPLC-3D-FL	Pulp	2010	[44]

Table 3 (continued)

Metabolites	Technique	Avocado part	Year	Reference
Phenolic compounds	FT-IR FL	Pulp	2010	[45]
Fatty acids	GC-MS	Pulp	2010	[10]
Procyanidins Pigments	HPLC-MS^n UV-Vis	Pulp, peel, seed	2010	[46]
Phenolic compounds	UHPLC-PDA-FL-MS	Peel, pulp, seed	2011	[47]
Fatty acids	CE-UV	Oil	2011	[48]
Fatty acids Sterols Tocopherols	GC-FID GC-MS HPLC-UV	Oil	2012	[49]
Proteins	Nano-LC-MS/MS	Pulp	2012	[50]
Phenolic compounds	UV/Vis HPLC-DAD-MS	Peel, seed	2012	[51]
Fatty alcohol derivatives Alkanols Fatty acids Sterols Coumarins	FT-IR NMR HPLC-MS	Unripe pulp	2012	[52]
Chlorophyll Carotenoids	UV-Vis	Pulp	2012	[53]
Lipids	HA-LAESI MS	Pulp	2013	[54]

As have been seen, avocado is a very complex matrix formed by a wide variety of compounds. Usually, it is highly recognized for being an excellent source of energy, fatty acids and vitamins. However, it also delivers certain kind of non-nutritive substances, such as phenolic compounds, carotenoids or alkanols among others, which are responsible for some of the organoleptic properties and, besides, they may contribute to enhance human health. Over the years, numerous researches have drawn attention to the connection between avocado consumption and a better health, finding that some of the numerous substances present in avocado fruit are closely related to several healthy effects for the human beings [55]. Some of these healthy benefits have been synthesized in ***Figure 4***.

Wilson Grant, in 1960, published the first clinical study where avocado intake was associated with the maintenance of normal serum cholesterol levels or even with their reduction [56]. Later on, in the 90s and beginning of 2000, there was an increase in the number of works that studied the relationship between avocado fat and its effect on

cardiovascular diseases, cholesterol, lipid profile, weight control and diabetes [22, 57-64]. Although the principal healthy benefits attributed to avocado fruit are the aforementioned, some others have been also evaluated, such as: prevention and treatment of osteoarthritis [65, 66], anticancer properties [67-72], protective activity against liver injury [26], skin protection [73-75], reduction of risk of macular degeneration [12], influence on short memory [76], antioxidant activity [10, 45-47, 77-79], reduction of metabolic syndrome risk [80, 81] or anti-inflammatory effects [77, 78, 82]. Studies reveal that all these effects are mainly due to the presence in avocado fruit, principally, of fatty acids, dietary fiber, D-mannoheptulose and perseitol, potassium, magnesium, vitamins C, E, K and B group, carotenoids, phenolics, phytosterols or terpenoids [55].

Figure 4. Principal healthy effects attributed to avocado consumption.

For all of the aforementioned, it can be concluded that the inclusion of avocado in the everyday diet can bring "positive" effects to the health of human beings, even though it is important to remember that a single food not provide all required nutrients and nutraceuticals, because no food is 100% complete from a nutritional point of view. Thus, it is recommendable that the diet includes several food items from all the different groups (fruits, vegetables, legumes and potatoes, fish and meat, etc.) in order to assure a good nutrition. Besides, the combined consumption of different foods can improve the bioavailability and absorption of specific nutrients or bioactive compounds. In this sense, it has been observed, for instance, that to consume carotenoid-rich fruits or vegetables together with avocado or avocado oil can significantly increase carotenoids absorption, fact that may help to strengthen health effects [83].

1.3. Cultivation and production

Although the origin of this crop is Central America, avocado is nowadays widely cultivated throughout the world [5, 84]. In 2012, the world production of avocado was of about 440,000 tonnes, decreasing a 3% over the previous year [85]. ***Figure 5*** shows the tendency of harvested area and avocado production all over the world since 2002 [85]. It is appreciated a clear increase of the cultivation, whilst the production does not present such marked behaviour. As this fruit has distinctive and pleasant sensory attributes, and it is perceived by buyers as beneficial to health, its demand has experimented an increase over the last years that has consequently caused an increase of the production [86].

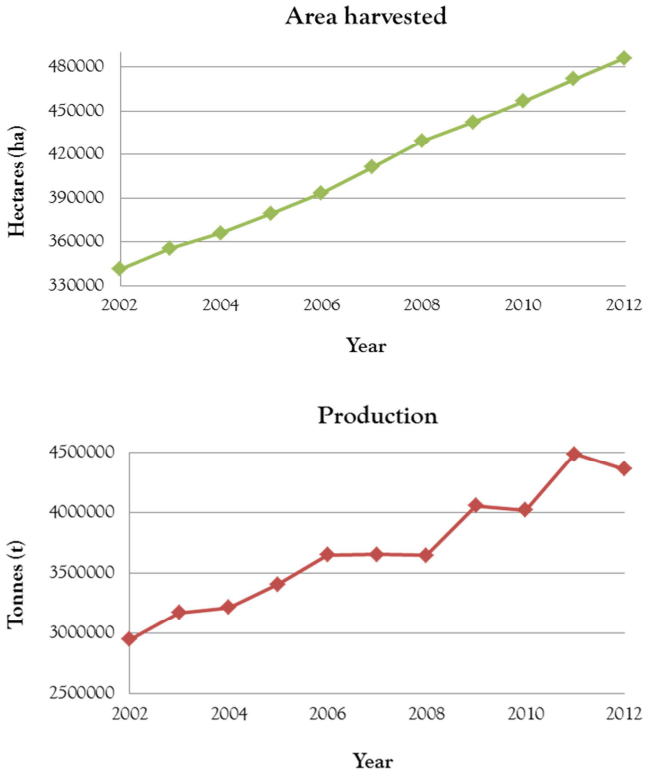

Figure 5. Tendency of the harvested area and production of avocado fruit in the world from 2002 to 2012 [85].

The world´s leading producers are Mexico (28.5%), Chile (8.3%), Dominican Republic (6.7%), and Indonesia (6.2%) [87]. Considering these numbers, it is evident that the American continent conquers the avocado production (67%), being also the continent with the highest amount of harvested hectares (ha), followed by Africa (17%), Asia (12%), Europe (2%) and Oceania (2%).

The main countries producing avocado fruit within each continent are highlighted in the following sector diagrams (***Figure 6***), observing that Kenya, Mexico, Indonesia, Spain and Australia are those with remarkably higher production.

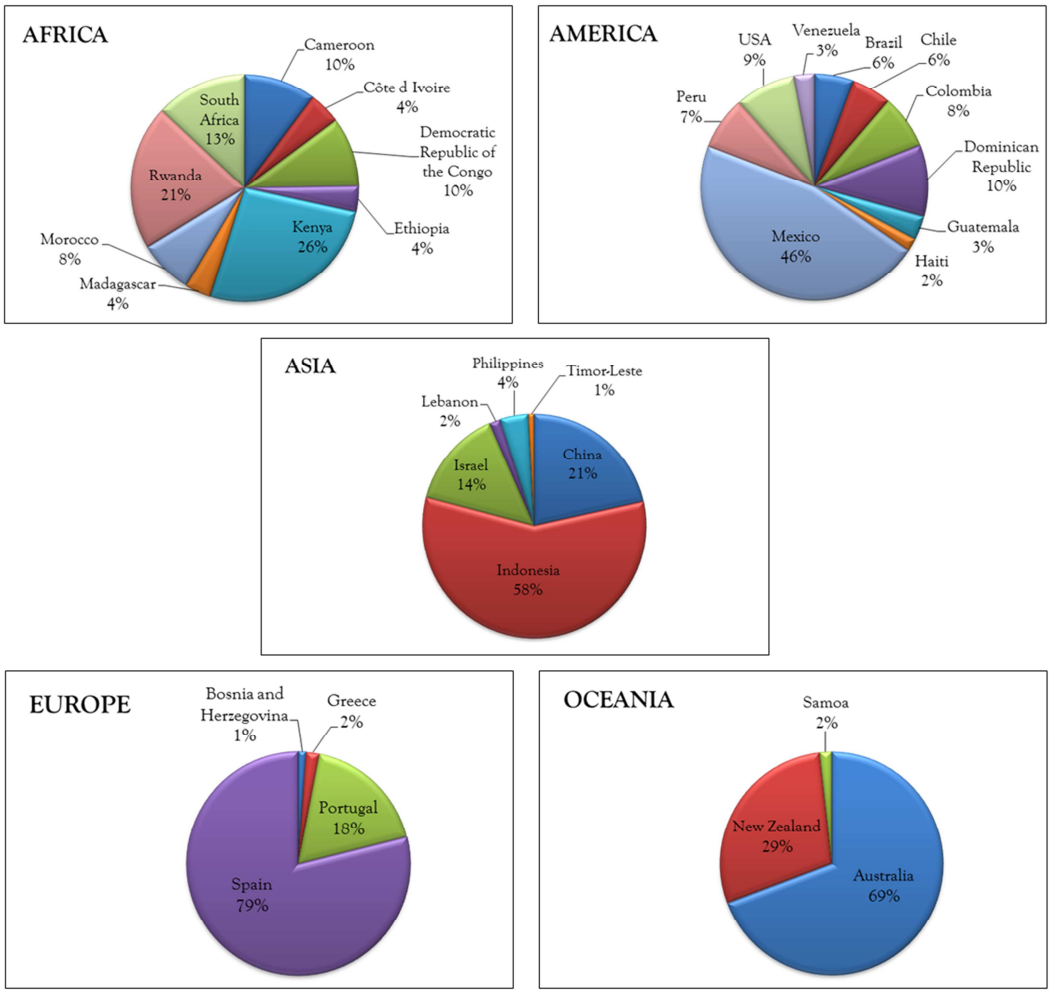

Figure 6. Sector diagrams representing the principal avocado producers by continent in 2012 [85].

Despite the fact that Spain does not represent a big percentage of the total world production (just a 2%) it is the main producer in Europe (79%). Besides, the characteristic climatic conditions of Spain facilitate the cultivation of this type of tropical-subtropical fruit, which requires specific edaphoclimatic conditions (temperatures of 20-21ºC, relative humidity during flowering >70%, frost free zones, etc.). Thus, it is a crop that can grow easily in the South and East of Spain, as well as in the Canary Islands. According to the "Ministerio de Agricultura, Alimentación y Medio Ambiente" of Spain [88], the total harvested area for avocado fruit was 10,558 ha in 2011 and the total production reached 98,535 t. As can be seen in ***Figure 7,*** Andalucia stands out as the principal avocado producer of Spain (89,216 t); and within this region, coastal areas of

Malaga and Granada occupy the first positions both in harvested area and in avocado production. This crop is also found in Huelva, Almeria, Cadiz and Sevilla, but in these places it does not play such a relevant role.

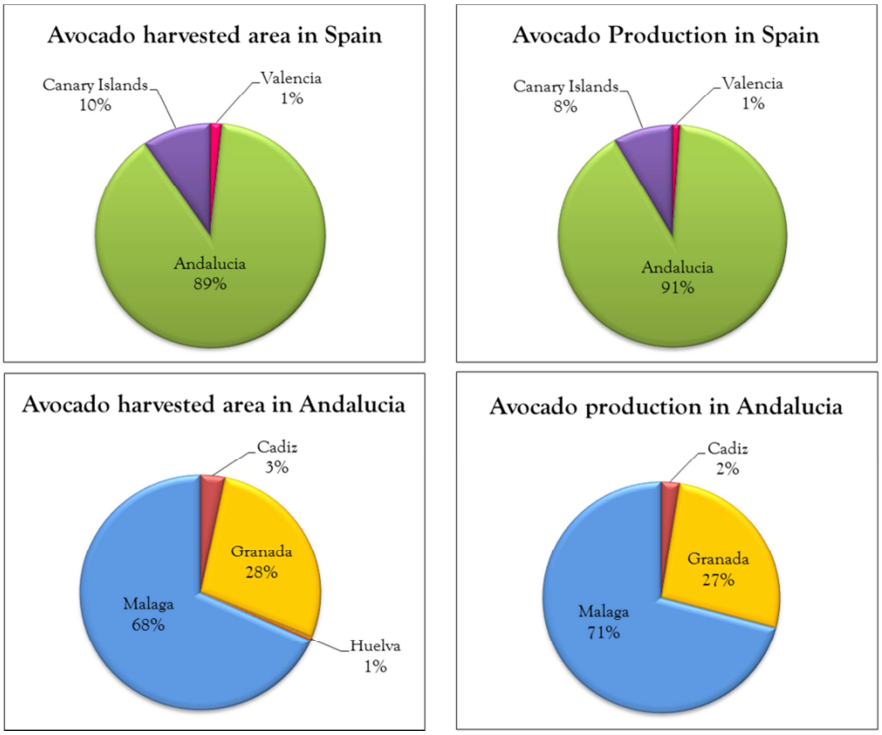

Figure 7. Principal zones of Spain where avocado fruit is cultivated and the influence to the total production of the country. Data related to 2011 [88].

An important agricultural parameter is the crop yield, which refers to the measure of the production of a specific crop per unit area of land cultivation. The higher the crop yields the better the land quality or the higher the cultivation efficiency. Globally, the crop yield for avocado fruit is of about 9.5 t/ha. The principal avocado producers present higher values; around 10-12 t/ha for Mexico, Chile and Indonesia, and 27.7 t/ha for Dominican Republic. The crop yield of Spain and Andalucia are similar to the global average (9.3 and 9.5 t/ha, respectively). In Andalucia, it is mainly due to the contribution of Malaga and Granada, because in Almeria, Cadiz, Huelva and Sevilla the crop yield is between 7 and 3 t/ha. A slight increase of the crop yields would generates a considerable impact on the avocado production.

1.4. World trade

In general, those countries that produce the biggest amount of avocado tend to be the highest consumers of this fruit. However, in the last decades the demand of this fruit

has considerably grown in several markets, causing an important increase of the volume of avocado that is intended for exporting.

The principal avocado exporter countries are Mexico, Chile, The Netherlands, Peru and Spain; whereas the main importers of this tropical fruit are USA, The Netherlands and France (***Figure 8***) [87]. It should be pointed out the appearance of The Netherlands and France both in the graph of the most important exporters and importers, which is due to the fact they re-export a significant percentage of their avocado imports to other countries in the European Union. Spain occupies the fifth place on the exporters list, being responsible of the 9% of the total avocado export that are mainly destined to the European market (see ***Figure 9***) [87]. One of the main reasons for which European countries prefer importing Spanish avocados is for the proximity among the countries, which represents a clear advantage both on fruit quality and smaller distribution costs.

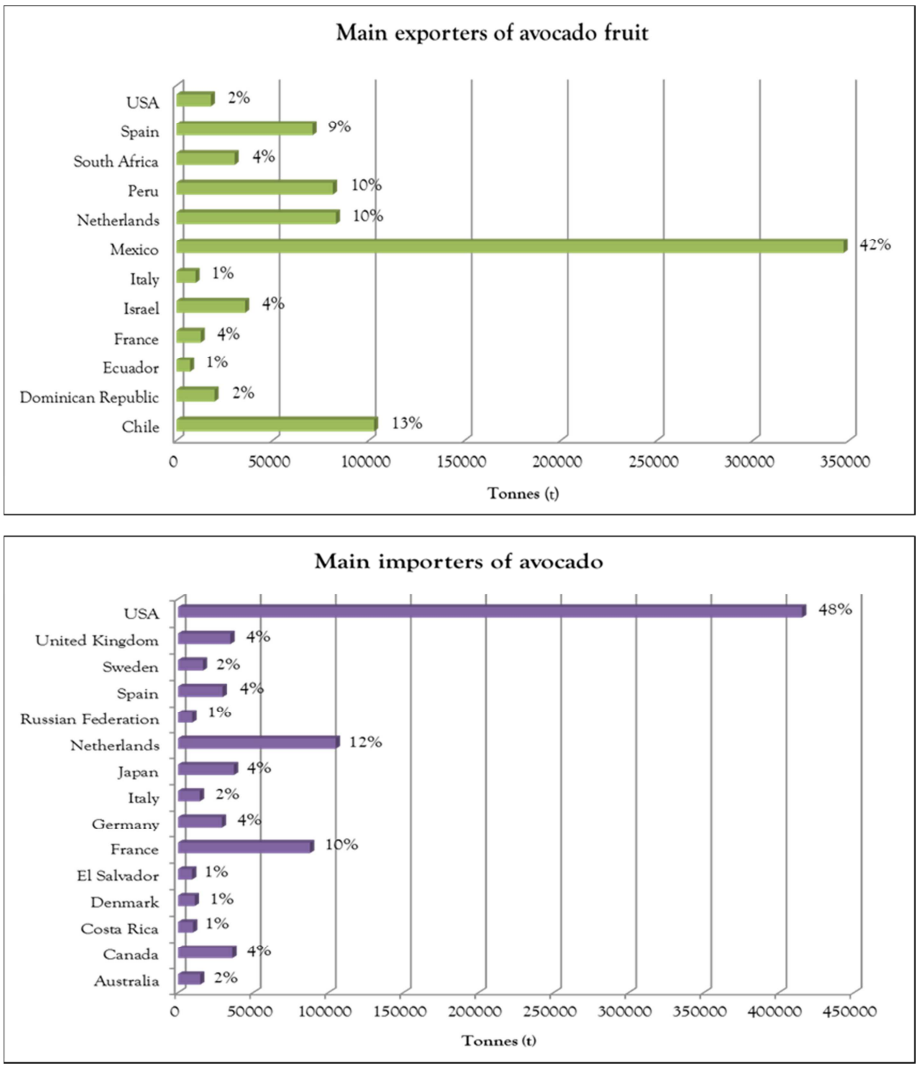

Figure 8. Principal avocado exporters and importers [87].

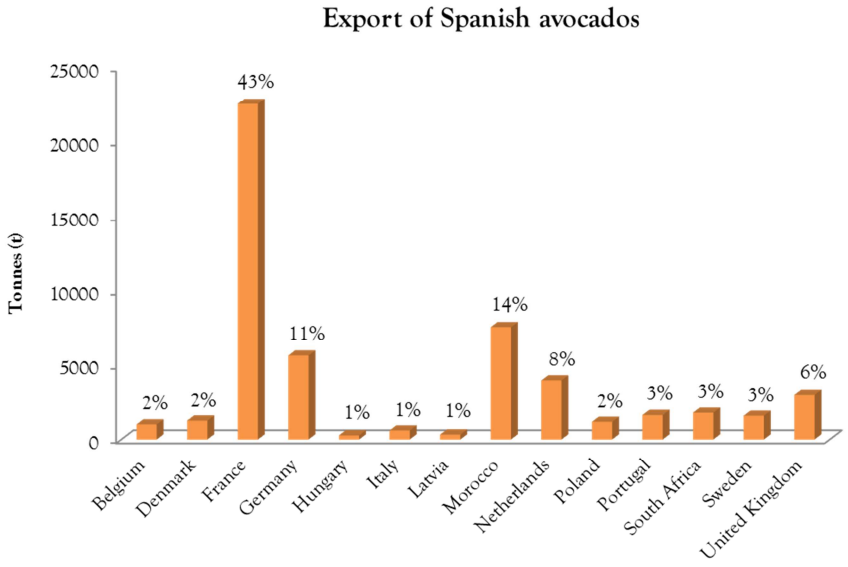

Figure 9. Main countries of Spanish avocado destination [87].

1.5. Socioeconomic impact

The presence of avocado in the world market has been growing steadily in the past decades, and it is no longer considered an exotic fruit, but part of the daily diet of many countries. Avocado has a large market as a fresh fruit; however, in order to increase commercialization on a larger scale and give avocado an added value, it is important to develop food products derived from this fruit with a long enough shelf life to assure their transportation and distribution to consumers. For these reasons, it is possible nowadays to find a wide variety of processed foods derived from avocado, such as guacamole, frozen products, refreshing drinks and avocado paste, as well as numerous uses of it in the cosmetic, soap, and shampoo industry. Besides, avocado oil is gaining interest in the fat and oil market, due to avocado fruit is the only one that can rival the olive and palm fruits in oil content, and also because diverse health effects have been attributed to its consumption –although it is used in cosmetic too– [89-91]. Furthermore, industries are taking advantage of avocado byproducts (seed and peel) to extract oil, as well as several interesting compounds that are present at important amounts and can be used as antioxidants, flavoring, colorants or texturizer additives, making possible a better exploitation of avocado fruit [92, 93].

The economic and social importance of avocado principally resides in the benefits that its cultivation gives to producers, processors, and consumers. The orchards create jobs by demanding labor for farming operations, harvest, packinghouse operations,

transportation, and marketing. In addition, the development of new products would also promote the creation of processing plants, which in turn would generate new jobs, and increase the farmer´s profit.

2. Metabolomics

During the 90s decade, concurring with the beginning of the Human Genome Project, different fields such as molecular biology or biochemistry experimented significant changes related to the techniques and approaches commonly used, being coined new concepts like genomics, transcriptomics, proteomics and metabolomics; the so-called *omics* sciences [94]. The main goal of all these *omics* sciences is the same: the comprehensive characterization of an organism, tissue or cell type.

The interest on metabolomics field emerged at the end of the 90s, being Nicholson and Fiehn the pioneers in this area. Although the basic idea of determining multiple analytes simultaneously appeared during the 1980s –when Nicholson and Wilson applied ^{1}H NMR spectroscopy to analyze the plethora of the metabolites found in biological fluids [95]–, the concept of metabonomics was not coined till 1999 by Nicholson and his research group at the Imperial College of London, who defined metabonomics as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" [96]. A year later, Fiehn et al. (Max-Planck Institute, Germany) published the first results about the study of plant metabolome by using GC-MS and they coined the term metabolomics, which was referred to the "comprehensive and quantitative analysis of all metabolites in a system" [97]. Originally, metabonomics and metabolomics represented different approaches to perform the determination of the metabolites present in a biological system; however it nowadays exists a great deal of overlap in methodologies and both terms are often used interchangeably. Therefore, from now on the term metabolomics will be used in its broadest meaning, including also the metabonomics sense.

Metabolomics appear as a complement to genomics, transcriptomics and proteomics, contributing to the understanding of the complex molecular interactions that take place in biological systems, because it is impossible to reach that understanding at the transcriptomic level and difficult at the proteomic level. The metabolome represents the

final expression of the genome, transcriptome and proteome, since the genetic information contained at the DNA –and transcribed through the RNA– determines the protein synthesis, and this, the metabolites present in a specific biological system [98]. These small molecules involved in biochemical processes provide a lot of information on the status and functioning of a living system, reflecting changes in phenotypes and functions (functional genomics) and showing the interrelationship among the different information flow levels in biology.

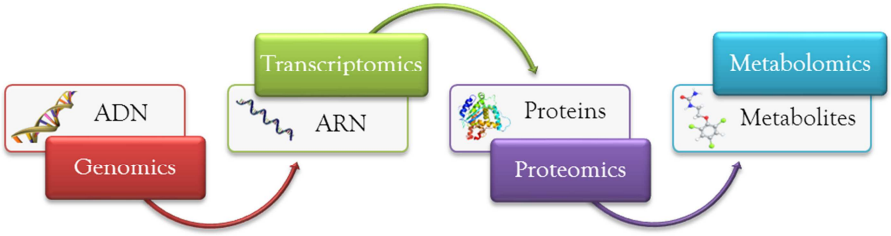

Figure 11. Information flow in biology.

The holistic knowledge of the metabolites implies a very important challenge from a technical point of view. Metabolomics does not only involve the exhaustive profiling of total metabolites in an organism, but also its dynamic variations and its biochemical implications, becoming to an interdisciplinary science that requires knowledge from different fields like organic chemistry, analitycal chemistry, chemometrics, informatics and bioscience.

2.1. Terminology

Metabolomics has been a concept widely defined by several authors; although it was Fiehn one of the first who tried to systematize metabolomics definition, specifying that it is a multifunctional approach which uses different analytical strategies depending on the biological system under study in each case [99]. According to him, metabolomics is "the quantitative and qualitative measure of the whole low molecular weight metabolites synthesized for a given biological system (cell, tissue, fluid, organ, organism, etc.) in a specific biological state"; in other words, it is "the high-throughput measure both qualitative and quantitative of the metabolome of a specific biological system".

Metabolome could be described as the collection of all the small molecules or chemical substances that can be found in a cell, tissue or organism, and that defines its

biochemical phenotypes in response to genetic or environmental changes [100]. These small molecules include a broad range of endogenous and exogenous chemical compounds –peptides, aminoacids, organic acids, vitamins, phenolic compounds, alkaloids, minerals, carbohydrates, etc.– used, consumed or synthesized by a given cell or organism. Theses compounds are very diverse in their physical and chemical properties and they occur in a wide concentration range.

The study of the metabolome can be achieved by using different metabolomics approaches [101-104], which are summarized in ***Figure 12***:

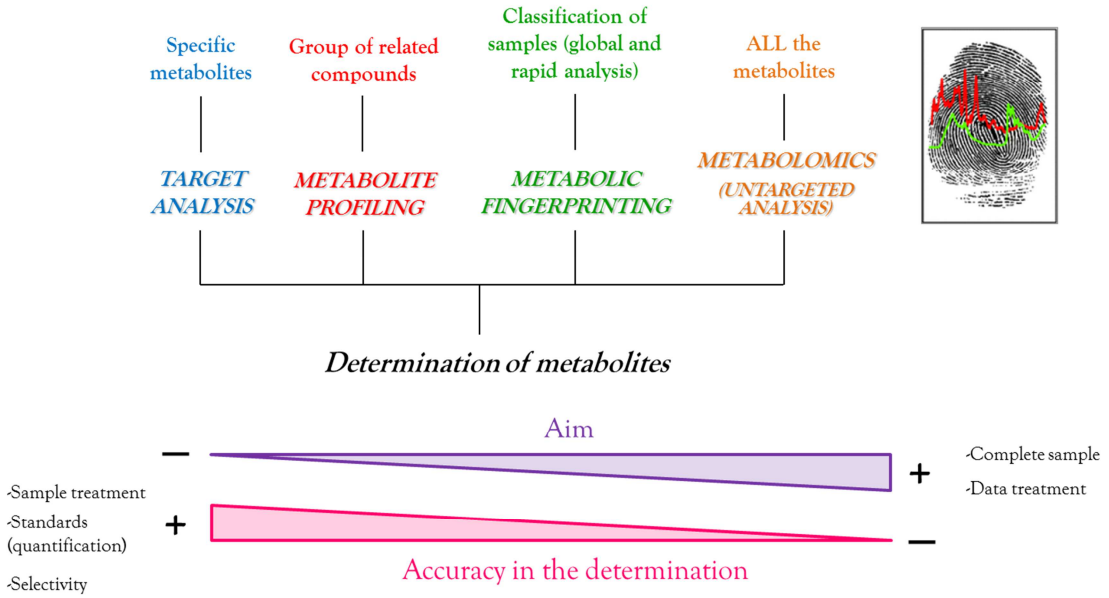

Figure 12. The approaches most used in metabolomics studies.

- Metabolite targeted analysis: It refers to the detection and precise quantification of a single or small group of specific metabolites. A multitude of different analytical methods might be used for this purpose.

- Metabolite/metabolic profiling: Identification and approximate quantification of a set of known and related metabolites, because of its structural or functional similarity, or because they are involved in specific metabolic pathways. A derivation of this term is global metabolic profiling, which refers to the detection of a wide range of metabolites making use of a single analytical technique or a combination of complementary platforms. It aims to detect as many metabolites

as possible, whether these are known or unknown. Profiling is typically done by using a separation technique in combination with MS.

- Metabolic fingerprinting: Rapid, global, high-throughput and direct analysis of crude sample extracts, typically made by NMR (MS and IR spectroscopy can be also used), for sample classification or screening, not being mandatory metabolite identification. Metabolite variation is observed principally on the total spectral pattern changes without the previous knowledge of the compounds investigated. It is extensively applied in biomedical and pharmaceutical investigations, as well as in quality assessment. A derivation of fingerprinting is footprinting, where the cell-free spend media (exometabolome) is analyzed for left metabolites.

- Metabolomics: Qualitative and quantitative analysis of the whole metabolome of a biological system.

2.2. Application fields

Metabolomics has been an application-driven science with broad range of applications in several disciplines. To date, most of the work in metabolomics has primarily focused on clinical or pharmaceutical applications such as drug discovery, drug assessment, clinical toxicology and clinical chemistry [105-107]. However, in the last years metabolomics has emerged as an interesting tool in fields such as plant and food sciences and nutrition research [100, 105]. ***Table 4*** shows some of the main application areas of metabolomics nowadays [108].

Table 4. Summary of the most important applications fields of metabolomics.

Metabolomics applications	
Toxicity assessment	Integrated systems biology
Medical diagnosis	Environmental adaptations
Pharmaceutical drug development	Nutrition assessment
Biomarker discovery	Food assessment and safety
Plant breeding and assessment of crop quality	Technological advances in Analytical Chemistry

Plant metabolomics goes beyond the simple identification of plant constituents. Recently, it has been used for the characterization of pathways and metabolite network behaviors in plants that suffered for environmental stress; for taxonomic or biochemical purposes; for discovery of gene functions and elucidation of regulatory metabolism in a metabolic network; as well as for studying developmental processes such as establishment of symbiotic associations [98, 102]. Furthermore, plants –being nature's most prolific biochemists– are a long-standing source of inspiration for industrial chemists in the search for novel bioactive compounds that may be the basis for the mass production of new synthetic drugs for the pharmaceutical industry, or biocides such as fungicides and insecticides for use in agriculture. In addition, one of the most important application fields of plant metabolomics is the quality assessment of crops, which is a direct function of metabolite content. There is therefore great interest in using metabolomics approaches to understand better what especially has occurred during crop domestication. This allows designing new concepts for more targeted crop improvement to current needs, not only from a product quality point of view, but also to tackle problems related to crop fitness (disease resistance and its molecular basis, plant–insect interactions, mycorhizal interactions, etc). Through these approaches we are gradually gaining a much more detailed picture of crop biochemistry and its link to nutritional, sensory and other crop-related characteristics [109, 110]. Furthermore, it is important to bear in mind that, nowadays, agricultural crops are increasingly viewed as an important pillar for a plant-based economy, because different parts of the plant –both the fruits (seed, peel, pulp) and the trees– can be processed and used to obtain industrial products (including polymers, fibers, latex, industrial oils, packaging materials, and basic chemical building blocks); fuels, biodiesel and biomass; and food (both traditional and those with enhanced nutritional, safety, stability, processability and other desirable characteristics).

Plant and food metabolomics are closely related since plant-based products comprise a significant part of the human food intake almost throughout the world [111, 112]. The edible plant metabolome contains more than 10,000 different detectable metabolites, of which 2,000 are nutrient compounds and more than 8,000 are non-nutrient phytochemicals. This fact reflects that the food metabolome is characterized by considerable chemical diversity of the compounds and also by considerable variations in

the dynamic concentration range in which these metabolites could be found (from millimolar to femtomollar) [100, 113].

Metabolomics tools have gained a very important role in food and nutrition science because they allow studying many aspects of "molecular nutrition", which could be classified in several groups: (i) Food component analysis; (ii) physiological monitoring in food intervention or diet challenge studies/bioavailability studies; (iii) food quality and safety; (iv) food authenticity. Some of the most relevant applications of metabolomics in food and nutrition area are listed in ***Figure 13*** [100, 114].

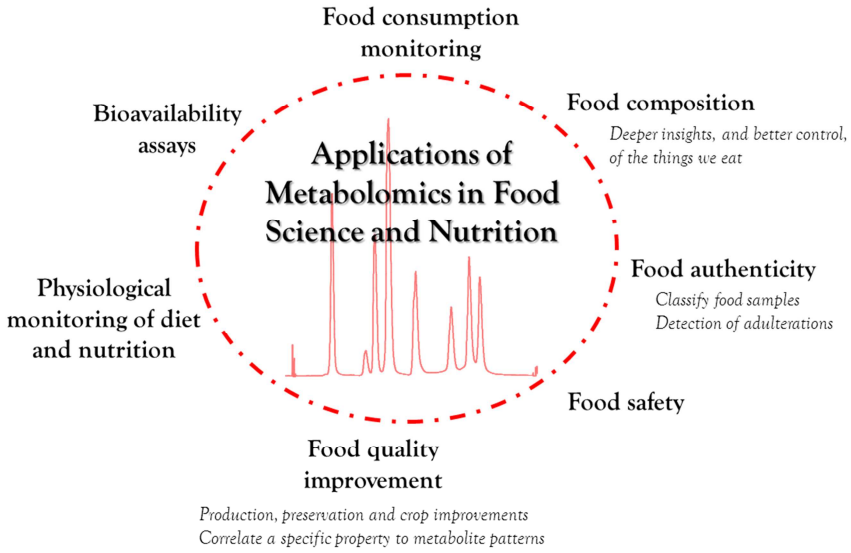

Figure 13. Applications of metabolomics in the field of nutrition and food science.

For food products, the biochemical composition determines all aspects of nutritional value, taste and fragrance, appearance, etc., as well as potentially negative aspects such as toxicity. Therefore, metabolomics offers food chemists the opportunity to deconstruct foods into their chemical constituents with more precision for a better knowledge and understanding of all the molecular details that are responsible of the specific foods and beverages properties. Besides, food composition can be also used to control food quality and thus to prevent frauds, problem that is significantly growing in today´s global market because of the different food adulterations that are performed to reduce the price of the production [115].

Nowadays, food metabolomics allows going deep into the study of how different cooking processes, preservation/storage conditions, etc., have influence on the

concentration of some of the food metabolites too. Furthermore, it will assist the food processing industry to improve further food processing strategies and optimize the individual steps involved. Consequently, the industry will gain a better position to serve the consumer by providing more nutritious, healthy and attractive products that may also have longer shelf lives and involve less waste.

Over the past century, food scientists and biochemists have made great advances in the identification of the essential nutrients needed for the human growth and maintenance of the organism. However, in the last decades the easier access to foods has favored nutritional "over-sufficiencies", which in many cases are related to several diseases such as obesity, diabetes, chronic inflammation or cardiovascular diseases, among others. For this reason, today, the main objective of food scientists is to try to identify bioactive food components that potentially increase life expectancy, reduce weight, enhance physical or mental performance and also that allow preventing or treating the above mentioned diseases [116, 117].

Fruits and vegetables have historically been considered rich sources of some essential dietary micronutrients and fibers, and, more recently, they have been recognized as important sources of a wide variety of phytochemicals, which are secondary metabolites (non-nutrient compounds) synthesized by plants that mediate its interaction with the environment [118, 119]. Numerous epidemiological studies have shown an inverse association between fruit and vegetable consumption and chronic diseases, mainly due to the protective effect of phytochemicals [120, 121]. It has been highlighted that the beneficial properties attributed to the consumption of fruits and vegetables are due to the existing synergy among some of the metabolites present in these matrices, rather than the effect caused by each individual compound [6, 121]. Therefore, it is important to link food and health from a global point of view and, to accomplish it, new trends in food science allow going from the deep characterization of food to the evidences about its healthy effects and understanding of its metabolism [122-124]. In this sense, a new concept has been defined: *Foodomics* [125], an emerging discipline that combines all of the modern *omics* technologies for the profiling of food compounds with other tools such as bioinformatics (detection of biomarkers for food quality or safety), toxicity assays (food

contaminants), *in-vitro/vivo* assays (bioactivity) or clinical trials to determine its possible healthy effects [126-128].

2.3. Workflow in metabolomics studies

Regardless of the approach used, the metabolomics workflow is composed by different stages (***Figure 14***), which includes different steps that should be carefully designed and executed to provide valid datasets and, subsequently, valid experimental conclusions to reach a final interpretation for an initial biological question [103].

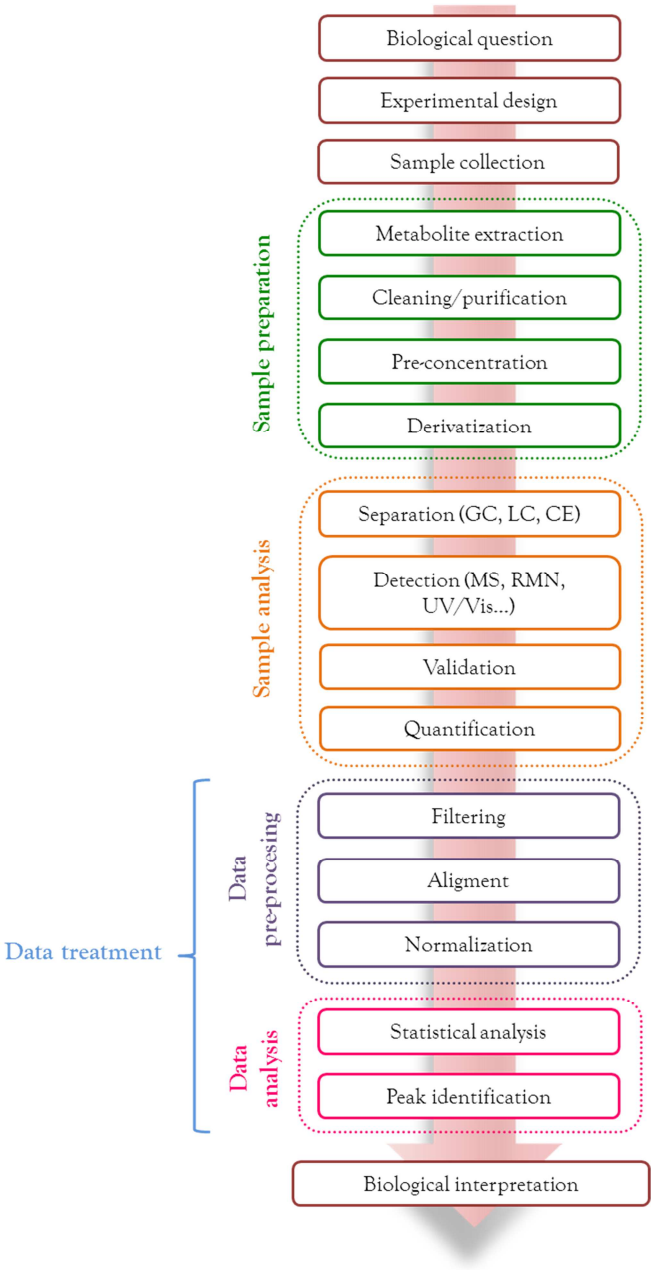

Figure 14. Diagram of the possible necessary steps to carry out a metabolomics experiment.

2.3.1. Experimental design

As it is appreciated in the previous figure, one of the first steps of the metabolomic workflow is the experimental design. The main goal of this phase is to plan experiments in the most efficient way to obtain data that describe the relationship between the different factors/variables affecting a process and its output. Thus, it should be considered the type of information needed, the kind of chemistry expected and the analytical facilities available [129].

Typically, once the biological question has been clearly formulated, one needs to choose the most appropriate biological resource and analytical technology. When a metabolomics study is designed, the analytical options are usually selected according to the type of metabolites that are most likely to be of interest to the analyst. It is necessary to understand the limitations of the methods we are using at each step of the experimentation, and then formulate the most appropriate experimental design [130]. ***Figure 15*** shows, as a particular example, the list of items to be considered before starting a plant metabolomics experiment.

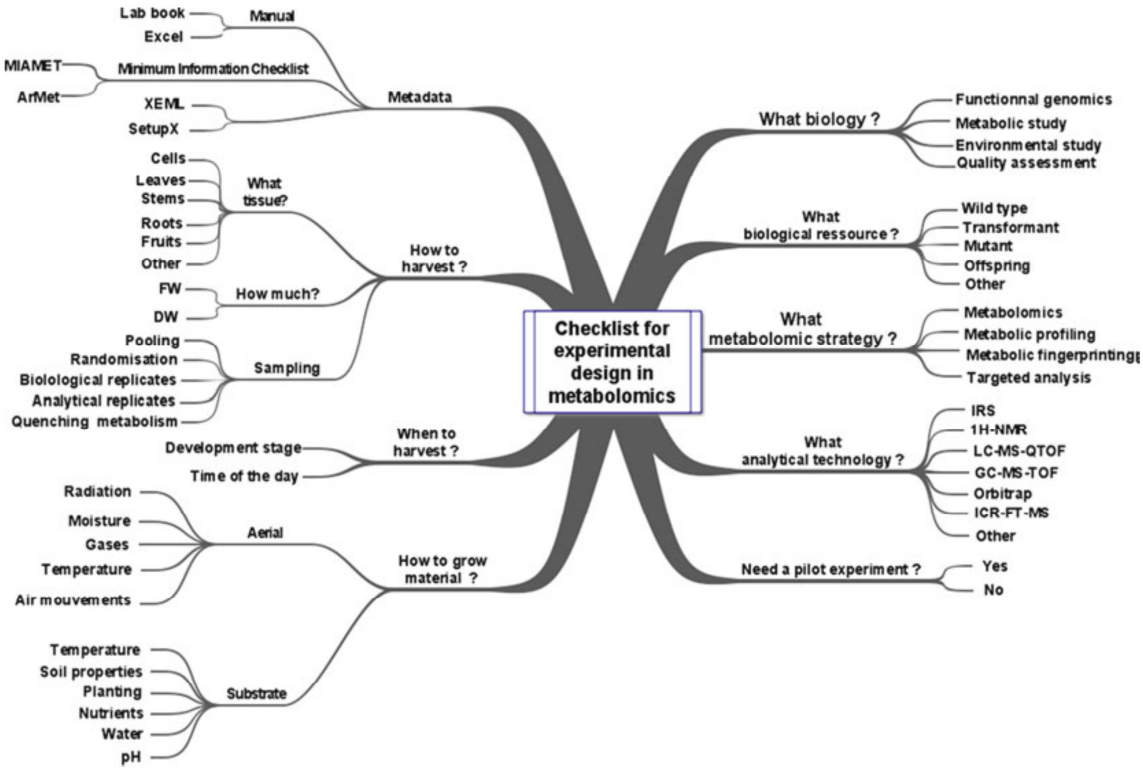

Figure 15. Example of an experimental design in plant metabolomics [131].

2.3.2. Sample collection

The following stage is the sample collection, which can greatly influence the reproducibility of the metabolomic analysis. Sampling aims to provide samples in a form and amount suitable for metabolomics analysis and which are representative of the total material to be analyzed. Representative means that the sample has to show a high degree of similarity to the total entity to be studied. Careful attention should be paid to sampling to reduce experimental errors. Sample collection not only involves sampling, but also their handling and storage. It is necessary that the conditions and duration of sample storage are properly controlled and recorded, since stability during sample storage is an important factor that is rarely measured. Any change in metabolite levels as a consequence of storage could therefore create unnecessary and unwanted trends across a sample dataset. Depending on the intended analyses, samples can be stored as fresh-frozen, freeze-dried, etc. Freeze-drying, also so-called lyophylization, is a common process of preservation and storage applied both in plant and food metabolomics, because it removes water from aqueous samples avoiding its degradation. Besides, the absence of water in samples makes possible storage almost indefinite –if a cold, dry and neutral atmosphere is maintained– and also a higher stability of the metabolites [131, 132].

2.3.3. Sample preparation

Among all the steps involve in a metabolomics workflow, it is noteworthy that the major cause of errors in the generation of analytical results is still linked to sample preparation. The preparation of the samples for metabolomics research highly depends on the purposes of the study. This stage comprises processes as metabolite extraction, cleaning, purification, derivatization and pre-concentration, although all of them are not always needed. Metabolite extraction and pre-concentration are, probably, the most frequent ones [114, 130]. The principal aims of the sample preparation are (i) to extract efficiently the metabolites present in a sample; (ii) to eliminate interferents (salts, proteins…) that can complicate the following analysis; (iii) to achieve an extract compatible with the analytical technique chosen, (considering for instance possible solvent restrictions); and (iv) to concentrate the metabolites [133].

Numerous metabolites contained in plants and food samples are dispersed within a complex solid or liquid matrix, and analytical instruments usually require liquid samples.

Thus, one of the aims of sample extraction is to transfer the metabolites of interest from the complex food/plant matrix into a homogeneous liquid phase suitable for instrumental analysis. To carry out a proper sample preparation it is important to bear in mind the type of metabolomics approach that is going to be followed. If it is a targeted analysis, the sample preparation should be selective for the metabolites under study and reproducible, and it should allow the incorporation of other steps (clean-up, pre-concentration, quenching, etc.) if they are required. However, if the metabolomics approach is untargeted, the preparation of the samples has to be unselective, simple, fast, and reproducible to assure the detection of a high number of metabolites [104], because as it has been mentioned throughout this section, plant and food metabolomes are characterized by the presence of a wide variety of compounds –chemically diverses– in a broad range of concentrations. For this reason, it is a challenge to carry out the sample preparation to extract the majority of them, because there is not any single extraction procedure that covers all the metabolites [110]. In general, it is also important that the sample preparation method is simple and fast (to avoid metabolite loss or degradation during the preparation), reproducible and robust [134]. The most common techniques applied for metabolite extraction are: SLE, LLE, PLE, SFE, SPE, SPME, MAE, membrane methods (dialysis or ultracentrifugation), etc. [101, 133, 135].

2.3.4. Sample analysis

After preparing the sample and extract the metabolites of interest, it is crucial to perform the sample analysis, which comprises a big group of procedures. Metabolite separation and detection are considered as two key steps in metabolomics research, and the instruments selected to carry out this kind of studies should present some general requirements [136]:

- ✓ Excellent sensitivity and resolution for a wide range of compounds.
- ✓ The ability to handle a large range of concentrations for metabolites belonging to different chemical groups.
- ✓ The ability to identify and quantify different molecules.
- ✓ Short analysis time.
- ✓ Reproducible measurements.

Usually, sample analysis combines the use of different analytical techniques, such as spectroscopic (MS, NMR, IR, FL, among others); electrochemical (voltammetry, conductimetry); separation (CE, GC, LC); or hyphenated techniques. All of them have been applied to a greater or lesser extent for food and plant analysis, even though NMR and hyphenated techniques –combining a separation technique with MS as detection system– are the most common ones [130, 137, 138]. Metabolomics was first developed with NMR, because it is a technique that presents numerous strengths, such as speed, non-discriminating and non-destructive to samples, provide qualitative and quantitative information on hundreds of different metabolites present in a sample, applicable to intact biomaterials and minimal sample preparation. However, it is also characterized by a low sensitivity, difficulty to detect low-abundance metabolites and a high cost [101, 117, 139]. On the contrary, MS is an analytical tool that offers quantitative analysis with high selectivity and sensitivity and the potential to identify metabolites. Unlike NMR, it is a destructive technique and requires a sample preparation step, which can cause metabolite losses. Besides, depending on the sample introduction system and the ionization source used, specific metabolite classes may be discriminated. Despite the "disadvantages" that MS possess, in the last few years, it has been the analytical technique most frequently used in metabolomics, both hyphenated or not [101, 117]. It is usually combined with a separation technique (LC, GC or CE) to reduce the complexity of the mass spectra, due to metabolite separation in a time dimension provides isobar separation and delivers additional information on the physicochemical properties of the metabolites [132, 140]. Among these couplings, GC-MS and LC-MS have been the most extensively used for metabolomics studies of different samples [139, 141-143]. However, CE-MS has been applied to a lesser extent and its use in metabolomics studies is still limited because of its inherent weakness; although the interest on this analytical technique to perform metabolic profiling has recently grown, mainly due to the technological and methodological developments related, above all, with new and novel interfaces for CE-MS coupling [144]. The selection of the most suitable technique is generally a compromise solution between speed, selectivity and sensitivity [98, 101, 103].

The combination of a separation technique and a mass spectrometer is possible making use of an interface, also called ionization source, where the analyzed samples are ionized prior to its detection on the mass spectrometer [145]. Ionization is a process by

which sample molecules or atoms are converted into gas-phase ionic species through different mechanisms [146]. There is a wide variety of available ionization techniques; some of them are very energetic –causing extensive fragmentation–, whilst others are softer and only produce ions of the molecular species. ***Table 5*** gathers some of the ionization modes most applied to couple GC/LC/CE to MS [134, 147].

Table 5. Ionization techniques most commonly used with GC-MS, LC-MS and CE-MS.

Ion source	*Description*
APCI	A corona discharge needle provides ionization of the solvent molecules, which are in excess. The plasma so formed, contains both solvent and oxygen and nitrogen radical ions, which react with traces of water to form $H3O^+$ and OH^-, as well as other ions. These undergo ion/molecule reactions with the analyte to form $[M+H]^+$ and $[M-H]^-$ species.
APPI	The analyte is sprayed into the source in a solvent frequently containing a dopant molecule. The spray is irradiated by a powerful UV source that forms excited species which undergo secondary ion/radical molecule reactions with the analytes to cause ionization.
ESI	The eluent of the capillary or chromatographic column is sprayed at atmospheric pressure from a fine needle held at a high voltage. With the addition of heat and drying inert gases, the solvent is evaporated and a series of fine charged droplets is formed. Then, the coulombic forces between ions of similar charge on the surface exceed the surface tension of the liquid and spawn ever smaller droplets. The successive desolvation processes provide transfer of ions from the liquid to the gas phase and their introduction into the MS vacuum.
CI	Chemical reagent gases are introduced into an EI source, in large excess over metabolite molecules, and the reagent gas molecules are ionized by electron bombardment. Ion or charge transfer induces ionization of metabolites. The product ion is an even-electron ion with low internal energy and undergoes little fragmentation.
EI	Bombardment of the molecules by high-energy electrons creates positively charged ions. It is characterized by a heavy fragmentation highly reproducible.

The selection of a specific ionization technique are mainly driven by the physicochemical properties of the samples under study, as well as by the type of information required. It is important to bear in mind that the ionization system chosen can considerably affect the detection of the metabolites. ***Figure 16*** shows a scheme of the ranges of molecular weights and polarities cover by different ionization systems.

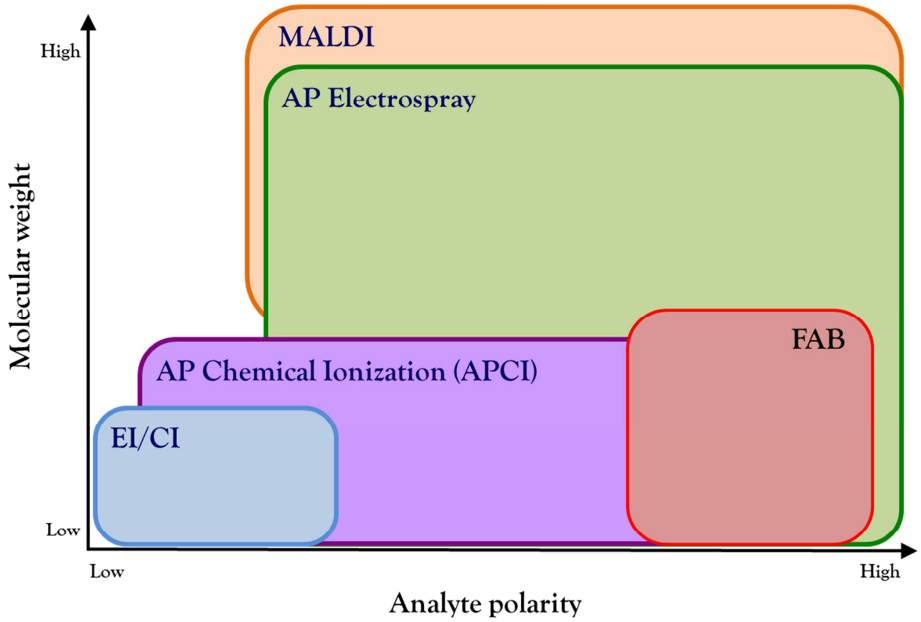

Figure 16. Range of work of some ionization sources.

In relation to the mass analyzer, there are numerous systems that can be used, which vary in terms of size, price, resolution, mass range, resolving power, scan rate, mass accuracy and the ability to perform tandem mass spectrometry experiments (MS/MS). Nowadays, the most frequently ones are Q analyzers, Q-IT, TOF, FT-ICR, orbitrap and IMS [134, 145].

At this point, it is very necessary to take again into account one of the most important limitations of metabolomics studies, which is related to the chemical complexity of the metabolome, the biological variance inherent in most living organism, and the dynamic range limitations of most instrumental approaches. The chemical properties of the metabolites range from ionic inorganic species to hydrophilic carbohydrates, hydrophobic lipids, and complex natural products. The chemical diversity and complexity of the metabolome make to profile all the metabolome simultaneously extremely challenging [139]. For this reason, currently, no single analytical technique provides the ability to profile all the metabolome [98, 103], being essential to make use of different analytical platforms, which can offer complementary information that allows enlarging the knowledge about the complete metabolome of different biological systems [148].

2.3.5. Data treatment

The final step on the metabolomics workflow is the data treatment, which comprises both data pre-processing and data analysis. It is a very important process in metabolomics research –as in all the *omics* sciences– because of the large amounts of data generated during the previous phase, meaning that it will rely heavily upon bioinformatics to convert the complex raw data achieved into useful information. Within data treatment, data pre-processing is essential to yield adequate data to work with, whereas data analysis will allow us to find significant changes and to validate the data obtained with the biological samples [98, 138, 149].

In chemometrics, pre-processing covers all editing of the data up to the point of starting the subsequent data analysis. There are several applicable methods or techniques to the dataset that will make samples analyzable and comparable: binning, peak alignment, baseline correction, noise reduction, normalization, scaling and smoothing. Usually, all this processes are carried out on computers by means of different commercial or homemade developed software. Depending on the analytical technique chosen for the metabolomic study, the number of pre-processing steps required for the following data analysis varies and its order of execution is not always obvious [149, 150].

Due to the generated datasets are quite complex and contain important amounts of information, data analysis cannot be handled manually and without the use of statistic tools. It is frequently performed through multivariate analysis, which is simply the simultaneous statistical analysis of more than one statistical variable, instead of traditional statistical methods that deal with single variables or small groups of variables. In this sense, multivariate analysis is extremely useful since it reduces the dimensionality of the data without losing information [150, 151].

Pattern recognition techniques are the chemometrics tools most extensively used among the multiple methods that multivariate statistics include, and its main objective is to identify similarities or ways of behavior of the analyzed samples or measured variables on a data matrix. It is possible to distinguish between unsupervised and supervised pattern recognition methods [152, 153].

Unsupervised methods try to establish the existence of groups of samples in the data matrix taking into account similarities between them, without prior information about these similarities; in other words, the modeling is done without user intervention and solely on the explanatory variables. Methods like PCA or HCA stand out among the different unsupervised approaches that are popularly applied for data analysis in metabolomics [98, 150]. By contrast, the aim of supervised pattern recognition methods is to establish implicit or explicit classification models. In this type of methods samples that belong to a particular class or group are known and they are used to create a sample set ("training" set) that allow building a classification model. This classification model is then used to predict membership in one sample which was not included in the training set. A validation set (independent of the training set) is used to validate the classification model. Within this group, the methods most commonly used are: DA, K-NN, SIMCA and PLS-DA [98, 150, 152].

Both plant and food metabolomics tend to make use of PCA and PLS-DA, which are essential tools for rapid interpretation of information-rich spectral datasets for inferring biological/chemical conclusions. In general, multivariate analysis can enormously facilitate the biological understanding and exploration of complex, multiparametric metabolic systems. Although bioinformatics tools have experienced an impressive progress in the last years, it is fundamental that the rest of the procedures/techniques involved in a metabolomics study continue improving and advancing to assure and enable the visualization of a greater proportion of the metabolome at greater speeds.

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EXPERIMENTAL PART, RESULTS AND DISCUSSION

In this section, "Experimental part, results and discussion", the results achieved during the realization of the present Doctoral Thesis are discussed. It is divided in 3 main parts: Sections I, II and III. Each of them is dedicated to one of the 3 different analytical techniques used to perform metabolomics studies in avocado fruit. A brief introduction –where the main objectives and motivations for carrying out each experimental work are summarized– and several chapters –describing the experimental work carried out– are included in every section. To conclude, at the end of this "Experimental part, results and discussion" a detailed comparison among the techniques is made, giving a global vision about the complementary information that it is possible to obtain by using different platforms.

To carry out separation processes, Analytical Chemistry can make use of numerous techniques, which allow separating different compounds found in a sample on the basis of the existing differences on their physicochemical properties. In this Doctoral Thesis, we are focused on the use of chromatographic (LC and GC) and non-chromatographic (CE) techniques, that separate different compounds according to a selective retention principle or to a size/charge ratio, respectively. Usually, LC, GC and CE are coupled to a detection system that facilitates the identification and quantification of the compounds under study. Among all the possible couplings, during this PhD work special attention has been paid to MS because of its versatility, sensitivity and specificity, among other advantages; but not leaving other "classical" detectors aside, such as UV detection or FID. All these analytical platforms can be applied, to a lesser or greater extent, for studying the metabolome of a plant or food. As has been described in the Introduction, there are several metabolomics approaches that can be followed. In the different metabolomics studies compiled in this section, targeted metabolite analysis of avocado are found in Section I, whereas untargeted analysis, principally as global metabolic profiling, are enclosed both in Section II and III.

The first section (I) deals with CE –which is a non-chromatographic separation technique– coupled to diverse detection systems (UV and MS). It is possible to find 3 chapters, being the first one a bibliographic revision. The other two chapters enclose the optimization of two electrophoretic methods for analyzing small groups of metabolites in avocado fruit and the study of its evolution during the ripening process.

Section II and III are dedicated to chromatographic techniques. In chromatography, compounds are distributed between two phases, one of which is stationary (stationary phase) whilst the other (the mobile phase) moves in a specific direction. Depending on the physical state of the mobile phase, different types of chromatographic techniques can be found. Two of them are extensively used along the Doctoral Thesis to perform some metabolic studies of avocado fruit, specifically LC and GC. The LC part (section II) contains 3 chapters where different methodologies are proposed for the study of the avocado metabolome. The third section presents 5 chapters about GC and its coupling to different detectors, such as MS and FID. In the case of GC-MS, the coupling is performed by using 2 ionization systems. One of them is quite novel, because it is not a very common interface for GC-MS (APCI), and for this reason a chapter of this section is dedicated to the development of a database for this new GC-APCI-MS platform that helps to study the metabolome of avocado in subsequent works.

As aforementioned, to conclude the "Experimental part, results and discussion" an overview of the principal advantages or disadvantages of each technique is given, primarily considering the results obtained in this PhD work. An exhaustive comparison between them is not pursued, but it is tried to show the existing complementarity and the beneficial use of different approaches to achieve a better knowledge about the complete metabolome of a fruit.

Section I: CAPILLARY ELECTROPHORESIS

CE is an analytical separation technique that emerged on 1930 as a result of the work of Arne Tiselius. It is based on the different mobility that the compounds present inside a capillary tube –filled with the buffer solution– under the effect of an electric field. Despite the apparition of new and novel analytical platform, CE is still a usual tool in analytical chemistry because of its high speed of analysis, its high efficiency, and its automation simplicity. Moreover, CE is also characterized for the necessity of small volumes of sample and solvent.

The numerous advantages of CE make possible its utilization in a wide variety of applications, many of them in the area of food science. For this reason, it can be beneficial to start performing an exhaustive bibliographic revision that allow us obtaining a holistic vision of all the applications –published till 2010 (date of the article publication)– of CE (coupled to different detection systems) for the determination of different phenolic compounds in food derived from plants, which is presented in ***Chapter 1***. The revision has focused on the phenolic compounds, because they are substances highly present in the vegetable kingdom due to its participation in the plant development and protection against oxidation.

Considering all the information compiled in chapter 1 and the scarce number of applications of CE for the analysis of phenolic compounds in matrices such as avocado fruit, ***Chapter 2*** is dedicated to the optimization of a CE-UV method to determine, both qualitative and quantitatively, several phenolic acids present in 13 varieties of avocado fruit at two different ripening degrees. A statistic treatment has applied trying to achieve valuable information that gives us information about the most influential compounds in the differentiation between varieties or ripening stages. It is a target metabolic analysis, because only a group of compounds chemically related is analyzed: phenolic acids, which have been chosen for being small and charged molecules that can be determined with by CE-UV, for being metabolites previously found in avocado fruit, and also for existing as pure standards commercially available, which helps to the identification of compounds by UV. The interest of this work lies in the fact that it is the first time that an electrophoretic method coupled to UV detection together with statistical tools is used to characterize the phenolic acid profile of *Persea americana*.

In ***Chapter 3*** the main objective is to study the metabolic evolution of an important number of samples, that belong to a single variety of avocado but they have been picked at different moments of the harvest time, taking into account the behavior of 10 specific metabolites from diverse chemical groups. To reach our goal, two different CE-MS approaches (using a *Full Scan* or a *MRM* MS method) have carefully optimized and applied to the analysis of the samples under study. The two methodologies have provided quantitative information about the evolution of the selected metabolites during the process of ripeness of Reed variety. Besides, LC-MS has been used with the intention to confirm the results achieved by CE-MS and also to identify some unknown metabolites whose concentration has remarkably varied during the ripening.

Chapter 1

Application and potential of capillary electroseparation methods to determine antioxidant phenolic compounds from plant food material

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Abstract

Antioxidants are one of the most common active ingredients of nutritionally functional foods which can play an important role in the prevention of oxidation and cellular damage inhibiting or delaying the oxidative processes. In recent years there has been an increased interest in the application of antioxidants to medical treatment as information is constantly gathered linking the development of human diseases to oxidative stress.

Within antioxidants, phenolic molecules are an important category of compounds, commonly present in a wide variety of plant food materials. Their correct determination is pivotal nowadays and involves their extraction from the sample, analytical separation, identification, quantification and interpretation of the data.

The aim of this review is to provide an overview about all the necessary steps of any analytical procedure to achieve the determination of phenolic compounds from plant matrices, paying particular attention to the application and potential of capillary electroseparation methods. Since it is quite complicated to establish a classification of plant food material, and to structure the current review, we will group the different matrices as follows: fruits, vegetables, herbs, spices and medicinal plants, beverages, vegetable oils, cereals, legumes and nuts and other matrices (including cocoa beans and bee products). At the end of the overview, we include two sections to explain the usefulness of the data about phenols provided by capillary electrophoresis and the newest trends.

Keywords: Phenolic compounds / Capillary electrophoresis / Plant food material

1.1. Introduction

Phytochemicals are a big group of non-nutrient substances present in plants, which are biologically active and have an important role in the interaction of plants with their environment as well as reveal health promoting impacts [1]. Several compounds included in this group are considered antioxidants, owing to the fact that these compounds have a capacity to protect cells and biomacromolecules [2] neutralizing free radicals and, for this reason, they may prevent against oxidative degradation and certain human diseases

(cancer, inflammatory disorders, neurological degeneration, coronary heart diseases, etc.) [3]. As a consequence, antioxidant compounds are nowadays gaining more and more interest and the consumption of food rich in antioxidants is increasing. Vegetables, fruits and other plant matrices are some of the most important sources of natural antioxidants [4].

Antioxidants can be classified taking into account their mechanism of action, although there are other possible classifications. Bearing in mind the mechanism of action, they can be divided into primary antioxidants, synergistic and secondary antioxidants [5]. Some substances considered as antioxidants are ascorbate, tocopherols, some enzymes, carotenoids and bioactive plant phenols. The health benefits of fruits and vegetables are largely due to the antioxidant vitamin content supported by a large number of phytochemicals, some with greater antioxidant properties. Sources of tocopherols, carotenoids and ascorbic acid are well known and there is a great number of publications related to their role in human health. However, plant phenols have not been completely studied because of the complexity of their chemical nature and the extended occurrence in plant materials.

Phenolic compounds are one of the most important, numerous and ubiquitous groups of compounds in the vegetable kingdom, being synthesized by plants during normal development and in response to different situations (stress, UV radiation, etc.) [6]. These substances are obtained from carbohydrates via the shikimate pathway and phenylpropanoid metabolism [7, 8] and there are more than 8000 different known structures [3]. However, this large range of structures possess a common structural feature: an aromatic ring with one or more hydroxy substituents. The way to classify these components it is not clearly established; a possible classification can be based on the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton, including for instance, simple phenols, phenolic acids, coumarins, flavonoids and stilbenes. [9] (see ***Figure 1.1***).

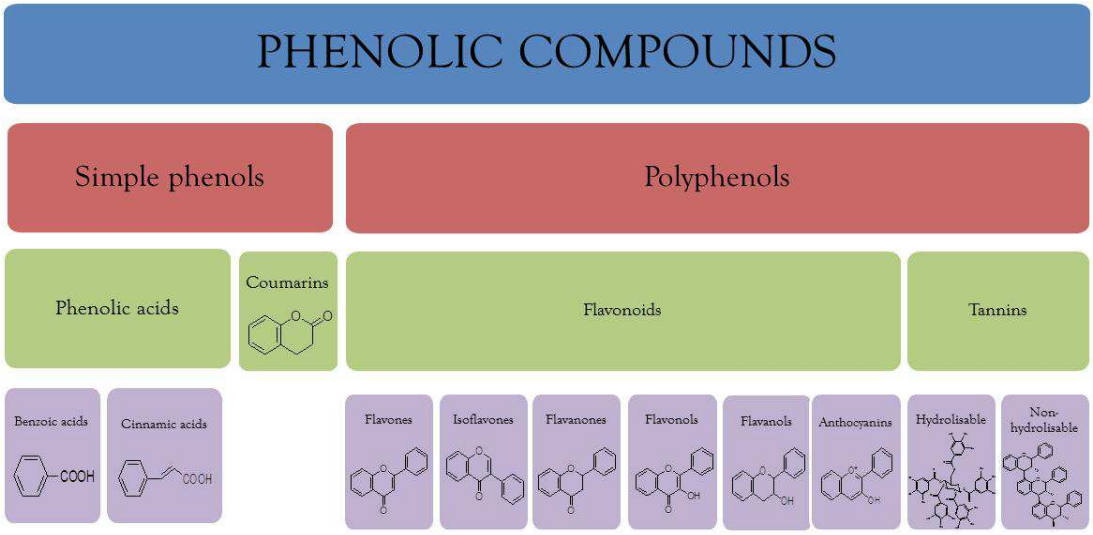

Figure 1.1. A simplified classification of phenolic compounds and representative structures belonging to benzoic acids, hydroxycinnamic acids, flavones, isoflavones, flavanones, flavonols, flavanols, anthocyanins and tannins. Only the basic skeletons from where phenolic compounds of vegetal origin are derived are shown.

Phenolic compounds play an important role in plants, foods and humans. In plants, these compounds carry out diverse functions, such as protective agents against UV light, take part in growth and reproduction, components of pigments, essences, flavours, etc. In food and beverages, phenolic compounds may contribute to the oxidative stability and organoleptic characteristics (bitterness, flavour, astringency...) [10]. Phenols act in human bodies providing numerous beneficial effects, due to the fact that they have anti-microbial [11], cardioprotectives, anti-allergenics and anti-inflamatories activities [12, 13]. Since 1980, several studies have shown that lower risk of chronic diseases was correlated with a diet rich in fruits and vegetables [14, 15].

Therefore, the determination of this family of compounds is of special relevance. In principle, a wide range of analytical methods can be used to determine these natural compounds, but it is important to bear in mind that the complexity of the matrix, which generally contains these compounds, makes mandatory the use of separative techniques with high resolving power. The most used techniques have been chromatographic techniques (TLC, HPLC, GC), electroseparation methodologies (MEKC, CITP, CE, CZE), combined methods (CEC) and spectroscopic methods (MS, NMR, UV, IR, NIR) [16].

The aim of this review is to describe the different CE methods that have been used so far to carry out the determination of phenolic compounds in plant food matrices. Since, in general, any analytical procedure for the determination of individual phenolic compounds involves four basic steps: extraction from the sample, analytical separation, quantification and interpretation of generated data (achieving worth conclusions). These steps will be explained in later sections of this article, paying particular attention to the separation step and the different CE methodologies (coupled to several detection systems) reported to date. In ***Figure 1.2***, a valid scheme for any method developed for the isolation and determination of polyphenols from plant food material using CE as analytical tool is presented.

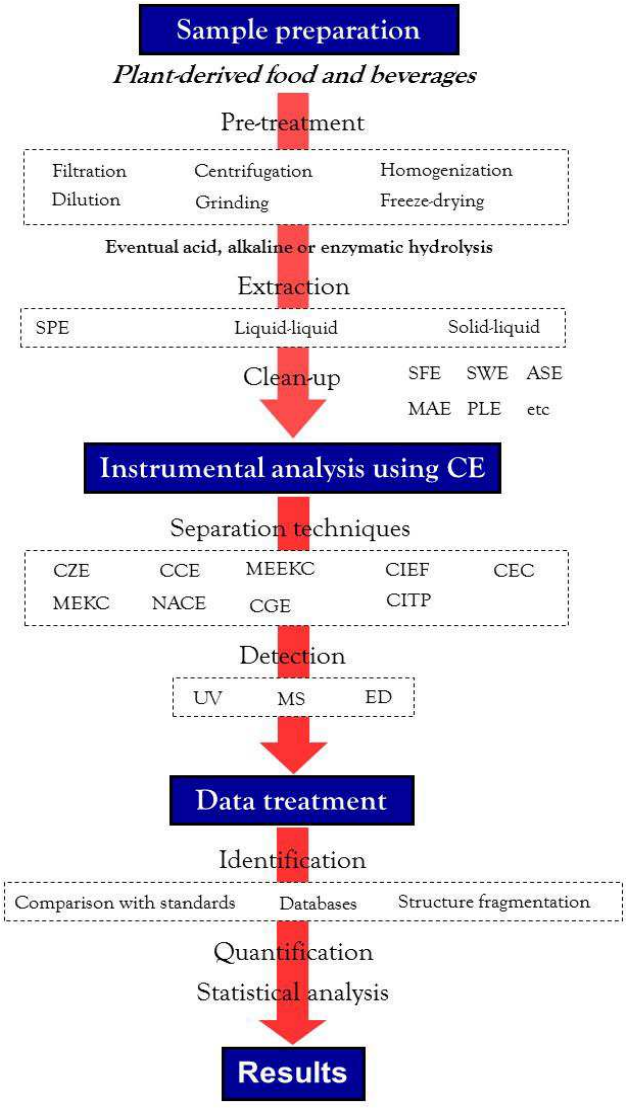

Figure 1.2. Complete analytical procedure to achieve the determination of phenolic compounds present in plant food material, including sample preparation, instrumental analysis (by means of electroseparation methods) and data treatment.

1.2. Sample preparation

Although the advances in modern analytical techniques have led to significant improvement in the quality of analysis, the importance of sample peparation should not be underestimated; isolation of the phenolic compounds from the sample matrix is generally necessary to succeed in any analysis. The ultimate goal when the analyst face the sample preparation step is clear: to achieve the preparation of a sample extract uniformly enriched in all components of interest and free from interfering matrix components [17]. It covers several steps ranging from exhaustive solvent extraction and preconcentration procedures to simple LLE or filtration [9]. The precise procedure will depend on the nature of both the analyte (for instance, total phenols, *o*-diphenols, specific phenolic classes or individual antioxidants), and the sample (fruit or vegetable type, or portion (skin, leaf, seed...)).

Traditional procedures include homogenization, filtration/centrifugation, distillation, solvent and Soxhlet extraction, among others. The introduction of SPE, SFE, SPME, PLE, SWE, MAE, membrane extraction and surfactant cloud point extraction have met the increasing demand for new and more sofisticated techniques, which can be fully automated and consume less amount of solvent [8, 18].

Isolation of phenolic compounds from fruits is further complicated by the unequal distribution in various structural forms; accumulation of soluble phenols is greater in the outer tissues (epidermal and subepidermal layers) of the fruit than in the inner tissues (mesocarp and pulp) [19].

Usually solid samples are first homogenized, which may be preceded by (freeze-) drying or freezing with liquid nitrogen. The next step is the analytical isolation; to achieve that purpose, solvent extraction, which may be followed by SPE, is still the most widely used technique due to its ease of use and wide-ranging applicability. In the case of liquid matrices, they are usually filtered and/or centrifugated; afterwards the sample is either directly injected into the separation systems or, more often, the sample undergo to LLE or SPE procedures.

Solubility of antioxidants is governed by their chemical nature in the plant that may vary remarkably; moreover there is a possibility of interaction between phenols and other

plant components, such as carbohydrates and proteins. Solubility of phenolic compounds is also affected by the polarity of the solvent(s) used; therefore it is very difficult to develop an extraction procedure suitable to recover all plant phenols [6].

Solvents, such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformamide and their combinations, have also been used for the extraction of phenolics, often with different proportions of water [8]. In those cases, the recovery of polyphenols from the plant material under study was influenced by the extraction time, number of extraction stages, ratio of solvent-to-sample, sample particle size, temperature, etc., and thus the analyst should check the influence of all the mentioned parameters to assure high recoveries.

Interesting applications can be mentioned as well for SPE [20, 21], SWE [22, 23], ASE or PLE [23, 24] or MAE [25].

In a number of instances, an hydrolysis step has to be included, mainly as an aid to structural elucidation and characterization of phenolic glycosides and phenolic choline esters [26]. Three types of hydrolytic treatment are used for this purpose: acidic, enzymatic and alkaline. Hydrolysis could also be used to minimise interferences in subsequent analytical separation and to simplify the data obtained.

An in-depth discussion about the diverse extraction systems, which can be used to carry out the sample preparation, is beyond the scope of this manuscript (please, check the following comprehensive reviews [6, 8, 9, 17, 18, 27]).

1.3. CE methods

CE is a micro-analytical technique applicable for analyses of a great range of analytes, above all polar and charged compounds [28]. The electrophoretic process is a differential movement or migration of solutes caused by the attraction or repulsion in an electric field [29]. The term “electroseparation methods” includes a wide variety of separation methodologies, which present different operational characteristics and are based on different mechanisms of separation. In general, these methods can replace chromatographic methods (such as HPLC) owing to the fact that they represent useful and powerful tools to analyze simultaneously several kinds of analytes [3], as well as they provide some advantages dealing with high efficiency, short analysis time, low solvent

volume consumption, versatility and simplicity [15, 30]. To understand the reason of the great versatility that CE offers to the analyst, ***Table 1.1*** includes the different CE separation techniques [29, 31]:

Table 1.1. Capillary electroseparation techniques, their acronym and separation principle.

Separation technique	Acronym	Separation principle
Capillary zone electrophoresis *Non-aqueous capillary electrophoresis*	CZE NACE	Charge/size *(different physicochemical properties of organic solvents)*
Micellar electrokinetic chromatography	MEKC	Interaction hydrophobic/ionic with surfactant micelles
Capillary electrochromatography	CEC	Mobility in a free solution and chromatographic retention
Capillary isotachophoresis	CITP	Migration capacity between tampons of different nature
Capillary isoelectric focusing	CIEF	Isoelectric point
Capillary gel electrophoresis	CGE	Molecular size
Chiral capillary electrophoresis	CCE	Formation of stereospecific complex
Microemulsion electrokinetic capillary chromatography	MEEKC	Hydrophobicities and electrophoretic mobilities

Several publications describe that CZE and MEKC are the "classical" CE modes and the most used to analyze phytochemicals in different plant materials [30, 32], and probably CZE is more widely used for its simplicity and versatility [33]. After carrying out the separation of the sample components (regardless the CE mode used), the detection of the analytes has to be made. Different detection systems can be coupled to CE; they can be classified in three groups: detection based on optical techniques (FL, phosphorescence, UV-Vis absorption, chemiluminiscence [34], IR spectroscopy, NRM [35, 36], Raman spectroscopy, refraction, etc.); electrochemical techniques (such as conductometric [37], potentiometric, amperometric [38] and voltametric detection [39]); and other techniques like MS [38, 40-42] and radiochemical techniques.

UV-Vis absorption is clearly the detection tecnique most widely used [32, 38, 41], although nowadays, CE coupled to MS is getting more popular and affordable. MS has a great potential and the advantages of MS detection include the capability for both determining molecular weight and providing structural information. In general, if a

separation technique is coupled with MS the interpretation of the analytical results can be more straightforward.

For obtaining a good separation in CE it is necessary to optimise several parameters, such as buffer type, pH and concentration, type and dimensions of capillary, additives (type and concentration), temperature, voltage and injection mode, etc. The influence of every parameter on the separation will be evaluated by the analyst and will depend on the CE methodology used, the kind of phenolic molecule under study and the matrix analyzed.

In the rest of the review we will try to summarize some of the most relevant applications in the field of capillary electroseparation methods to determine phenolic compounds from plant food material, as well as give to the reader an idea about the usefulness of the data achieved and the new trends in CE analytical separations.

1.4. CE analysis of plant food material

As was already mentioned, the phenolic compounds are characteristic of many plants, and they are found practically in all food of vegetable origin constituting an integral part of our diet. Nevertheless, it is not easy to establish a phenolic distribution owing to the fact that the quantity of phenols depends on the site of their ultimate accumulation in the fruit as well as on the type of the fruit we wish to study. This variability in terms of distribution and concentration is essentially due to a variety of factors such as climatic conditions, genetics, and cultivation treatment. Moreover, in the case of plant-derived food origin that has undergone a certain technological treatment or more specifically food processing, the qualitative and quantitative variability is intimately related to the nature of the mentioned process.

It is really complicated to establish a classification of plant food material, since there are several ways of doing it. For instance, there is a culinary classification which is not the same as the botanical distinction [43, 44]. Trying to structure properly the current review and do it easy to understand to the reader, we will divide this section in the following parts: fruits, vegetables, herbs, spices and medicinal plants, beverages, vegetable oils, cereals, legumes and nuts and other matrices (including cocoa beans and bee products).

1.4.1. Fruits

Botanically, a fruit is a ripened ovary with seeds and any other structure that enclose it at maturity. This definition of a fruit means that many 'vegetables' are fruits (squash, tomatoes, beans, corn) and many 'grains' are also fruit (rice, wheat, etc.). Although this definition is correct, we are going to consider the "culinary" distinction. Traditionally, "fruits" are the edible pulpy tissue without seeds that are used as desserts or as sweet side dish to a meal, among others, due to their sweet or tart taste [44].

Fruits can be classified on the basis of their seeds, the harvest, the number of ovaries and the number of flowers involved in their formation, and other characteristics. The easiest classification could be the one which is based on common characteristics, finding: citrus fruits, pome fruits (apples and pears), stone fruits, tropical fruits, berries and others.

All of them are rich in vitamins, minerals and water, whereas they contain a few amount of fibre, proteins and fat. Carbohydrates vary in each fruit (5% - 20%) and the majority are sugars. Fruits have also an important content of antioxidants, specifically, phenolic compounds. According to Robards et al. [7], fruits are a great source of cinnamic acids (chlorogenic, ferulic, sinapic, *p*-coumaric and caffeic acids) and flavonoids (flavanols, flavonols and anthocyanins), finding mainly the glycoside forms of these compounds.

Paying attention to the different groups, citrus fruits are quite rich in cinnamic acids, which are conjugated with glucaric, galactaric acid, some lactones and other sugars more common. Specifically, they have a big amount of ferulic acid. Stone fruits, such as apricots, peachs, plums, etc., have significant amounts of kaempferol, quercetin, caffeoylquinic acids and *p*-coumaroylquinic acids; whereas pome fruits contain chlorogenic acids, caffeoylquinic acids and *p*-coumaroylquinic acids, but smaller amounts of caffeoyl-, *p*-coumaroyl- and feruloyl-glucoses. Anthocyanins are the predominant group of flavonoids present in berries and the rest of phenolic compunds depend on the variety and the family of berry fruits [11, 45]. The following table (***Table 1.2***) shows some of the most relevant articles where phenolic compounds were determined in fruits by using CE methods. All the optimum parameters used to carry out the analysis (instrumental and experimental variables), the extraction system used (initial amount of sample and final amount of solvent) and the name of the compounds under study are included.

Table 1.2. Summary of the most relevant articles where phenolic compounds were determined in fruits by using CE methods. The optimum parameters used to carry out the analysis (instrumental and other variables), the extraction system used (initial amount of sample and final amount of solvent) and the name of the compounds under study are included.

					Instrumental variables						**Chemical variables**			
	References	**Sample**	**Extraction system**	**Initial quantity→Final quantity of solvent in the extraction process**	**λ_d [nm]**	**V [kV]**	**T [°C]**	**i.d. [µm]**	**L_{ef} [cm]**	**t_{inj} [s]**	**Type of buffer**	**[Buffer] [mM]**	**pH**	**Detected compounds**
Citrus fruits	K. Kanitsar et al. [46]	Orange Grapefruit	– (dilution)	Fruits were hand-squeezed, centrifuged and supernatant diluted. Sample cleanup process with continuous flow system	200	20	20	75	67	5	Boric acid	100	9.5	Hesperidin, sinapic acid, ferulic acid, *p*-coumaric acid, caffeic acid.
	T. Wu et al. [47]	Grapefruit	SLE	1 g peel→ 10 mL 99.7% EtOH (3 times) → 80 µL→ 1 mL 60 mM borate buffer. 80 µL pulp juice → 1 mL 60 mM borate buffer	– (ED)	12	Room temp.	25	75	6	Borate	60	9.0	Hesperidin, naringin, hesperedin, naringenin, rutin.
	J. M. Herrero-Martínez et al. [48]	Orange	SLE	3.5 g → 4 mL MeOH+1 mL 12 M HCl+12 mg BHT → 10 mL MeOH	295	20	25	50	40	5	Phosphate + SDS + SC + MeOH	50 + 25 + 25 + 10%	7.0	Naringenin
	S. M. S. Sawalha et al. [49]		SLE	0.2 g dried simple → 10 mL MeOH → 2 mL MeOH:H_2O (50:50, v/v) → diluted 1:1 in water	– (MS)	25	Room temp.	50	100	5	Boric acid	200	9.5	Naringin, neohesperidin, hesperidin, narirutin.
Tropical fruits	M. Kofink et al. [50]	Guaraná	SLE	50 mg seeds → 5 mL purified water. 50 mg powder extract → 5 mL puriefied water	280	18	20	75	40	3	Borate + (2-hydroxypropyl)-γ-CD	100 + 12	8.5	(–)-catechin, (+)-catechin, (+)-epicatechin, (–)-epicatechin
	T. S. Fukuji et al. [51]	Abiu-roxo Wild mulberry	SLE	Whole fruit → 1 mLl 1:1 EtOH:deionized water Same process described above + hydrolisation in 4 M NaOH + 10 mM EDTA + 1% ascorbic acid	200	30	25	50	30	5	Sodium tetraborate + MeOH	50 + 7.5 % (v/v)	9.2	Chlorogenic acid, ferulic acid, *p*-coumaric acid, caffeic acid, gallic acid, protocatechuic acid.

Table 1.2. (continued)

	References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
Berries	P. Bridle et al. [52]	Strawberries Edelberries	SPE	Pigments eluted 3% formic acid in MeOH → dissolved in 25 mM phosphate buffer (pH 2.5) + MeOH (3:1)	560	25	25	75	50	2	Sodium borate	150	8.0	Cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside, pelargonidin 3-succinylglucoside, cyanidin 3-sambubioside-5-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-sambubioside.
	C. T. da Costa et al. [53]	Blackcurrant	SLE	1 g powder → 25 mL water → partitioned against 50 mL $CHCl_3$, Et_2O, EtOAc and MeOH → redissolved in 30 mL water	520	25	20	50	70.4	4	Sodium phosphate + ACN	25 + 30% (v/v)	1.5	Cyanidin 3-glucoside, cyanidin 3-rutinoside, delphinidin 3-glucoside.
	D. J. Watson et al. [54]	Cranberries	SLE SPE	5 g → 20 mL 95% EtOH:1.5 M HCl 85:15 (v/v) (3 times) → 3 mL + 15 mL 1% HCl in MeOH → cartridge →elution with 2 mL 1% HCl in MeOH → dried → 2 mL 50:50 water:MeOH	525	20	27	75	48	5	Phosphoric acid + Urea + β-CD	150 + 3000 + 50	2.11	Peonidin, cyanidin.
	S. Ehala et al. [55]	Bilberry Cowberry Cranberry Strawberry Blackcurrant Redcurrant	Ultrasonic extraction	50 g frozen berries → 100 mL $MeOH/H_2O$ (70:30)+1% HCl+20 mg L-ascorbic acid (3times) → Final amount extracts 150 mL	210	20	25	50	39	20	Sodium tetraborate	35	9.3	*Trans*-resveratrol, cinnamic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, quercetin, (+)-catechin, caffeic acid.
			SPE	(off-line preconcentration) 5 mL sample solution (ultrasonic extraction) → 0.5 mL MeOH										

Table 1.2. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
E. Dadáková et al. [56]	Apple	SLE	0.5 g grinded freeze-dried + ascorbic solution (80 mg in 7.5 mL water) → 12 mL MeOH + 5 mL 6M HCl → Neutralized with 2 g $NaHCO_3$ → 7.5 mL MeOH and 100 mL water → made up to 200 mL by water	270	20	25	75	67	2	Boric acid + Sodium tetraborate + SDS + MeOH	10 + 10 + 20 + 15% (v/v)	9.2	Quercetin
H. Y. Huang et al. [57]	Grape Apple	SLE	25 g fresh fruit milled and blended →25 mL MeOH	200	-27	30	50	40	3	Phosphate + Heptane + Cyclohexano + SDS + ACN	25 + 1.36% (w/v) + 7.66% (w/v) + 2.89% (w/v) + 2% (w/v)	2.0	(+)-catechin,(–)-epicatechin, caffeic acid, (–)-epigallocatechin, gallic acid.
F. Priego Capote et al. [58]	Grape	Superheated ethanol-water leaching	1-3 g milled skins → extracting with 0.8% HCl in different EtOH-water mixtures	220 285 (FL)	25	15	50	56	6	Sodium tetraborate + MeOH	50 + 10% (v/v)	8.4	Resveratrol, (–)-epicatechin, (+)-catechin, malvidin-3-glycoside, peonidin-3-glycoside, cyanidin-3-glycoside, delphinidin-3-glycoside, kaempferol, myricetin, quercetin.
F. Berli et al. [59]		SLE	50 g skin grape berries → 50 mL EtOH 12% + tartaric acid 6 mg/mL + SO_2 100 µg/mL → supernatant diluted 1:100 (v/v)	280	30	15	75	50	5	Sodium tetraborate + MeOH	20 + 30% (v/v)	9.0	Resveratrol, catechin, quercetin.

1.4.2. Vegetables

The term "vegetable" usually refers to the fresh edible portion of a herbaceous plant (fruits, tubers, bulbs, leaves) consumed either raw or cooked. Vegetables generally contain little amount of protein or fat and varying proportions of minerals, fibre, carbohydrates or antioxidant phytochemicals, such as polyphenolic compounds.

Fruit as the edible part of the vegetable

Solanaceae represents one of the largest and most diverse plant families including vegetables (tomato, potato, capsicum, eggplant) and commercial (tobacco) and ornamental (petunia) crops. The plant species of Solanaceae used as food are rich in healthy components and therefore they are also widely consumed. Tomato consumption, either fresh or processed, is higher than that of all other fruits and vegetables. Helmja et al. [60] have determined the phenolic composition and vitamin content of the skin extracts of tomato, chilli pepper, eggplant and potato (the latter not being a fruit, but a tuber) by CZE with UV detection at 210 nm. The separation of polyphenols was performed in a 75 cm x 75 μm i.d. fused-silica capillary (effective length 50 cm) using 25 mM sodium tetraborate (pH 9.3) as separation buffer and 25 kV. Different phenolic acids and flavonoids were identified with the spectra of the reference compounds and by spiking the standard solutions in the extracts: genistein, rutin, naringenin, myricetin, quercetin and chlorogenic and caffeic acids were identified in tomato; cinnamic, chlorogenic, caffeic and ferulic acids were detected in eggplant, and luteolin, quercetin and caffeic acid in chilli pepper. Additionally, the antioxidative capability of the phenolic compounds in the tomato skin extract was monitored and evaluated by CZE. The electropherogram recorded 5 min after the reaction between the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the tomato extract was compared with the electropherogram of the original tomato extract. In later studies, using the same CZE conditions, these researchers were able to identify additional phenolic compounds: naringenin chalcone in the tomato skin [61] and dihydroxycinnamoyl amide, cinnamic acid derivative and isomers of chlorogenic acid in the eggplant skin extract [62]. In both extracts, the evaluation of the antioxidative capability was also carried out by HPLC-DAD-ESI-MS/MS. Due to MS detection, HPLC proved to be advantageous over CE, enabling the identification of a higher number of phenolic compounds in the original extracts (16 and

10 in tomato and eggplant, respectively). Nonetheless, the speed, resolution and low amount of sample and reagents consumed on CE, made it an attractive method for reaction monitoring.

The composition of tomato extracts is very complex. Simplification of the electropherograms and a higher sensitivity and selectivity were achieved coupling CZE to ED [4] since only electroactive analytes could be detected. Peng et al. [4] accomplished the quantitative analysis of ascorbic acid, naringenin, rutin, myricetin and chlorogenic acid in the peel, pulp and seeds of three tomato varieties. Separations were undertaken in a 80 cm x 25 μm i.d. fused-silica capillary maintained at 20 °C, under the following conditions: 50 mM borate (pH 8.7) running buffer; 16 kV separation voltage; and potential of the working electrode 0.90 V (vs SCE).

Tubers

In the global diet, potato is another important species of the Solanaceae family. These carbohydrate-rich tubers are a staple food in many places, being one of the most grown crops today, with over 100 edible varieties. Nevertheless, the sweet potato is not one of them, belonging to a different plant family, the Convolvulaceae.

CZE coupled to UV detection [63, 64] has been applied to quantify several phenolic acids (chlorogenic acid and some of its positional isomers, caffeic and ferulic acids) in different potato varieties, giving comparable results to those found analysing the same samples by HPLC. Fernandes et al. [63] tested several electrophoretic conditions. Chlorogenic acid isomers separation was carried out in a fused-silica capillary (72 cm x 50 μm i.d.; effective length 50 cm) maintained at 30 °C, applying a voltage of 20 kV with a 100 mM sodium dihydrogen phosphate buffer (pH 4.44). The method was applied as well in a preliminary study to evaluate the effect of light on potato tubers. The results indicated that prolonged exposure to light resulted in a significant change in the isomeric ratios and in an increase in the total chlorogenic acid content.

As already described in the previous section [60], potato skin has been as well analyzed by CZE-UV detection, identifying catechin, rutin, chlorogenic acid, quercetin and caffeic acid in the methanol/water extract.

On the other hand, CZE coupled to ED [65] has been used for the quantification of rutin, quercetin and chlorogenic and caffeic acids together with vitamin C in sweet potato. In particular, the quantity difference in the peel and pulp and the variation between fresh and cooked sweet potato of these active compounds were investigated, finding lower contents in the pulp and when the samples were cooked (except for quercetin). All five analytes were well-separated within 20 min utilising 18 kV and a 60 mM borax running buffer (pH 9.0) in a 75 cm x 25 µm i.d. fused-silica capillary. The applied voltage to the working electrode was 0.95 V (vs SCE).

Bulbs

The bulb onion (Alliaceae family) constitutes a key part of the daily diet in most countries for its distinctive taste and flavour. The number of studies using CE for the detection of phenolic compounds in onion has been scarce; however, they revealed the presence of several flavonoids.

The major flavonoids of mature onion bulb are quercetin 3,4'-diglucoside and quercetin 4'-monoglucoside [66, 67]. As could be expected, significant differences in the levels of both flavonols were observed between the six onion varieties analyzed (including white, red and brown bulbs); concretely, only trace amounts were detected in the white variety [66]. Separations were performed in a 57 cm x 75 µm uncoated fused-silica capillary (effective length 49.4 cm) at 25 °C and applying a voltage of 25 kV. The buffer consisted of 10 mM boric acid, 10 mM sodium tetraborate and 15 mM EDTA dissolved in 15% v/v methanol/water (pH 10.2 adjusted with 1 M hydroxide solution) and the detector was set at 280 nm. The use of HPLC-UV and H^1-NMR confirmed the identification of both flavonols and allowed the identification of two minor isorhamnetin derivatives. Price and Rhodes [67] investigated the changes in composition resulting from autolysis experiments during 24 h in four different onion varieties (white, brown, hybrid pink and red skinned). HPLC-UV was used to study the onion extracts before autolysis, identifying 17 additional minor components (representing only 16% of the total peak area) besides the two main quercetin derivatives mentioned. During the autolysis period, a small loss in the total flavonol content and a significant change in the flavonols conjugation were observed. The deglucosidation products were studied in more detail using CE-UV.

The second most important class of flavonoids in pigmented onion varieties are anthocyanins. Petersson et al. [68] developed a CE-ESI-TOF-MS method for the identification of anthocyanins employing silica capillaries coated with poly-LA 313 (polycationic amine-coating polymer) and an MS-compatible volatile separation buffer, consisting in 15 mM formic acid (pH 1.9). A total of ten anthocyanins, containing the aglycon ion of either cyanidin, peonidin, delphinidin, petunidin or malvidin, were identified. CE was coupled to ESI-TOF-MS by a sheath-flow interface with acidified methanol/water as sheath liquid. Positive detection was used due to the better ionisation of the analytes in this ion mode.

Leaves

A validated method for the determination of quercetin was applied to two plant species from the Brassicaceae family, white cabbage and cauliflower [56]. Due to their four-petaled flowers, which look like a cross, this family is also known as Cruciferae. Typically, the flower heads of cauliflower and the leaves of cabbage are the parts of the plant eaten. The method was based on the acid hydrolysis of bonded quercetin in the samples followed by SPE and a MECK-UV determination at 270 nm. Separations were achieved in fused-silica capillaries of 70 cm (67 cm effective length) x 75 μm i.d. maintained at 25 °C and applying a voltage of 20 kV. The running buffer consisted of 10 mM boric acid, 10 mM sodium tetaborate, 20 mM SDS and 15% methanol (pH 9.2). The quercetin content in cabbage was 7±1 mg/kg, whereas the cauliflower samples did not contain detectable amounts of that compound.

1.4.3. Herbs, spices and medicinal plants

Spices are natural and common food additives, which have been used, throughout thousand of years, to increase or to improve the food flavour, colour and taste, as well as to preserve the quality of food [69, 70]. Several spices possess beneficial effects on human health, so they can be used, besides flavoring/coloring/seasoning, like natural treatment in different diseases [71].

On the other hand, for centuries, medicinal plants have been used to treat human health problems in many countries of Asia, specifically in China [72]. Almost all these plants are consumed as beverages, i.e. the leaves or other parts of the plants are mixed,

usually, with hot water for extracting the compounds which could have beneficial properties [69, 71]and people drink the infusion originated.

The number of articles related to phenolic compounds present in different spices or plant matrices is quite impressive, although the percentage of papers in which electroseparation methods were used is quite low (in most cases, HPLC was the method of choice).

The following table (***Table 1.3***) contains information about different important aspects involved in the determination of phenols in these matrices, such as extraction system and separation and detection optimum parameters, compounds under study, etc. Considering the fact that plants, herbs and spices contain very similar compounds or closely related ones, only some examples have been included in order to contain the size of this review.

Table 1.3. Important aspects involved in the electrophoretic determination of phenols in herbs and spices: extraction system, separation and detection optimum parameters and compounds under study.

				Instrumental variables						**Chemical variables**			
References	**Sample**	**Extraction system**	**Initial quantity→Final quantity of solvent in the extraction process**	**λ_d [nm]**	**V [kV]**	**T [°C]**	**i.d. [µm]**	**L_{ef} [cm]**	**t_{inj} [s]**	**Type of buffer**	**[Buffer] [mM]**	**pH**	**Detected compounds**
E. Ibañez et al. [73]	Rosemary	SFE	60 g → unspecified	230	10	25	50	20	1	Sodium deoxycholate + Boric acid-sodium tetraborate + ACN	50 + 20 + 15% (v/v)	9.0	Rosmanol, carnosol, carnosol isomer, carnosic acid, methyl carnosate.
R. Sáenz-López et al. [74]		SLE	150 mg → 2 mL MeOH	250	30	25	50	56	3	Disodium tetraborate	50	10.1	Carnosol, carnosic acid.
M. Bonoli et al. [75]		SLE	1 g → 10 mL organic solvent ($MeOH/CHCl_3$/isopropilic alcohol and 1:1 $MeOH:CHCl_3$, v/v) → four 100 fold diluted extract in isopropanol	200	30	35	50	40	3	Sodium tetraborate	20	9.0	Carnosol, carnosic acid, rosmarinic acid.
A. L. Crego et al. [76]		SWE	1 g → 3.47 mL water → freeze-drying	200	30	25	50	45	10	Sodium tetraborate + ACN	50 + 10%	9.5	Carnosol, rosmarinic acid, carnosic acid, genkwanin, rosmanol
M. Herrero et al. [77]		PLE (SWE)	2 g → 6.6 mL water → freeze-drying →10 mg dry extract → 1 mL water	200	20	Room temp.	50	67	10	Ammonium acetate	40	9.0	Isoquercetin, homoplantagenin, gallocatechin, carnosic acid, rosmarinic acid.
Y. Peng et al. [78]		SLE	2 g → 10 mL 70% EtOH	– (ED)	16	Room temp.	25	75	8	Borate buffer	80	9.0	Hesperetin, acacetin, diosmetin, ferulic acid, apigenin, luteolin, rosmarinic acid, caffeic acid.
A. Ben Hameda et al. [79]	Sage	SLE	Leaf + water 1:10 w/w ratio	280	20	25	75	35	5	Borate buffer	40	9.2	Epicatechin, catechin, vanillic acid, rosmarinic acid, caffeic acid, galli acid.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
S. Başkan et al. [80]	Sage	SLE	1 g → 10 mL MeOH	210	28	25	50	45	5	Borate buffer	40	9.6	Carnosic acid, rosmarinic acid.
J. Petr et al. [81]	Marjoram	PSE	1 g dried plant → 22 mL acetone → Diluted in water-MeOH (90:10, v/v) → Diluted in 50 mM sodium borate, pH 9.5 (electrokinetic accumulation CE injection)	200	-10	20	50	40	1800	Sodium phosphate + SDS	50 + 60	2.5	Sinapic acid, ferulic acid, coumarinic acid, caffeic acid, syringic acid, vanillic acid, 4-hydroxybenzoic acid.
T. Nhujak et al. [82]	Turmeric	SLE	100 mg → 10 mL EtOH → ethanolic extract diluted 1 mL solution containing 300 mg/L dodecylbenzene and 300 mg/L 2,4-Dinitrophenyl phthalimide	214 425	- 15	25	50	30	3	Phosphate buffer + n-octane + SDS + 1-butanol + 2-propanol	50 + 1.1% (v/v) + 180 + 890 + 25% (v/v)	2.5	Curcumin, demethoxycurcumin, bis-demethoxycurcumin.
Q. Chu et al. [83]	Dandelion	SLE	2 g dried powder → 10 mL anhydrous EtOH and deionized water (4:1) → to 5 mL in volume 3 g powder granules → 10 mL anhydrous EtOH and deionized water (4:1) → made up to 5 mL in volume	– (AD)	16	Room temp.	25	75	8	Borate buffer	50	8.0	Diosmetin, ferulic acid, chlorogenic acid, luteolin, caffeic acid.
F. N. Fonseca et al. [84]	Chamomile	SLE	16 g dried plant → 400 mL 1:1 MeOH:H_2O (3 times) → Residue of recovered fractions → 24 mL MeOH (solution) 10 mL solution → 20 mL H_2O (stock solution) 2.5 g stock solution → 10 mL 1:1 MeOH:H_2O	337	25	25	50	25	CZE 3 24 CEC	Phosphate buffer + ACN	50 + 50%	2.8	Herniarin, umbelliferone, chlorogenic acid, caffeic acid, apigenin, apigenin-7-*O*-glucoside, luteolin, luteolin-7-*O*-glucoside, quercetin, rutin, naringenin.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
A. Kulomaa et al. [85]	*Eucommia ulmoides*	LLE	Mixture of boiling water-MeOH (30:70, v/v) Boiling water	220	30	25	50	80	5	Sodium dihydrogen phosphate + Disodium hydrogen phosphate	30 + 30	7.0	Flavone, rutin, quercitrin, chlorogenic acid, ferulic acid, caffeic acid, protocatechiuc acid.
		Soxhlet	3.5 g leaves → acetone-dichloromethano										
		SFE	1 g powder → MeOH-water (2:1, v/v) → extracts collected into 3.5 mL acetone										
H. Y. Cheung et al. [86]	*Eucommia ulmoides*	SLE	10 g (bark/leaves) → 150 mL MeOH → 0.2 g dried bark/0.1 g dried leaves → 50 mL MeOH	214	20	20	50	50	5	Boric acid + SDS + 1-butanol	50 + 50 + 4%	9.5	Geniposide, geniposidic acid, pyrogallol, rutin, chlorogenic acid, ferulic acid, *p*-coumaric acid, quercetin, caffeic acid, protocatechuic acid.
J. Šafra et al. [87]	*Melissae herba*	SLE	1 g dried pulverized plant → 50 mL MeOH → Supernatant diluted with water to contain 20% (v/v) MeOH.	270/ 320	–	Room temp.	300	16	–	MOSPO + Tris + Hydroxyethylcellulose + Boric acid + MeOH (for CZE) (electrolyte system for ITP–CZE in column coupling mode)	25 + 50 + 0.2% + 40 + 20%	8.1	Protocatechuic acid, caffeic acid, rosmarinic acid, *p*-coumaric acid, chlorogenic acid, ferulic acid, quercitrin, apigenin.
G. Chen et al. [88]	Sophora flower *Ligustrum lucidum* Camphor laurel	SLE	0.5 g Sophora flower → 50 mL MeOH 2 g *Ligustrum* and camphor → 50 mL MeOH Residues → washed with 20 mL MeOH Extracts+washings → concentrated to 40 mL → diluted to 50 mL with MeOH Extracts diluted with running buffer	– (ED)	12	20	25	40	6	Borate buffer	100	9.0	Daidzein, rutin, quercetin.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
K. Helmja et al. [2]	Hop	SLE (SFE as well)	0.7 g → 7 mL MeOH:water 70:30	210	20 or 30	Room temp.	75	50	15	Sodium tetraborate	25	9.3	Naringin, resveratrol, catechin, rutin, naringenind, chlorogenic acid, quercetin, myricetin, *p*-coumaric acid, caffeic acid.
D. Arráez-Román et al. [89]		SLE	2.5 g hop pellets → 30 mL hexane (x3) → 30 mL MeOH (x3)→ combination extracts → evaporation organic solvent → residue + 2 mL MeOH:water 50:50 v/v	– (MS)	25	Room temp.	50	100	10	Ammonium acetate/ammo_nium hydroxyde	80	10.5	Kaempferol-3-*O*-rutinoside, hesperidin, rutin, luteolin-7-*O*-glucoside, kaempferol-3-*O*-glucoside, quercetin-4´-*O*-glucoside, chlorogenic acid.
R. Pomponio et al. [90]	Echinacea	LLE	Sample solution (prepared in MeOH-water 70:30, v/v) → diluted with water to obtain a final MeOH-water ratio of 10:90	300	20	20	50	43.5	2	Sodium tetraborate + Sodium deoxycholate	40 + 70	9.2	*p*-nitrobenzoic acid, vanillic acid, caffeic acid, cichoric acid, caftaric acid.
R. Pomponio et al. [91]	*Cistus*	SLE	4 g → 150 mL boiling water (3 times) → Lyophilized residues dissolved in water	200	- 10	40	50	19.5	1	Heptane + SDS + Butan-1-ol + Sodium phosphate	1.36 % (w/v) + 2.31% (w/v) + 9.72% (w/v)+ 50	2.5	Catechin, gallocatechin.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
X. K. Wang et al. [92]	*Cortex fraxini*	SLE	1.250 g →10 mL 80% (v/v) MeOH → Precipitated → 8 mL 80% (v/v) MeOH Filtration residue → 5 mL 80% (v/v) MeOH Collected solution → diluted to 25 mL 80% (v/v) MeOH → diluted with running buffer	214	22	25	50	50	15	Sodium tetraborate + Tween 20 + MeOH	20 + 64 + 9% (v/v)	9.3	Scopoletin, rutin, esculetin, chlorogenic acid, caffeic acid.
Y. Peng et al. [93]	Honeysuckle	SLE	0.2 g → 10 mL 80% EtOH	– (ED)	16	Room temp.	25	75	8	Borax	50	8.7	Hyperoside, chlorogenic acid, luteolin, caffeic acid.
R. Hamoudová et al. [94]	St. John´s wort	SLE	0.5 g → 50 mL MeOH 0.5 mL supernatant → 2 mL IS stock solution (50 µg/mL kaempferol in MeOH) and diluted with water to 10 mL	270	driving current 200 mA	Room temp.	300	16	–	MOSPO + Tris + Boric acid	25 + 50 + 65	8.3	Quercetin, quercitrin, chlorogenic acid, isoquercitrin, hyperoside, rutin.
A. Segura-Carretero et al. [95]	*Hibiscus sabdariffa* L.	SPE	25 g homogenized dried calyces → 1 L acetic acid (15% v/v) → filtrate mixed 40 g Amberlite XAD-2 → Amberlite particles packed into a glass column → washed with deionized water → Anthocyanins renained absorbed on the column → eluted with 1 L ethanol (70% v/v)-acetic acid (1% v/v). Red solution → concentrated to dryness → 2 mL water	– (MS)	25	25	50	80	20	Boric acid + ammonia	200	9.0	Delphinidin-3-sambubioside, cyanidin-3-sambubioside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside, cyanidin-3,5-diglucoside, chlorogenic acid.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
S. J. Sheu et al. [96]	Artemisiae capillaris herba	SLE	4 g → 20 mL MeOH (2 times) → combined extract → reduced to 5 mL	254	20	25-26	75	MEKC 92.4 72.4 CZE	2	MEKC Sodium borate + SDS Sodium borate + 2,3,6-tri-O-methyl-β-ciclodextrin CZE	MEKC 20 + 20 25 + 6.75 mg/mL CZE	9.82 –	Scopoletin, 6,7-dimethyl esculetin, chlorogenic acid, caffeic acid,capillaris in.
J. Šafra et al. [97]	*Herba Epilobi*	SLE	1.5 g → 50 mL MeOH → Supernatant diluted with water + 20% (v/v) MeOH	270	ITP–CZE driving current 50-200 mA	Room temp.	300	16	30 µl	MES + Tris + Boric acid + α-CD + HEC + MeOH	25 + 50 + 30 + 10 + 0.2% + 20% (v/v)	8.31	Protocatechuic acid, caffeic acid, gentisic acid, cinnamic acid, coumaric acid, ferulic acid.
M. Vaher et al. [98]	Sweet gale Sea buckthorn Hiprose Knotweed	SFE	Unspecified	240	18	Room temp.	50	70	–	Disodium tetraborate in water	25	9.4	Resveratrol, catechin, quercetin, flavone.
M. E. Yue et al. [99]	*Hippophae rhamnoides* (sea buckthorn)	Soxhlet extraction	2 g air-dried podwer → 50 mL MeOH (with or without 5 mL 25% (v/v) hydrochloric acid)	270	15	25	50	30	1	Borate	20	10.0	Quercetin, kaempferol, isorhamnetin.
J. Gorbatšova et al. [100]		SLE	10 g frozen squeezed berries → 20 mL CH_3OH/H_2O (70:30)	210 265 280	16	25	50	35	20	Borate	20	9.3	Myricetin, *trans*-resveratrol, catechin, quercetin, caffeic acid, *p*-coumaric, gallic acid.
M. Vaher et al. [101]		SLE	50 g raw material squeezed → 50% MeOH	210 265	+20	25	50	41	–	Sodium tetraborate	20	9.3	Rutin, chlorogenic acid, quercetin, caffeic acid, gallic acid.

Table 1.3. (continued)

References	Sample	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
G. Chen et al. [102]	Puerariae Radix *Puerirae lobata*	SLE	2 g → 50 mL 95% EtOH. Residues → 10 mL 95% EtOH (2 times) Extract+washings → concentrated to 40 mL and diluted to 50 mL with 95% EtOH. Extracts diluted with running buffer	– (ED)	9	20	25	40	6	Borate	50	9.0	Puerarin, daidzein, rutin.
K. L. Li et al. [103]	Scute-coptis herb	SLE	0.4 g pulverized herb couple → 10 mL 70% MeOH (3 times) → Extracts combined → addition 2 mL IS solution (5.2 mg umbelliferone in 1 mL 70% MeOH) → diluted to 50 mL with 70% MeOH	270	30	30	50	71.5	5	Sodium borate + Sodium dihydrogenphosphate + SC + ACN	5 + 15 + 50 + 65%	6.97	Wogonin 7-O-glucuronide, oroxylin A-7-O-glucuronide, baicalin, oroxylin A, wogonin, baicalein.
C. O. Okunji et al. [104]	*Garcinia kola* seed	SLE	2.15 g ground sample → 110 mL water at 90°C	325	28.5	25	50	55	2	Borate	100	9.5	Biflavanones (GB1, GB2, GB1-glycoside, kolaflavanone).

(ASE → Dionex commercial name for PLE (Pressurized liquid extraction)).

1.4.4. Beverages

Since we are aware that the current review can not cover all the developments concerning the determination of antioxidants in all the beverages consumed nowadays, we will focus on those beverages which could be consider more important. Therefore, we include the following beverage samples: tea, coffee, juice, wine and beer. In the last part belonging to this section, we include some interesting methods developed to carry out the determination of antioxidants in other beverages, such as cider, cognac and other liquors. For each matrix surveyed, methods are tabulated (or at least described in detail) in order to assist the method selection.

Tea

The term "tea" is referred to the plant, leaf, or beverage originated from the species *Camellia sinensis*. There are a lot of types of commercial tea, but the most abundant are two: *sinensis* and *assamica* [105]. One of the most significant differences between them is the size of its leaves, being *assamica* leaves bigger than *sinensis* leaves. Despite that the origin of this plant is the Southeast of Asia, it is cultivated in about 30 countries in the tropical and subtropical regions [105]. It is consumed by a large number of people and, after water, is one of the most important or relevant beverages [43].

Tea is an excellent natural source of antioxidants, and phenolic compounds represent the most abundant group. Indeed, tea is particularly rich in terms of flavanoids, such as flavanol monomers (catechin, epicatechin) and flavanol gallates (epicatechin gallate, epigallocatechin gallate) [106, 107]. Apart from the mentioned analytes, tea contains as well other polyphenols to a lesser amount, such as gallic acid, coumaric acid and caffeic acid, as well as purine alkaloids, theobromine and caffeine [108].

There are different types of tea according to the manufacture: green, black, white and oolong teas. Green tea (non-fermented beverage) is elaborated with young leaves which are submitted to several processes, like withering, steaming or pan firing, drying and grading in order to deactivate the enzymes which are responsible for the degradation of catechins in thearubigins and theaflavins [109]. White tea (considered non-fermented or semi-fermented beverage) is produced using leave buds with white trichomes. In black tea production the leaves undergo plucking, withering, maceration and drying. This type of

tea is fermented for several hours and during this step occurs the oxidation of polyphenols, altering the chemical composition and changing leaf colour and aroma. Oolong tea is a semi-fermented tea, owing to the fact that the manufacture includes a short period of oxidation. There are different methods for carrying out its production, and thus, a wide variety of products on the basis of the catechins oxidation degree [105, 106].

Liquid chromatography with UV absorbance or MS detection has been the method selected more often for analyzing phenolic compounds in tea [110], but there are several publications in which CE methods (such as CZE or MEKC) are used to determine the compounds mentioned above. The best resolution, separation and quantitation for catechins are usually achieved by MEKC methods, whereas CZE methods are more rudimentary [111, 112].

Table 1.4 summarizes some of the CE methods regarding determination of phenolic compounds from tea. Information about the type of samples used in every study, separation and detection optimum parameters, detected compounds, etc. are included.

Table 1.4. CE methods developed to carry out the determination of phenolic compounds from tea. Information about the type of samples used in every study, separation and detection optimum parameters and detected compounds has been included.

				Instrumental variables						**Chemical variables**			
Reference	**Sample tea**	**Extraction system**	**Initial quantity→Final quantity of solvent in the extraction process**	**λ_d [nm]**	**V [kV]**	**T [°C]**	**i.d. [µm]**	**L_{ef} [cm]**	**t_{inj} [s]**	**Type of buffer**	**[Buffer] [mM]**	**pH**	**Detected compounds**
L. P. Wright et al. [113]	Black	SLE	CZE 6 g → 5 mL aqueous MeOH	280 380	CZE 22.5	CZE 25	50	CZE 51	5	Boric acid + Potassium sulfate	600 + 50	7.0	Theaflavin-3,3´-digallate, free theaflavin, theaflavin-3-monogallate, theaflavin-3´-monogallate.
			NACE 2 g → 100 µL ACN-MeOH-acetic acid (74.5:25:0.5, v/v)		NACE 27.5	NACE 18.5		NACE 32		ACN + MeOH+ Acetic acid + Ammonium acetate	71 v/v+ 25 v/v+ 4 v/v + 90		
H. Horie et al. [114]	Green Oolong Black	SLE	250 mg → 50 mL ACN-2% metaphosphoric acid (1:1, v/v) → diluted 10 times with water	194 270	25	30	75	70	5	Sodium tetraborate + Boric acid + SDS	20 + 80 + 50	8.4	(–)-Epigallocatechin, (–)-epicatechin, (–)-epigallocatechin gallate, (–)-epicatechin gallate.
M. Bonoli et al. [115]	Green	SLE	100 mg →100 mL water-formic acid solution (99.7/0.3, v/v) 99 mL green tea extract → 1 mL acetone	200	30	29	50	40	1	Potassium dihydrogen phosphate + Sodium tetraborate + SDS	20 + 50 + 200	7.0	(–)-Gallocatechin, (+)-catechin, (–)-epigallocatechin, (–)-epigallocatechin-3-gallate, (–)-gallocatechingallate, (–)-epicatechingallate, (–)-epicatechin, gallic acid.
H. Y. Huang et al. [116]	Tea beverage	Direct injection	–	200	- 27	30	50	40	3	MEEKC Heptane + Cyclohexanol + CAN + SDS + Phosphate solution	1.36% + 7.66% + 2.0% + 2.89% + 25	2.0	(–)-epigallocatechin gallate, (–)-epicatechin, (–)-epigallocatechin.
	Tea leave	SLE	1 g → 20 mL 100°C hot water							MEKC SDS + MeOH + Phosphate solution	2.89% + 2.0% + 25		

Table 1.4. (continued)

Reference	Sample tea	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
D. Stach et al. [107]	Green Black	SLE	1.4 g → 400 mL 90°C hot water 1 mL → 50 µL 80 mM HCl + 20 µL AHBA	200	18.2	25	50	45.4	1	SDS + Sodium phosphate buffer + MeOH	100 + 20 + 10% (v/v)	2.7	(–)-epicatechin, (–)-epigallocatechin, (+)-catechin, (–)-catechin gallate, (–)-epicatechin gallate, (–)-epigallocatechin gallate.
L. Arce et al. [112]	Green	SLE	1.75 g → 100 mL boiling water	210	20	20	75	57	10	Boric acid	150	8.5	(–)-epicatechin, (+)-catechin, gallic acid, (–)-epigallocatechin, (–)-epigallocatechin-3-gallate, (–)-epicatechin-3-gallate.
M. B. Barroso et al. [117]	Green Black	LLE	0.5% solution → diluted 1:2 with Mili-Q water	200	30	21	50	70	–	Borate.phosphate + SDS	20 + 25	7.0	(+)-catechin, (–)-epigallocatechin, (–)-epigallocatechin gallate, (–)-epicatechin, (–)-epicatechin gallate.
R. Gotti et al. [118]	Green	SLE	1 g → 60 mL 85°C hot water → diluted 1:2 with IS (syringic acid) aqueous solution (100 µg/mL)	200	15	25	50	8.5	2	Borate-phosphate buffer + SDS + Hydroxypropyl-β-CD	25 + 90 + 25	2.5	(–)-epicatechin, (+)-catechin, (–)-catechin, (–)-epigallocatechin, (–)-epicatechin gallate, (–)-gallocatechin gallate, (–)-epigallocatechin gallate.
S. Kodama et al. [119]	Green Oolong Black	SLE	1 g → 60 mL 85°C hot water → diluted five times with purified water	210	25	20	50	56	2	Borate + Phosphate + 6-O-α-D-glucosyl-β-CD + SDS	200 + 20 + 25 + 240	6.4	(–)-catechin, (+)-catechin, (–)-catechin gallate, (–)-epigallocatechin gallate, (–)-epicatechin gallate, (–)-epigallocatechin, (+)-epicatechin, (–)-epicatechin.
L. A. Kartsova et al. [120]	Green Black	SLE	200 mg → 20 mL hot water	200	20	20	75	50	10	Phosphate buffer + SDS + Urea	25 + 20 + 10	7.0	(+)-Catechin, (–)-epigallocatechin, (–)-epigallocatechin gallate, (–)-epicatechin, gallic acid, (+)-gallocatechin gallate, (–)-epicatechin gallate.

Table 1.4. (continued)

Reference	Sample tea	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
M. Bonoli et al. [121]	Green Black	Dilution / Normal infusion (to drink)	1 mg green tea extract → 1 mL water/formic acid solution (99.7:0.3 v/v) 2 g dried leaves → 250 mL boiling water All samples → diluted 2 times with destilled water → additon of acetone (1%, v/v)	200	30	29	50	40	1	Potassium dihydrogenphosphate + Sodium tetraborate + SDS	10 + 8.3 + 66.7	7.0	(–)-gallocatechin, (+)-catechin, (–)-epigallocatechin, (–)-epigallocatechin-3-gallate, (–)-gallocatechingallate, (–)-epicatechingallate, (–)-epicatechin, gallic acid.
H. Zhang et al. [122]	Jasmine Green	SLE	3 g → 10 mL EtOH (3times) → 20 mL water	211	27.5	25	50	40	99	Borate buffer + SC + Ethylene glycol	10 + 90 + 40% v/v	–	(–)-epigallocatechin, (–)-epicatechin, (+)-catechin, (–)-epigallocatechin gallate, (–)-epicatechin gallate.
P. J. Larger et al. [123]	Green	SLE	0.2 g → 100 mL boiling water → 5 mL ACN	278	30	25	50	56	15	Monosodium dihydrogenphosphate + Sodium tetraborate + SDS + ACN	50 + 50 + 20 + 10%	6.0	(–)-epigallocatechin, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin gallate, (–)-epicatechin gallate, chlorogenic acid, kaempferol derivative, diverses flavonoids and their glycosides.
	Black		4 g → 500 mL boiling water 100 mL → 5 mL ACN										
T. Watanabe et al. [124]	Green Black	Direct injection	–	280	15	20	50	32	1	Phosphate + Borate + SDS	25 + 50 + 25	7.0	(+)-catechin, (–)-epigallocatechin, (–)-epicatechin, (–)-epigalocatechin gallate, (–)-gallocatechin gallate, (–)-epicatechin gallate, (–)-catechin gallate.
J. P. Aucamp et al. [125]	Black	LLE	1% (w/v) tea solution → 0.5% (w/v)	200	14	25	50	50	2	Phosphate + MeOH + SDS	25 + 6% (v/v) + 100	7.0	(+)-catechin, (–)-epigallocatechin, (–)-epigallocatechin gallate, gallic acid, (–)-epicatechin, (–)-epicatechin gallate.

Table 1.4. (continued)

Reference	Sample tea	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
L. Chi et al. [126]	Chinese herbal	LLE	150 mL → 1000 µL 20% 500 mM KH_2PO_4-250mM $Na_2B_4O_7$ and 30% EtOH	– (AD)	15	Room temp.	25	60	12	Potassium dihydrogenphosphate + Sodium tetraborate + β-CD	400 + 200 + 0.20	7.6	Kempferol, apigenin, rutin, ferulic acid, quercetin, luteolin.
H. Horie et al. [127]	Green	SLE	3 g → 180 mL boiling water → Diluted 10 times with 0.1% metaphosphoric acid	280	30	23	50	70	5	Borax buffer	20	8.0	(–)-epigallocatechin gallate, (–)-epicatechin gallate, (–)-epigallocatechin, (–)-epicatechin, (+)-catechin.
B. C. Nelson et al. [128]		SLE	0.2 g → 20 mL water + L-tryptophan (IS)	280	20	20	50	60	4	Tetraborate buffer + SDS + MeOH + Urea + β-CD	20 + 110 + 14% + 1500 + 1	8.0	(–)-epigallocatechin, (–)-epicatechin, (–)-epigallocatechin gallate, (–)-epicatechin gallate.

Coffee

Coffee is an alcohol-free beverage and one of the most important natural drinks widely consumed in the world [129]. There are many species of coffee, but as far as commercial production is concerned, the most important are *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) [43]. Brazil is one of the main producer and exporter (35% world production) [130], followed by Colombia, Indonesia, Mexico and Uganda [131].

Coffee is a natural source of antioxidants and contains plenty of phenolic compounds, being chlorogenic acid the most abundant [132].

Chromatographic techniques have been traditionally used for determining these compounds in coffee samples; electrophoretic methods only represent the 1% of total number of publications found in the scientific literature. Risso et al. [133] developed a method by MEKC to determine some phenolic acids, such as chlorogenic acid (resolving two positional isomers), caffeic acid and ferulic acid. The optimum conditions were a capillary of 22.5 cm x 75 µm i.d., 10 mM phosphate + 70 mM SDS + 5% MeOH like running buffer, pH 2.5, voltage applied of -17 kV, 22ºC, hydrodynamic injection at 25 mbar for 3 s. The results obtained were satisfactory and the method allowed the rapid and simultaneous determination of the phenolic acids previously mentioned.

Zhu et al. [134] investigated two stacking methods in MEKC in an attempt to increase the amount of sample injected, as well as to focus analytes onto a small zone; one employed a "high-conductivity zone", which was inserted between the sample zone and background solution to build an unequal conductivity gradient. The other employed a "low-temperature bath". Doing that they were able to separate three phenolics acids (ferulic, caffeic and chlorogenic).

Chu et al. [129] determined catechin, rutin, o-dihydroxybenzene and ferulic, chlorogenic, caffeic, gallic and protocatechuic acids in samples of instant coffee by CE with AD. They used a voltage of 16 kV for the separation and 80 mM borate (pH 8.4) as running buffer. The dimensions of capillary were 75 cm length and 25 µm i.d. The injection was made by means of electrokinetic injection at 16 kV for 8 s. The results proved that the method was accurate, reproducible and sensitive for the analysis of natural antioxidants in coffee samples.

Juice

Juice is a beverage obtained from fruits or vegetables, since it is the liquid naturally contained in their tissues. The traditional way to extract the juice is mechanically squeezing or macerating fresh fruits/vegetables (without heat or solvents). The juice is composed by different substances, such as amino acids, carbohydrates, organic acids and phenolic compounds, among others.

O´Shea et al. [135] used CE with ED and employing an on-column Nafion joint to identify chlorogenic, *p*-coumaric and caffeic acids in samples of apple juice. The conditions were: 10 mM sodium borate (pH 9.5) as running buffer, capillary of 65-70 cm effective length and 50 μm of i.d., a effective voltage of 425 V/cm (≈ 28 kV) and detection potential of 650 mV.

Cancalon et al. [136] resorted to CE for the determination of flavonoids such as didymin, hesperidin, narirutin, neohesperidin and naringin in citrus juice, specifically this method was applied to compare pure orange juice and pulpwash (a major adulterant of orange juice). The capillary used had 70 cm of effective length and 50 μm i.d. The optimum conditions were sample injection for 15 s, a voltage of 21 kV, 25ºC of temperature and 35 mM solution of borax at pH 9.3 as background electrolyte. The detection range wavelength was set between 200 and 500 nm. The addition of pulpwash, a lower-quality juice product, by comparison with a pure citrus juice, can be detected with this method.

Gel-Moreto et al. [137] analyzed the diastereomers of naringin, prunin, narirutin, hesperidin, neohesperidin and eriocitrin in lemon juice. They used CCE for being a good method for the separation of stereoisomers. The running background electrolyte was 200 mM borate buffer with 5 mM γ-CD, pH 10.0. Uncotaed, fused-silica capillary had an effective length of 60 cm and an internal diameter of 75 μm. The separation voltage was 15 kV, the detection was carried out at 290 nm and the temperature was maintained at 25ºC. Finally, the samples were injected by pressure during 2 s. This method highlighted that CCE could achieve the separation of the diastereomers, above all of eriocitrin and hesperidin. Following the same approach, Aturki *et al.* [138] determined the aforementioned compounds (flavanone-7-*O*-glycosides) in citrus juices. This type of

compounds can be separated only if there was a chiral environment. Lemon, orange and grapefruit juices were the samples used, which were extracted by SPE. They carried out the optimisation of different electrophoretic parameters and, finally, the best conditions were: capillary of 50 µm i.d. x 40 cm effective length, thermostated at 20°C, the applied voltage was 20 kV, samples were injected by pressure (5 kPa x 10s), and the background electrolyte consisted of 20 mM of sodium tetraborate buffer pH 7.0 containing 5 mg/mL of sulfobutyl ether-β-CD. The analyses were recorded at 205 nm, providing this wavelength the highest sensitivity for these compounds. The method allowed a qualitative analysis of the diastereomers of naringin, neohesperidin, narirutin, eriocitrin and hesperidin contained in several citrus juices, and the results showed good resolution in less than 10 min and high peak efficiency.

Simó et al. [139] used a combination of MEKC and reverse-phase liquid chromatography for analysing narirutin, hesperidin, naringin, benzoic acid and a compound belonging to the family of flavanones (no fully characterised). The orange juice samples were injected at pressure (0.5 psi for 2 s) and analyzed in a capillary of 20 cm effective length x 50 µm i.d., using a buffer of 50 mM boric acid/sodium tetraborate and 100 mM SDS at pH 8.0. The temperature was 25°C and the detection system was set at 280 nm. The best results were obtained with those conditions for MEKC and they could identify several compounds, although with LC-DAD-MS a higher number of analytes could be detected. LC method showed better peak area reproducibility than MEKC, but the later presented the highest analysis speed. Both of them had similar analysis time reproducibility, and could be used as complementary techniques.

Desiderio et al. [140] determined several flavanone-7-*O*-glycosides in orange, lemon and grapefruit juice by isocratic reversed phase CEC using a 75 µm i.d. silica fused column packed with 5 µm ODS silica gel. Optimum separation conditions were found using a mixture of ammonium formate (pH 2.5)-acetonitrile (8:2, v/v) as the mobile phase by the short-end injection mode. Under these conditions all the investigated flavanones were baseline-resolved within short analysis time (between 5 and 10 min).

Peng et al. [141] evaluated the analytical performance of CE with ED for analysing phloridzin, epicatechin, chlorogenic acid and myricetin in apple juice. The capillary used had 75 cm and 25 µm i.d., and CE was performed using a running buffer of 50 mM

borate buffer (pH 8.7) at 18 kV. The apple juice was electrokinetically injected at 18 kV for 6 s. They saw that the amounts of phenolic compounds in different juices were not the same, because, according with literature, the levels of phenolic compounds in juices had an enormous dependence on the fruit varieties used and the process to obtain the juice. CE-ED demonstrated to be a very powerful technique which had high resolution and sensitivity and good reproducibility.

Other phenolic compounds identified in juice were anthocyanins, which are responsible of their different colours (red, purple, blue, yellow). J. Sádecká and Polonský [142] enclosed several publications about determination of anthocyanins by electrophoretic methods, specifically, CZE and MEKC. One of these articles was published by Watanabe et al. [143] who identified cyanidin-3-sambubioside-5-glucoside, cyanidin-3-glucoside-5-glucoside, cyanidin-3-sambubioside and cyanidin-3-glucoside in elderberry juice using MEKC with SDS solution in a phosphate buffer at pH 7.0.

Wine

Wine is a fermented beverage with a minimal alcohol level (8.5-17% by volume) obtained from grape juice. There are a wide range of varieties of grapes, such as Merlot, Pinot Noir, Cabernet Sauvignon, Syrah, Cinsault, Rondinella, Sangiovese, Nebiolo, Grenache, Tempranillo, Carignan, etc [43]. In spite of the different grape varietals grown all over the world that produce hundreds of types of wine, these may be grouped into a small list of basic categories: red, white, sparkling, rosé and fortified wine.

Wine is considered as a rich source of flavonoids and resveratrol and its contents vary depending on the wine origin [144], but it is well known that red wine has higher amounts of phenolic compounds than white or rosé wine.

A huge number of worth publications about wine and phenolic compounds can be found, however, only few of them (less than 10%) are referred to electrophoretic methods. Several articles compare chromatographic techniques, usually HPLC, with CE. The most used methodologies are CZE and MEKC, although CITP is also used and, in a lesser extend, NACE. The next table (***Table 1.5***) outlines some publications including information about the different methodologies used, with the most important parameters and the compounds identified in each case.

Tabla 1.5. Summarized information about different methods to determine phenolic compounds in several wine beverages.

				Instrumental variables						Chemical variables			
Reference	**Sample wine**	**Extraction system**	**Initial quantity→Final quantity of solvent in the extraction process**	**λ_{d} [nm]**	**V [kV]**	**T [ºC]**	**i.d. [µm]**	**L_{ef} [cm]**	**t_{inj} [s]**	**Type of buffer**	**[Buffer] [mM]**	**pH**	**Detected compounds**
R. Sáenz-López et al. [145]	Red	–	Centrifugation→ upernatant isolated and Potassium metabisulphite added (amounts non specified)	280	25	10	75	56	6	Sodium tetraborate + MeOH	50 + 10%	9.4	Malvidin-3-O-(6-coumaroyl)-glucoside, malvidin-3-O-(6-acetyl)-glucoside, malvidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside catechin dimer, malvidin-3-O-glucoside and pyruvic acid derivative, petunidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-glucoside.
C. García-Viguera et al. [146]		LLE	5 mL extracted with diethyl ether→ 0.5 mL MeOH	280	20	30	75	50	2	Sodium borate	100	9.5	Gallic acid, 3,4-dihydroxybenzoic acid, 4-hydroxyphenethyl alcochol, *cis*-CAFTA, catechin, vanillic acid, *trans*-COUTA, caffeic acid, syringic acid, *p*-coumaric, epicatechin, myricetin, quercetin, kaempferol, isorhamnetin.
P. Andrade et al. [147]	Port	LLE	20 mL → 0.5 mL MeOH	280	20	30	75	50	2	Sodium borate	100	9.5	Tyrosol, epicatechin, catechin, syringic acid, *p*-coumaric acid, caffeic acid, gallic acid, 3,4-diOH-benzoic, *cis*-COUTA, *trans*-COUTA.
P. Andrade et al. [148]	White	LLE	20 mL extracted with diethyl ether → 1 mL MeOH	280	20	30	75	50	2	Sodium borate	100	9.5	Tyrosol, epicatechin, syringic acid, ferulic acid, *p*-coumaric acid, caffeic acid, 3 nonidentified hydroxycinnamic esters, gallic acid, 3,4-diOH-benzoic, *cis*-COUTA, *trans*-COUTA, *trans*-CAFTA.

Table 1.5. (continued)

Reference	Sample wine	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
M. I. Gil et al. [149]	Red	LLE	5 mL → 1 mL MeOH	280	20	30	75	75	2	Sodium borate	100	9.5	Epicatechin, catechin, epigallocatechin, syringic acid, *p*-coumaric acid, vanillic acid, caffeic acid, gallic acid, *cis*-COUTA, 3,4-diOH-benzoic acid, *trans*-COUTA, *cis*-CAFTA.
Z. Guadalupe et al. [150]	Red	GPC	2 mL → 500 µL 12% (v/v) EtOH in aqueous solution containing 6 g/L tartaric acid, pH 3.5	280 420 520 599	25	10	75	56	6	Sodium tetraborate + MeOH	50 + 10%	9.4	Malvidin-3-O-(6-coumaroyl)-glucoside, malvidin-3-O-(6-acetyl)-glucoside, malvidin-3-O-glucoside, peonidin-3-O-glucoside, malvidin-3-O-glucoside catechin dimer; malvidin-3-O-glucoside, pyruvic acid derivative, petunidin-3-O-glucoside, delphinidin-3-O-glucoside, cyanidin-3-O-glucoside.
J. Pazourek et al. [151]	Red White	SPE	2 mL → 0.2 mL MeOH	305	20	25	75	30	4	Borate	25	9.4	*Cis*-resveratrol, *trans*-resveratrol.
X. Gu et al. [152]	Red	Direct injection	–	310	20	Room temp.	50	30	–	Sodium borate + Sodium phosphate + SDS	25 + 25 + 75	9.0	*Trans*-resveratrol.
		SPE	1 mM in ACN	200-400	25	20	50	30	3	Boric acid + Dibasic phosphate + SDS + ACN	30 + 30 + 75 + 15%	9.2	*Cis*-resveratrol, *trans*-resveratrol.
R. Hamoudová et al. [153]		–	Diluted with water and directly injected (30µL)	254	– –	25	ITP 800 CZE 300	ITP 9 CZE 16	–	Water- MeOH + MOSPO + Tris + Boric acid + 2-HEC + β-CD	4:1 + 25 + 50 + 15 + 0.2% + 5	8.5	Protocatechuic acid, gallic acid, caffeic acid, vanillic acid, syringic acid, ferulic acid, *p*-coumaric acid, quercitrin, rutin, kaempferol, quercetin.

Table 1.5. (continued)

Reference	Sample wine	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
B. C. Prasongsidh et al. [154]	Non specified	–	Dilution of 100 µL sample in 900 µL buffer	220	20	40	50	60	7	Sodium deoxycholate + Disodium hydrogen phosphate + Disodium tetraborate + phophoric acid	0.05 + 0.01 + 0.006 M each	9.3	*Cis*-resveratrol, *trans*-resveratrol, quercetin, catechin, gallic acid.
G. Cartoni et al. [155]	Red	LLE	2 mL → 50 µL water-MeOH 1:1	210	15	25	50	36	2-5	Hydrogencarbonate	50	8.3	Syringic acid, *p*-coumaric acid, vanillic acid, caffeic acid, 3,4-diOH-benzoic acid, gallic acid.
G. Vanhoenacker et al. [156]		LLE	50 mL → 2.5 mL MeOH-water 1:1	280	20	25	50	65	5	Ammonium tetraborate	18.75	9.3	Gallic acid, 3,4-diOH-benzoic acid, catechin, caffeic acid, epicatechin, *p*-coumaric acid, myricetin, quercetin, kaempferol, vanillic acid, syringic acid, trans-polydatin, ferulic acid, rutin, cis-polydatin.
S. P. Wang et al. [157]	Grape	SPE	20 mL → 3 mL ACN	250	16.8	Room temp.	75	45	1	Borax	35	8.9	Myricetin, luteolin, quercetin, apigenin, naringenin, kaempferol, hesperetin, baicalein, galangin.
Y. Peng et al. [158]	Red	LLE	1 mL → 2 mL buffer	– (ED)	12	Room temp.	25	70	6	Borate	100	9.2	*Trans*-resveratrol, epicatechin, catechin
L. Arce et al. [159]	Red White	SPE	2 mL → 2 mL MeOH	280	20	20	75	67	5	Sodium borate	100	9.5	*Trans*-resveratrol, epicatechin, catechin, *p*-coumaric, caffeic acid, gentisic acid, quercetin, salycilic acid.

Table 1.5. (continued)

Reference	Sample wine	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
R. Saénz-López et al. [160]	Red	–	Centrifugation→ upernatant isolated and Potassium metabisulphite added (amounts non specified)	599	25	10	75	46	6	Sodium tetraborate + MeOH	50 + 15%	8.4	Malvidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, petunidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside.
Q. Chu et al. [161]		LLE	30 µL → 5 µL 2-[(2-aminoethyl)amino]-5-nitropyridine hydrochloride	310	20	20	50	30	-	Sodium borate + Sodium phosphate + SDS	25 + 25 + 75	9.0	*Trans*-resveratrol.
R. G. Peres et al. [162]	Red White Blended	LLE	1 mL → 2.5 mL EtOH-water 60:40 v/v	280	25	25	75	40	3	Sodium tetraborate + MeOH	17 + 20%	Sodium tetraborate range	Resveratrol, catechin, rutin, syringic acid, kaempferol, p-coumaric acid, myricetin, quercetin, caffeic acid, gallic acid.
Z. Demianová et al. [144]	Red White	LLE	1 mL → 2 mL MeOH	230	30	25	50	8.5	1.5	Malonic acid + TBAOH in MeOH	5 + 9.6	13.5	Resveratrol, quercetin, myricetin, catechin, epicatechin.
M. A. Rodríguez-Delgado et al. [163]	Spanish	LLE	5 mL → 0.5 mL MeOH	280	20	25	75	50	2	Boric acid + SDS + MeOH	150 + 50 + 5%	8.5	Catechin, epicatechin, quercetin, rutin, protocatechuic-aldehyde, syringic-aldehyde, ferulic acid, *p*-coumaric acid, vanillic acid, myricetin, kaempferol, caffeic acid.
J. Woraratphoka et al. [164]	Red White Blended	LLE	1 mL → 1 mL EtOH (50%)	206 217	15	25	50	56	7	Phosphate + Borate	25 + 10	8.5	Resveratrol, epicatechin, catechin, rutin, quercetin, syringic acid, cinnamic acid, *p*-coumaric acid, gentisic acid, *p*-hydroxybenzoic acid, salicylic acid, caffeic acid, gallic acid, protocatechuic acid.

Table 1.5. (continued)

Reference	Sample wine	Extraction system	Initial quantity→Final quantity of solvent in the extraction process	λ_d [nm]	V [kV]	T [°C]	i.d. [µm]	L_{ef} [cm]	t_{inj} [s]	Type of buffer	[Buffer] [mM]	pH	Detected compounds
R. C. Minussi et al. [165]	Red Rosé White	LLE	1 mL/2 mL → 100 µL buffer + 10% MeOH	206 217 312	15	20	75	50	7	Phosphate + Borate	25 + 10	8.8	Tyrosol, *cis*-resveratrol, *trans*-resveratrol, catechin, epicatechin, hydroxytyrosol, sinapic acid, epicatechin gallate, syringic acid, *o*-coumaric acid, p-coumaric acid, vanillic acid, gentisic acid, *p*-hydroxybenzoic acid, salicylic acid, caffeic acid, gallic acid, protocatechuic acid.
J. Pazourek et al. [166]	Red White Rosé	Direct injection	Diluted 5 times with water	190	20	25	75	36.5	5	Sodium tetraborate	25	9.5	Epicatechin, catechin, gentisic acid, *p*-coumaric acid, caffeic acid, gallic acid.
		SPE	5 mL → 2 mL MeOH + 5 mL water										
J. J. Berzas Nevado et al. [167]	Red	SPE	25 mL → 4 mL ACN-water (3+7)	320	5	25	75	25	5	Sodium tetraborate	40	9.5	*Cis*-resveratrol, *trans*-resveratrol.
Z. Dobiášová et al. [168]	Red White	SPE	2 mL → 0.3 mL 100% MeOH	305	20	25	75	36.5	5	Sodium tetraborate	25	9.5	*Trans*-resveratrol.
J. Hernández-Borges et al. [169]	Red	SPE	5 mL → 1 mL MeOH	280	15	20	75	40	2	Borate + Phosphate + Hexadimethrine bromide + α-CD	125 + 49 + 0.002% (w/v) + 2.5	7.5	Protocatechuic acid, salicylic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, ferulic acid, sinapic acid.

Beer

Beer is one the most ancient alcoholic beverages in the world. It was consumed by Mesopotamian, Egyptians, Greeks and Romans. Nowadays, the consumption of beer in the world continues increasing, because several studies over the last years have shown the beneficial effects of this beverage [170-172]. It is a fermented and flavoured alcoholic drink [173] made from malted grains, hops, yeast and water [43]. Beer components are ethanol and other alcohols, polyphenols, organic acids, vitamins, inorganic ions and bitter acids, among others [142].

In literature, only about 2% of the total number of scientific papers regarding phenolic compounds used electrophoretic methods as analytical tools.

Moane et al. [174] described the use of CE with ED and they achieved the separation of 7 phenolic acids (chlorogenic, sinapic, ferulic, caffeic, p-coumaric, vanillic, and protocatechuic acids) in different types of beer. To achieve that purpose, they used the following capillary electrophoretic conditions: a running buffer 25 mM sodium phosphate and 1M nitric acid pH of 7.2, a fused silica capillary of 65 cm length x 50 µm i.d., voltage of 25 kV and hydrodynamic injection (applying 20 psi for 30 s). The described method demonstrated to be a very useful and powerful tool in the determination of active compounds in complex samples.

Holland et al. [175] carried out the detection of phenolic acids by CE-ED on-capillary dual electrode system consisting of two platinum wires. The integrated dual electrode configuration was evaluated for direct AD in which the current response at both electrodes was monitored, using phenols as model analytes. The method demonstrated high effectiveness and selectivity, and good collection efficiencies for chemically reversible compounds. The CE separation conditions were: 25 µm i.d. x 32 cm fused silica capillary, 20 mM phosphate, pH 7, applied voltage of 15 kV, hydrodynamic injection at 10 psi, for 1s.

According to Hernández-Borges et al. [169], a beer sample was analyzed by coelectroosmotic CE method and they could identify five phenolic compounds, particularly, salicylic, p-hydroxybenzoic, vanillic, p-coumaric and ferulic acids. They used the following conditions: 75 µm i.d. capillary with 40 cm of effective length, run potential

15 kV, temperature 20ºC, hydrodynamic injection for 2 s, buffer formed by 125 mM borate, 49 mM phosphate, 0.002% (w/v) hexadimethrine bromide, 2.5 mM α-CD, at pH of 7.5. The method achieved, in less than 3.5 min, the separation of the phenolic compounds with a good resolution.

Other beverages

In this section we include some other beverages, like cider, cognac and other liquors; only a few papers in which phenols were analyzed in the mentioned samples can be found. Other infusions/tisanes besides tea could be included, but the most part of the plants used for doing these beverages are medicinal; therefore we have already spoken about them in section 1.4.3.

Peng et al. [141] described the use of CE with ED for the analysis of myricetin, chlorogenic acid, (–)-epicatechin and phloridzin in cider. The fused silica capillary had a dimension of 75 cm length x 25 μm i.d. CE was carried out in a 50 mM borate buffer with pH of 8.7 and the separation voltage applied was 18 kV. The injection of cider samples was electrokinetically for 6 seconds. The results showed that this method was optimum to determine the compounds mentioned above and each variety had the same phenolic acid and flavonoid profile (qualitative profile), but the amount of them was different on the basis of the fruit varieties and the process.

Panosyan et al. [176] carried out the detection of phenolic aldehydes, which could serve as quality and age markers of cognac. These phenolic aldehydes (vanillin, syringaldehyde, coniferaldehyde and sinapaldehyde) were determined by HPCE, using a 50 mM borate buffer with pH 9.3 and a capillary of 45 cm x 50 μm i.d. The temperature was maintained at 20ºC and the voltage was 30 kV. Detection was performed at different wavelengths: 348 nm, 362 nm, 404 nm and 422 nm. The duration of the analysis was 10 min, time enough to obtain good separation and reasonably high detection sensitivity.

Watanabe et al. published two articles related to Japanese liquors, such as sake and sochu. In the first one [177], the author determined tyrosol, tryptophol and ferulic acid in different commercial sake samples by MEKC, using the following conditions: fused-silica capillary (50 μm x 31.4 cm) thermostated at 20ºC, potential of 15 kV, injection of samples by pressure at 350 mbar for 1 s, UV detection at 280 nm. The running buffer

used was 20 mM SDS solution in 30 mM borate buffer (pH 8.5). The second article [178] showed the application of the same method (MEKC) to determine vanillic acid, vanillin, ferulic acid and 4-vinylguaiacol in sochu samples. The conditions were exactly the same apart from the running buffer, which in this case consisted on 25 mM SDS solution in 25 mM phosphate-50 mM borate buffer (pH 7.0).

Both of them proved to be a very useful tool for the analysis of phenols in comparison with other techniques, as HPLC, and for this reason they can be applied to routine quality control of liquors. The major difference with other research works is the sample preparation, since in these two papers the extraction of phenolic compounds was performed by solid-phase instead of liquid-liquid.

1.4.5. Vegetable oils

Among the different vegetable oils, VOO is unique because it is obtained from the olive fruit (*Olea europaea L.*) solely by mechanical means, without further treatment other than washing, filtration, decantation, or centrifugation [179].

Its chemical composition consists of major and minor components. The major components, that include glycerols, represent more than 98% of the total weight. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1 n-9) ranges from 56 to 84% of total fatty acids [180], while linoleic acid (18:2 n-6), the major essential fatty acid and the most abundant polyunsaturate in our diet, is present in concentrations between 3 to 21% [181]. Minor components, that amount to about 2% of the total oil weight, include more than 230 chemical compounds, e.g., aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants [182]. The main antioxidants of VOO are carotenes and phenolic compounds, including lipophilic and hydrophilic phenols [183]. While the lipophilic phenols, such as tocopherols, can be found in other vegetables oils, some hydrophilic phenols of VOO are not generally present in other oils and fats [183, 184]. The phenolic fraction of VOO consists of a heterogeneous and very complex mixture of compounds, mainly simple phenols, lignans, flavonoids and secoiridoids each of which varies in chemical properties and impacts in different ways on the quality of VOO [182, 185]. There is evidence that phenolic compounds could play a major role in the healthy effects of VOO, besides to be responsible of its antioxidant

activity and organoleptic properties (flavour, astringency,...) [186-188]. Therefore, the determination of this family of compounds in olive oil is of special relevance.

So far, different analytical methods (GC, HPLC, CE) coupled to different detectors (UV, fluorescence, MS) have been developed to analyze olive oil phenolic compounds. Although HPLC is the most frequent method of choice, very interesting results have been achieved by using other analytical techniques; obviously in the current review we focus on CE methodologies.

CE was used for the first time in 2003 for analysing extracts from VOO by Bendini et al. [189]; they published a very interesting paper in which LLE and SPE were compared and the analytical separations were made by HPLC and CZE. Since that moment, CE has become more and more popular in the analysis of phenols from vegetable oils (mainly olive oils) [190, 191]. ***Table 1.6*** summarizes the most important applications in the field; the differences in internal diameter of the capillary and time of injection, as well as the extraction system (the quantity of olive oil used in the extraction protocol and the volume of solvent for redissolving the phenolic compounds extracted) cause the differences in sensitivity among these methods.

All the gathered methods without MS detection used simple CZE methodologies based on a borate run buffer at alkaline pH, since the most efficient operative mode to separate phenolic compounds has been found to be borate-based CZE.

In 2006, Carrasco-Pancorbo et al. [200] developed the first CE-ESI-IT MS method offering to the analyst the chance to study important phenolic compounds, such as phenolic alcohols (TY, HYTY and 2-(4-hydroxyphenyl)ethyl acetate), lignans ((+)-pinoresinol and Ac Pin), complex phenols (Lig Agl, Ol Agl, their respective decarboxylated derivatives and several isomeric forms of those compounds and 10-hydroxy-Ol Agl) and another phenolic compound (EA) in extra-virgin olive oil (EVOO), using a simple SPE before CE-ESI-MS analysis.

Table 1.6. Relevant examples of separation of phenolic compounds from the polar fraction of olive oil using CE methods.

			Instrumental Variables						Chemical Variables			
Reference	**Extraction system and amounts**	**Detected compounds in olive oil**	**λd [nm]**	**V [kV]**	**T [°C]**	**i.d. [μm]**	**Lef [cm]**	**t_{inj} [s]**	**Type of Buffer**	**[Buffer] [mM]**	**pH**	**Other relevant aspects**
Bendini et al. [189]	LLE (Pirisi et al. [192]) 2 g → 1mL	HYTY, TY, unidentified secoiridoids compounds	200	27	30	50	40	3s (0.5 p.s.i)	Sodium Tetraborate	45	9.6	1st paper where CE is used for the analysis of phenolic compounds from oils
Bonoli et al. [193]	LLE (Pirisi et al. [192]) 2 g → 1mL	HYTY, TY, DHPE, unidentified oleuropein aglycone derivatives	CZE method of Bendini et al. [191]									
Bonoli et al. [194]	LLE (Pirisi et al. [192], modified by Rotondi et al. [195]) 2 g → 0.5mL	HYTY, TY, DAOA, vanillic acid, Ac Pin	CZE method of Bendini et al. [191]									
Carrasco-Pancorbo et al. [196]	60 g → 0.5mL	13 phenolic acids + taxifolin (flavanonol)	210	25	25	75	50	8s (0.5 p.s.i)	Sodium Tetraborate	25	9.6	Potent extraction system which permits to detect little quantities of phenolic acids
Buiarelli et al. [197]	10 g → non specified (Combination of LLE-SPE)	5 phenolic acids	200	18	25	50	36	2s (1.5 p.s.i)	Sodium Tetraborate	40	9.2	
Carrasco-Pancorbo et al. [198]	60 g → 0.5mL [196]	13 phenolic acids + taxifolin (flavanonol)	210	-25	25	75	50	8s (0.5 p.s.i)	Sodium Tetraborate (20% 2-propanol)	50	9.6	Co-electroosmotic CE
Gómez-Caravaca et al. [199]	60 g → 2mL (SPE-Diol)	TY, Pin, Ac Pin, DOA, Lig Agl, HYTY, Ol Agl, EA	214/250	25	25	75	100	8s (0.5 p.s.i)	Sodium Tetraborate	30	9.3	Use of standards obtained by semipreparative-HPLC
Carrasco-Pancorbo et al. [200]	60 g → 2mL (SPE-Diol [199])	11 phenols (simple phenols, lignans, complex phenols and EA)	214/**MS (ESI-IT)**	25	25	50	100	10s (0.5 p.s.i)	NH_4OAc (5% 2-propanol)	60	9.5	1st paper in which CZE-ESI-IT MS is used for the analysis of phenolic compounds from oils

Table 1.6. (continued)

Reference	Extraction system and amounts	Detected compounds in olive oil	λd [nm]	V [kV]	T [°C]	i.d. [μm]	Lef [cm]	t_{inj} [s]	Type of Buffer	[Buffer] [mM]	pH	Other relevant aspects
Carrasco-Pancorbo et al. [201]	60 g → 2mL (SPE-Diol [199])	26 compounds belonging to all the different families of phenolic compounds present in olive oil	200/240/280/330	28	22	50	40	8s (0.5 p.s.i)	Sodium Tetraborate	45	9.3	26 compounds in less than 10 min. 1st paper in which flavonoids are detected by CE, and 1st method "multicomponent" for the phenols of olive oil
Carrasco-Pancorbo et al. [202]	60 g → 2 mL (SPE-Diol [199])	Applicative work using a method previously described [203]	CZE method of Carrasco-Pancorbo [203]									Interesting from a quantitative and applicative point of view
Carrasco-Pancorbo et al. [203]	60 g → 2 mL (SPE-Diol [199]) and diluted 1:10	All the phenolic compounds "well-knonw" and other 28 analytes	**MS (ESI-TOF)**	30	25	50	85	10s (50 mBar)	Ammonium hydrogen carbonate	25	9.0	1st paper in which CZE-ESI-TOF MS is used for the analysis of phenolic compounds from oils. TOF permits the "identification" of new compounds in the profile of the oils
Aturki et al. [204]	LLE (Pirisi et al. [192]) with minor modifications 2 g → 0.5 mL and diluted 1:5	7 phenolic acids and HYTY, TY and Oleuropein	200	22	20	75	33 (eff. packed lengths of 24.5 and 23cm)	Combination of pressure and Voltage (10 bar x 10kV x 0.5min)	Mobile phase: Ammonium formate buffer/Water/ACN (5:65:30 v/v/v)	100	3.0	Reversed-phase capillary electrochromatography. Complete method validation
Carrasco-Pancorbo et al. [205]	60 g → 2 mL (SPE-Diol [199]) and diluted 1:8	Applicative work using a method previously described [201]	CZE method of Carrasco-Pancorbo [203]									Quantitative comparison of phenolic profiles of two PDOs and their relation to sensorial properties with statistical analysis
Gómez-Caravaca et al. [206]	60 g → 2 mL (SPE-Diol [199]) and diluted 1:10 or redissolved in 10 g oil+10 mL 1-propanol	All the phenolic compounds "well-knonw" and other 28 analytes	**MS (ESI-TOF)**	30	25	50	85	10 s (50 mBar)	NH_4OAc/AcH in methanol/ACN (1/1 v/v)	25	5.0	Direct injection of the investigated matrix introducing a plug of olive oil directly into the capillary. Comparison between CZE and NACE

After showing the potential of IT as mass analyzer, another publication compared the capabilities of a HPLC-ESI-TOF MS method with those of a CE-ESI-TOF MS [203], showing that the two methodologies were able to determine many well-known phenolic compounds present in olive oil and provided information about the presence and relative concentration of minor phenolic compounds. Both CE-MS and LC-MS could determine more than 45 analytes in each run.

Aturki et al. [204] achieved the simultaneous separation of ten phenolic compounds (protocatechuic, *p*-coumaric, *o*-coumaric, vanillic, ferulic, caffeic and syringic acids, HYTY, TY and oleuropein) in EVOOs by isocratic reversed-phase CEC, demonstrating that the mentioned methodology can be successfully employed for the separation of polar compounds with high precision, linearity and sensitivity.

One of the most promising newest applications involved the use of NACE-ESI-TOF MS [206]; the authors compared the results achieved by NACE with those obtained by CZE (with aqueous buffers) both coupled to ESI-TOF MS. Both methods offer the chance to study phenolic compounds from EVOO belonging to different families by injecting methanolic extracts with efficient and fast CE separations. Apart from that, in the case of the NACE method, the direct injection of the investigated matrix introducing a plug of olive oil directly into the capillary was also checked, producing very interesting results.

In ***Figure 1.3***, a comparison of the analysis of the phenolic fraction of an EVOO analyzed by HPLC (the technique most widely used) with the profiles achieved by three different CE methodologies are showed. The comparison between HPLC and CE results in terms of analysis time is quite clear, although the truth is that nowadays the improvement in the chemistry of LC columns and LC systems (withstanding higher pressures) could provide shorter analysis time. Anyway, what is more interesting about ***Figure 1.3*** is to observe the evolution/improvement of the CE method. The first example (A) is the electropherogram obtained with a simple CZE-UV method [201]. The method was quite powerful, being able to separate, identify and quantify about 20 compounds in 6 min. The second example (B) [203] illustrates a CZE-ESI-TOF MS separation of the polar extract from an EVOO. TOF MS facilitated the identification of a great number of compounds. In (C), the reader can have a look at the profile achieved when direct

injection of EVOO diluted in an organic solvent was introduced inside capillary and a NACE separation carried out. Despite that analysis time is still better for aqueous CE (B), the fact of doing direct injection of olive oil in NACE is one of the greatest advantages that can be mentioned.

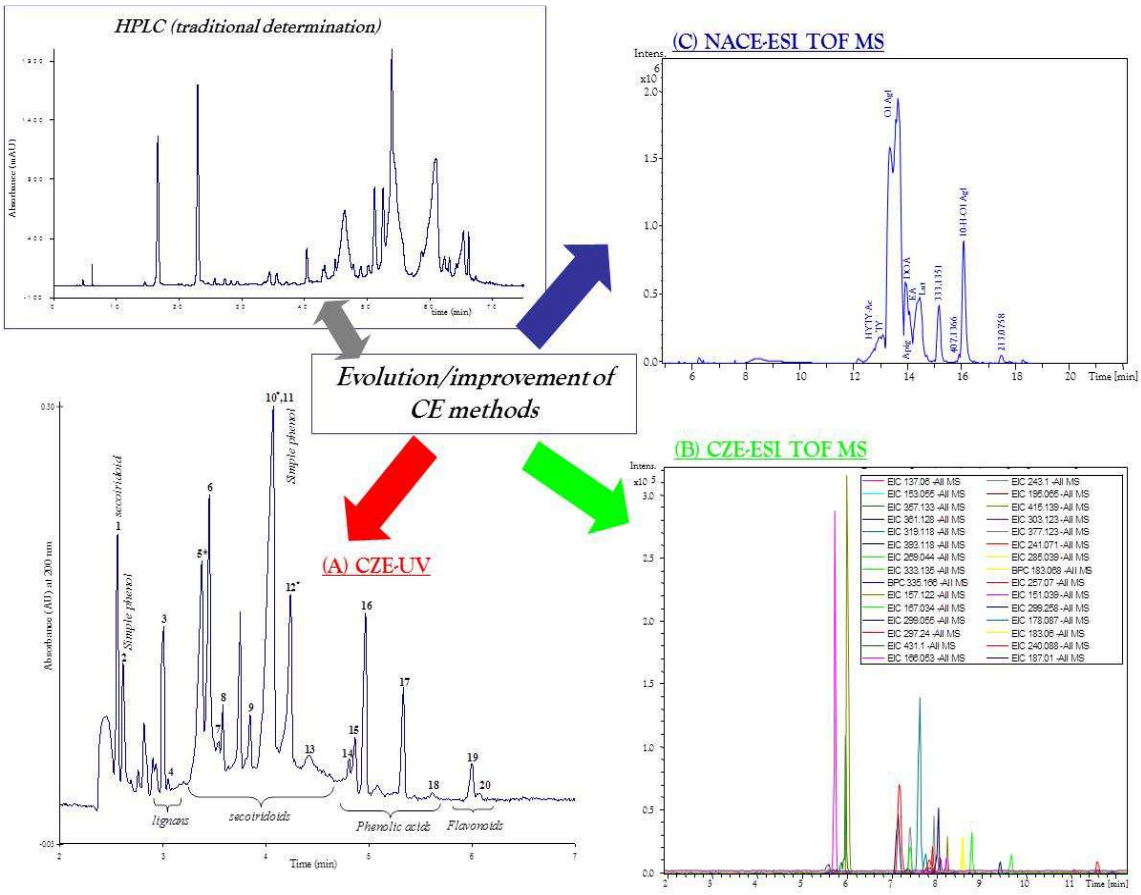

Figure 1.3. Comparison between HPLC and CE as analytical tools for analysing polyphenols from EVOO. The figure shows as well the evolution/improvement of the CE methods. (A) CZE-UV analysis of an extract from an EVOO. Peak identification numbers: 1, Lig Agl (a); 2, TY, 3, Pin; 4, Ac Pin; 5, Ol Agl (a) + DOA (a); 6, DOA (b); 7, Lig Agl (b); 8, Ol Agl (b); 9, EA (a); 10, Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b,c); 11, HYTY; 12, DOA (d) + EA (d); 13, EA (e); 14, trans-cinnamic acid; 15, 4-hydroxyphenylacetic acid; 16, sinapinic acid; 17, gentisic acid; 18, o-coumaric acid; 19, luteolin; and 20, apigenin. (B) EIEs as obtained by CZE-ESI-TOF-MS of an extract from EVOO at the optimum electrophoretic and MS conditions. (C) BPE obtained by NACE-ESI-TOF-MS after direct injection of EVOO sample inside the capillary.

1.4.6. Cereals, legumes and nuts

Cereals, legumes and nuts are matrices that have been studied together in some publications, which is why we decided to include them in the same section.

Cereals have an important role in human nutrition, since they constitute one of the most pivotal pillars on the diet. The term "cereal" includes wheat, barley, maize, oats,

millet, rye, sorghum, malt and rice. Cereals are a source of phenolic acids [207] and flavonoids in the free and conjugated forms, which are usually located in the outside layers [208]. Maize, rice, wheat and sorghum have been described also for containing anthocyanins [209].

Several research groups have worked in the field of the determination of phenolic compounds from cereals by using electrophoretic methods. For instance, Hernández-Borges et al. [169] described a rapid separation by coelectroosmotic CE of 7 phenolic acids (protocatechuic, salicylic, *p*-hydroxybenzoic, vanillic, syringic, ferulic and sinapic acids) present in corn flour samples. The phenols were extracted by LLE and these samples were hydrodynamically injected for 2 second. The separation was carried out with a fused-silica capillary of 75 µm i.d. x 40 cm effective length, at 20°C, using as running buffer 125 mM borate, 49 mM phosphate, 0.002% (w/v) hexadimethrine bromide, 2.5 mM α-CD, at 7.5 pH and applying a separation voltage of 15 kV. Finally, compounds were detected at 280 nm by DAD. The same research group [210] developed a CE method to determine the phenolic acids mentioned above in gofio, a typical food produced in Canary Islands (Spain) that consists of roasted flours, alone or mixed, of barley, wheat and corn. In this case, they used the following conditions: 40 cm effective length x 75 µm i.d. capillary, run voltage -15 kV, temperature 20°C, hydrodynamic injection for 2 s and detection at 280 nm. The running buffer was 125 mM borate, 48.6 mM phosphate, 0.002% (w/v) hexadimethrine bromide, 2.5 mM α-CD and pH 7.5. This method proved to be reproducible and provided good separation.

According to Vaher et al. [211], wheat is an important source of sinapic, syringic, ferulic, *p*-coumaric, vanillic and caffeic acids. The analyses were carried out at 25°C with a fused silica capillary (50 µm i.d. x 52 cm effective length), using 50 mM sodium tetraborate (pH 9.3) as separation buffer. Detection was made at 210 nm and the voltage was set at 20 kV. Several wheat samples were analyzed and the method demonstrated its usefulness.

Peng et al. [212] analyzed the hull and flour of buckwheat by CE-ED. They could identify (–)-epicatechin, rutin, hyperoside and quercetin using 50 mM borate buffer with pH 8.7 and a fused silica capillary of 75 cm length and 25 µm i.d. Samples were injected for 6 s and the separation voltage was 16 kV. The method was satisfactory. A similar study

was carried out by Kreft et al. [213] and they determined rutin in seeds of buckwheat, using an uncoated capillary of 50 µm i.d. x 57 cm effective length. The temperature was maintained at 18ºC and the applied voltage was 25 kV. Samples were injected for 20 s, the detection was carried out at 380 nm and the running buffer was 50 mM borate and 100 mM sodium dodecyl sulfate (pH 9.3).

Barley is other cereal which has been studied, indeed Samaras et al. [214], Kvasnička et al. [215] and Bonoli et al. [216] paid attention to it. The first author identified ferulic, *p*-coumaric, vanillic, homovanillic, and *p*-hydroxyphenyl-acetic acids, catechin and 4-vinylguaiacol in barley and different types of malts. For the CE analysis, they used a fused silica capillary (40 cm effective length x 50 µm i.d.), the temperature was maintained at 25ºC, the applied voltage was 25 kV, the running buffer was formed by 50 mM borate containing 20% methanol (pH 9.5), samples were injected for 5 s and separations were monitored at 200, 280, 325 and 420 nm. Kvasnička et al. [215] resorted to CZE to identify *p*-coumaric, caffeic, ferulic and sinapic acids, using the following conditions: the background electrolyte was 50 mM acetic acid, 95 mM 6-aminocaproic acid, 0.1% polyacrylamide, 1% polyvinylpyrrolidone and 10% methanol; capillary of 28 cm effective length x 50 µm i.d.; constant voltage of -30 kV; temperature 30ºC; detection at 230 and 315 nm; and samples injection for 25 s by pressure. Finally, Bonoli *et al.* [216] analyzed wholemeal barley flour using MEKC but, in contrast to the previous, they determined other different phenolic compounds besides phenolic acids. They carried out SLE and PLE, using different mixtures of solvents and methods. The electrophoretic conditions were: a 20 mM sodium tetraborate, 5 mM potassium dihydrogen phosphate and 10 mM SDS buffer (pH 9.0), 50 µm i.d. x 40 cm effective length capillary, voltage of 30 kV, temperature 30ºC, injection for 3 s and detection at 200 nm. Results showed that the method was efficient, reliable and suitable to determine some flavan-3-ol compounds, proanthocyanidins, hydrolysable tannins and hydroxycinnamic acids.

Nuts are a group of food consumed traditionally in the Mediterranean area, Asia and South America. Nowadays, they are eaten all around the world like snacks or appetizers, but they are also included in different desserts, sauces, etc. [217]. Nuts are a great source of energy, providing proteins, essential unsaturated fatty acids, vitamins and minerals

[218]. They also contain several compounds with beneficial effects in human health, such as tannins and polyphenols, among others [219].

Cifuentes et al. [220] was one of the first authors who identified phenolic compounds in nuts, particularly in almond peels, by CE. They used MEKC with acidic buffers to determine procyanidins and other compounds. The optimum conditions used for the analyses were: fused-silica capillary 30 cm effective length x 50 µm i.d., applied voltage of 14 kV, sample injection for 1.5 s, temperature 25°C, detection at 200 and 280 nm, and running buffer formed by 50 mM acetic acid/sodium acetate and 100 mM SDS (pH 5.0). The results showed the presence of catechin and procyanidin B2 in almond peel samples.

Cashew nut has been other matrix analyzed, specifically by Česla et al. [221]. They used MEKC to carry out the separation of different anacardic acids and chose MS as detector. They employed two different methodologies, since they used bare fused silica capillaries and polydimethylacrylamide-coated capillaries. Conditions were the same for both methodologies, except the effective length of capillaries, the applied voltage and the background electrolyte. The temperature was maintained at 15°C, the detection was carried out at 214 and 305 nm, and samples were injected hydrodynamically for 10 s. Non-coated capillaries had an effective length of 40 cm, the voltage used with them was 20 kV and the running buffer was formed by 40 mM borate pH 9.2 with 1 M urea, 27 mM SDS and 24% acetonitrile. On the other hand, polydimethylacrylamide-coated capillaries had 50 cm of effective length, the separation voltage was -17.5 kV and the background electrolyte was 10 mM phosphate, 1 M urea, 20% acetonitrile, 20 mM SDS, 10 mM β-CD and 1 mM heptakis-6-sulpho-β-CD. The best results were obtained with polydimethylacrylamide-coated capillaries, since separation selectivity and efficiency were better.

Gómez-Caravaca et al. [222] developed a CE method coupled to MS for determining catechin, digalloylglucose, glansreginin B, ferulic acid glucoside, vanillic acid glucoside, glansreginin A, cumaroylquinic acid, chlorogenic acid, ellagic acid and ellagic acid pentoside dimer. The optimum conditions were uncoated fused silica capillary 50 µm i.d. x 90 cm effective length, voltage 30 kV, running buffer 40 mM ammonium acetate/NH_3 (pH 9.5) and sample injection for 20 s. The method proved to be successful, as it allowed

to identify and quantify compounds which were present in the polar fraction of walnuts in a short analysis time (less than 15 min).

Karamać [223] resorted to CZE to separate tannin fraction of walnut, hazelnut and almond kernels. This author employed an uncoated silica capillary with 50 µm i.d. x 40 cm effective length, temperature 40ºC, a 100 mM boric buffer (pH 8.5), a constant voltage of 20 kV, injection for 3 s and detection at 280 nm. The results showed that the profiles of hazelnuts and almonds were quite similar, whereas the walnut analyses were qualitatively different. The tannin fraction of walnut mainly consisted of hydrolysable tannins, whereas condensed tannins were typical in the fractions of hazelnuts and almonds.

Legumes are dried seeds or fruit of different plants, which have been part of the human diet, together with the cereals, for centuries. There are a wide range of legumes, for instance, lentils, chickpeas, white and black beans, peas, soybean, lupin, etc. Only a few of them have been analyzed by CE and soy is, undoubtedly, the most studied.

Shihabi et al. [224] used CE to identify daidzin, genistin, biochanin A, daidzein, genistein and coumestrol in samples of soybean. To carry out the separation, they employed a capillary of 50 cm x 50 µm i.d., a running buffer formed by 200 mM borate (pH 8.6) and a voltage of 13 kV. The samples were injected for 10 s by pressure and the detector set at 254 nm. The method proved to have good reproducibility in a short time.

The method developed by Cifuentes et al. [220] for determining procyanidins and other phenolic compounds in almond peels, was applied to lentils, white beans and black beans. The compounds under study were not found in white beans, while lentils presented 6 compounds (procyanidin B_3, procyanidin B_1, (+)-catechin, *cis p*-coumaric acid, *trans p*-coumaric acid and (–)-epicatechin) and black beans contained 5 of the analytes studied (procyanidin B_1, (+)-catechin, procyanidin B2, *trans p*–coumaric acid, (–)-epicatechin). This method allowed a good separation and good reproducibility (intra- and interday) in less than 5 min.

Peng et al. [225] analyzed different soy products by CE with ED. They achieved daidzein and genistein separation using the following conditions: capillary of 70 cm x 25

μm i.d., sample injection for 6 s, voltage of 12 kV and 100 mM borate buffer (pH 11.0). It proved to be a useful qualitative method, simple and rapid.

Dinelli et al. [226] developed a CE-DAD method to identify and quantify different flavonoids (daidzein, genistein, glycitein and kaempferol) in soybean and three common kinds of beans. The aim of this work was to compare the compositional changes in those samples when they were treated with UV-B radiation. CE analyses were carried out with capillaries of 75 μm i.d. x 30 cm effective length, at 25°C, applying a voltage of 15 kV and using 50 mM ammonium acetate buffer (pH 10.5) with 20% (v/v) methanol. Samples were injected for 10 s and the electropherograms were recorded at 214 and 260 nm. The results showed that these common beans could be used as a potential dietary source of isoflavones.

García-Villalba et al. [227] carried out a comparison between transgenic and conventional soybean using CE-UV and CE-MS. They identified several plant metabolites and some of them were phenolic compounds, like genistein, daidzein, formononetin, kushenol B, exiguaflavanone D, genistin, daidzin sophora-iso-flavanone D, kushenol M, 6-methoxytaxifolin, genistein 7-*O*-malonylglucoside, daidzein 7-*O*-malonylglucoside, naringenin 7-*O*-glucoside and taxifolin 3-rhamnoside. To achieve that, they employed a fused-silica capillary of 50 μm i.d. and 84 cm of effective length, a 50 mM ammonium hydrogencarbonate (pH 9.0), a voltage of 28 kV. Samples were injected for 10 s and the wavelength chose in UV-Vis was 200 nm. One of the most significant results obtained in this work was the presence of one compound in traditional soybean that was not found in the transgenic one.

There are other publications analyzing soy and other legumes used in different applications. For example, Micke et al. [228] used MEKC to identify isoflavones in soy germ pharmaceutical capsules, and Mellenthin et al. [229] resorted to CZE to analyze polyphenols of soy, lupin and pea for determining the presence of proteins of those legumes in meat products.

1.4.7. Others

As commented before, we have decided to include a section entitled "Other matrices" which will enclose information about cocoa beans and bee products.

Cocoa beans and products

Cocoa beans, the seeds from *Theobroma cacao*, are a rich source of polyphenols, in particular flavan-3-ols (or catechins) and procyanidins, representing about 10% of the whole bean's dry weight and its derivative chocolate, especially dark chocolate [230]. Geographic and climatic factors can affect the content of flavonoids in cocoa beans, as well as changes in their quantities are occurring during the chocolate and/or cocoa manufacture. Different electroseparation techniques have been used for the analysis of phenolic compounds in cocoa beans and its products.

A patented MEKC method [231] was applied in the separation of cocoa procyanidin oligomers. The buffer consisted of 200 mM boric acid and 50 mM SDS (pH 8.5 adjusted with sodium hydroxide), and the uncoated fused-silica capillary maintained at 25°C was 50 cm length x 75 µm i.d. Analytes were separated in 12 min and detected by DAD at 200 nm.

Modified MEKC methods, using SDS as a principal component of the running buffer, were developed for the determination of catechins and xanthines in cocoa extracts [232, 233]. The separations were obtained on fused-silica capillaries of 50 µm i.d. with a total length of 38.5 cm, used in the "short-end" mode (8.5 cm of effective length). The applied voltage was maintained at 15 kV (anodic detection), the temperature at 30 °C and compounds were detected at 220 nm. Owing to the reported poor stability of catechins in alkaline solutions, MEKC under acidic conditions was preferred. As a result, the EOF was significantly suppressed, resulting in a fast migration of the SDS micelles into the anode and making necessary the use of additives into the buffer to modulate selectivity. In a first study [232], three different systems for the separation of (+)-catechin, (–)-epicatechin, caffeine, and theobromine in four different commercial chocolate types (black and milk) and two cocoa powder samples were optimised. 3-[(3-cholamidopropyl)dimethylammonio]-1-propansulfonate and taurodeoxycholic acid sodium salt were individually supplemented to the SDS solution to obtain binary mixed micelles with varied hydrophobicity. A further cyclodextrin-modified MEKC approach was developed by addition of hydroxypropyl-β-cyclodextrin (HP-β-CD) to the SDS-based buffer. This system resulted more suitable to analyze the cocoa samples than those based on micelles. Under the optimum conditions (50 mM Britton-Robinson buffer at pH 2.5,

90 mM SDS, 10 mM HP-β -CD), the method was applied to improve the extraction procedure, choosing water under sonication for 10 min at 60 °C; then it was validated and used for the quantitative determinations.

In a subsequent study [233], (+)-catechin, (–)-epicatechin and theobromine from different cocoa beans and commercial black chocolate were quantified using an enantioselective CD-MEKC method in order to evaluate the possible epimerisation of epicatechin during chocolate manufacture. The method conditions were similar to those described previously from the same authors, changing the buffer composition (12 mM of HP-β-CD instead of 10) and the extraction protocol (29% ethanol under sonication for 15 min at 65 °C). The analysis of cocoa beans showed remarkable differences in (+)-catechin and (–)-epicatechin contents depending on the provenience and maturation degree of the beans. (-)-Catechin was not detected in any of the analyzed samples. Interestingly, in black chocolate very small amounts of the native (+)-catechin were found, whereas a clear peak of (–)-catechin was detected. This results confirmed the hypothesis of an epimerisation at the C-2 position of (–)-epicatechin probably occurring during heat processing and storage of the beans.

Kofink et al. [234] obtained a similar conclusion regarding (–)-epicatechin epimerisation during chocolate production; (–)-epicatechin and small amounts of (+)-catechin were detected in unfermented, dried, unroasted cocoa beans, while increased levels of (–)-catechin were found in roasted cocoa beans and in commercial cocoa products. The high temperature during the bean roasting process and particularly the alkalisation of the cocoa powder were the main factors inducing the epimerisation reaction. The enantioselective separation was achieved by CCE-UV detection using 100 mM borate buffer (pH 8.5) with 12 mM (2-hydroxypropyl)-γ-cyclodextrin as chiral selector, an applied voltage of 18 kV and a fused-silica capillary (50 cm effective length x 75 μm i.d) maintained at 20 °C.

Ohashi et al. [235] did not analyzed the phenolic compounds coming from cocoa, but those added as flavouring material. In particular, the vanillin and ethylvanillin included in three types of cocoa drinks and their metabolites 2-methoxyphenol and 2-ethoxyphenol after the addition of the bacterium *Bacillus firmus* were determined by CE-DAD. Those metabolites were responsible of off-flavours in the cocoa drinks. The

running buffer system comprised 50 mM phosphate buffer and 2 mM cetyltrimethylammonium hydroxide at pH 10.0 with 10% acetonitrile and sorbic acid as the internal standard. Samples were simply diluted before their analysis.

Bee products

Honeybee products have been employed since ancient times with both domestic and medical purposes, being subjected to extensive clinical studies during the last few decades. The phytochemical composition of hive products depends on their floral origin and thus on the geographic and climatic characteristics at the site of collection. Such composition differences are likely to affect the biological and clinical properties of bee products. Major hive products are honey, propolis, royal jelly, bee pollen, bee bread and beeswax. Among them, only the phenolic profiles of honey, propolis and pollen have been studied by CE methods.

To make honey, bees gather the nectar from various flowers, ingest and regurgitate it several times before being stored in the comb. Then, considerable amounts of water are evaporated, raising honey's sugar concentration and preventing its fermentation. Monofloral honeys are appreciated by consumers, and their price is often related to this floral origin. The analysing methodology to prove the origin of monofloral honeys is not fully established. However, the study of phenolic compounds has resulted in a useful tool for the determination of honey's botanical and geographical origins [236-238].

Delgado et al. [236] made the first attempt to provide an alternative methodology based on CE for the characterization of honey flavonoids, comparing the separation obtained by MEKC with that achieved by HPLC. Fourteen different flavonoids isolated from honey were analyzed by MEKC with UV-Vis detection. However, it was difficult to find specific experimental conditions to separate all flavonoids in a single run, optimising three different electrophoretic methods, depending on the flavonoid markers sought in honey. These authors achieved the complete separation of 13 flavonoids from honey in a single run [237] (12 of them also included in the preceding work) by adding 10% methanol as organic modifier to the MEKC running buffer, which consisted of 200 mM sodium borate and 50 mM SDS (pH 8.0). The optimised method was applied to study honey samples with different botanical (lavender, rosemary, orange tree and heather) and

geographical (Spain, Mexico and Canada) sources to asses the use of flavonoids as possible markers for honey origins. Citrus honey was characterised by the accumulation of hesperetin, lavender by luteolin, rosemary by 8-methoxykaempferol and heather by some unidentified flavonoids. Regarding geographical origin, no significant differences were found on the honey flavonoid pattern.

A more extensive study about the use of phenolic compounds as floral markers for honeys was carried out using CZE with UV detection at 280 nm [238]. A total of 26 phenolic compounds, including 16 phenolic acids and 10 flavonoids, together with hydroxymethylfurfural were analyzed in 11 monofloral honey samples (heather, lavender, acacia, rape, sunflower, rosemary, citrus, rhododendron, thyme, chestnut-tree and calluna). The analysis were carried out on a fused-silica capillary (50 cm × 50 μm) at 30 °C using 100 mM sodium borate buffer with 20% methanol (pH 9.5), within 18 min. Phenolic profiles of the analyzed honeys showed significant differences. Total phenolic acid content or the relative amount of one individual phenolic acid derivative could be related to the floral origin of the honey. The higher contents of phenolic acid derivatives were found in heather honey whereas citrus and rosemary honeys were characterized for having small amounts. On the other hand, heather honey had a considerable amount of phenylethylcaffeate, while lavender honey contained *m*-coumaric acid as the principal phenolic compound. In some cases, a single phenolic compound was detected in only one unifloral honey type, and could be considered as a potential floral marker. Thus, thyme honey was characterised by the presence of rosmarinic acid, heather honey by ellagic acid, citrus honey by hesperetin, and lavender honey by naringenin.

Gómez-Caravaca et al. [239] studied the polyphenolic profile of 6 different honeys using as well CZE with UV detection. Standard solutions of 22 phenolic compounds previously found in honey were prepared and spiked individually in the honey extracts. Good resolution in a satisfactory time was achieved with the developed CZE method. Nonetheless, only 5 phenolic compounds were identified in the studied samples, of which 2 coeluted, enabling the quantification of only 3 of them. Due to the problems found with the UV detection, the use of MS was recommended and, in fact, used in another work for the identification of phenolic compounds in rosemary honey [240]. CZE and ESI-MS parameters (i.e. running buffer composition and pH, applied voltage,

sheath liquid composition and flow rate, drying gas flow rate and temperature, and nebulisation gas pressure) were optimised. This methodology allowed the identification of 13 flavonoids in rosemary honey, being one of them the flavonol kaempferol, potential floral marker for rosemary honey.

Propolis, or bee glue, is a dark-coloured resinous substance collected by honeybees from leaf buds and cracks in the bark of various tree species and enriched with salivary and enzymatic secretions. Bees use propolis to seal holes in their hives, make stronger the borders of the comb or make the entrance weather-proof. It is also used as an "embalming" substance to cover the killed invaders that can not be transported out of the hive. The modes of operation used for the determination of phenolic compounds in raw propolis and commercial propolis preparations have been CZE [241-243], MEKC [48, 244, 245] and a combination of both [246, 247] coupled to DAD, with the exception of a work employing CZE-MS [248]. Besides the well-known advantages of CE over HPLC for the characterization of compounds (analysis time, solvent consumption, sample quantity), CE allowed the analysis of propolis samples without any pre-cleaning, since contaminants can be removed from the capillary by rinsing with the appropriate solvents after each analysis.

Chi et al. [241] developed the first CE method to determine the composition of a propolis sample collected in China. The electrophoretic conditions were optimised using 12 phenolic standards occurring in propolis. To achieve a good separation, 0.7 mM β-ciclodextrin was added to the buffer solution consisting of 25 mM sodium dihydrogenphosphate and 25 mM sodium tretraborate at pH 10.1. Caffeic, 3,4-dimethylcaffeic and isoferulic acids and quercetin were identified and quantified in the ethanolic extracts of the real propolis sample.

Cao et al. [242] developed a CZE method that could separate ferulic and caffeic acids, rutin, apigenin, luteolin and quercetin within 12 min with no need of incorporating additives to the buffer. The effects of several factors such as acidity and concentration of the background electrolite, separation voltage, injection time and UV wavelength were investigated, being the optimum values borax buffer with pH 9.2 and 50 mM, 23 kV, 12 s and 262 nm, respectively. The 6 active constituents were determined in raw propolis and propolis capsules from China. Capsules were indeed more concentrated than raw

propolis, except for caffeic acid that was not detected. Rutin content increased notably more than the rest of the ingredients.

A broad study of the levels of phenolic compounds in other pharmaceutical products containing propolis was accomplished using as well CZE-UV by Volpi [243]. With a buffer constituted by 30 mM of sodium tetraborate (pH 9) and applying 15 kV, 15 phenolic standards were separated on a uncoated fused-silica capillary 70 cm (50 cm effective length) x 50 µm i.d. within 40 min, although not all could be identified in the samples. Commercial propolis preparations were in the form of ethanolic, aqueous-ethanolic, and aqueous-glycolic extracts used to prepare oral sprays, tablets, and syrups. The aqueous-ethanolic preparation mainly contained caffeic acid, galangin, quercetin and chrysin, whilst the ethanolic extract was mostly composed of resveratrol, chrysin and caffeic acid, with 12.4% and 6% of nonidentified compounds, respectively. On the contrary, the aqueous-glycolic propolis preparation contained 11% of caffeic acid and approx. 85% of nonidentified compounds.

There is only one report using CZE-ESI-MS for the identification of propolis antioxidant compounds [248]. Therefore, the separation parameters and the MS conditions were carefully optimised using directly the ethanolic extract of raw propolis. Due to volatility requirements, the most commonly used buffers or additives for CZE-DAD were not suitable. Separation was achieved in a 90 cm x 50 µm i.d. bare fused-silica capillary with a running buffer of 80 mM ammonium acetate/NH_3 (pH 10.5) at 30 kV. 11 flavonoids previously reported in propolis were identified by comparing both migration times and MS data of the peaks in the propolis extract with those of pure standards, when available. Identification was confirmed by the data provided by a TOF mass spectrometer, which enabled formula generation for each mass.

The usefulness of both CZE and MEKC-DAD for the profiling of phenolic compounds in 10 raw propolis samples was demonstrated by Hilhorst et al. [246]. First, the samples were extracted with water to isolate the more polar constituents, such as phenolic acids. The extracts were subsequently analyzed by CZE with borate buffer at pH 7.0 and 9.3, and MEKC (borate, pH 9.3, 10% acetonitrile), using 50 mM SDS as surfactant. The selectivity of both systems differed considerably but the information obtained was similar. The dry residues from the water extraction were extracted with

ethanol/water (70:30, v/v) and analyzed by MEKC only, to enable the identification of the more hydrophobic constituents of propolis. In this way, pinocembrin, chrysin and galangin could be identified in all the samples. On the basis of the recorded CZE and MECK electropherograms, samples could be clearly divided into two different groups, probably due to their different origin.

In the later work, CZE and MEKC methods were developed independently and the obtained results compared, being able to identify a different number of compounds in the water extracts using CZE or MECK. In the following investigation [247], the MEKC method was supplemented to separate two phenolics, quercetin and myricetin, that could not be separated by CZE. Therefore, the combination of CZE, utilizing 100 mM borate buffer (pH 9.5) containing 5% methanol under a voltage of 18 kV, and MEKC, utilizing 0.03 M sodium borate (pH 8.5) containing 50 mM SDS under a voltage of -15 kV, allowed the separation of 15 flavonoids, of which 12 were detected in raw propolis and 11 in the commercial propolis products. The total contents of the identified flavonoids in raw and commercial propolis ranged from 254 to 19147 mg/kg and from 1228 to 7985 mg/kg, respectively, suggesting that both origin and processing were important factors affecting the flavonoid content.

MEKC itself has also being employed in the determination of several compounds in propolis. After the systematic optimisation of the analytical conditions of the borax buffer concentration (30 mM) and pH (9.0), surfactant concentration (12 mM), organic modifier (5% ethanol), applied voltage (14 kV), and injection time (8 s), Lu et al. [245] established a MEKC method for the determination of hesperetin, cinnamic and nicotinic acids in a propolis oral liquid preparation. The method was validated in terms of linearity, reproducibility and detection limits.

Propolis and other complex food samples were analyzed by a mixed MEKC-DAD method developed for the separation of ten flavonoid aglycones [48]. The micellar system composed of 25 mM SDS and 25 mM sodium cholate buffered at pH 7 provided the separation of all the analytes in less than 20 min. In raw propolis, chrysin and galangin were the flavonoids with the highest concentration, identifying naringenin, quercetin, and kaempferol as well.

Fontana et al. [244] described a MEKC method for profiling flavonoids and phenolcarboxilic acids from propolis collected in different Brazilian provinces. The authors did not state clearly in the text which compounds were identified in the samples, however they recommended the use of CZE if the profiling of phenolics in propolis, when it is limited only to phenolcarboxilic acids analysis.

Bee pollen is the male seed of a flower blossom collected by honey bees on their legs as they move from flower to flower, mixed with their digestive enzymes and some nectar, accumulating many flavonoids and phenolic acids [249]. In the hive, pollen is used as a protein source for the brood-rearing. To our knowledge, three studies have been carried out in relation to the phenolic content of pollen using CE.

Yang et al. [250] developed a MEKC-UV methodology to determine two flavonoids, isorhamnetin-3-O-neohesperidoside and typheneoside, in pollen typhae (pollen of several species of the genus Typhaceae, an important traditional Chinese medicine). Later on, the use of CZE coupled to ED [251] and MS detection [249] allowed the identification of 13 phenolic acids and flavonoids and 10 flavonoids, respectively, in various pollen samples. The CZE-ED method was validated in terms of repeatability, linearity, and accuracy and subsequently used for the analysis of the phenolic content of honeybee-collected pollen from natural and broken pine, buckwheat, corn, rape, papaver, camellia, basswood, Chinese gooseberry and mixed plant sources. The pollen samples were rich in phenolic compoundss, particularly chrysin, rutin, baicalein, kaempferol, apigenin, vanillic acid and luteolin. Rape, Chinese gooseberry and papaver pollens contained the highest levels of the assayed compounds. The authors stated that the data presented was insufficient to identify unknown samples based on their phenolic profiles, needing additional samples and statistical methods to indicate possible floral markers for pollen.

A CE-ESI-TOF-MS method [249] allowed the identification of several phenolic compounds, mainly flavonoids and glucoside derivatives, in the honeybee-collected pollen from *Ranunculus petiolaris* HKB, a medicinal plant that grows in Durango, Mexico. The separation parameters as well as the MS conditions were systematically investigated. The employed conditions were as follows: 95 cm x 50 μm i.d. fused-silica capillary; 80 mM ammonium acetate buffer (pH 10.5); 30 kV voltage; sheath liquid, 2-propanol/water 60:40 (v/v) plus 0.1% (v/v) triethylamine at 6 μL/min; ESI 4 L/min at 130 °C of drying

gas and 0.3 bar of nebulizing gas pressure; m/z 50–800 MS scan at a spectral rate of 1 Hz in the negative ion mode. Optimisation was carried out using the real sample. The identification of compounds was done without the use of standards due to the mass accuracy and isotopic pattern provided by TOF-MS, which allowed the determination of the elemental composition of the detected compounds.

1.5. Usefulness of generated data

After having a look at the previous sections of the current review, it seems quite clear that the determination of phenolic compounds is awaking a lot of interest from researchers, indeed phrases like the following one can be read quite often nowadays: "Widely distributed in the plant kingdom and abundant in our diet, plant phenols are today among the most talked about classes of phytochemicals". To answer the question of "why are phenolic compounds so interesting?", we can summarize several issues which have been studied in depth during the last decade [252]:

❖ The levels and chemical structure of antioxidant phenols in different plant foods, aromatic plants and various plant materials.

❖ The probable role of plant phenols in the prevention of various diseases associated with oxidative stress, such as cardiovascular and neurodegenerative diseases and cancer.

❖ The ability of plant phenols to modulate the activity of enzymes, a biological action not yet understood.

❖ The ability of certain classes of plant phenols such as flavonoids (also called polyphenols) to bind to proteins.

❖ The stabilisation of edible oils, protection from formation of off-flavours and stabilization of flavours.

❖ The antimicrobial activity.

❖ The bitter-tasting and the pungency of phenols.

❖ The preparation of food supplements.

From our point of view, the development and validation of methodologies for the determination of polyphenols are required. These methods could give us comprehensive information about the composition of the sample under study.

Having detailed information about the phenolic compounds which are present in an extract from plant food material, we could understand better the properties of that extract. All the compounds present in a particular sample will be responsible of its characteristics (antioxidant activity, anticancer activity, etc); therefore, it is not enough to have an idea about the major components of the sample, but about all the minor components as well.

Producing detailed data about the phenolic composition of a sample could help to refine the existing databases. Reliable data on the nutrient composition of foods consumed by people are critical in many areas –health assessment, the formulation of appropriate institutional and therapeutic diets, nutrition education, food and nutrition training, epidemiological research on relationships between diet and disease, plant breeding, nutrition labeling, food regulations, consumer protection, and agricultural goods and products, as well as for a variety of applications in trade, research, development, and assistance.

It is well-know that phenolic compounds appear to offer promising chances as chemical "markers" in biosystematic studies of plants. Essentially, the generally agreed requirements for a chemical character to be in use in taxonomy are as follows: chemical complexity and structural variation, physiological stability, widespread distribution and easy and rapid detection, and phenolic compounds match up well to these criteria.

All these areas of interest and the ones included in ***Figure 1.4*** try to point out the importance of the determination of this kind of analytes. CE methods can obviously help a lot in these areas giving complementary information to that achieved by other analytical platforms, such as HPLC or GC coupled to different detectors, and NMR, among others.

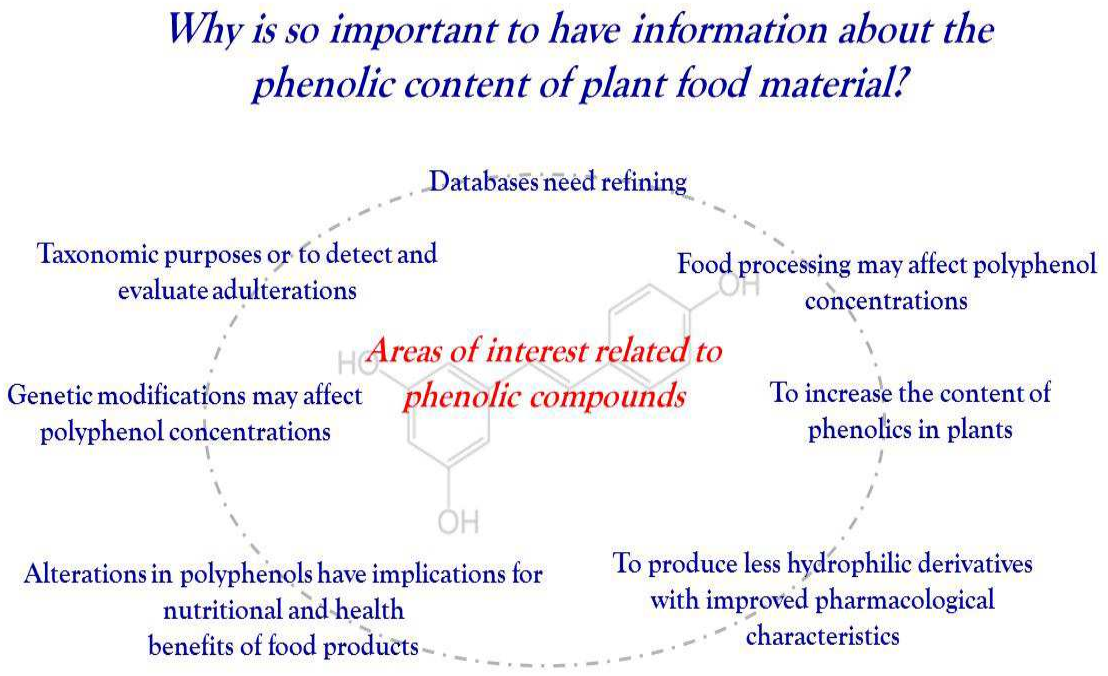

Figure 1.4. Why is so important to have information about the phenolic content of plant food material? Usefulness of generated data about polyphenolic content in plant food matrices.

1.6. New trends and perspectives

Despite the very interesting results achieved so far about the antioxidant phenolic compounds from plant food material by capillary electroseparation methods, there is no doubt about the fact that more potent CE methods will be developed in the near future giving answer to the necessity of the analyst to face complicated problems. It is expected that nanotechnology will play a main role in the evolution of CE, improving its results in terms of analysis time, selectivity, sensitivity, etc. Indeed, in the last ten years CE has already undergone several transformations related to miniaturisation and undoubtedly, nowadays the use of minidevices is significantly increasing.

In that respect, Wang et al. [253] developed a pressurized CEC method with gradient elution to analyze different flavonoids in several corn samples; Polášek et al. [254] demonstrated that the addition of molybdate to the CE background electrolyte affect the selectivity of separation of polyhydric phenols; and Dong and et al. [255] used CZE with an electrode modified with vitamin B_{12}.

CE microchips constitute the first group or generation of μ-TAS (micrototal analysis systems) or lab-on-a-chip in the miniaturisation scene of food analysis [256]. This type of

analytical technologies can be coupled to different detection systems [257], such as ED, LIF, MS, UV, SPR sensor or CL [258].

This new methodology has been applied in several areas, like bioanalytical and clinical analyses, and environmental and food applications among others [256]. It allows the separation and detection of DNA, proteins and peptides, amino acids, nitroaromatics, carbohydrates, hydrazines and antioxidants (catechols and phenols) [259]. Several authors has developed CE-microchip methods to identify phenolic compounds in food samples, such as green tea [260], wine [261], pears and apples [262] and a commercial food sample (Country Gravy Mix) [263].

Novel nanomaterials with unique mechanical, geometrical, chemical and/or electronic properties will enlarge the potential of this technology for CE analysis, including microchip-CE, on-column concentration and clean-up applications.

Apart from the use of minidevices, microchips and novel nanomaterials, the use of MS as detection system is becoming mandatory. The number of applications of CE-MS in the analysis of phenolic compounds from plant food material is constantly increasing, showing the potential of this coupling and the different performance of the developed methods depending on the ionisation source and the mass analyzer used. ESI is still one of the most versatile ionisation methods and is the natural method of choice for the detection of ions separated by CE. The trend is to use more and more sophisticated analyzers, being QqQ, TOF, Q-TOF, FT-ICR, orbitrap and IT-orbitrap those more powerful regarding the m/z range that can be covered, the mass accuracy and the achievable resolution. It is expected that new technological advances, as well as novel instrument configurations would make this technique more robust and useful for plant matrices analysis.

One of the most important limitations of CE is its sensitivity, which could be overcome using two different approaches: improving the on-line preconcentration strategies or using new CE interfaces. Both sheath liquid and sheathless interfaces have been proven to enable the coupling of CE with ESI-MS. For several reasons, the first category has been the most popular to date, however it is commonly acknowledged that such interfaces have much lower sensitivity than sheathless one. From our point of view, it is very likely that new and improved sheathless interfaces will be developed soon.

The fact of being nowadays in the -omics era is relevant and has some influence in the food analysis as well. Terms such as genomics, transcriptomics, proteomics and metabolomics are quite used in science; however, another term should be defined, since it is getting more attention by the scientific community: Foodomics. It can be defined as a discipline that studies the Food and Nutrition domains through the application of -omics technologies [264]. Thus, Foodomics would cover, for instance, the metabolomic study of foods toward compounds profiling, or new investigations on food functions via nutrigenomics or nutrigenetics approaches. In other words, it means that the application of -omics technologies can be very helpful to carry out the comprehensive characterization of food and even to understand the possible role of that foodstuff in the human health. Using this approach to study, for instance, plant food material, will result in a plethora of data that can be overwhelming in its abundance. For meaningful interpretation, the appropriate statistical tools must be employed to manipulate the large raw data sets in order to provide a useful, understandable, and workable format. Different multidimensional and multivariate statistical analyses and pattern recognition programs have been developed to distill the large amounts of data in an effort to interpret the complex information achieved from the measurements. Improvements are also expected in this field in the near future.

Meaningful interpretations of -omic data are often limited by poor spatial and temporal resolution of the acquired data sets, and one way to remedy this is to limit the complexity of the samples being studied. An alternative approach is to implement multidimensional systems that may be classified in various ways. For example, based on the type of displacement used, such systems are either simultaneous or sequential. The latter is achieved using two or more separation processes, which occur in different media, under different conditions (for example LC-GC). Such processes may be conducted either on-line or off-line, and we expect that CE will be more used in this kind of bidimensional systems.

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Chapter 2

Merging a sensitive CE-UV method with chemometric exploratory data analysis for the determination of phenolic acids and subsequent characterization of avocado fruit

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Abstract

Herein we present the development of a powerful CE-UV method able to detect and quantify an important number of phenolic acids in 13 varieties of avocado fruits at 2 ripening degrees. All the variables involved in CE separation were exhaustively optimized and the best results were obtained with a capillary of 50 μm i.d. x 50 cm effective length, sodium tetraborate 40 mM at a pH of 9.4, 30 kV, 25°C, 10 s of hydrodynamic injection (0.5 psi) and UV detection at 254 nm. This optimal methodology was fully validated and then applied to different avocado samples. The number of phenolic acids determined varied from 8 to 14 compounds; in general, they were in concentrations ranging from 0.13 ppm to 3.82 ppm, except for *p*-coumaric, benzoic and protocatechuic acids, which were found at higher concentrations. Principal component analysis was applied to highlight the differences between varieties and ripening degrees, looking for the most influential analytes.

Keywords: Phenolic acids / Avocado / *Persea americana* / Capillary electrophoresis / Principal component analysis

2.1. Introduction

Avocado (*Persea americana*) is a tropical or sub-tropical fruit native from South America, but it is widely cultivated throughout the world [1]. In fact, there are extensive crops of avocado in the coasts of Granada and Málaga (located in the south of Spain). According to FAOSTAT data for 2010 [2], the harvested area in Spain have increased around 500 Ha since 2005 and Spain occupies the 11th position on the world list of avocado production, being Mexico the first one.

The consumption of avocado fruit is increasing because of the numerous healthy benefits that it possesses, related to its composition [3-5]. This fruit is considered an important source of energy [1, 6], mainly for its high amounts of lipids [7]; specifically avocado is rich in monounsaturated fatty acids, being oleic acid the main fatty acid [8, 9]. It also contains carbohydrates (C7); proteins; dietary fibre; vitamins E, C, B_2, B_5 and B_6; potassium; magnesium and phosphorus [10-12]. Moreover, it has considerable amounts of pigments [carotenoids (α-carotene, β-carotene, cryptoxanthin, lutein, isolutein,

zeaxanthin, etc.) [13], chlorophylls (chlorophylls a and b) and anthocyanins (cyanidin 3-*O*-glucoside) [14]]; sterols (β-sitosterol, campesterol, stigmasterol) [15-17]; and phenolic compounds (phenolic acids and some flavonoids) [18-24].

Phenolic acids are secondary metabolites present in plants, usually conjugated to sugars or to other molecules as proteins, cellulose or lignins; and they constitute a big class of compounds included into the group of phenolic compounds [25, 26]. According to its origin, phenolic acids can be classified as derived from benzoic acid or from cinnamic acid [27, 28]. These compounds are related to different functions in plants [29, 30]; besides they have been associated with color and organoleptic properties of foodstuffs and the food industry has investigated the effect of the phenolic acids on fruit maturation and their roles as food preservatives [31]. Moreover, the relationship between the intake of phenolic acids and its health benefits has been extensively described in literature [32], since they have antioxidants properties and antibacterial, anticarcinogenic and anti-inflamatory activities, among others [25, 33].

Both phenolic compounds [34-36] and phenolic acids [28, 32, 37] have been traditionally determined in plant matrices by using different separative techniques coupled to diverse detection systems. Liquid chromatography is normally the technique of choice [28, 37], because it offers a wide range of advantages. At the end of the 90s, CE started to receive increased attention [32, 38], because it is a powerful tool that provide high efficiency, short analysis time, versatility and the consumption of solvent is low [39, 40]. However, after this promising starting, some analysts could say that CE did not achieve the expectations that the scientific community had. Although it is quite clear that the reproducibility and the sensitivity are the two the main problems of CE, there are some ways to solve these drawbacks and there are interesting applications in which this technique can get very valuable results.

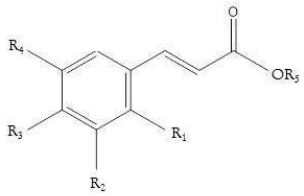
The objective of this study was to demonstrate the usefulness of CE to identify and quantify the phenolic acids present in the pulp of different avocado fruit. PCA was applied to highlight the differences between varieties and ripening degrees. To the best of our knowledge, this is the first time in which a CE-UV method together with statistical tools has been used for the determination of phenolic acid profile and analytical characterization of *Persea americana*.

2.2. Material and methods

2.2.1.Chemicals and standards

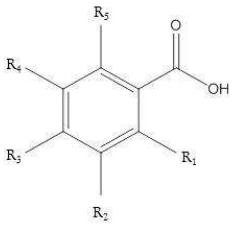
Standards of phenolic acids (vanillic, homovanillic, isovanillic, ferulic, 4-hydroxybenzoic, benzoic, *trans*-cinnamic, syringic, caffeic, gallic, gentisic, *p*-coumaric, *m*-coumaric, *o*-coumaric, protocatechuic, *o*-pyrocatechuic, sinapic, chlorogenic, *α*-resorcylic, *β*-resorcylic, *γ*-resorcylic acids) were purchased from Extrasynthese (Lyon, France), Sigma-Aldrich (St. Louis, EEUU) and Fluka (St. Louis, EEUU); its structures are showed in ***Figure 2.1.*** The stock solution containing the 21 compounds was prepared in MeOH/water (50:50, v/v) at a concentration of 9.5 ppm.

HYDROXYCINNAMIC ACIDS SKELETON



Compound	R_1	R_2	R_3	R_4	R_5
trans-cinnamic acid	–	–	–	–	–
Chlorogenic acid	–	–	OH	OH	$C_7H_{12}O_6$
Sinapic acid	–	OCH_3	OH	OCH_3	–
m-coumaric acid	–	OH	-	–	–
Ferulic acid	–	OCH_3	OH	–	–
o-coumaric acid	OH	–	-	–	–
p-coumaric acid	–	–	OH	–	–
Caffeic acid	–	OH	OH	–	–

HYDROXYBENZOIC ACIDS SKELETON



Compound	R_1	R_2	R_3	R_4	R_5
Homovanillic acid	–	OCH_3	OH	–	–
Isovanillic acid	–	OH	OCH_3	–	–
Gentisic acid	OH	–	–	OH	–
Benzoic acid	–	–	–	–	–
Syringic acid	–	OCH_3	OH	OCH_3	–
γ-resorcylic acid	OH	–	–	–	OH
α-resorcylic acid	–	OH	–	OH	–
Vanillic acid	–	OCH_3	OH	–	–
o-pyrocatechuic acid	OH	OH	–	–	–
4-hydroxybenzoic acid	–	–	OH	–	–
Gallic acid	–	OH	OH	OH	–
β-resorcylic acid	OH	–	OH	–	–
Protocatechuic acid	–	OH	OH	–	–

Figure 2.1. Structures of the phenolic acids included in the standard mix used for the optimization.

Taxifolin was purchased from Extrasynthese (Lyon, France) and it was used as IS to evaluate the reproducibility of the extraction system and the electrophoretic procedure.

MeOH, used as solvent for the sample extraction and the preparation of stock solutions, was purchased from Panreac (Barcelona, Spain). Hydrochloride acid was acquired from VWR (West Chester, EEUU) and it was used, together with NaOH (from Panreac), to adjust the pH of running buffers. Sodium tetraborate (borax), sodium carbonate anhydrous and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO)

were obtained from Sigma-Aldrich (St. Louis, EEUU) and from Panreac (Barcelona, Spain); all of them were used as running buffers at different concentrations and pH values. All the solvents were HPLC grade and they were used as received.

Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q system (Millipore, Bedford, MA, USA).

All the solutions were filtered with 0.2 μm disposable syringe filters of regenerated cellulose (Symta, Madrid, Spain) before the injection in the instrument.

2.2.2. Avocado samples

Thirteen different varieties of avocado fruit ('Harvest', 'Sir Prize', 'Hass', 'Jiménez 1', 'ColinV 33', 'Tacambaro', 'Lamb Hass', 'Hass Motril', 'Pinkerton', 'Nobel', 'Jiménez 2', 'Marvel' and 'Gem') were collected at two different ripening degrees; the first one was the fruit just harvested and the second one when the avocado was ready for consumption. All the varieties were grown under identical environmental conditions (soil, rain, light, etc.).

According to the information provided by Alcaraz et al. [41], four of the samples used in this study ('Hass Motril', 'Jiménez 1', 'Jiménez 2', and 'Tacambaro') present the same genetic profile as Hass cultivar, showing the same accession and being possible to think that they come from mutations of this avocado variety.

All the samples were provided by the IHSM La Mayora (CSIC), located in Algarrobo-Costa (Málaga, Spain). Once that the samples were in our lab, they were processed; the pulp of 2-3 pieces of fruit was homogenized, frozen and freeze-dried.

The extraction of phenolic acids in these avocado samples was carried out taking into account our previous experience with this matrix [42, 43]. The extracts were prepared using 4 g of each sample (with 100 ppm of IS added) and 40 mL of MeOH. The mix was put in a falcon tube and it was shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 2500 rpm for 20 min. After that, the supernatants were evaporated to dryness with a rotary evaporator and reconstituted in 1 mL of MeOH/water (50:50 v/v). Each sample was prepared by triplicate.

A mixture of the extracts from the 26 avocado samples under study (mixing an equivalent volume of each extract) was used as QC sample to evaluate trueness and

matrix effect. Apart from that, QC samples were injected after the optimization studies to corroborate the chosen conditions.

2.2.3. CE-UV analysis

CE determination of phenolic acids was made with a Beckman Coulter P/ACE™ MDQ Capillary Electrophoresis System equipped with an UV detector, which allowed us to detect individual compounds using their optimum wavelengths. All capillary tubing (uncoated fused silica) were from Beckman Instruments (Fullerton, CA, USA) and they had an internal diameter of 50 μm, a total length of 60 cm and 50 cm of L_{ef}. Before the use of a new capillary, it was necessary to condition it by flushing with 0.5 M of NaOH during 20 min, followed by a rinse with Milli-Q water for 10 min and 15 min with buffer. At the beginning of every day, capillary was undergone to a washing routine with NaOH 0.1 M during 10 min and then with Milli-Q water for 5 min. Before each sample injection, the capillary was equilibrated with running buffer (40 mM sodium tetraborate adjusted to pH 9.4) for a period of 5 min.

Samples were injected hydrodynamically in the anodic end at 0.5 psi (1 psi= 6895 Pa) for 10 s. Each electrophoretic run was carried out applying a voltage of 30 kV for 30 min and maintaining the capillary temperature at 25°C (controlled by using a fluorocarbon-based cooling fluid), resulting in a current of ~75 μA.

To assure a good repeatability, between runs the capillary tubing was flushed with Milli-Q water, NaOH 0.1 M and Milli-Q water during 1 min, 2 min and 1 min, respectively. The running buffer was changed after four analyses.

UV detection was set at 254 nm, although during the preliminary studies 200, 214, 280 and 300 nm were used as well. Both migration times (of the detected peaks in the avocado samples compared with those of the pure standards) and spiked samples were used to achieve the identification of phenolic acids.

System control, data acquisition and handling were made with 32 Karat software, which was installed in a personal computer.

2.2.4. Multivariate statistical analysis

An exploratory analysis of the data was carried out through PCA, which was used to display a natural grouping tendency and outliers between avocado samples. Data were analyzed by using The Unscrambler® v9.7 software (CAMO Software AS, Oslo, Norway).

2.3. Results and discussion

2.3.1. Optimization of the method

The separation and detection conditions have to be optimized to achieve the best resolution in the shortest time. There are different variables (experimental and instrumental) to be considered in any optimization process. The choice of the best conditions was based on several criteria as analysis time, sensitivity, separation selectivity and peak shape.

An univariate optimization was carried out, changing one of the parameters and keeping constant the rest of them, using the mixture of 21 phenolic acids at a concentration of 9.5 ppm in methanol/water (50:50, v/v) (see *Section 2.2.1.*).

Effect of the experimental variables

Three different aqueous buffers were used: sodium tetraborate, sodium carbonate and CAPSO, all of them at the same concentration. In a first approach, two different pH values were tried for each one: the pH value that the buffer gives naturally and, taking into account the pk_a values of the compounds under evaluation and some preliminary studies, the pH adjusted to 9.3 by adding proper amounts of 1.0 M bicarbonate or 1.0 M HCl. It was observed that CAPSO was not a good option as running buffer because neither in the first case nor at pH of 9.3, the resolution and peak shape were good enough as a result of the short analysis time.

A more detailed study was developed trying to choose between sodium tetraborate and sodium carbonate. For that, different values of pH within the range from 8.0 to 10.5 (step of 0.5) were tested. pHs of 10.0 and 10.5 needed more than 60 min to separate all the peaks, which was an excessive time. On the other hand, with a pH of 8.0, the overlapping of some peaks was observed. At 8.5, 9.0 and 9.5, analysis times were reasonable, but the best resolution was achieved with pH values of 9.0 and 9.5. For this

reason, in a second stage, pH values within the range of 9-9.6 were tested, specifically, 9.0, 9.2, 9.4 and 9.6. Sodium tetraborate at pH of 9.4 was chosen as a proper compromise solution, keeping in mind the fact that resolution and analysis time were more adequate than those achieved by using sodium carbonate (***Figure 2.2***).

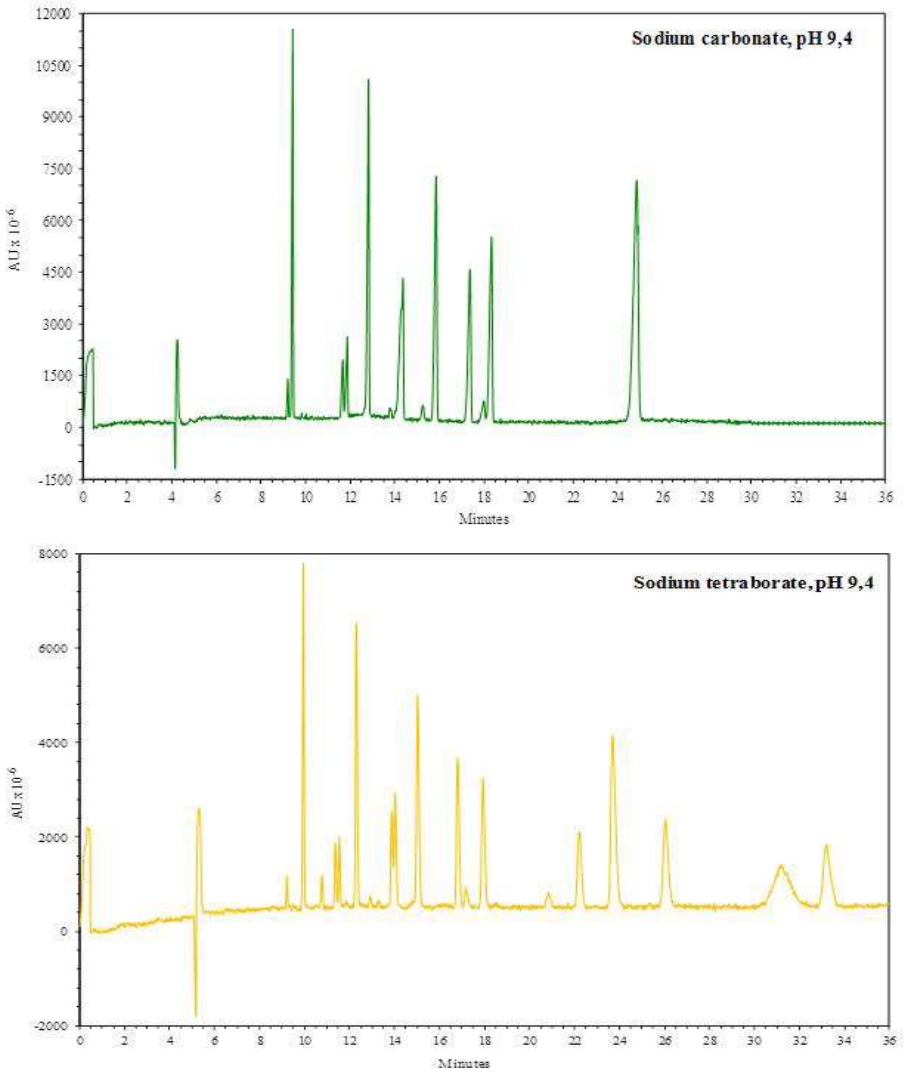


Figure 2.2. Different buffers tested at 50mM and the same pH value (9.4). Instrumental conditions were kept constant**:** 25 kV of voltage, temperature of 25ºC, capillary with 50 µm i.d x 50 cm L_{ef}, hydrodynamic injection at 0.5 psi for 10 s, UV detection at 280nm.

To evaluate the influence of the ionic strength, different buffers were prepared at the following concentrations: 12.5, 25, 50, 75, 100 and 150 mM. After the preliminary studies, it was found that the optimum value was between 25-50 mM, therefore some intermediate concentrations, such as 30, 35, 40 and 45 mM, were tried. At 30 and 35 mM the analysis time was longer than at 25 mM, but a good separation was not still reached in the electropherograms. Between 40 and 45 mM, there were not too many

differences in terms of resolution. Therefore, the optimum running buffer was 40 mM of sodium tetraborate adjusted to pH of 9.4.

Effect of the instrumental variables

After the optimization of the buffer composition, its pH and ionic strength, the conditions related to the instrument were studied. The first one was the voltage and values of 15, 20, 25 and 30 kV were tested. When higher voltages were applied, migration times were shorter and efficiencies were higher than when lower voltages were tested, owing to the fact that the electrophoretic and electroosmotic velocities are directly related to the electric strength. One of the main problems of using higher voltages is the generation of Joule heat in the capillary, causing broader peaks, the possibility of sample decomposition, or the formation of bubbles inside the capillary [44]. In this work, it was possible to work at 30 kV, having a good resolution and reducing the duration of the runs, because the generated heat was properly dissipated by the equipment.

The temperature of the instrument is also related to the heat dissipation, which is more effective as the temperature decreases; it is connected to the pH and viscosity of the buffer as well. High temperatures involve a drop in the viscosity, which in turn, increases the electrophoretic mobility. The best results were obtained maintaining the temperature at 25ºC.

The most common mode to inject the sample is hydrodynamically, and it could be done by gravity, pressure or vacuum [45]. This type of injection was used applying 0.5 psi of pressure for a period of 10 s, because higher injection times increased the plug of sample decreasing the efficiency and broadening the peaks [38].

Finally, it was necessary to establish the optimum UV detection wavelength. Phenolic acids present a characteristic band of absorption depending on its structure; hydroxybenzoic acids have their maximum absorbance band from 200 to 290 nm and hydroxycinnamic acids present an additional one in 270-360 nm range [46]. The detector used only had five available channels: 200, 214, 254, 280 and 300 nm. The best results were obtained at 254 and 280 nm. Taking into account the most problematic peaks in terms of resolution (isovanillic and sinapic acids, and syringic and ferulic acids), 254 nm

was chosen as the optimum detection wavelength, despite the fact that the sensitivity of the method for some compounds under study decreased a little.

Figure 2.3 shows the electropherogram obtained for the standard mixture analyzed under the optimal conditions.

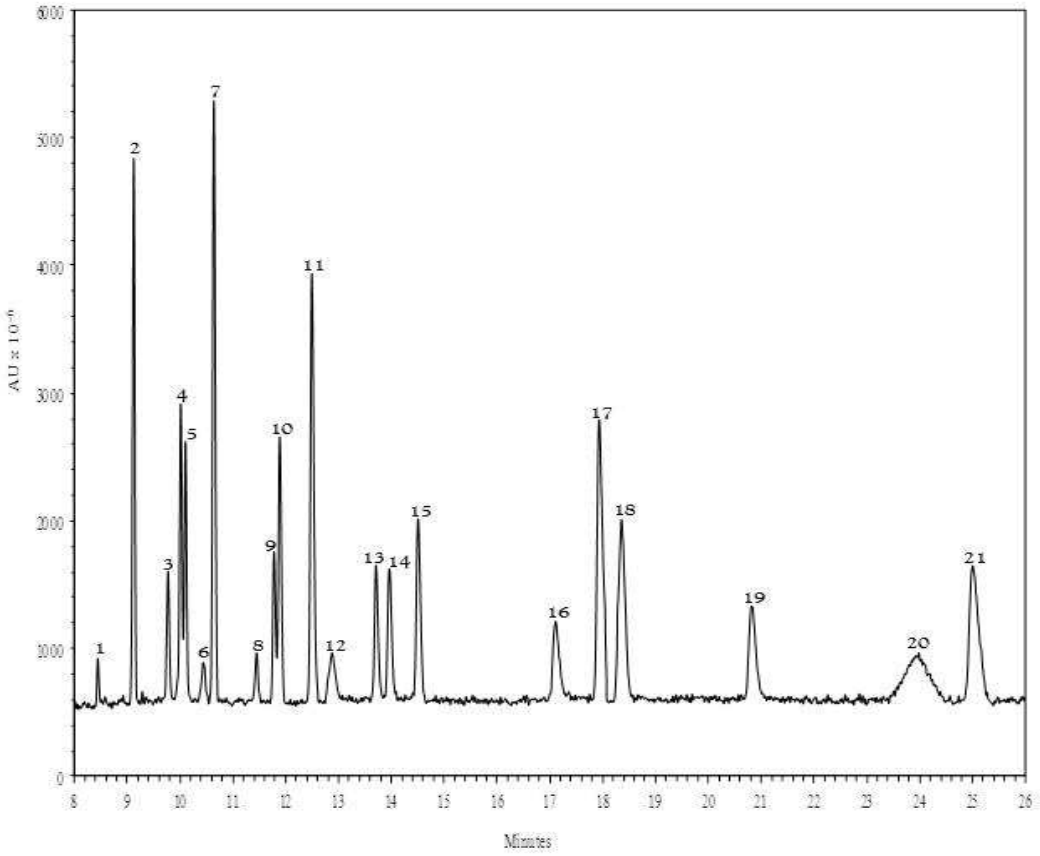


Figure 2.3. Electropherogram achieved for the mixture of 21 phenolic acids by using the optimized method: buffer, 40mM sodium tetraborate (pH 9.4); capillary, 60 cm (50 cm of L_{ef}) x 50 µm i.d.; applied voltage, 30 kV; temperature, 25ºC; hydrodynamic injection at 0.5 psi for 10 s. UV detection at 254 nm. Peaks identification: **1**, homovanillic acid; **2**, *trans*-cinnamic acid; **3**, chlorogenic acid; **4**, isovanillic acid; **5**, sinapic acid; **6**, gentisic acid; **7**, *m*-coumaric acid; **8**, benzoic acid; **9**, syringic acid; **10**, ferulic acid; **11**, *o*-coumaric acid; **12**, *γ*-resorcylic acid; **13**, *p*-coumaric acid; **14**, *α*-resorcylic acid; **15**, vanillic acid; **16**, *o*-pyrocatechuic acid; **17**, 4-hydroxybenzoic acid; **18**, caffeic acid; **19**, gallic acid; **20**, *β*-resorcylic acid; **21**, protocatechuic acid.

2.3.2. Method validation

A study to calculate different analytical parameters of the proposed method was carried out to check the repeatability, reproducibility and accuracy of the method, and also to establish the calibration curves that allow the quantification of the compounds under study and the calculation of the LOD and LOQ.

Both the standard mix containing the 21 phenolic acids and QC samples (spiked at different concentration levels) were used for the method validation. All the data obtained in this study are summarized in ***Table 2.1 (A)*** and ***(B).***

Table 2.1(A). Analytical parameters of the developed method.

Compound	External calibration curve	r^2	LOD (ppb)	LOQ (ppb)	Lineal range (ppm)	MATRIX EFFECT	
						RF (neat solution)[a]	RF (with matrix)[a]
Homovanillic acid	y= 52.820x + 161.993	0.991	145	483	LOQ-60	60.09	63.20
	y= 60.454x - 327.651	0.995			60-500		
trans-cinnamic acid	y= 716.008x + 120.758	0.993	12	39	LOQ-60	726.80	711.47
	y= 749.937x – 3669.680	0.995			60-500		
Chlorogenic acid	y= 185.246x – 48.570	0.993	42	139	LOQ-60	199.25	193.89
	y= 211.659x – 2423.999	0.996			60-500		
Isovanillic acid	y= 359.272x + 99.654	0.996	43	142	LOQ-15	202.27	222.71
	y= 200.709 + 1641.264	0.990			15-500		
Sinapic acid	y= 354.181x – 160.881	0.995	22	73	LOQ-30	389.10	391.38
	y= 408.207x – 3805.369	0.995			30-250		
Gentisic acid	y= 90.270x – 23.608	0.992	89	297	LOQ-60	99.88	102.67
	y= 97.505x – 455.358	0.993			60-500		
m-coumaric acid	y= 763.941x – 31.386	0.993	11	37	LOQ-60	772.19	787.49
	y= 776.608x – 2077.637	0.994			60-500		
Benzoic acid	y= 70.850x – 11.504	0.994	111	371	LOQ-60	75.49*	78.54*
	y= 78.796x – 715.941	0.993			60-500		
Syringic acid	y= 213.164x + 98.230	0.993	39	131	LOQ-60	229.60	234.50
	y= 220.749x – 541.778	0.997			60-500		
Ferulic acid	y= 391.546x – 153.416	0.993	20	66	LOQ-60	413.84	409.95
	y= 443.610x – 5303.829	0.990			60-500		
o-coumaric acid	y= 789.674x – 47.834	0.998	12	39	LOQ-15	738.36	749.30
	y= 766.564x + 9671.030	0.992			15-500		
γ-resorcylic acid	y= 388.439x – 44.832	0.984	18	60	LOQ-15	429.98*	452.86*
	y= 478.880x – 2573.050	0.998			15-500		
p-coumaric acid	y= 287.579x – 97.871	0.994	29	96	LOQ-60	283.93	298.62
	y= 304.135x – 1914.705	0.990			60-500		
α-resorcylic acid	y= 319.578x – 203.981	0.984	25	85	LOQ-30	345.07	333.23
	y= 342.411x – 2305.386	0.990			30-500		

Table 2.2(A) (continued)

Compound	External calibration curve	r^2	LOD (ppb)	LOQ (ppb)	Lineal range (ppm)	MATRIX EFFECT	
						RF (neat solution) [a]	RF (with matrix)[a]
Vanillic acid	y= 395.147x – 99.361	0.994	20	67	LOQ-60	422.17	413.80
	y= 436.980x – 4218.301	0.994			60-500		
o-pyrocatechuic acid	y= 271.064x – 257.867	0.990	27	90	LOQ-60	292.29	287.99
	y= 319.570x – 3325.884	0.992			60-500		
4-hydroxybenzoic acid	y= 685.245x – 223.442	0.997	12	39	LOQ-15	737.60	730.00
	y= 739.109x – 84.910	0.989			15-125		
Caffeic acid	y= 849.862x + 70.935	0.998	9	30	LOQ-15	960.34	929.36
	y= 958.640x + 2.585	0.986			15-125		
Gallic acid	y= 407.545x – 334.929	0.992	19	64	LOQ-30	405.75	408.98
	y= 452.597x – 4075.963	0.991			30-500		
β-resorcylic acid	y= 904.991x – 1495.049	0.990	9	31	LOQ-60	928.57	922.00
	y= 930.901x – 4302.023	0.990			60-500		
Protocatechuic acid	y= 752.895x – 564.509	0.996	11	37	LOQ-60	783.62	776.39
	y= 794.346x – 5304.318	0.985			60-500		

[a] Response factors were calculated to evaluate the possible matrix effect at 3 concentration levels (5, 30 and 125 ppm). Only the intermediate value (30 ppm) of the neat solution and of the spiked avocado extract is showed, due to the other concentrations present the same behaviour. Values with asterisk (*) means that the concentration considered was 125 ppm.

Table 2.3(B). Analytical parameters of the developed method.

Compound	ACCURACY						
	Repeatability[b]		Intermed. Precision[b]		Reproducibility[c]		Trueness[d]
	Peak area/ migration time	Migration time	Peak area/ migration time	Migration time	Peak area/ migration time	Migration time	
trans-cinnamic acid	7.8	1.4	9.5	2.7	11.0	5.5	105.2
m-coumaric acid	7.6	2.0	10.3	2.9	11.9	5.6	107.8
p-coumaric acid	10.1	2.6	11.5	3.6	17.7	6.5	109.0
o-pyrocatechuic acid	12.4	2.2	13.3	4.6	18.7	7.8	106.6
Protocatechuic acid	10.5	3.0	12.8	6.6	13.1	10.0	106.4

[b] RSD values (%) for peak area/migration time ratio corresponding to some analyte; measured from six consecutive injections of each analyte within the same day (repeatability or intra-day precision) and on six different days (intermediate or inter-day precision), by using the phenolic acid mix at 9.5 ppm.

[c] RSD values (%) for peak area/migration time ratio corresponding to some analyte; measured from ten injections on ten different days and by using different capillaries and the phenolic acid mix at 9.5 ppm.

[d] The trueness of the assay is the closeness of the average test value obtained to the nominal value. It was measured by calculating the recovery (%), spiking a blank matrix with 5, 30 and 125 ppm of each compound.

Calibration curves and figures of merits

External calibration curves were prepared using the standard mix of 21 phenolic acids at 9 different concentration levels (1, 2.5, 5, 15, 30, 60, 125, 250 and 500 ppm) to check the linearity and the sensitivity of the developed methodology. They were obtained for each standard by plotting the peak area as a function of the concentration. Each point of the calibration graph was made by duplicated. Two calibration curves were obtained for each compound: the first one for the lowest levels of concentration (from 1 to 30-60 ppm) and the other one for the highest levels (30-60 to 500 ppm). All the standard calibration curves showed a good linearity in the studied range of analyte concentrations; regression coefficients (r^2) were higher than 0.982 in every case.

Detection (LOD=$3S_0/b$) and quantification limits (LOQ=$10\ S_0/b$) were determined as well. These values were calculated taking into account the IUPAC recommendations (IUPAC technical report, 2002), where S_0 is the standard deviation of the blank and *b* the slope of each calibration equation. Caffeic acid showed the lowest LOD (9 µg/L) and, consequently, the lowest LOQ (30 µg/L). On the other hand, homovanillic acid is the compound with the highest LOD and LOQ, 145 µg/L and 483 µg/L, respectively.

Matrix effect

A standard-added calibration was used to evaluate the possible matrix effect [47]. Known amounts of our standard mix solution (5, 30 and 125 ppm) were added to the QC sample, and the responses of these compounds were compared with those of the phenolic acid solution (neat solution) at the same concentration. The comparison between the neat solution and the spiked extract was made with the RFs, which were calculated as a ratio between the peak area obtained and the concentration of the analyte.

A student-t conventional test was used to determine if significant differences exist between both values. In ***Table 2.1***, RFs for the intermediate value of concentration are presented as an example; the same behavior was observed for the other two concentration levels. As a result, no statistical differences were found for the studied compounds and that means that the presence of avocado extract does not interfere in the response of the analytes.

Accuracy

The accuracy of the assay is the conformity between the obtained result and the reference value. It is calculated by determining trueness and precision [48].

The precision of the analytical procedure was measured as repeatability, intermediate precision and reproducibility, which were expressed as the relative standard deviation (RSD) in the peak area/migration time and migration times. Repeatability and intermediate precision was determined, respectively, by analyzing the mix of phenolic acids, at a concentration of 9.5 ppm, within the same day (repeatability, n=6) and on six consecutive days (intermediate precision, n=6), considering five of the phenolic compounds under study (*trans*-cinnamic, *m*-coumaric, *p*-coumaric, *o*-pyrocatechuic and protocatechuic acids). These compounds were chosen because they presented different migration times, fact which allowed checking the repeatability and the reproducibility at different points of our analytical window. The reproducibility study was carried out by performing ten separations of the same mix under the same conditions, but changing the capillary. As we can see in ***Table 2.1***, the results obtained for the precision, in terms of repeatability, were acceptable; being in all the cases lower than 13.3%, bearing in mind the ratio peak area/migration time; or lower than 6.6% if only migration time is considered. The reproducibility values for the total peak area/migration time were between 11.0 and 18.7% (an average value of 14.5% for the five compounds), and within the range from 5.5 to 10.0% (average of 7.1%) for the migration times. According to AOAC International recommendations [49], the acceptable repeatability and reproducibility values (RSD) are, respectively, 6 and 11 % (at a concentration level of 10 ppm). As observed in ***Table 2.1***, the obtained values of repeatability were found within the range 1.4-3.0% (considering migration time) and 7.6-12.4% (when area/migration time was taken into account); intermediate precision values were between 2.7-6.6% (migration time) and 9.5-13.3% (area/migration time); and reproducibility varied from 5.5-10.0% (when migration time was observed) and from 11.0-18.7% (area/migration time). In every case, when RSD values were calculated considering migration time, our values of precision fulfilled the AOAC requirements. However, when peak area/migration time ratios were evaluated, RSD values increased (as expected; in particular when CE is used), exceeding the acceptable levels; therefore to correct the

fluctuations of peak area values and to assure an adequate quantification, an IS was added to the samples under study.

The trueness of a method describes how close a test result is to the accepted reference value for the quantity measured. Lack of trueness indicates systematic error which is usually expressed as the "bias" or the "recovery". Because suitable *Certified Reference Materials* (CRMs) were not available, recovery was estimated by analyzing a sample before and after the addition of a known amount of analytes (by spiking or standard addition). In this case, the recovery is calculated from the difference between the results obtained before and after spiking as a fraction of the added amount.

For that, a QC sample was spiked with different concentrations (5, 30 and 125 ppm) of the same five phenolic acids chosen for the precision study. ***Table 2.1*** shows the recoveries (%) obtained for each individual compound and it is observed that they are between 105.2% and 109.0%, which means that our method is truthful, taking into account the AOAC guidelines, which establishes a good trueness from 80% to 115%.

2.3.3 Identification and quantification of phenolic acids in avocado samples

Once the method was optimized, it was applied to the analysis of 13 varieties of avocado at two different ripening degrees. In section *2.2*, the varieties used and the extraction procedure were shown.

As it can be seen in ***Figure 2.4*** (only includes the profile of 6 avocado extracts to contain the size of the figure) the richness of the electropherograms highlights the complexity of this matrix. The proposed method is focused on the separation of phenolic acids, which start to migrate from minute 8. Before this time, some other peaks appeared, identifying some of them as quinic acid, epicatechin, succinic acid or pantothenic acid.

The identification was achieved by comparing the migration times of the detected peaks in the avocado samples profiles with those of the reference compounds and by spiking the mixture of the phenolic acids and the real avocado samples with standard compounds.

Phenolic acids were found in all the varieties under study, although qualitative and quantitative differences could be observed when the phenolic acid profiles of the 13

varieties at the same ripening degree were compared; the same was seen comparing the profiles of the each variety at the two different ripening degrees.

In general terms, Jiménez 1 was the richest variety, having 14 of the 21 phenolic acids tested, and Harvest was the poorest one with only 8 phenolic acids. *Trans*-cinnamic, *p*-coumaric and protocatechuic acids were found in all the studied samples (at the first and at the second ripening degree), but on the contrary, homovanillic, isovanillic, *o*-coumaric and *α*-resorcylic acids were not detected in any of them. Other compounds, such as benzoic and ferulic acids, were observed at least in one of the degrees of ripeness; for instance ferulic acid was present at the second ripening stage of all the varieties.

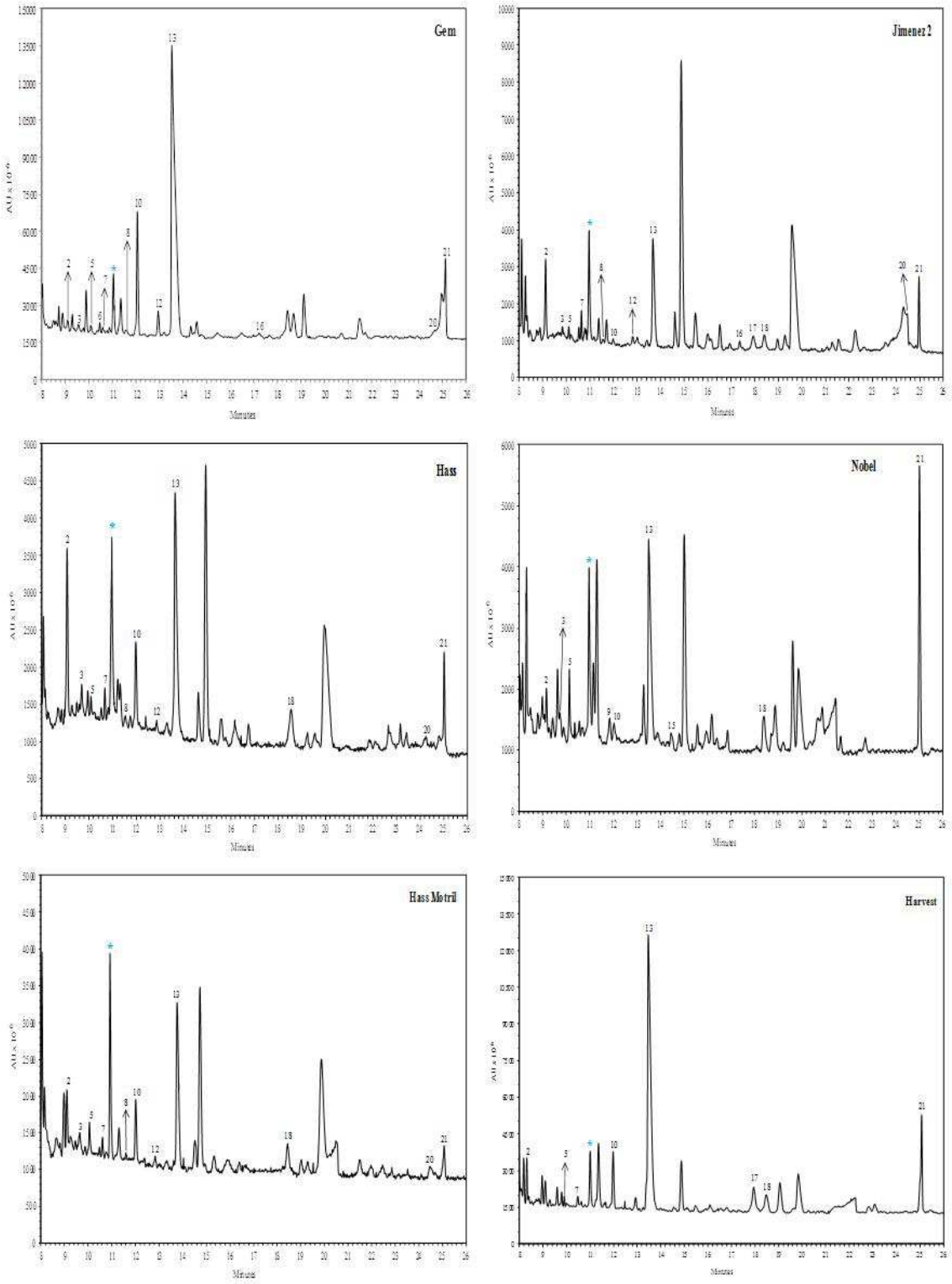


Figure 2.4. Electropherograms obtained for 6 varieties of avocado fruit at the second ripening degree (to contain the size of the picture), analyzed under the optimal conditions. Peak identification is showed in ***Figure 2.2***. *Peak corresponding to the IS (taxifolin).

It can be also emphasized the fact that some compounds are characteristics for a variety, for example gallic acid was only present in Sir Prize; or for a specific ripening degree (*e.g.* 4-hydroxybenzoic was solely found at the second degree of ripeness). To illustrate these variations related to the ripening process, ***Figure 2.5*** shows the comparison of Marvel variety extracts at both ripening degrees. Chlorogenic, sinapic, benzoic, ferulic and *β*-resorcylic acids are only present in the CE profiles when the analyzed fruit is ripe or ready for consumption. However, the opposite occurs for gentisic acid; which is only found in extracts from the fruit just harvested.

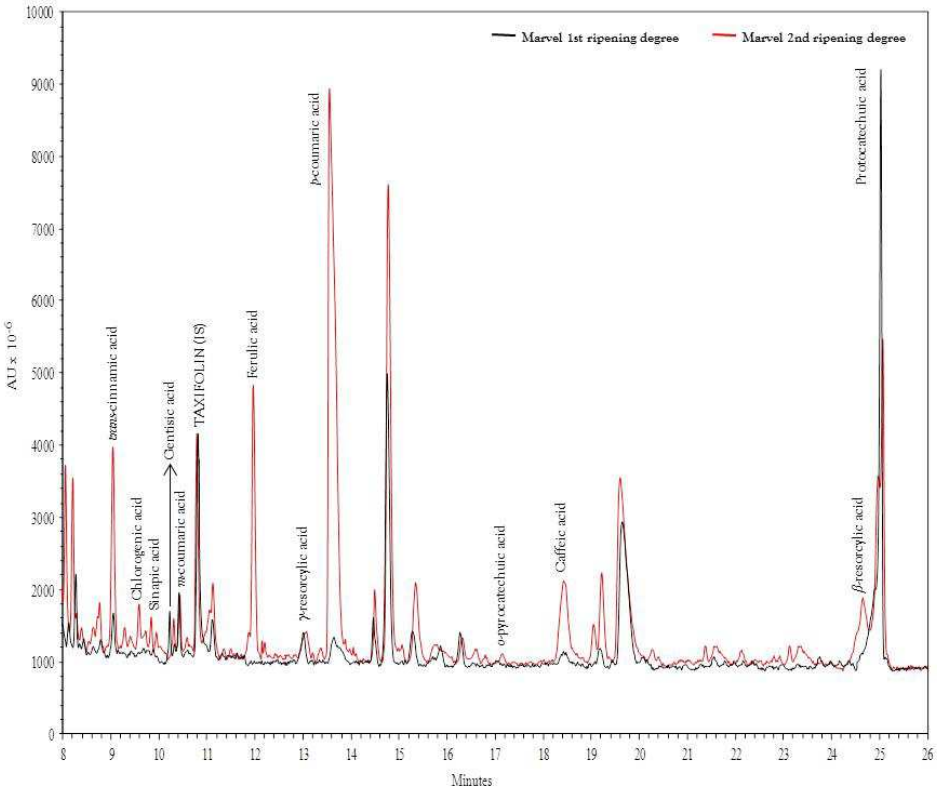


Figure 2.5. Comparison of the electropherograms obtained for *cv.* Marvel at two different ripening degrees, by using the optimal conditions.

It has been previously stated that samples were analyzed by triplicate. The results were studied and compared by using ANOVA and no statistically significant differences were found among the three measurements of each variety of avocado fruit. ***Table 2.2*** shows the arithmetic mean value obtained for each compound and also an estimation of the total content of hydroxycinnamic (HCA) and hydroxybenzoic acids (HBA). Overall, the concentration of HCA increases with the ripening process, while the presence of HBA tends to be lower as the fruit ripens.

Table 2.2. Quantitative results (mg analyte/kg dry sample) obtained for different avocado varieties by using CE-UV method.

Compound	Colin V 33		Gem		Harvest		Hass		Hass Motril		Jiménez 1		Jiménez 2	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
trans-cinnamic acid	0.22 (0.01)	0.53 (0.03)	0.84 (0.06)	0.74 (0.07)	1.48 (0.07)	1.05 (0.07)	2.10 (0.21)	2.78 (0.28)	1.24 (0.06)	0.88 (0.09)	1.63 (0.11)	1.83 (0.09)	1.45 (0.11)	2.22 (0.16)
Chlorogenic acid	–	1.58 (0.11)	1.77 (0.09)	1.02 (0.10)	–	–	1.72 (0.13)	1.39 (0.08)	0.24 (0.01)	0.91 (0.07)	0.94 (0.07)	0.95 (0.10)	0.81 (0.04)	0.58 (0.04)
Sinapic acid	–	–	0.33 (0.03)	0.56 (0.06)	0.93 (0.09)	0.80 (0.08)	–	0.27 (0.02)	–	0.53 (0.03)	–	0.29 (0.02)	–	0.30 (0.03)
Gentisic acid	–	–	3.83 (0.31)	2.19 (0.18)	–	–	–	–	3.40 (0.17)	–	3.41 (0.24)	n.q	–	–
m-coumaric acid	–	n.q	0.61 (0.06)	0.23 (0.02)	0.65 (0.05)	0.61 (0.03)	0.87 (0.06)	0.58 (0.04)	0.31 (0.03)	0.30 (0.02)	0.83 (0.07)	1.21 (0.06)	0.82 (0.06)	0.62 (0.06)
Benzoic acid	–	4.74 (0.33)	1.13 (0.09)	1.62 (0.08)	1.87 (0.11)	–	9.84 (0.49)	1.24 (0.07)	1.40 (0.11)	0.69 (0.07)	4.21 (0.25)	2.30 (0.17)	1.79 (0.15)	1.07 (0.09)
Syringic acid	3.84 (0.19)	3.57 (0.18)	–	–	–	–	–	–	–	–	–	–	–	–
Ferulic acid	0.43 (0.04)	1.25 (0.06)	–	3.98 (0.22)	0.96 (0.07)	3.52 (0.21)	–	1.96 (0.15)	–	1.60 (0.11)	–	1.59 (0.08)	–	0.52 (0.05)
γ-resorcylic acid	–	–	–	–	–	–	0.57 (0.06)	n.q	0.65 (0.05)	1.54 (0.11)	n.q	0.25 (0.02)	n.q	0.68 (0.05)
p-coumaric acid	5.91 (0.41)	6.84 (0.48)	5.51 (0.47)	33.63 (1.68)	6.72 (0.67)	33.75 (1.69)	0.63 (0.06)	8.33 (0.58)	0.77 (0.08)	8.45 (0.42)	2.13 (0.17)	7.88 (0.51)	2.32 (0.17)	7.93 (0.48)
Vanillic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
o-pyrocatechuic acid	–	–	0.63 (0.03)	0.61 (0.05)	–	–	–	–	–	–	–	0.90 (0.05)	0.42 (0.04)	0.62 (0.04)
4-hydroxybenzoic acid	–	–	–	1.80 (0.13)	–	1.74 (0.09)	–	–	–	–	–	0.32 (0.03)	–	0.61 (0.03)
Caffeic acid	1.28 (0.09)	3.82 (0.27)	–	–	–	1.42 (0.09)	0.22 (0.02)	1.36 (0.07)	0.13 (0.01)	0.77 (0.04)	n.q	0.55 (0.04)	0.48 (0.05)	0.73 (0.04)
Gallic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
β-resorcylic acid	0.72 (0.07)	0.49 (0.05)	0.43 (0.02)	n.q	–	–	0.26 (0.02)	0.40 (0.03)	–	0.37 (0.04)	–	1.07 (0.05)	n.q	1.15 (0.07)
Protocatechuic acid	15.17 (0.76)	15.06 (0.75)	9.55 (0.67)	3.59 (0.31)	5.21 (0.26)	3.99 (0.26)	1.28 (0.11)	1.40 (0.07)	1.24 (0.09)	0.63 (0.06)	2.72 (0.16)	1.65 (0.13)	2.85 (0.14)	1.58 (0.12)
TOTAL	27.57 (1.58)	37.88 (2.26)	24.63 (1.83)	49.97 (2.88)	17.82 (1.33)	46.88 (2.52)	17.49 (1.16)	19.71 (1.39)	9.38 (0.61)	16.67 (1.05)	15.87 (1.08)	20.79 (1.36)	10.94 (0.77)	18.61 (1.25)
HCA	7.84 (0.49)	14.02 (0.66)	9.06 (0.39)	40.16 (1.36)	10.74 (0.61)	41.15 (1.59)	5.54 (0.23)	16.67 (0.82)	2.69 (0.12)	13.44 (0.57)	5.53 (0.25)	14.30 (0.47)	5.88 (0.21)	12.90 (0.75)
HBA	19.73 (0.77)	23.86 (0.99)	15.57 (1.07)	9.81 (0.68)	7.08 (0.33)	5.73 (0.35)	11.95 (0.43)	3.04 (0.06)	6.69 (0.35)	3.23 (0.19)	10.34 (0.65)	6.49 (0.20)	5.06 (0.26)	5.71 (0.31)

Table 2.2 (continued)

Compound	Lamb Hass		Marvel		Nobel		Pinkerton		Sir Prize		Tacambaro	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
trans-cinnamic acid	1.13 (0.06)	2.23 (0.11)	0.84 (0.08)	3.21 (0.16)	0.62 (0.03)	1.15 (0.08)	0.58 (0.06)	0.50 (0.04)	2.03 (0.14)	1.50 (0.17)	0.81 (0.05)	0.91 (0.06)
Chlorogenic acid	0.82 (0.08)	1.67 (0.08)	–	1.60 (0.08)	0.45 (0.05)	1.42 (0.10)	2.15 (0.11)	1.34 (0.07)	1.11 (0.08)	0.89 (0.09)	1.08 (0.08)	–
Sinapic acid	–	1.89 (0.13)	–	0.91 (0.09)	1.13 (0.06)	1.40 (0.07)	1.10 (0.08)	1.86 (0.09)	2.24 (0.11)	0.77 (0.08)	1.04 (0.07)	0.50 (0.04)
Gentisic acid	5.48 (0.27)	2.07 (0.21)	4.70 (0.33)	–	–	–	–	–	–	–	–	n.q
m-coumaric acid	1.14 (0.06)	0.96 (0.07)	0.88 (0.07)	0.79 (0.05)	–	–	0.76 (0.05)	0.39 (0.04)	1.53 (0.08)	0.59 (0.04)	0.64 (0.04)	0.72 (0.04)
Benzoic acid	5.24 (0.26)	6.18 (0.31)	–	0.69 (0.03)	0.81 (0.08)	–	6.52 (0.33)	3.80 (0.32)	15.89 (0.79)	8.27 (0.58)	8.82 (0.44)	4.32 (0.35)
Syringic acid	–	–	–	–	3.85 (0.27)	3.25 (0.23)	–	–	–	12.12 (0.61)	–	–
Ferulic acid	–	2.52 (0.18)	–	4.59 (0.23)	–	0.51 (0.05)	–	0.91 (0.07)	–	4.86 (0.24)	0.49 (0.05)	1.06 (0.07)
γ-resorcylic acid	–	–	1.48 (0.15)	1.74 (0.09)	1.27 (0.09)	–	0.65 (0.04)	0.86 (0.05)	0.63 (0.04)	–	0.84 (0.05)	1.56 (0.08)
p-coumaric acid	4.27 (0.34)	15.32 (0.92)	2.41 (0.12)	22.88 (1.60)	1.14 (0.11)	8.54 (0.60)	3.50 (0.23)	5.22 (0.26)	1.16 (0.12)	27.68 (1.38)	1.46 (0.12)	6.67 (0.33)
Vanillic acid	–	–	–	–	–	0.55 (0.04)	–	–	0.50 (0.04)	6.64 (0.33)	–	–
o-pyrocatechuic acid	0.40 (0.03)	0.62 (0.06)	0.41 (0.02)	0.83 (0.06)	–	–	–	0.63 (0.03)	–	–	–	–
4-hydroxybenzoic acid	–	0.61 (0.05)	–	–	–	–	–	0.69 (0.04)	–	–	–	–
Caffeic acid	0.52 (0.03)	0.88 (0.05)	0.41 (0.03)	3.09 (0.15)	n.q	1.03 (0.07)	–	0.49 (0.02)	0.55 (0.03)	0.59 (0.04)	–	0.25 (0.01)
Gallic acid	–	–	–	–	–	–	–	–	–	8.41 (0.42)	–	–
β-resorcylic acid	–	n.q	–	1.82 (0.13)	–	–	–	–	–	–	n.q	0.14 (0.01)
Protocatechuic acid	9.89 (0.49)	1.52 (0.11)	7.74 (0.43)	5.24 (0.34)	1.50 (0.08)	5.00 (0.43)	0.71 (0.07)	1.64 (0.11)	1.73 (0.11)	1.02 (0.05)	1.44 (0.11)	0.89 (0.09)
TOTAL	28.89 (1.63)	36.47 (2.28)	18.87 (1.23)	47.39 (3.02)	10.77 (0.76)	22.85 (1.65)	15.97 (0.96)	18.33 (1.16)	27.37 (1.55)	73.34 (4.02)	16.62 (1.01)	17.02 (1.08)
HCA	7.88 (0.41)	25.47 (0.98)	4.54 (0.09)	37.07 (1.90)	3.34 (0.18)	14.05 (0.58)	8.09 (0.31)	10.71 (0.39)	8.62 (0.20)	36.88 (1.46)	5.52 (0.12)	10.11 (0.26)
HBA	21.01 (0.52)	11.00 (0.27)	14.33 (0.48)	10.32 (0.44)	7.43 (0.41)	8.80 (0.57)	7.88 (0.41)	7.62 (0.12)	18.75 (0.90)	36.46 (0.50)	11.10 (0.55)	6.91 (0.40)

-The showed value is the average of three different analysis (n=3) with the standard deviation between brackets.
-Each compound was quantified in terms of its own commercial standard and using the appropriate calibration curve.
-Total content of phenolic acids was the result of a summation of the amount of every analyte. For the calculation of HCA and HBA contents, only hydroxycinnamic or hydroxybenzoic acids, respectively, were considered.

Phenolic acids detected in avocado fruits were generally in concentrations ranging from 0.13 ppm to 3.82 ppm, but the concentration of some compounds covered wider ranges, such as *p*-coumaric acid (0.63 ppm to 33.75 ppm), benzoic acid (0.69 ppm to 15.89 ppm) or protocatechuic acid (from 0.63 ppm to 15.17 ppm), among others. Harvest (at the 2nd ripening degree) turned out to be the variety where *p*-coumaric acid was found at the highest concentration (33.75 ppm), followed by Gem (33.63 ppm) and Sir Prize (27.68 ppm). However, fruits from Pinkerton and ColinV 33 (at the 2nd ripening degree) were the varieties with the lowest concentrations of this acid (5.22 and 6.84 ppm, respectively).

As it was mentioned in *Section 2.2*, four of the studied varieties (Hass Motril, Jiménez 1, Jiménez 2 and Tacambaro) are mutants from Hass *cv.* and some interesting differences were found among them, both qualitative and quantitative. *Trans*-cinnamic, *m*-coumaric, benzoic, *γ*-resorcylic, *p*-coumaric and protocatechuic acids were detected in the 10 genetically related samples (considering the two degrees of ripeness), being benzoic acid the predominant one when the fruit was just harvested (except for Jiménez 1), and *p*-coumaric acid the most abundant phenolic acid at the second ripening degree. It is remarkable that chlorogenic and caffeic acids were present in fruits of Hass, Hass Motril, Jiménez 1 and Jiménez 2 at both ripening degrees, however in the case of Tacambaro cultivar, they were just determined in one of the evaluated ripening degrees (chlorogenic acid at the first one and caffeic acid at the second one). Sinapic and ferulic acids only appeared at the second stage of ripeness, except for Tacambaro where they were detected when the fruit was just harvested as well. Ripe fruits from Jiménez 1 and Jiménez 2 contained compounds such as 4-hydroxybenzoic and *o*-pyrocatechuic acids, being the last one also found at the first ripening degree of Jiménez 2.

It is interesting to highlight the fact that, to the best of our knowledge, this is the first report in which an exhaustive quantification (in terms of pure standards) of 17 phenolic acids present in avocado extracts is carried out. Torres et al. [20] determined the total phenolic content of some avocado samples (mesocarp, cotyledon, young and mature leaves) and identified 16 phenolic acids in four different avocado varieties. Later on, Golukcu and Ozdemir [22] studied the changes in the phenolic composition of four avocado cultivars during harvesting, quantifying 12 phenolic compounds (phenolic acids

and flavonoids). The current work can enlarge the knowledge about this matrix, since 13 varieties (grown under identical environmental conditions) at two ripening degrees were compared. Among the 13 varieties included in our study, only one of them (Hass cv.) was previously studied in the two reports aforementioned.

More recently, Hurtado-Fernández et al. [43] using a powerful UHPLC method identified a great number of compounds (belonging to different chemical families, such as nucleosides, vitamins, isoprenoids, phenolic acids, amino acids, flavonoids, fatty acids, etc.) and quantified some of them (20 analytes; 9 phenolic acids). All the phenolic acids quantified by means of UHPLC-MS have been confirmed with CE-UV; moreover, this CE methodology has allowed the identification and quantification of some more phenolic acids because of the specificity of the method.

2.3.4. Exploratory data analysis: PCA results

Chemometric studies were performed to evaluate the whole structure of the data set and general trends in the metabolite fluctuations during the ripening process of avocado fruit. The main objective of the PCA is to reduce the number of variables that should be included in the models, eliminating those parameters that are not providing relevant information for the discrimination. The principal components (PCs) are obtained combining linearly the original variables and each of these components is able to explain the points presenting the highest variability in the data.

Two matrices were generated for the study. The first matrix (A) was built to obtain a global vision of the influence that the groups of HCA and HBA had on the discrimination. Thus, matrix A includes only 2 variables, the total concentration of HCA and HBA, and 78 samples (26 samples by triplicate). On the other hand, with matrix B the pursued objective was to observe the individual implication of each phenolic acid and for this reason it was formed by 17 variables (the number of phenolic acids that were quantified in the avocado extracts) and 78 samples.

Figure 2.6 shows the score and loading plots of PC1 vs. PC2 for matrix A. It can be seen that PC1 was built considering the influence of HCA, whereas PC2 was made taking into account HBA. HCA were responsible of the grouping between ripening degrees and HBA differentiated among varieties.

In matrix B the first two PCs explained an 81.96% of total variance in raw data, whereas PC3 and PC4 accounted for 9.11% and 5.05% respectively. All the possible combinations of PCs were studied; however, ***Figure 2.7*** only shows PC2 vs. PC1, since they were those which provided the best separation. The ripening degree is the variable with the highest influence in the data variability and it is related with the concentration of *p*-coumaric acid. All the varieties are well differentiated between ripe (number 2) and unripe (number 1), except for ColinV 33, which has a similar composition regardless the ripening degree and both samples are grouped in the same area of the plot. As it was expected, Hass Motril, Jiménez 1, Jiménez 2 and Tacambaro (mutants from Hass cultivar) are laying quite close in the graph, because these varieties present a similar phenolic acid profile for its common origin.

The second component was built considering the influence of benzoic and protocatechuic acids. Those varieties with positive scores, present the highest content of protocatechuic acid (ColinV 33 at first and second ripening degree), while negative scores mean that the avocado sample are rich in benzoic acid, being Sir Prize 1 the richest one.

In both figures, it was logically observed, the fact that the higher the concentration of the analyte found in the sample, the bigger the dispersion among the three replicates. Examples of that could be unripe Colin V 33 (the richest one in terms of protocatechuic acid content); unripe Sir Prize (sample with the highest concentration of benzoic acid); and ripe fruits of Sir Prize, Gem, Harvest, Marvel and Lamb Hass, which present the highest amounts of *p*-coumaric acid and HCA. Besides, both PCAs showed a quite similar grouping, being Sir Prize, Gem, Harvest, Marvel and Lamb Hass (all of them at the second stage) the varieties with the highest metabolic differences between first and second ripening degree.

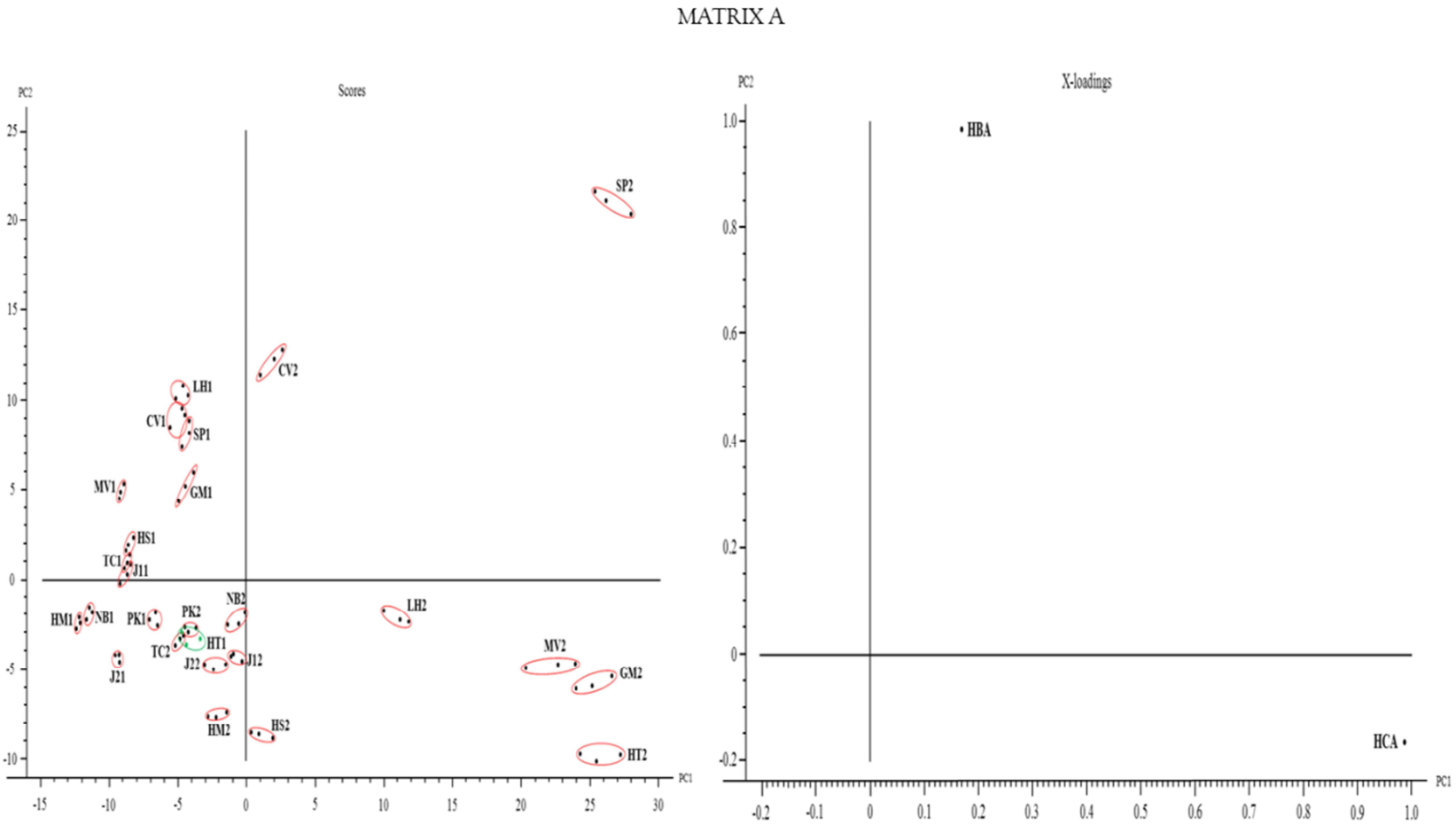


Figure 2.6. Score and loading plots of PCA modelling of CE-UV data considering total content of HCA and HBA. **CV**: Colin V 33; **GM**: Gem; **HT**: Harvest; **HS**: Hass; **HM**: Hass Motril, **J1**: Jiménez 1; **J2**: Jiménez 2; **LH**: Lamb Hass; **MV**: Marvel; **NB**: Nobel; **PK**: Pinkerton; **SP**: Sir Prize; **TC**: Tacambaro. **1**: first ripening degree; **2**: second ripening degree. The replicates samples of HT1 are in green to distinguish them properly from replicate samples of PK2 y TC2.

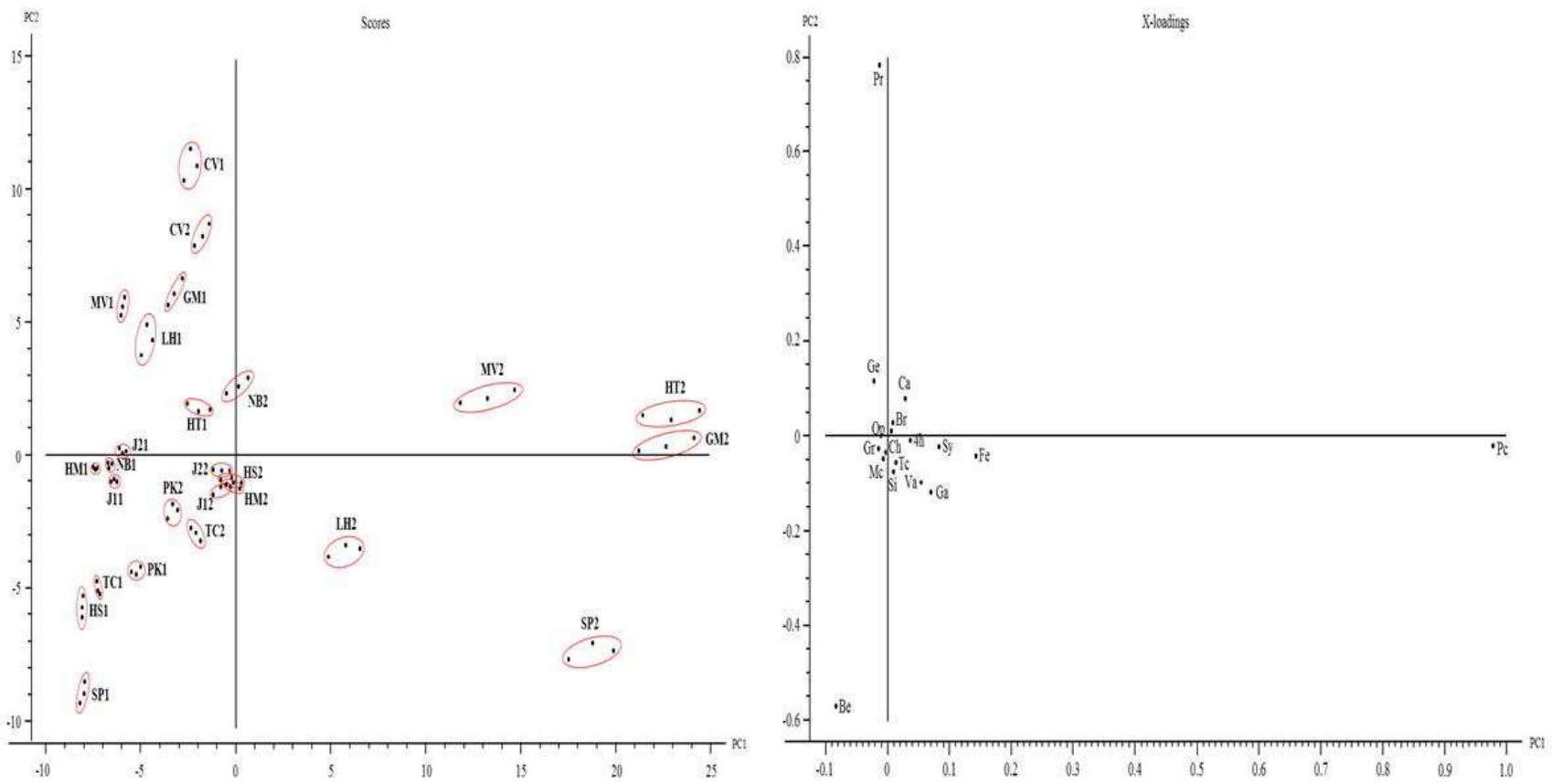


Figure 2.7. Score and loading plots of PCA modelling of CE-UV data considering the individual concentration of each quantified phenolic acid. Identification legends of varieties as in ***Figure 2.6.*** **Tc**: *trans*-cinnamic acid; **Ch**: chlorogenic acid; **Si**: sinapic acid; **Ge**: gentisic acid; **Mc**: *m*-coumaric acid; **Be**: benzoic acid; **Sy**: syringic acid; **Fe**: ferulic acid; **Gr**: *γ*-resorcylic acid; **Pc**: *p*-coumaric acid; **Va**: vanillic acid; **Op**: *o*-pyrocatechuic acid; **4h**: 4-hydroxybenzoic acid; **Ca**: caffeic acid; **Ga**: gallic acid; **Br**: *β*-resorcylic acid; **Pr**: protocatechuic acid.

2.4. Conclusions and perspectives

An effective and sensitive CE-UV method was developed and successfully applied to the analysis of phenolic acids extracted from different avocado varieties at two ripening degrees. From 8 to 14 compounds were identified and quantified, depending on the variety, being Jiménez 1 the richest one. Three of the phenolic acids (*trans*-cinnamic, *p*-coumaric and protocatechuic acids) were common for all the samples under study and ferulic acid was detected at the second ripening degree of every variety.

The analytical parameters of the proposed method were calculated, obtaining good values for repeatability and reproducibility, with RSDs of peak areas/migration time between 7.6% and 12.4% for repeatability, 11.5% and 13.3% for intermediate precision, and between 11.0% and 18.7% for reproducibility.

The statistical studies stood out the fact that HCA, in particular *p*-coumaric acid (whose concentration increases during ripening) was the variable with the highest influence in the data variability. HBA composition seemed to be more cultivar-related.

The results are very promising, since keeping in mind some of the health-related attributes of the phenolic acids and their influence in the sensory properties and stability of food; it could be interesting to harvest the fruit strengthening the presence of certain analytes.

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Chapter 3

Determination of changes in the metabolic profile of avocado fruits (*Persea americana*) by two CE-MS approaches (targeted and non-targeted)

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Abstract

A CZE method with two different MS detection conditions (*MRM* and *Full Scan*) was developed to determine qualitative and quantitative changes in the metabolic profile of avocado fruits (*Persea americana*). LODs in *MRM* approach were found between 20.1 and 203.0 ppb for abscisic acid and perseitol, respectively, whilst in *Full Scan* varied within the range 0.22-1.90 ppm for the same metabolites. The RSDs for reproducibility test did not exceed 11.45%. The two MS approaches were used to quantify 10 metabolites (phenolic acids, flavonoids, a carbohydrate, an organic acid, a vitamin and a phytohormone) in 18 samples of avocado at different ripening states, and the achieved results were compared. Perseitol, quinic, chlorogenic, *trans*-cinnamic, pantothenic and abscisic acids, as well as epicatechin and catechin decreased during the ripening process, whereas ferulic and *p*-coumaric acids showed the opposite trend. Moreover, some other unknown compounds whose concentration changed largely during ripening were also studied by MS/MS and Q-TOF MS to get a tentative identification.

Keywords: Avocado / *Persea americana* / Capillary electrophoresis / Mass spectrometry

3.1. Introduction

Fruits and vegetables are extremely relevant in the human diet since they contain most of the essential components for human nutrition [1]. They serve as an important source of fiber, vitamins and other phytochemicals, therefore being the target of several studies that focus on food quality and nutritional value. In other words, we can say that knowing what we eat is nowadays essential to dietetics and food science, as well as for biodiversity, agriculture production, and food industry.

In the area of metabolite characterization of fruits, it is possible to highlight that the studies on two cultivated fruit crops (tomato and strawberry) dominate the field; however, other fruits such as avocado (*Persea americana*) are increasingly consumed, in part because of its high nutritional value and because of its reported health-benefits (anticancer activity, *in vivo* antioxidant and antimicrobial effects, inhibition of lipid and protein oxidation… [2-7]), arousing scientific interest for determining its composition.

The avocado is a fruit with a high nutrition profile and an unusual composition; the outstanding compositional feature is its high fat content, which varies significantly between different cultivars [8]. Avocado is a complex mixture of proteins, sugars, vitamins, sterols, tannins, fatty acids, amino acids, alkanols, terpenoid glycosides, various furan ring-containing derivatives, phenolic compounds, coumarins, etc [8-10]. This chemical diversity, together with the fact that levels of fruit metabolites are strongly affected by genetic and environmental factors as well as transportation and storage conditions, makes the determination of the whole metabolome of avocado fruit very complex and difficult to assess. Indeed, most of the studies published so far focused in the determination of a particular group of compounds [9, 11-13]; only in a recent report, a global UHPLC-MS foodomics approach has been used [10].

Modifications in any of the aforementioned compounds can also lead to changes in the overall nutritional quality of the fruit. An increasing understanding of the metabolic changes underlying fruit development and ripening may help in developing, for instance, new strategies to identify horticultural maturity, which is difficult for avocado, because ripening is not accompanied by drastic changes in external appearance. Already in 1983, Lee et al. [14] examined the feasibility of establishing picking dates and proposed dry weight analysis as an index of maturity. Oil content is also used as an indicator of fruit maturity, and thus, commonly helps to define the optimum harvest period. In a wide number of studies the variation in the composition of avocado fruit during ripening or storage has been the parameter checked, determining fatty acids [5, 8, 9, 15, 16], sugars [9, 17], total protein [17], carbohydrates [15], phenolic, ascorbic acid and glutathione contents [18], aroma volatiles [19], carotenoids, tocopherols [15], etc.

Bearing in mind the complexity of the avocado metabolome, the factors affecting it, and the fact that some compounds are closely related to others, it is possible to think that a non-biased, exploratory type of analysis could fit very well to study changes in the metabolic profile of avocado fruits. There are different technologies available for metabolomics, being mass spectrometry (MS)-based technologies the most popular nowadays (GC-MS, LC-MS and CE-MS), together with NMR spectroscopy. Although not used as extensively as GC-MS and LC-MS, CE-MS has demonstrated to be a powerful

technique for the profiling of polar metabolites in complex samples [20-24], providing complementary or additional information on the composition of the sample under study.

This study aims to characterize biochemical changes during the development and ripening of the avocado fruit by using two different CE-MS approaches (non-targeted and targeted; in other words, using a *Full Scan* or a *MRM* MS method). Combining the information achieved by the two methodologies, we will get quantitative information about 10 metabolites belonging to 6 different families (flavonoids, phenolic acids, phytohormones, vitamins, organic acids and carbohydrates), as well as qualitative information about some other compounds whose concentration widely varied during ripening. The CE-MS quantitative results were compared with those achieved by LC-MS; LC-Q-TOF MS analyses were carried out as well to facilitate the identification of some relevant metabolites.

3.2. Materials and methods

3.2.1. Chemicals and standards

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogen carbonate and ammonium hydroxide were from Panreac (Barcelona, Spain), and ammonium acetate from Merck (Darmstadt, Germany). Buffers were prepared by weighting the quantity indicated in doubly distilled water and adding ammonium hydroxide to adjust the pH. The buffers were stored at 4ºC and warmed to room temperature before use. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). 2-propanol of HPLC grade from Lab-Scan (Dublin, Ireland) was used in the sheath liquid, and NaOH solution (1 M) from Panreac (Barcelona, Spain) was used for capillary cleaning procedures and activation of the capillary wall. All solutions and buffers were degassed by ultrasonication before use.

ACN, from Lab-Scan (Dublin, Ireland), and Milli-Q water and acetic acid, from Panreac (Barcelona, Spain), were used in the LC mobile phases.

Standards of perseitol (from Carbosynth (Comptom, UK)), taxifolin and chlorogenic acid from Extrasynthese (Lyon, France), quinic acid from Fluka (St. Louis, EEUU), and catechin and epicatechin, pantothenic, abscisic, *trans*-cinnamic, *p*-coumaric, ferulic and folic acids from Sigma-Aldrich (St. Louis, EEUU) were used in this study. Taxifolin and

folic acid were used as ISs, the first one to evaluate the efficiency of the extraction protocol, and the second one to check the repeatability between runs and, if necessary, for quantification purposes (by using an internal standard calibration).

3.2.2. Samples

In the preliminary studies fruits of 4 varieties of avocado (*Persea americana*) were used: Hass, Bacon, Reed and Fuerte. All the varieties were grown under identical environmental conditions (soil, rain, light, etc.). The samples used in this study were part of the germplasm collection from the IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). For each variety, the pulp of 3-4 pieces of fruit, which were weighted, peeled, sliced, frozen to be further freeze-dried, was used. Fruits were processed when they reached the edible ripening stage.

For the second part of the study, once the CE-MS method was fully optimized, we focused our attention in Reed *cv.* and worked with 18 samples of that variety, which were handpicked at different maturity stages (between June and October) of 2011 season. The aim was to evaluate the metabolic changes that occur in this variety during its main harvesting season in Southern Spain.

The harvest dates were the following: 27/06/2011, 06/07/2011, 18/07/2011, 01/08/2011, 17/08/2011, 31/08/2011, 16/09/2011, 31/09/2011, and 17/10/2011. Two different samples coming from the same batch were studied for each harvest date.

A pooled sample of the 18 avocado extracts (e.g. mixing an equivalent volume of each one) was used as analytical QC sample. For validation purposes, the mentioned QC samples and a standard mix composed by the 10 compounds previously mentioned in the Chemicals and standards section were used. Both samples were injected every 4 samples throughout the batch. Data derived from these samples were used to assess system stability.

3.2.3. Extraction procedure

Extracts were prepared according to Hurtado-Fernández et al. [13]; briefly, 4 g of the freeze-dried (and homogenized) sample were put inside a falcon tube adding 40 mL of pure MeOH. The tubes were shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 3000 rpm for 10 min. After that, the supernatants were

evaporated to dryness and redissolved in 1 mL of MeOH/water (50/50, v/v). ISs were added to evaluate the efficiency of the extraction protocol (taxifolin), and to check the repeatability between runs (folic acid). Therefore, taxifolin was added to the sample before the extraction procedure, whilst folic acid was added just before the injection into the CE-MS system.

3.2.4. Capillary electrophoresis-mass spectrometry analyses

Analyses were performed in a P/ACE™ MDQ System (Beckman Coulter, Fullerton, CA, USA) coupled to the mass detector using an orthogonal ESI from Agilent (see below). The CE instrument was controlled by a personal computer running 32 Karat System Software from Beckman. Bare fused-silica capillaries with 50 μm i.d. and a total length of 85 cm from Beckman Coulter Inc. (Fullerton, CA, USA) were used. The running buffer was 40 mM ammonium acetate at pH 9.5, voltage was set at 30 kV, temperature at 25°C, and 5 s hydrodynamic injections were made at the anodic end using N_2 at a pressure of 0.5 psi. Before their first use all new capillaries were conditioned by rising with 1 M sodium hydroxide for 20 min, followed by a rinse with water for 3 min and then running buffer for 15 min. Capillary conditioning at the beginning of the day consisted of 3 min with 1 M sodium hydroxide, then 2 min with water and, finally, 10 min with running buffer. Capillary conditioning between runs simply consisted of 5 min with running buffer. At the end of the day the capillary was rinsed with water for 60 min.

MS experiments were performed on a Bruker Daltonics Esquire 2000™ IT mass spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal ESI (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA). The composition of this sheath liquid was 60:40 (v/v) 2-propanol/water. For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation capillary was fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid established the electrical contact between capillary effluent and the electrospray needle. The mass spectrometer was run in negative and positive ion modes. In negative polarity, the capillary voltage was set at +3500 V. The IT scanned at 50-800 *m/z* range, the maximum accumulation time for the ion trap was set at 100 ms, the target count at

20,000 and the trap drive level at 100%. ESI operating conditions were optimized as described in Results and Discussion, and the optimum values of the ESI-MS parameters were: drying gas temperature, 250ºC; drying gas flow, 5 L/min; nebulizing gas pressure, 5 psi; and sheath liquid flow, 0.24 mL/h. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonics.

In MS the analyses were carried out in both *Full Scan* and *MRM*. When the second approach was selected, different segments were created along the analytical window to improve the performance in the determination of the compounds under study, changing the MS conditions, in particular, octopole, skimmers voltages and trap drive. In the final step of this study, MS/MS analyses (Auto MS/MS) were performed as well.

Linearity of the MS response was verified with solutions containing 10 standards at 6 different concentration levels over the range from the quantification limit to 250 or 500 ppm, depending on each analyte. For those compounds found in the avocado samples at higher concentrations, the linearity was tested in the wider range (from quantification limit to 500 ppm). Each point of the calibration graph corresponded to the mean value from three independent injections. LODs and LOQs of the individual analytes in standard solutions were calculated according to the IUPAC recommendations [25].

The precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatabilities in the peak areas/migration time were determined as the RSD obtained for 5-10 consecutive injections of each standard at a selected concentration value, carried out within the same day and on five different days, respectively.

To evaluate the matrix effect, we compared the responses of the analytes under study spiked post-extraction with those in a neat solution (in triplicate), calculating the response factors (RF, which is considered to be the ratio between the peak area and the concentration of the analyte) when the analytes were in presence of the avocado matrix and in a neat solution. Whether significant differences between both values could be found was checked using ANOVA.

3.2.5. High performance liquid chromatography-mass spectrometry analyses

HPLC-ESI-IT MS was used as complementary methodology to confirm, when possible, the quantitative results obtained by CE-MS. Moreover, HPLC-ESI-Q-TOF MS was used to achieve a tentative identification of some metabolites whose concentration seemed to change widely during the ripening process.

An Agilent 1260-LC 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. Avocado metabolites were separated on a Zorbax C18 analytical column (4.6 x 150mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at 25ºC and a flow rate of 0.8 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and ACN (Phase B), and the solvent gradient changed according to the following conditions: 0 to 30 min, 5-100% B, then, the B content was lowered to the initial conditions (5%) and the column re-equilibrated for 5 min. A volume of 10 μL of the avocado extracts was injected. The separated compounds were monitored in sequence first with DAD (240 and 280 nm) and then with an IT MS detector. MS experiments were performed on a Bruker Daltonics Esquire 2000™ ion trap (see section 2.4.). The IT MS was run in the negative ion mode and the capillary voltage was set at +3200 V. The IT scanned at 50-800 *m/z* range, and the optimum values of the ESI-MS parameters were: drying gas temperature, 300ºC; drying gas flow, 9 L/min; and nebulizing gas pressure, 30 psi.

For identification purposes, we used HPLC-ESI-Q-TOF MS. The LC system was coupled to an ultra high resolution Q-TOF MS (Bruker Daltonik, Bremen, Germany). The LC method, in this case, was slightly modified to get a proper separation of the unknown compounds of interest (section 3.5); the column and the mobile phases were the same as above, but the gradient changed according to the following: 0 to 22 min, 5-30% B, 22 to 25 min, 30-100% B, then, the B content was lowered to the initial conditions and the column re-equilibrated for 5 min.

The mass spectrometer was operated in negative and positive ionization modes and acquired data in the mass range from *m/z* 50 to 800 with a spectra rate of 1 Hz. In negative mode, the capillary was set at +4000V, the End Plate offset at −500V, the Nebulizer Gas at 2 bar and the Dry Gas at 9 L/min at 250°C. The accurate mass data of

the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provides a list of possible elemental formulas by using the SmartFormula™ Editor tool.

The polarity of ESI and all the detection parameters of both IT and Q-TOF mass spectrometers were optimized using the height of the MS signal for the compounds included in our standard mix and some other compounds found in the avocado QC samples.

3.3. Results and discussion

3.3.1. Preliminary studies with 4 different avocado varieties

In the first stage of this study, we compared the metabolic profiles of 4 different avocado varieties (Hass, Bacon, Reed and Fuerte). The aim was double: make a selection of metabolites present in all the tested varieties (which could be analyzed with our methodology) and select the richest variety. The presence of 36 compounds in the avocado extracts was checked by using pure commercially available standards of: glucose, sinapic, succinic, stearic, 2,3-dihydroxybenzoic, ferulic, gallic, protocatechuic, 3-hydroxy-4-methoxybenzoic, folic, *p*-coumaric, *trans*-cinnamic, caffeic, chlorogenic, homovanillic, syringic, quinic, benzoic, citric, abscisic, pantothenic, and ascorbic acids, scopoletin, riboflavin, chrysin, tryptophan, tyrosine, phenylalanine, thiamine, catechin, epicatechin, naringenin, kaempferide, laricitrin, perseitol and β-carotene. Migration times and m/z signals were used to checked their presence or absence in the avocado samples. This list includes amino acids, flavonoids, vitamins, phenolic acids, fatty acids, sugars, etc.

Figure 1 shows the BPEs of the extracts of the 4 varieties included in the preliminary studies. The variability in the profiles seemed to be more quantitative than qualitative, although some m/z signals were detected only in one of the varieties.

The compounds selected for further stages of this study, since they were present in every avocado sample analyzed, were: perseitol (carbohydrate or polyalcohol), catechin and epicatechin (flavonoids), *trans*-cinnamic, *p*-coumaric, ferulic and chlorogenic acids (phenolic acids and derivatives), abscisic acid (phytohormone), pantothenic acid (vitamin), and quinic acid (organic acid).

Once those 10 compounds in particular were selected, we proceeded to carry out a proper optimization of the CE and MS conditions.

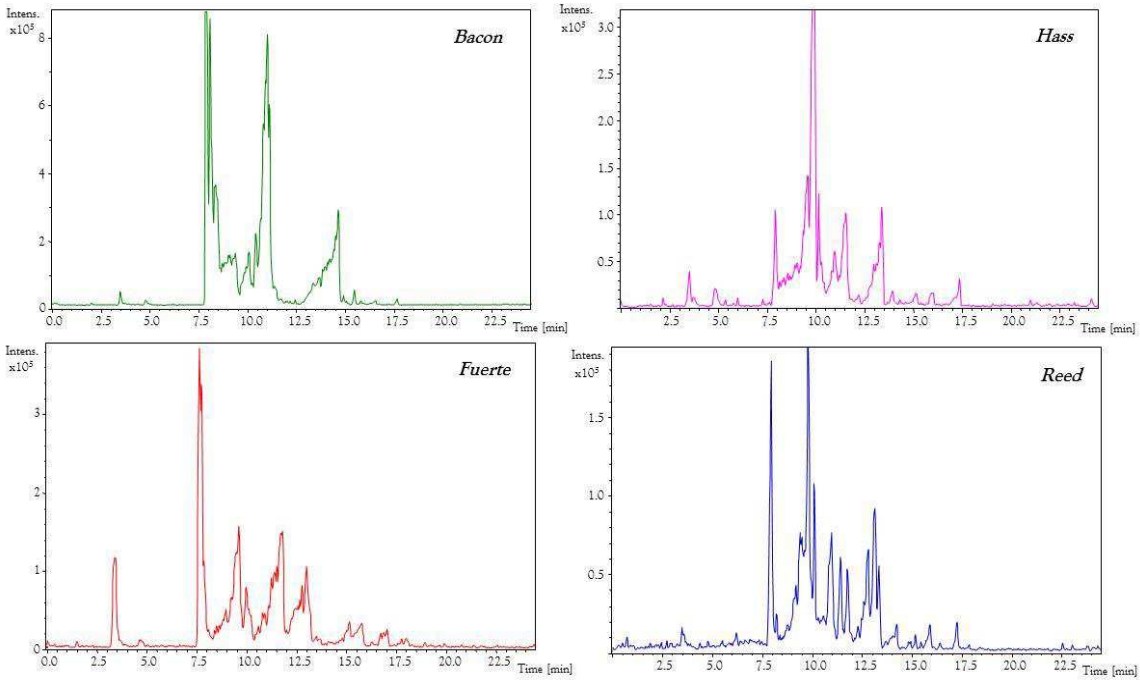


Figure 3.1. BPEs of the extracts of avocado fruits from 4 varieties: Bacon, Hass, Fuerte and Reed. Capillary 80 cm x 50 µm; ammonium acetate at pH 9.4 and 25ºC; sheath liquid isopropanol/water (60/40, v/v) at 0.24 ml/h; injection time 5s at 5 psi.

3.3.2. Optimization of CE separation and MS detection conditions (*Full Scan* and *MRM*)

First of all, some direct infusion experiments were performed to understand the MS signal that the 10 selected metabolites produced and to optimize the MS conditions looking for the maximum sensitivity.

Table 3.1 shows the signals produced by each compound in positive and negative polarity as well as in MS/MS. The information summarized in ***Table 3.1*** can be completed with ***Figure 3.2***, which includes examples of MS/MS spectra of several different standards and their dimmers (at least one example for family of compounds included in the standard mix).

Table 3.1. MS signals produced by each metabolite in positive and negative polarity and MS/MS mode.

	Compound	MW	MS Mode	Major *m/z* signals in MS *	Precursor ions and fragments in MS/MS **	Cut off energy ♦
1	Perseitol	212.20	–	**210.8** $[M-H]^-$/422.9 $[2M-H]^-$/ 300.9/495.1	**211**: 192.7 $[M-H-18]^-$/ 100.7/ 88.7/ 118.7 **423**: 210.7	57 113
			+	**234.8** $[M+Na]^+$/447.0 $[2M+Na]^+$/337.9/477.0	**235**: 222.8/ 213.8 **447**: 234.8/ 252.8	60 123
2	Epicatechin	290.27	–	**288.9** $[M-H]^-$/ 579.0 $[2M-H]^-$/ 325.0/ 553.1	**289**: 244.7 $[M-H-44]^-$/ 204.7 $[M-H-44-40]^-$/ 270.7 $[M-H-18]^-$ **579**: 288.8/ 244.7/ 202.7/ 468.8	79 161
			+	**312.9** $[M+Na]^+$/ 304.1/ 290.9 $[M-H]^+$/ 230.0/ 603.0	**313**: 138.7/ 272.8/ 122.7/ 290.8 **305**: No fragmentation **603**: 312.8 / 483.3	85♦ 83♦ 167
3	Catechin	290.27	–	**288.8** $[M-H]^-$/ 579.0 $[2M-H]^-$/ 325.0/ 339.0/ 480.9	**289**: 244.7 $[M-H-44]^-$/ 204.7 $[M-H-44-40]^-$/ 270.7 $[M-H-18]^-$ **579**: 288.8/ 244.7/ 202.7/ 468.9	79 161
			+	**312.9** $[M+Na]^+$/ 360.1/ 290.9 $[M-H]^+$/ 230.0/ 603.0	**313**: 172.7/ 112.9/ 256.9/ 272.9/ 290.8 **360**: 268.0/ 207.8/ 324.9/ 340.1 **603**: 312.9	85♦ 99 167
4	Abscisic acid	264.32	–	**262.9** $[M-H]^-$/ 527.1 $[2M-H]^-$	**263**: 152.7/ 218.8 $[M-H-44]^-$/ 110.8 $[M-H-152]^-$ **527**: 262.8/ 152.8/ 218.9	71 146
			+	**302.9**/ 286.9 $[M+Na]^+$/ 551.1 $[2M+Na]^+$/ 416.0/ 529.1 $[2M+H]^+$	**303**: 276.9 **287**: 274.9/ 246.9 $[275-28]^+$/ 184.8/ 228.8 $[247-18]^+$ **551**: 286.9	83 78 153
5	Pantothenic acid	219.23	–	**217.8** $[M-H]^-$/ 437.0 $[2M-H]^-$/ 694.2	**218**: 87.7 $[M-H-130]^-$/ 145.7 $[M-H-72]^-$ **437**: 217.7 $[M-H]^-$	59 120
			+	**257.8** $[M+K]^+$/ 477.0 $[2M+K]^+$/ 367.4 $[M+148]^+$/ 275.8 $[258+18]^+$/ 496.0	**258**: 217.8 $[258-40]^-$/ 127.7 $[M-H-90]^-$/ 145.6 $[M-H-72]^-$ **447**: 275.8 $[M+K+18]^+$/ 459.0 $[447-18]^-$/ 346.9 $[447-130]^-$/ 257.8 $[447-219]^+$/ 219.8 $[447-258]^+$ **367**: 201.8 $[367-147-18]^+$/ 219.8 $[367-147]^+$/ 257.8 $[M+K]^+$/ 302.4 $[258+44]^+$	70 132 101
6	Quinic acid	192.17	–	**190.7** $[M-H]^-$/ 382.8 $[2M-H]^-$/ 339.0 $[2M-H-44]^-$	**191**: 84.7 $[M-H-106]^-$/ 126.6 $[M-H-64]^-$/ 172.6 $[M-H-18]^-$/ 92.7/ 110.6 **383**: 190.6 $[383-192]^-$	51 112
			+	**406.9** $[2M+Na]^+$/ 214.8 $[M+Na]^+$/ 307.9/ 241.9 $[M+K+H]^+$	**407**: 214.8 $[407-192]^+$/ 310.7 $[407-96]^+$/ 388.9 $[407-18]^+$ **215**: 196.8 $[215-18]^+$	112 58
7	*trans*-cinnamic acid	148.16	–	**146.7** $[M-H]^-$/ 339.0	**147**: 102.6 $[147-44]^-$	39
			+	**170.8** $[M+Na]^+$/ 316.0 $[171+145]^+$	**171**: 152.8 $[171-18]^+$/ 138.9 $[171-32]^+$ **316**: 148.8 $[316-167]^+$/ 130.8 $[149-18]^+$/ 102.8 $[149-18-28]^+$	45 86

Table 3.1 (continued)

	Compound	MW	MS Mode	Major *m/z* signals in MS *	Precursor ions and fragments in MS/MS **	Cut off energy ♦
8	Chlorogenic acid	354.31	–	**352.9** $[M-H]^-$/ 707.1 $[2M-H]^-$/ 190.7 $[quinic-H]^-$	**353**: 190.7 $[M-163]^-$ **707**: 352.8 $[707-M]^-$	97 197
			+	**376.9** $[M+Na]^+$/ 354.9 $[M+H]^+$/ 731.1 $[2M+Na]^+$	**377**: 358.9 $[377-18]^+$/ 214.8 $[377-162]^+$/ 330.9 $[377-46]^+$/ 184.7 $[377-192]^+$/ 162.8 **355**: 162.7 $[M+H-quinic]^+$ **731**: 376.8 $[731-M]^+$	103 97 203
9	Ferulic acid	194.18	–	**192.7** $[M-H]^-$/ 339.0 $[M-H+146]^-$/ 133.7 $[M-H-59]^-$/ 288.8 $[M-H+96]^-$/ 482.8 $[M-H+96+194]^-$	**193**: 133.6 $[M-H-59]^-$/ 148.6 $[M-H-44]^-$/ 177.6 $[M-16]^-$ **339**: 192.6 $[339-147]^-$/ 133.6 $[193-59]^-$	52 93♦
			+	**216.8** $[M+Na]^+$/ 410.9 $[2M+Na]^+$/ 194.8 $[M+H]^+$	**217**: No fragmentation **411**: 216.8 $[411-194]^+$/ 194.8 $[217-Na]^+$/ 176.8 $[195-18]^+$	58♦ 113
10	*p*-coumaric acid	164.16	–	**162.7** $[M-H]^-$/ 190.7 $[M-H+28]^-$/ 118.7 $[M-H-44]^-$/ 324.9 $[M-H+162]^-$	**163**: 118.6 $[M-H-44]^-$ **191**: 84.7 $[191-106]^-$/ 126.6 $[191-64]^-$/ 172.7 $[191-18]^-$	43 51
			+	**186.7** $[M+Na]^+$/ 146.8 $[M+H-18]^+$/ 347.9 $[187-161]^+$	**187**: 168.7 $[187-18]^+$ **348**: 164.7 $[348-183]^+$/ 146.7 $[M+H-18]^+$/ 118.8 $[M+H-18-28]^+$	50♦ 95

* In bold letter we indicate the predominant *m/z* signal in the MS spectrum.

** Fragments appear in decreasing order of intensity in the MS/MS spectrum. Some of the common fragments found in the MS spectra were: 59=CH_3COO, 28=CO or C_2H_4, 44=HCOO and 18=H_20.

♦ The parameters which were changed in order to get high quality MS/MS spectra were Amplitude (V) and Cut Off, being width (*m/z*) set at 4. Ampl was set at 1.0; for those compounds that were not fragmented by applying that energy (♦), Ampl was changed being set at 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5. On the contrary, when an excessive fragmentation was get with Ampl at 1.0, this value was decreased.

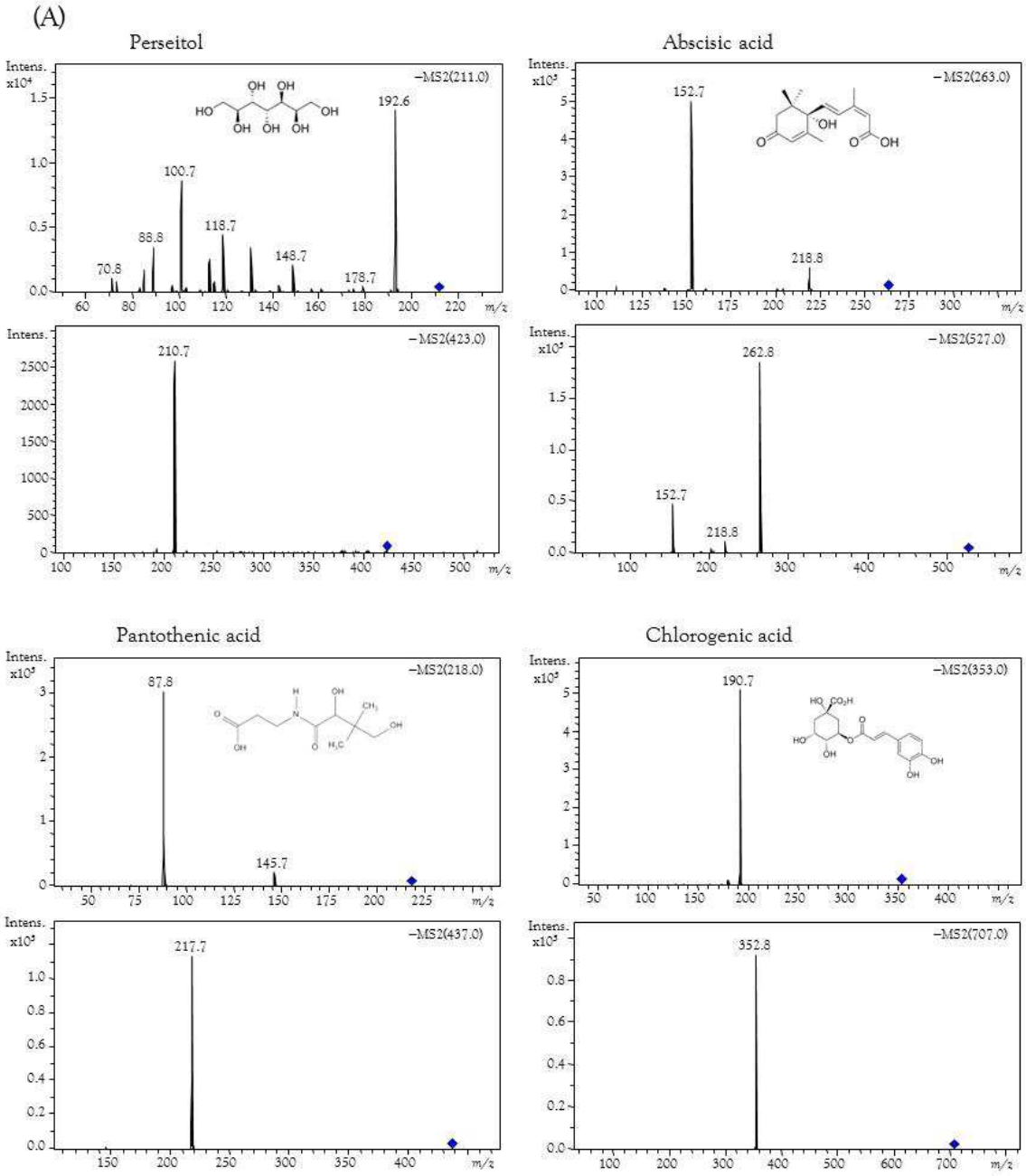


Figure 3.2. (A) MS/MS spectra in negative polarity of perseitol (precursor ions *m/z* 211 $[M\text{-}H]^-$ and 423 $[2M\text{-}H]^-$), abscisic acid (precursor ions *m/z* 263 $[M\text{-}H]^-$ and 527 $[2M\text{-}H]^-$), pantothenic acid (precursor ions *m/z* 218 $[M\text{-}H]^-$ and 437 $[2M\text{-}H]^-$) and chlorogenic acid (precursor ions *m/z* 353 $[M\text{-}H]^-$ and 707 $[2M\text{-}H]^-$).

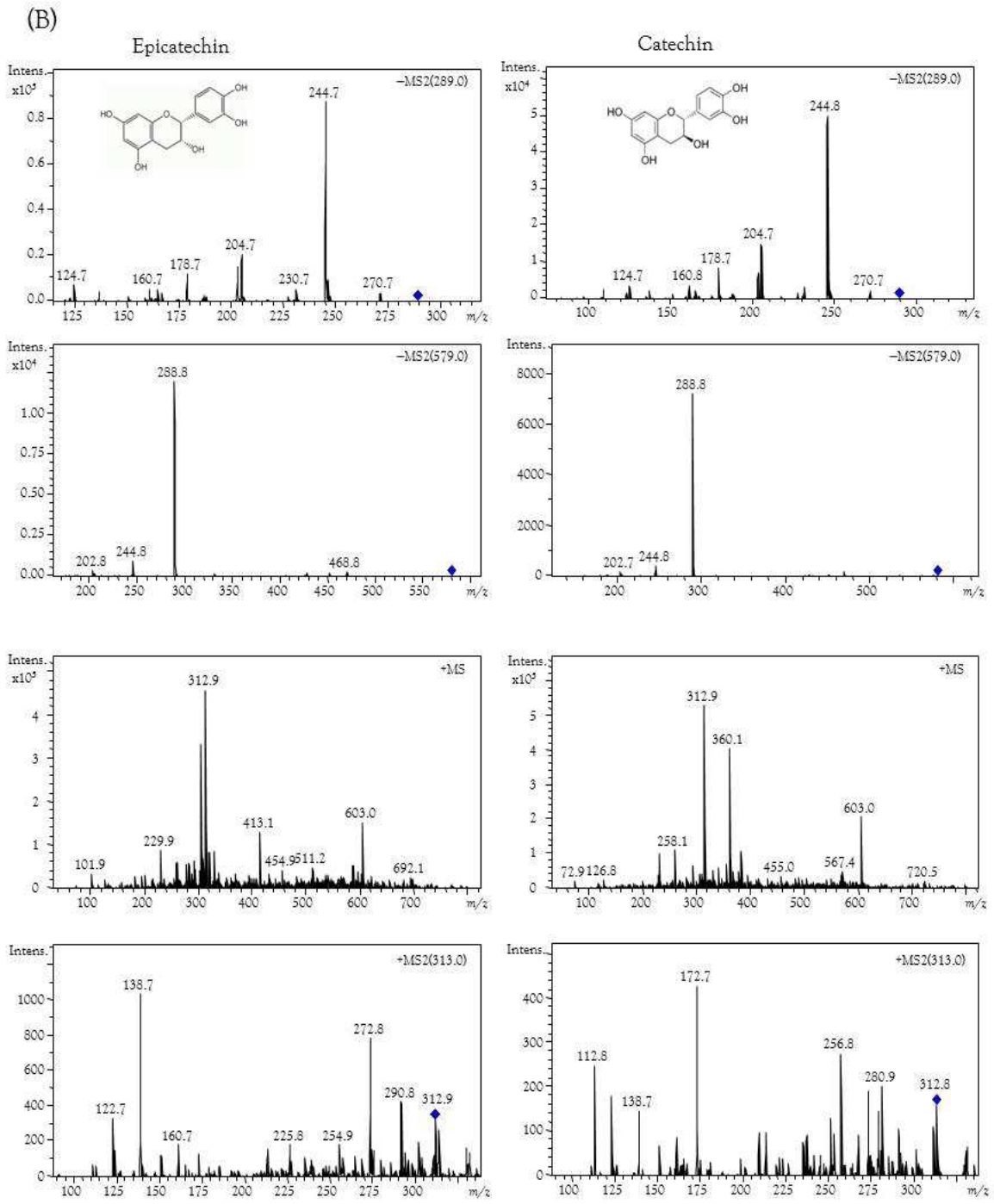


Figure 3.2. Continued. (B) MS (in positive polarity) and MS/MS spectra of flavonoids catechin and epicatechin in both polarities (precursor ions *m/z* 289 $[M\text{-}H]^-$ and 579 $[2M\text{-}H]^-$ and 313 $[M+H]^+$).

It seemed very interesting to study the MS and MS/MS behavior of catechin and epicatechin, isomers which produce very similar signals in MS. Indeed, the MS and MS/MS spectra in negative polarity are practically the same, being easier to distinguish them in positive polarity.

When the optimal MS parameters were selected to carry out the most sensitive possible detection, we proceeded to optimize the CE conditions. Initially, the electrophoretic conditions were optimized according to the following criteria: separation selectivity, analysis time and peak shape. We used both the standard mix of 10 compounds and the QC avocado sample.

Separation was evaluated using acetate- (NH_4OAc) and carbonate- (NH_4HCO_3) containing background electrolytes, observing in the preliminary studies that the profiles obtained using acetate-containing background electrolytes were better than those obtained with buffers based on carbonate. Therefore, ammonium acetate was chosen as optimum buffer.

The pH value was one of the variables with more influence in the separation. Based on our preliminary studies and the reports for successful separation of some of our compounds of interest, the optimum value of pH to get the best separation had to be between 8.5-10.0; thus, pH values in 0.25 steps within this range were tested. pH values lower than 9.0 gave a short analysis time, but poor resolution, whilst pH values higher than 9.5 enlarged considerably the analysis time, without enhancing resolution. ***Figure 3.3*** shows the results achieved for pH values of 9.0, 9.25 and 9.5. At pH values of 9.0, it was not possible to separate catechin and epicatechin, and abscisic and pantothenic acid; moreover, the separation between ferulic and *trans*-cinnamic acids was not good enough. When a pH value of 9.25 was tested, the separation between the two flavonoids (catechin and epicatechin) and pantothenic and abscisic acids improved, being the critical pair in this case *trans*-cinnamic and chlorogenic acids. Ammonium acetate buffer at pH 9.5 seemed to be very appropriate, since it lead to a proper resolution among the compounds under study within a reasonable analysis time; this value was considered as the optimum compromise solution.

To evaluate the influence of the ionic strength, buffers at concentrations ranging from 30 to 45 mM were prepared, since the optimum value in the preliminary studies was

found in that range. At 30 and 35 mM separation in the electropherograms was poor, being the separation obtained by using 40 and 45 mM very similar. 40 mM at pH 9.5 was finally chosen. Different organic modifier content (i.e. 5-10% of methanol and 2-propanol) did not lead to better results.

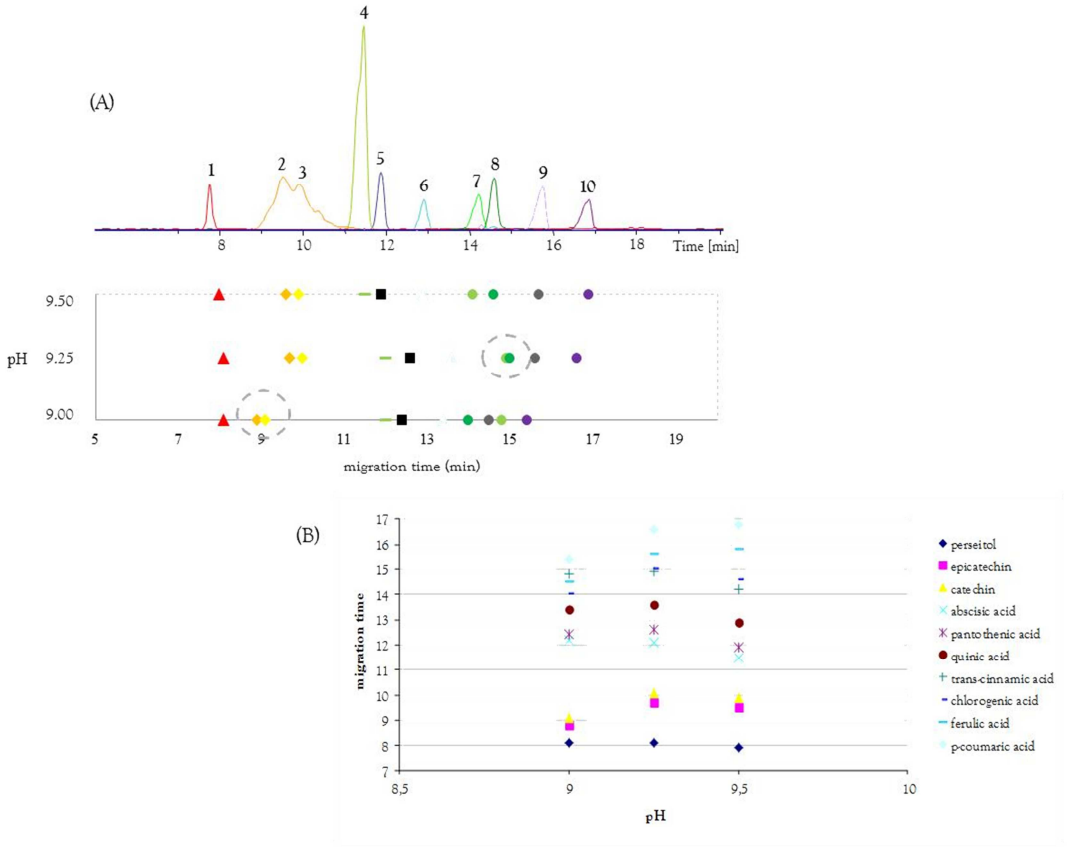


Figure 3.3. Migration time at different pH values of the 10 metabolites under study. (A) Optimum separation at pH 9.5 together with a plot of migration time *versus* pH indicating the most critical pairs. (B) pH *versus* migration time of the analytes included in this study.

The voltage applied was varied between 20 and 30 kV; a voltage of 30 kV was chosen in order to shorten the analysis time together with good separation and acceptable current. The injection time was tested between 3 and 10 s. Using longer injections, the detection limits were logically lower. However, when the avocado extracts were analyzed injecting longer than 5 s, the current values fluctuated in intervals that were wider than usual (±3-5 μA). The injections were made at the anodic end for 5 s (at 5 psi).

The sheath liquid flow and composition were also optimized, testing the intervals 0.20-0.28 mL/h for sheath liquid flow and 70:30 (v/v) 2-propanol/water to 50:50 as sheath liquid composition. Small amounts of volatile basic compounds (such as

triethylamine) can be used for negative MS detection, but in the current case this additive did not lead to a better sensitivity. Therefore, 60:40 (v/v) 2-propanol/water without any additive was selected as sheath liquid at a flow rate of 0.24 mL/h.

When all the separation and detection conditions were fully optimized, a standard mix and a QC avocado sample (mix of all the samples which were going to be analyzed in further stages of this study) were analyzed. The results are show in ***Figure 3.4.***

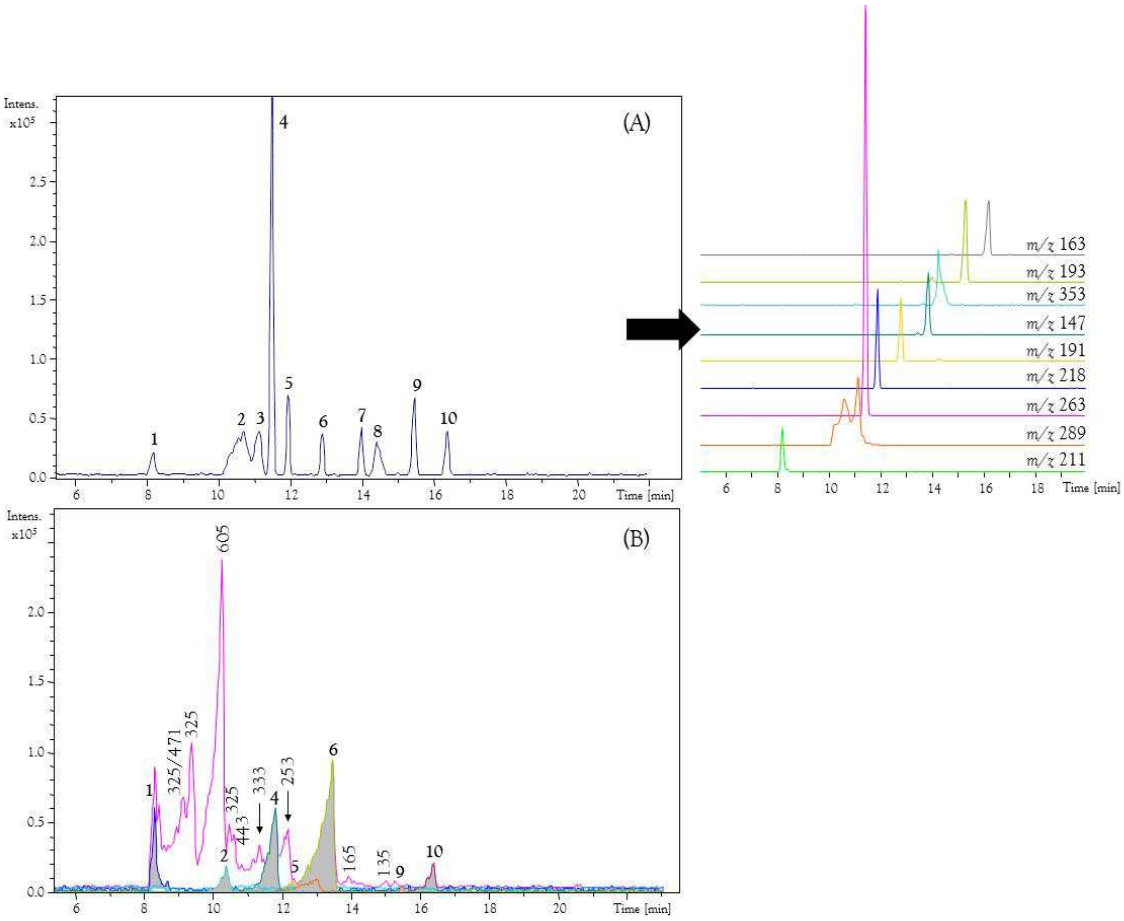


Figure 3.4. BPEs in negative mode of (A) a mixture of the standard mix together with the EIEs of each analyte and (B) avocado QC sample. In (B) some other *m/z* signals, apart from those of the compounds identified with standards are shown. Peak identity: 1. Perseitol, 2. Epicatechin, 3. Catechin, 4. Abscisic acid, 5. Pantothenic acid, 6. Quinic acid, 7. *Trans*-cinnamic acid, 8. Chlorogenic acid, 9. Ferulic acid, and 10. *p*-coumaric acid. Conditions: see ***Figure 3.1.***

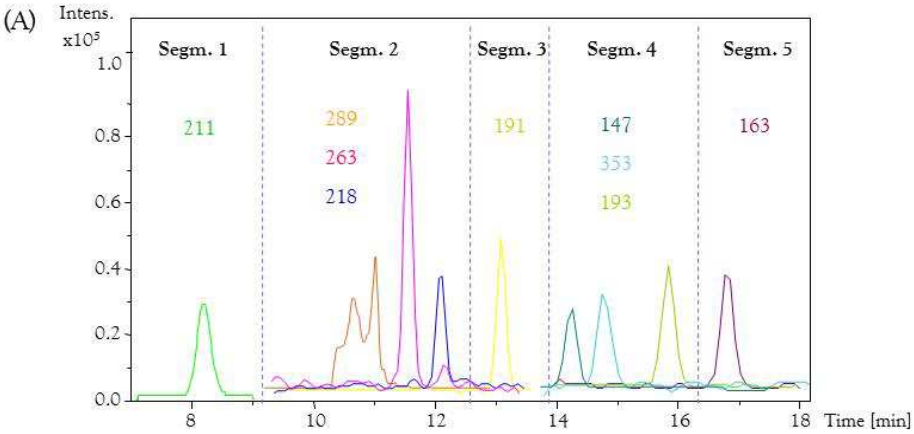
The separation of the compounds under study was carried out in about 16 min with a good resolution. In the BPE obtained for the avocado QC sample it can be seen that, besides the compounds that could be identified and quantified with standards, there were

others in the profile with noticeable intensity which migrated within the same analytical window.

The result obtained for the avocado QC sample suggested, from our point of view, that using two different CE-MS approaches, non-targeted and targeted, could be advisable. In the first one, MS detection could be done in *Full Scan*, detecting every *m/z* within the range 50-800; in the second, we could optimize the MS conditions to have a *MRM* method, looking for the selective determination of the compounds present in the standard mix.

MS conditions for *Full Scan* had already been optimized. For the targeted approach, five different segments were created and skimmers voltages, octopoles, trap drive, lens voltages, etc. were optimized. The parameters most strongly affecting ion transportation, hence sensitivity, were: Skim 1, Cap ExitOffset, Cap Exit, Octopole and trap drive. In ***Figure 3.5(A)*** we include the *MRM* electropherogram for the standard mix, together with the MS conditions for each segment.

When the same conditions were used to analyze the avocado QC sample (***Figure 3.5(B)***), the obtained results were very promising, making this approach an attractive alternative to carry out the selective determination of the compounds of interest, avoiding possible effects of ionic suppression.



Skim 1 (V)	- 11.6	- 12.3	- 11.0	- 10.3	- 10.1
Cap Exit Offset (V)	- 66.3	- 66.5	- 66.2	- 66.1	- 66.0
Cap Exit (V)	- 77.9	- 78.8	- 77.3	- 76.4	- 76.2
Opt (V)	- 2.36	- 2.38	- 2.34	- 2.32	- 2.30
Trap Drive	42.0	42.5	41.8	41.2	40.7

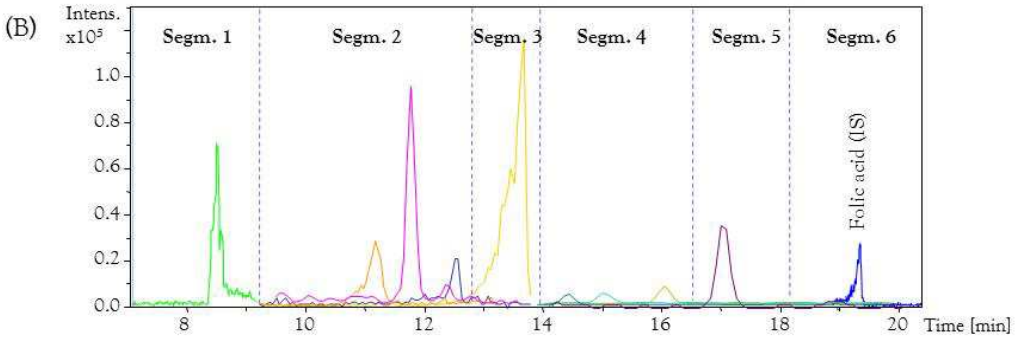


Figure 3.5. MRM electropherogram obtained for (A) a mix of standards and (B) avocado QC sample, together with the MS conditions for each segment and the *m/z* signals detected in each one. MS conditions in segment 6 were: Skim 1= −17.4 V; Cap Exit Offset= −67.5 V; Cap Exit= −84.9 V; Optopole= −2.54 V; Trap Drive= 46.6.

3.3.3. Determination of the analytical parameters of the two CE-MS approaches

Before starting the analysis of the 18 Reed avocado samples at different ripening stages, the analytical parameters of each methodology were evaluated. MS detection was done in positive and negative polarity, obtaining better results in negative polarity.

Table 3.2 summarizes the analytical parameters of the method in *Full Scan* and MRM in negative polarity, and ***Table 3.3*** includes the figures related to precision and matrix effect.

Table 3.2. Analytical parameters of the *Full Scan* and *MRM* CE-MS methodologies.

Compounds	Molecular formula	$[M-H]^-$	Migration time (min)	*Full Scan*				*MRM*			
				Calibration curve	r^2	LOD (ppm)	LOQ (ppm)	Calibration curve	r^2	LOD (ppb)	LOQ (ppb)
Perseitol	$C_7H_{16}O_7$	211.2	8.2	y = 1531x + 7386	0.9970	1.90	6.33	y = 3310x + 27733	0.9940	203.0	677.7
Epicatechin	$C_{15}H_{14}O_6$	289.0	10.5	y = 9947x – 19443	0.9990	0.30	1.00	y = 17576x + 49179	0.9980	36.7	122.3
Catechin	$C_{15}H_{14}O_6$	289.3	10.6	y = 7853x – 10519	0.9990	0.37	1.23	y = 12858x + 67071	0.9990	53.3	177.7
Abscisic acid	$C_{15}H_{20}O_4$	263.3	11.5	y = 13340x – 15776	0.9990	0.22	0.73	y = 35266x + 3E+06	0.9980	20.1	67.0
Pantothenic acid	$C_9H_{17}NO_5$	218.2	12.2	y = 2963x + 16898	0.9930	0.98	3.27	y = 9799x + 30130	0.9960	70.1	233.3
Quinic acid	$C_7H_{12}O_6$	191.2	13.2	y = 1756x + 27560	0.9870	1.66	5.53	y = 5568x + 24786	0.9960	120.5	401.7
Trans-cinnamic acid	$C_9H_8O_2$	147.1	14.4	y = 1592x + 13498	0.9940	1.83	6.10	y = 4000x + 77666	1.0000	166.7	555.7
Chlorogenic acid	$C_{16}H_{18}O_9$	353.3	14.8	y = 6480x – 42425	0.9880	0.45	1.50	y = 6931x + 34978	0.9870	96.7	322.3
Ferulic acid	$C_{10}H_{10}O_4$	193.2	16.0	y = 3937x + 15590	0.9960	0.74	2.47	y = 10098x + 38317	0.9950	66.6	221.9
p-coumaric acid	$C_9H_8O_3$	163.2	17.0	y = 2338x + 14524	0.9950	1.24	4.13	y = 7164x + 21816	0.9980	93.3	311.0

Table 3.3. Analytical parameters of the method CE-MS related to precision and matrix effect (only included those of *Full Scan*).

Compounds	Intra-day precision[a] (Area/migration time)	Inter-day precision[b] (Area/migration time)	Reproducibility[c] (Area/migration time)	Response factor (in solution)[d]	Response factor (in matrix)[d]
Perseitol	7.12	7.74	9.12	1520	1474
Epicatechin	8.34	8.45	10.09	9901	8654*
Catechin	6.78	7.33	11.45	7765	7223*
Abscisic acid	8.13	8.54	10.98	13245	13567
Pantothenic acid	7.01	7.99	9.78	2889	2657
Quinic acid	8.45	9.12	9.43	1654	1599
***Trans*-cinnamic acid**	7.88	9.01	9.65	1576	1377*
Chlorogenic acid	6.99	8.66	8.99	6555	6300
Ferulic acid	8.24	8.19	10.64	4034	4101
***p*-coumaric acid**	8.53	9.86	11.44	2399	2289

[a] RSD values (%) for peak area/migration time ratio corresponding to some analyte; measured from 5 consecutive injections of each analyte within the same day (repeatability or intra-day precision) by using the standard mix at 50 ppm.

[b] RSD values (%) for peak area/migration time ratio corresponding to some analyte; measured from 10 injections of each on 5 different days (intermediate or inter-day precision) by using the standard mix at 50 ppm.

[c] RSD values (%) for peak area/migration time ratio corresponding to some analyte; measured from 10 injections on 5 different days and by using different capillaries and the standard mix at 50 ppm.

[d] Response factors were calculated (peak area/concentration) to evaluate the possible matrix effect at 3 concentration levels (5, 25 and 50 ppm). Only the intermediate value (25 ppm) of the neat solution and of the spiked avocado extract is showed. Values with asterisk (*) means that there were significant differences between both RFs.

In general, detection and quantification limits were significantly lower when the *MRM* conditions were applied, being 10.2-fold lower than in *Full Scan* (in average). LODs were found within the range 0.22 ppm (abscisic acid) and 1.90 ppm (perseitol) in *Full Scan* and 20.1-203.0 ppb for the same compounds, respectively, in *MRM*. R^2 values were very close to 1 in both cases, being found in the interval 0.9870-0.9990 in *Full Scan* and 0.9870-1.000 in *MRM*.

The precision of the analytical procedure was measured as repeatability (intra- and inter-day) and reproducibility, which were expressed as the relative standard deviation (RSD) in the peak area/migration time. Repeatability (intra- and inter-day) were determined, respectively, by analyzing the mix of 10 metabolites at a concentration of 50 ppm, within the same day (repeatability, n=5) and on five consecutive days (intermediate precision, n=10). The reproducibility study was developed by performing ten separations of the same mix (at 50 ppm) on five different days, under the same conditions, but changing the capillary. As stated in ***Table 3.2***, the results obtained for the precision, in terms of repeatability, were acceptable; being in all the cases within the range 6.78-8.53% for intra-day precision and 7.33-9.86% for inter-day precision, respectively. The reproducibility varied between 9.12 and 11.45%.

When RFs (at 25 ppm) in neat solution and in the presence of matrix were compared, significant differences were observed between both values in 3 compounds, epicatechin, catechin and *trans*-cinnamic acid. At lower concentrations (5 ppm), significant differences were found for the same compounds and pantothenic and chlorogenic acids. Bearing this in mind, it becomes evident the fact that these 5 compounds cannot be properly quantified in *Full Scan*. As expected, when the response factors (in neat solution and in matrix) where calculated in *MRM*, no significant differences were detected at any concentration.

3.3.4. Application of the targeted and non-targeted approach to study the metabolic changes during ripening in avocado cultivar Reed

The two described approaches were used at this point to analyze the 18 avocado samples of cv. Reed trying to achieve several aims:

(i) to quantify the 10 metabolites under study and observe their evolution through the ripening process;

(ii) to compare the results achieved by *MRM* and *Full Scan* (for those compounds not suffering matrix effect);

(iii) and to observe (by using *Full Scan* data) the evolution of other unknown metabolites during ripening, giving a tentative identification for them (with MS/MS and Q-TOF analyses).

Table 3.4 summarizes the quantitative results. For all the compounds, the *MRM* approach was used to carry out the quantification. ANOVA analysis was carried out to establish the statistical differences of the *MRM* data included in the table, checking the differences among the ripening degrees under study. When matrix effect was not detected, the *Full Scan* approach was applied too. For some of the compounds, the quantitative results obtained by HPLC-ESI-IT MS were included to carry out a comparison in terms of quantitative capabilities of CE-MS and HPLC-MS for these metabolites.

Several of the compounds under study could be properly determined by *MRM* and *Full Scan* approaches; this is the case of perseitol, abscisic, quinic, chlorogenic, ferulic and *p*-coumaric acids, being both results in good agreement. When the metabolites could be determined by RP-HPLC-MS too, the LC results were very similar to the electrophoretic ones, but this was only possible for abscisic, pantothenic, ferulic and *p*-coumaric acids. Perseitol and quinic acid, even if they had good intensity in the BPC in LC, they appeared with the column-dead time, undergoing ionic suppression phenomena.

Table 3.4. Quantitative results (mg analyte/kg dry sample) obtained for the avocado samples of Reed variety at different ripening degrees (ripening stages) by means of the developed CE-MS method (when possible, we include *MRM* and *Full Scan* results, as well as HPLC-ESI-IT MS). Every results is the average of three independent determinations (n=3). RSD in all the cases within the range 5-10%.

Compound	RIPENING STAGE									
	1	1b	2	2b	3	3b	4	4b	5	
Perseitol	40.54	42.76	41.87	39.11	34.59	35.91	35.65	35.76	38.22	CE-MS Full
	41.99	43.11	40.55	40.44	35.22	36.22	35.43	35.55	34.50	CE-MS MRM
Epicatechin	14.12	14.87	15.60	15.76	14.04	15.37	11.62	11.77	11.04	CE-MS MRM
Catechin	1.64	1.87	1.66	1.66	1.86	1.75	1.65	1.55	1.51	CE-MS MRM
Abscisic acid	13.41	5.21	5.13	5.32	5.81	6.35	4.26	4.25	4.51	CE-MS Full
	13.63	4.81	5.05	5.29	5.63	6.10	4.04	4.11	4.33	CE-MS MRM
	12.52	4.53	5.16	5.01	5.52	6.25	3.75	3.76	4.51	HPLC-MS
Pantothenic acid	3.52	2.90	3.35	3.29	3.01	3.13	3.03	3.11	2.22	CE-MS MRM
	3.51	2.88	3.19	3.37	3.11	2.91	3.33	3.25	2.53	HPLC-MS
Quinic acid	158.88	162.49	125.65	115.65	118.36	119.55	110.58	110.12	95.76	CE-MS Full
	160.11	164.98	123.80	120.09	117.37	118.97	112.00	111.45	93.08	CE-MS MRM
trans-cinnamic acid	6.01	5.76	5.67	5.54	4.55	4.22	5.33	5.01	6.05	CE-MS MRM
Chlorogenic acid	2.15	2.55	2.56	2.45	2.12	2.43	1.65	1.76	1.61	CE-MS Full
	2.23	2.45	2.62	2.55	2.28	2.58	1.50	1.66	1.48	CE-MS MRM
Ferulic acid	1.45	1.67	1.55	1.73	1.59	1.82	1.13	1.12	0.89	CE-MS Full
	1.50	1.78	1.66	1.81	1.66	1.84	1.21	1.32	0.91	CE-MS MRM
	1.56	1.70	1.55	1.76	1.49	1.77	1.19	1.21	1.01	HPLC-MS
p-coumaric acid	5.21	4.65	4.77	5.02	5.11	4.76	4.12	4.02	4.55	CE-MS Full
	4.75	4.36	4.87	4.87	4.89	4.77	4.22	4.31	4.67	CE-MS MRM
	4.51	4.54	4.87	4.59	5.01	4.44	3.98	3.87	4.65	HPLC-MS

Table 3.4 (continued)

Compound	RIPENING STAGE										
	5	5b	6	6b	7	7b	8	8b	9	9b	
Perseitol	38.22	38.54	39.95	35.78	34.00	33.76	32.12	32.88	32.55	31.83	CE-MS Full
	34.50	33.89	33.78	33.66	33.52	33.93	32.14	31.76	32.41	32.55	CE-MS MRM
Epicatechin	11.04	9.89	8.62	8.32	7.36	6.64	5.46	4.89	3.81	3.43	CE-MS MRM
Catechin	1.51	1.45	1.35	1.21	1.23	1.20	1.09	1.25	1.43	1.13	CE-MS MRM
Abscisic acid	4.51	5.37	4.39	4.10	5.65	4.54	4.04	3.02	3.52	1.91	CE-MS Full
	4.33	5.10	4.61	4.12	5.81	4.83	3.80	3.11	3.43	1.75	CE-MS MRM
	4.51	4.63	4.57	4.25	5.71	4.63	3.81	3.25	3.38	1.62	HPLC-MS
Pantothenic acid	2.22	2.43	2.51	2.48	2.27	2.64	1.95	1.87	1.85	1.59	CE-MS MRM
	2.53	2.53	2.55	2.68	2.37	2.84	2.14	2.12	2.07	1.52	HPLC-MS
Quinic acid	95.76	92.24	88.56	85.67	81.98	82.33	76.65	77.89	70.41	70.52	CE-MS Full
	93.08	91.09	86.54	84.66	80.96	80.01	75.75	77.82	69.86	70.23	CE-MS MRM
trans-cinnamic acid	6.05	5.88	5.87	5.39	3.73	4.01	2.88	2.95	4.01	4.33	CE-MS MRM
Chlorogenic acid	1.61	1.52	1.43	1.73	1.10	1.26	1.09	1.07	1.00	1.17	CE-MS Full
	1.48	1.38	1.65	1.75	1.20	1.33	1.08	1.11	1.14	1.25	CE-MS MRM
Ferulic acid	0.89	0.77	0.88	0.89	1.44	1.79	2.15	2.35	2.76	2.88	CE-MS Full
	0.91	0.95	0.84	0.99	1.58	1.86	2.18	2.40	2.87	2.97	CE-MS MRM
	1.01	0.93	0.91	1.05	1.47	1.78	2.33	2.76	2.81	3.15	HPLC-MS
p-coumaric acid	4.55	4.99	7.55	5.48	9.65	13.79	13.65	13.11	15.45	16.66	CE-MS Full
	4.67	4.78	7.44	5.65	8.99	13.92	13.55	13.44	15.87	16.22	CE-MS MRM
	4.65	4.87	8.22	5.11	9.42	14.12	13.21	12.44	14.75	15.66	HPLC-MS

During ripening different metabolic processes take place, which require some energy, which in general, is supplied by the oxidation of carbohydrates and organic acids oxidation, which turn into CO_2 and water. This could explain the decrease of the concentration of perseitol meanwhile the avocado fruit is growing and ripening [26], varying from 41.99 to 32.55 mg/kg (*MRM*) in the samples 1 and 9b, respectively. Likely, as a consequence of the decreasing of the sugar content as the harvest date draws on, the fruits harvested at the beginning of the season need longer time to reach the climacteric point than the avocado fruits harvested later in the season. The avocado fruit may need a continuous supply of sugars and some other compounds from the leaves, and a reduction of this supply could begin the ripening process; harvest of the fruits could have the same effect inducing ripening. Moreover, according to Cowan [27] and Tesfay [28], one of the functions of C7-sugars is the protection of some key enzymes for the growth and development of the fruit and for avoiding damage caused by reactive oxygen species.

A decrease of the concentration of quinic, chlorogenic and *trans*-cinnamic acids (compounds involved in oxidative processes) was observed as well; similar behavior for organic acids has been observed in other climacteric fruits [29]. Quinic acid was one of the compounds found in higher concentration (160.11-70.23 ppm in *MRM*). When the avocado extracts were analyzed by HPLC-MS, although it was not possible to quantify it, it was observed that there were 2 isomers with *m/z* 191.055 and $C_7H_{12}O_6$ as molecular formula. In CE-MS, we probably determined both together. Chlorogenic acid was found in concentrations ranging from 2.62 to 1.08 mg/kg, being data from *MRM* and *Full Scan* very similar (matrix effect was observed for this compound in *Full Scan* at concentrations lower than 5 ppm (in solution)). In HPLC-MS, this compound had low intensity (in comparison with the BPC) and could not be properly quantified because of matrix effect. *Trans*-cinnamic acid was only quantified by CE-MS in *MRM*, undergoing the same problem as chlorogenic acid. On the contrary, an increase in the concentration of ferulic and *p*-coumaric acids was observed during ripening. According to several authors, both compounds are related to browning reactions of fruits and similar trends have been observed in other fruits during ripening [30, 31]. Hurtado-Fernández et al. [32] determined 17 phenolic acids in 13 avocado varieties (at two different ripening degrees), observing as a general behavior that the phenolic acid concentration (of compounds belonging to hydroxycinnamic acids) increase with the ripening process, showing the

hydroxybenzoic acids the opposite trend. In the current case, although the concentration of *trans*-cinnamic acid did not increase during ripening (being found between 6.05 and 2.88 mg/kg at different stages of the season), the decreasing trend was not very marked. Ferulic and *p*-coumaric acids could be quantified by the two CE approaches as well as by HPLC-MS, producing in all the cases very similar values. Decreasing of flavonoids during ripening process has been observed in other fruits [33, 34]. The drop in epicatechin concentration during ripening in avocado fruit, fact that is observed in this study, had been previously described [35]. This information is complemented in this work, since catechin has also been determined with a concentration that ranged from 1.87 to 1.09 mg/kg. The quantification of these two compounds resulted to be particularly complicated, being carried out reliability only by CE-MS in *MRM* mode. The plant hormone abscisic acid (ABA) is the major player in mediating the adaptation of the plants to stress; in this case its concentration decreased during ripening. Similar results were found when some other fruits were evaluated [36, 37]. This phytohormone was determined by CE-MS and LC-MS, with a good agreement of the results obtained by both methods. Its concentration ranged approximately from 13 to 2 mg/kg. When the first batch of samples was analyzed (samples 1 and 1b), a significant difference in ABA concentration (13.63 mg/kg for sample 1 and 4.81 mg/kg for sample 1b) was found. In the rest of batches, the results for samples belonging to the same harvest date were very similar. Vitamin B_5 or pantothenic acid decreased in content during ripening, fluctuating within the range 3.52-1.59 mg/kg (in *MRM*); this compound was also successfully quantified by HPLC-MS.

To complete the work, we proceeded to investigate the CE-MS data obtained in *Full Scan* to check whether there was any other compound whose concentration changed significantly during ripening. The comparison between sample 1 and 9b can be observed in ***Figure 3.6***.

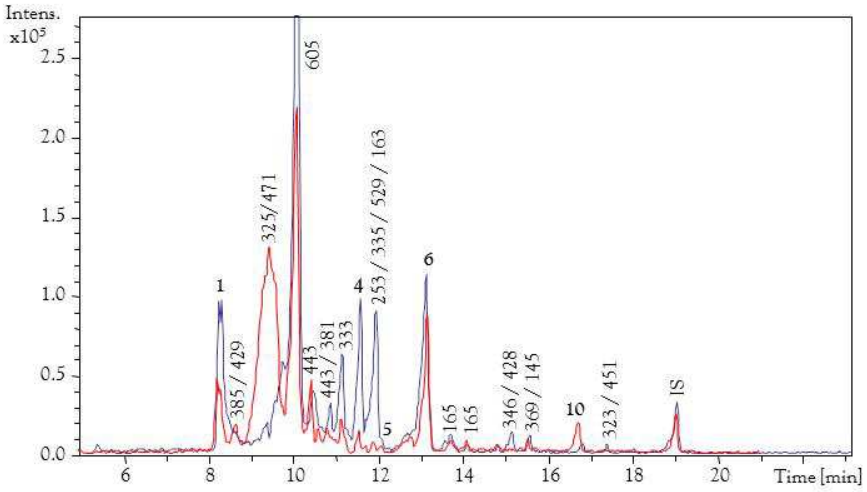


Figure 3.6. BPEs in negative polarity of sample 1 (blue line) and sample 9b (red line). Peak identity: see ***Figure 3.4.*** To those compounds not identified with standards, the *m/z* signal was written on top of the peak.

Taking advantage of the capabilities of ion trap, MS/MS experiments were performed to get as much information as possible regarding the compounds not identified in the first stage of this study (see ***Table 3.1. Supporting information***).

Compounds with *m/z* 325, 471 and 605, in the negative ion mode, resulted to be particularly interesting, firstly because they had the highest intensities in the electrophoretic profiles and, secondly, because their concentration fluctuated markedly during the season. The metabolite with *m/z* 605 is one of the predominant peaks in the profile, both at initial stages of ripening and at the end of the harvesting season. However, the metabolite with *m/z* 471 was at low concentration in sample 1 and its presence increased very sharply. This compound and the one with *m/z* 325 have several isomers, showing more than one peak when their EIEs are drawn.

To get a tentative identification of the peaks included in ***Figure 3.1. Supporting information***, LC-ESI-Q-TOF MS experiments were carried out. The compound with *m/z* 605 could not be properly detected in LC-MS, even if different MS conditions were assayed, showing the complementarity of both separation techniques when studying the metabolome of avocado. Therefore, we focused in the identification of the peaks with *m/z* 325 and 471.

3.3.5. HPLC-ESI -Q TOF MS analyses to identify two metabolites of interest

Table 3.5 contains information about the compounds with *m/z* 325.0929 and 471.1511, including MS signal detected, tolerance to predict molecular formula in TOF, molecular formula, error (ppm), mSigma value (which gives an idea about the reliability of the predicted molecular formula), possible candidates and MS/MS signals (obtained with Q-TOF MS).

The predicted identity for the two compounds included in ***Table 3.5*** was coumaric acid-hexose (*m/z* 325.0929) and rhamnosyl hexose of coumaric acid (*m/z* 471.1508), respectively. The MS/MS spectra (signals included in Table 4) showed the similarity of both structures (both are related with coumaric acid), since some fragments are exactly the same. The concentration of rhamnosyl hexose of coumaric acid increased markedly during the season, whilst coumaric acid-hexose levels kept quite stable during ripening.

Table 3.5. Information obtained by Q-TOF MS (in MS and MS/MS mode) about the compounds with *m/z* 325.0929 and 471.1511.

***m/z* experimental**	**Signal**	**Tolerance (ppm)**	**Molecular formula**	***m/z* theoretical**	**Error (ppm)**	**mSigma value**	**Possible candidate**	**MS/MS signal**
325.0930	$[M-H]^-$	20	$C_{15}H_{18}O_8$	325.0929	- 0.2	2.2	coumaric acid-hexose	145.0317 [325-180 (hexose)] 163.0420 [325-162 (rhamnose)] 117.0359 [325-208] 145.0316 [471-326 (coumaric acid-hexose)]
471.1511	$[M-H]^-$	25	$C_{21}H_{28}O_{12}$	471.1508	- 1.6	3.4	rhamnosyl hexose of coumaric acid	163.0420 [471-308]=[rhamnose] 205.0595 [471-266] 117.0362 [471-354] 187.0408

Table includes MS signal detected (experimental and theoretical m/z), tolerance to predict molecular formula in TOF, molecular formula, error (ppm), mSigma value, possible candidates and MS/MS signals. Databases checked: Phenol Explorer, PubChem, CehmSpider, KNApSAcK, METLIN.

3.4. Conclusions

The quality of a fruit is a combination of characteristics, attributes, and properties that have significance for product acceptability by the consumer. Therefore, it is quite important to determine fruit composition, not only to assure quality and determine the decision of the customer to repurchase the product, but also because nowadays consumers are increasingly concerned about the composition of what they eat. In this study, CE-MS, by using two different MS approaches (targeted and non-targeted), has demonstrated to be a powerful technique for the profiling of polar metabolites in complex samples such as avocado fruit, determining the quantitative evolution of 10 metabolites belonging to different chemical families and identifying two phenolic acid-related sugars, which change their levels during ripening. A comparison of the quantitative results of *MRM* and *Full Scan* approaches was carried out, being both in good agreement when no matrix effect was detected in *Full Scan*. HPLC-MS was also used in the current study, firstly to quantify the metabolites under study and compare its results with those of CE-MS and then, coupled to Q-TOF MS, to get a tentative identification of two interesting metabolites.

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Supporting information

Table 3.1. Supporting information. Other compounds detected in *Full Scan* (apart from those identified with standards).

Migration time	NEGATIVE POLARITY			POSITIVE POLARITY
	m/z	Other signals in MS	MS/MS *	*m/z* ♦
9.0-10.5	325	134/ 385	145/ 163/ 187/ 265/ 119/ 295	
9.0-10.0	471	457	163/ 187/ 351/ 411	490/ 511/ 449/ 406/ 311
10.0	605	533/ 647/ 687	573/ 415/ 283/ 265/ 543	689/ 625/ 312/ 265
10.5	443	503/ 325	381/ 232/ 267	
11.0	333		153/ 241/ 271	
11.9	253	335/ 529/ 163	163/ 133/ 101/ 149/ 193/ 235/ 172	277/ 293
13.8	165	109/ 315		280/ 350/ 249/ 185
14.1	165	115/ 149		
15.2	346	428		
15.5	369	145		
17.4	323	451/ 691		

* When the intensity was high enough, MS/MS analyses were carried out. When a particular compound had low intensity, we used a *MRM* approach specific for it. If no fragments are included means that intensity was too low for getting MS/MS spectra with good quality.

♦ The *m/z* signal of some compounds is not included in positive polarity. These compounds were poorly ionized in positive mode, being the MS spectra in this polarity of low quality when the concentration of the compound was not high enough.

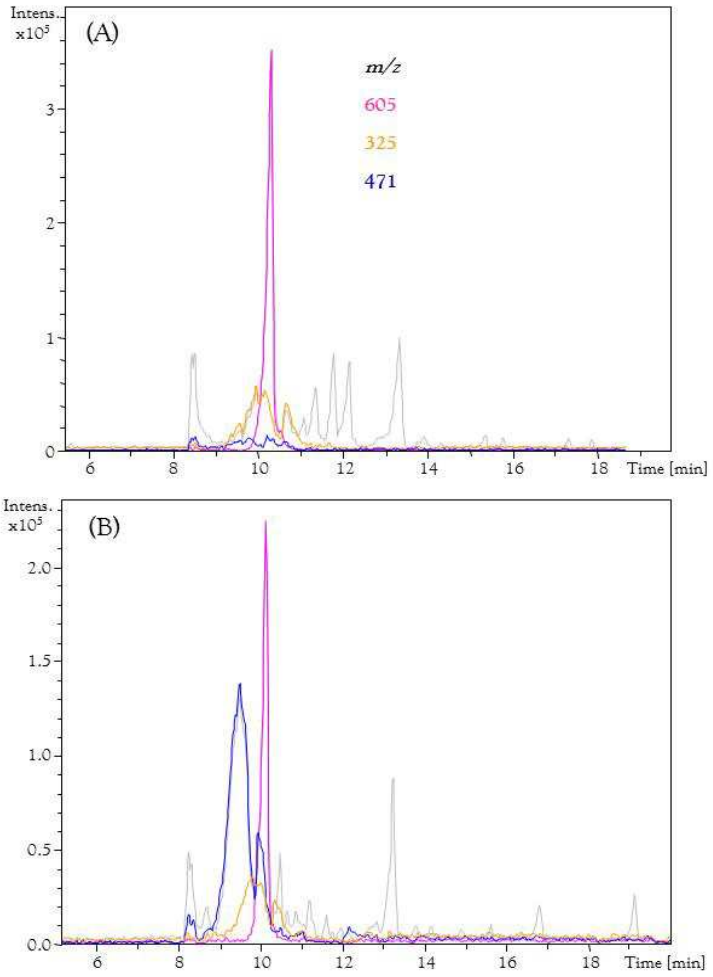

Figure 3.1. Supporting information. BPE in negative polarity of sample 1 (A) and sample 9b (B) in grey, together with the EIEs of signals *m/z* 605, 325 and 471.

Section II: LIQUID CHROMATOGRAPHY

LC is probably one of the most used analytical separation techniques for plant metabolic profiling. In 1906, Tswett developed the first application of LC. Since that date, the technique has experienced numerous changes and progresses, and the classical LC has been transformed into a modern one, emerging on the 60s HPLC, sometimes also named high pressure liquid chromatography. It is characterized by a high resolution and appropriate separation times, as a consequence of the use of packed columns with small particle sizes and pumping systems that maintain a constant flow. HPLC has become a routine technique in laboratories of analysis, because it is robust, reproducible and sensitive, and it can be reasonably fast. However, nowadays the use of UHPLC is gaining interest over conventional HPLC, primarily due to the utilization of stationary phases with smaller particle size (>2 μm) in combination with ultrahigh-pressure pumps –which overcome the high backpressure associated with such columns– helps to produce narrower peaks, rapid analysis times and lower detection limits, besides the solvent volume consumed is lower, decreasing the volumes of waste.

This section encloses three different works where LC has been used as separation technique coupled to different detection systems, prevailing in all the cases the use of MS.

In the first chapter of this part, ***Chapter 4***, a simple method for LC-DAD-ESI-TOF MS is described to carry out the characterization of three different avocado varieties (Hass, Lamb Hass and Rugoro) in terms of phenolic compounds, including both phenolic acids and flavonoids. The choice of this family of metabolites has been based on the fact that the phenolic fraction of avocado fruit has not been deeply studied, finding only a few publications in literature about that. Thus, the main motivation developing this methodology has been to enlarge the knowledge about the phenolic composition of avocado fruit. To assure the achievement of the best results, the method has been fully optimized and evaluated by using a standard mixture that contains all those phenolic compounds that can be potentially found in a complex matrix such as avocado. After this step, the method has been applied for the avocado extract analysis, accomplishing the identification of an important number of phenols.

Taking into account the valuable information obtained in chapter 4 and being aware about the richness and complexity that avocado profiles present, it has been decided to make use of a very potent analytical tool that allow carrying out a global metabolic

profiling study of avocado fruit. Thus, ***Chapter 5*** describes the optimization process of an UHPLC-UV/ESI-TOF MS method for the characterization of 13 different avocado samples at two ripening degrees, which has been validated considering diverse analytical parameters such as linearity, precision, accuracy, etc. It has been possible to identify tentatively around 200 metabolites from diverse structural classes; the identity of 60 of them was unequivocally confirmed, being 20 compounds properly quantified. In addition, the utilization of several statistic tools has provided significant information about the process of ripeness and the differentiation among samples.

Considering the promising results achieved by using the aforementioned approach, a method for HPLC-DAD/FL/ESI-IT MS has been developed to perform another global metabolic study of avocado fruit, which is summarized in ***Chapter 6***. In this occasion, the comprehensive identification and quantification of the complete fruit metabolome is not intended; we are looking for a comprehensive metabolic profiling of avocado fruit that can give us, in a very simple way, worthy information about the evolution of 4 avocado varieties collected at different time points over their harvest season. All the samples has been analyzed by HPLC-DAD/FL/ESI-IT MS, and the obtained data has been subsequently treated with chemometrics tools to evaluate those changes produced in avocado composition depending on fruit harvesting date. The information achieved by this interesting approach can offer a real alternative to farmers for an existing problem, because the specific and adequate avocado picking time tend to be established according to the oil content and dry matter in the avocado flesh. Besides, nowadays there are numerous evidences about the "positive" influence that some of the avocado compounds have on human health. Thus, being aware about how the ripening process affects the metabolites, it is possible to know the optimum time to harvest the fruits for getting the maximum benefits.

Chapter 4

Profiling LC-DAD-ESI-TOF MS method for the determination of phenolic metabolites from avocado (*Persea americana*)

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Abstract

A powerful HPLC-DAD-ESI-TOF MS method was established for the efficient identification of the chemical constituents in the methanolic extracts of avocado (*Persea americana*). Separation and detection conditions were optimized by using a standard mix containing 39 compounds belonging to phenolic acids and different categories of flavonoids; analytes which could be potentially present in the avocado extracts.

Optimum LC separation was achieved on a Zorbax Eclipse Plus C18 analytical column (4.6 x 150 mm, 1.8 μm particle size) by gradient elution with water + acetic acid (0.5%) and ACN as mobile phases, at a flow rate of 1.6 mL/min. The detection was carried out by ultraviolet-visible absorption and ESI-TOF MS.

The developed method was applied to the study of three different varieties of avocado, and 17 compounds were unequivocally identified with standards. Moreover, around 25 analytes were tentatively identified taking into account the accuracy and isotopic information provided by TOF MS.

Keywords: Liquid chromatography / Time of flight-mass spectrometry / Phenolic compounds / Avocado / *Persea americana* / Secondary metabolites

4.1. Introduction

Phenolic compounds are a wide group of secondary metabolites which can be found in plants, such as simple phenols, phenolic acids, flavonoids, coumarins, tannins, stilbenes, lignans, lignins, etc. [1]. These compounds can perform several functions in plants, such as protective agents against UV light, taking part in fertilization attracting pollinators, being components of pigments, essences, flavors, etc. In food and beverages, phenolic compounds may contribute to the oxidative stability and organoleptic characteristics (bitterness, flavor, astringency…) [2-4]. Numerous investigations have shown that, in humans, phenols exhibit potentially positive effects like anti-microbial [5], cardioprotectives, anti-allergenics and anti-inflamatories activities [6, 7], among others. These beneficial effects of phenols have been traditionally related to their strong antioxidant activity, that is, their ability to scavenge oxygen radicals and other reactive species [8, 9].

Important source of phenolic compounds are the fruits and vegetables produced in the Mediterranean basin. In the current study, we focused on avocado fruit (*Persea americana*) because it is an important crop of the tropical coast of Granada (Spain) and it is increasingly valued by consumers, not only for its unique flavor and texture, but also for its reported health benefits [10-12]. Avocado is an evergreen tree [13] native of Colombia, Mexico and Venezuela. There are many varieties (e.g. Fuerte, Hass, Pinkerton, Bacon, Azteca, Ettinger or Rincon) and all of them show common characteristics: oval form, green pale pulp, only one and big seed, rough or smooth surface, etc.

The qualitative and quantitative determination of these phenolic compounds in fruits and vegetables is therefore very important and several methods have been already described in literature [1, 2, 14, 15]. Traditionally, spectrophotometric methods and TLC were used, but later on, the need to separate and identify phenols individually caused the replacement of traditionally methods by other techniques, such as GC and HPLC coupled to different detectors. Furthermore, CE has been recently applied to the analysis of phenolic compounds from fruits and vegetables [16].

The phenolic fraction from avocado has not been studied in depth so far; there are several very interesting reports where the reader can find some information about the composition of avocado concerning phenolic compounds [11, 17-21], but there is not a detailed description about the pseudo-polar fraction of this important fruit. In some of the mentioned papers, the authors studied the phenolic composition of different fruits, vegetables or plant food material in general, and they determined just few compounds in avocado samples. Torres et al. [17] were pioneers describing the phenolic acids which can be found in avocado fruit. Ding et al. [11], however, in a very complete article, established a possible connection between some phytochemicals present in avocado and its chemopreventive characteristics.

The aim of this work was to develop a powerful HPLC-DAD-ESI-TOF MS method for the characterization of phenolic compounds from avocado fruit. The potential of this separation technique coupled to DAD and TOF detectors was evaluated by using a standard mix containing 39 compounds belonging to different families of phenols. After optimizing the separation and detection conditions, the optimum method was applied to the analysis of three varieties of avocado fruit ('Hass', 'Lamb-Hass' and 'Rugoro')

achieving the identification of an important number of compounds in the analyzed extracts.

4.2. Materials and methods

4.2.1. Chemicals and standards

The solvents used for the sample extraction were MeOH and EtOH, which were purchased from Panreac (Barcelona, Spain); and AcOH and EtOAc, which were obtained from Lab-Scan (Gliwice, Sowinskiega, Poland). For preparing the mobile phases, we used ACN and acetic acid, and they were purchased from Lab-Scan (Dublin, Ireland) and Panreac (Barcelona, Spain), respectively. All the solvents used were HPLC grade and they were used as received. Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q system (Millipore, Bedford, MA, USA).

Standards of phenolic compounds (vanillin, vanillic acid, homovanillic acid, ferulic acid, 4-hydroxybenzoic acid, benzoic acid, *trans*-cinnamic acid, syringic acid, caffeic acid, gentisic acid, *p*-coumaric acid, protocatechuic acid, sinapinic acid, gallic acid, ellagic acid, chlorogenic acid, 3-hydroxycinnamic acid, taxifolin, quercetin, luteolin, kaempferol, naringenin, apigenin, myricetin, laricitrin, galangin, chrysin, pinocembrin, pinocembrin-7-methyleter, poncirin, naringin, kaempferide, quercetin-3-O-glucose-6´´-acetate, isorhamnetin, catechin, narirutin, rutin, epicatechin, neohesperidin, myrtillin, kuromanin, cyanin, cyanidin-3-O-β-glucopyranoside, cyanidin-3-O-β-galactopyranoside, syringetin, cyanidin-3-O-2´´-O-β-xylopyranosyl-β-glucopyranoside, delphinidin-3-O-2´´-O-β-xylopyranosyl-β-glucopyranoside, delphinidin-3-O-β-glucopyranoside and delphinidin-3-O-6´´-O-α-ramnopyranosyl- β-glucopyranoside) were mostly purchased from Extrasynthese (Lyon, France), Sigma-Aldrich (St. Louis, EEUU) and Fluka (St. Louis, EEUU). All of them were used during the preliminary studies.

Taking into account the previously published literature about the determination of phenols from avocado in particular, or fruit, in general [17, 22-26], the availability of standards, and our preliminary studies (checking the phenols which were present in every avocado variety we had) we tried to prepare a mixture to carry out the method optimization. The idea was to create a representative mix which could be useful regardless the avocado variety under study. Moreover, the standard mix was created to cover a wide

range of polarities and molecular weights mimicking as close as possible what we could find in avocado samples.

Finally, this mixture was made with 39 phenolic compounds belonging to phenolic acids or related compounds, flavones, flavanones, flavonols, dihydrochalcones and dihydroflavonols (see ***Table 4.1***). We put the same amount of each standard and we added the necessary volume of methanol to have stock solutions containing 100 ppm of each compound.

All the stock solutions, samples, solvents and reagents were filtered with a 5 µm membrane filter (Millipore, Bedford, MA, EEUU) before separation or injection in the instrument.

Table 4.1. MS signal observed for the 39 compounds included in the standard mixture in negative and positive ion polarity.

	t_r (min)	Compound	Formula	Selected ion	m/z experimental	m/z theoretical	Error (ppm)	mSigma Value	Fragments in source
♣	3.4	*Gallic acid*	$C_7H_6O_5$	$[M-H]^-$	169.0147	169.0142	- 2.6	9.5	125.0270
				$[M+H]^+$	171.0286	171.0288	1.1	5.1	–
♣	5.2	*Protocatechuic acid*	$C_7H_6O_4$	$[M-H]^-$	153.0191	153.0193	1.8	7.7	109.0262
				$[M+H]^+$	155.0337	155.0339	1.3	7.1	137.0238
♣	6.4	*Gentisic acid*	$C_7H_6O_4$	$[M-H]^-$	153.0187	153.0193	4.5	7.2	109.0259
				$[M+H]^+$	155.0341	155.0339	- 1.3	4.1	–
♣	6.9	*4-hydroxybenzoic acid*	$C_7H_6O_3$	$[M-H]^-$	137.0245	137.0244	- 0.7	5.6	93.0274
				$[M+H]^+$	139.0386	139.0389	2.1	6.1	121.0305
♣	7.5	*Chlorogenic acid*	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0886	353.0878	- 2.4	7.1	191.0560
				$[M+H]^+$	355.1021	355.1024	0.8	24.9	163.0380/218.9902/291.0845
▲	7.6	*Catechin*	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0712	289.0718	2.2	10.2	245.0818
				$[M+H]^+$	291.0863	291.0863	0.0	11.0	123.0447/139.0387/163.0385
♣	8.1	*Vanillic acid*	$C_8H_8O_4$	$[M-H]^-$	167.0347	167.0350	1.6	4.8	–
				$[M+H]^+$	169.0498	169.0495	- 1.8	5.1	151.0385
♣	8.3	*Caffeic acid*	$C_9H_8O_4$	$[M-H]^-$	179.0355	179.0350	- 2.9	9.3	135.0440
				$[M+H]^+$	181.0493	181.0495	1.5	33.4	135.0445/163.0384
♣	8.9	*Syringic acid*	$C_9H_{10}O_5$	$[M-H]^-$	197.0447	197.0455	4.2	4.2	–
				$[M+H]^+$	199.0611	199.0601	- 5.0	7.1	–
♣	8.9	*Homovanillic acid*	$C_9H_{10}O_4$	$[M-H]^-$	181.0504	181.0506	1.0	8.9	137.0618/122.0372
				$[M+H]^+$	183.0654	183.0652	- 1.1	5.9	137.0619
▲	9.2	*Epicatechin*	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0715	289.0718	0.9	1.9	245.0818
				$[M+H]^+$	291.0866	291.0863	1.1	12.6	123.0449/139.0387
♣	10.0	*Vanillin*	$C_8H_8O_3$	$[M-H]^-$	151.0396	151.0401	3.0	3.1	136.0158
				$[M+H]^+$	153.0536	153.0546	6.6	51.5	137.0252
♣	10.3	*p-coumaric acid*	$C_9H_8O_3$	$[M-H]^-$	163.0396	163.0401	3.1	3.6	119.0479
				$[M+H]^+$	165.0544	165.0546	1.2	4.8	147.0436/119.0492

Table 4.1 (continued)

	t_r (min)	Compound	Formula	Selected ion	m/z experimental	m/z theoretical	Error (ppm)	mSigma Value	Fragments in source
♣	11.5	*Ferulic acid*	$C_{10}H_{10}O_4$	$[M-H]^-$	193.0506	193.0506	0.1	6.2	178.0273/134.0361
				$[M+H]^+$	195.0652	195.0652	0.1	7.6	177.0541/149.0593
▬	11.6	*Rutin*	$C_{27}H_{30}O_{16}$	$[M-H]^-$	609.1470	609.1461	- 1.5	2.1	–
				$[M+H]^+$	611.1606	611.1606	0.1	52.5	303.0499
♣	11.7	*Sinapinic acid*	$C_{11}H_{12}O_5$	$[M-H]^-$	223.0620	223.0612	- 3.4	4.8	193.0147/208.0381
				$[M+H]^+$	225.0759	225.0758	- 4.5	6.5	207.0652
♣	11.7	*Ellagic acid*	$C_{14}H_6O_8$	$[M-H]^-$	301.0005	300.9990	- 5.1	3.8	–
				$[M+H]^+$	303.0135	303.0135	- 0.1	2.9	–
♣	11.8	*3-hydroxycinnamic acid*	$C_9H_8O_3$	$[M-H]^-$	163.0402	163.0401	- 0.9	6.1	119.0479
				$[M+H]^+$	165.0545	165.0546	0.1	6.1	–
▪	11.9	*Taxifolin*	$C_{15}H_{12}O_7$	$[M-H]^-$	303.0510	303.0510	0.2	5.1	607.1061
				$[M+H]^+$	305.0640	305.0656	3.2	71.4	231.0647/259.0598
♣	12.5	*Benzoic acid*	$C_7H_6O_2$	$[M-H]^-$	121.0292	121.0295	2.4	11.8	96.0593
				$[M+H]^+$	123.0444	123.0441	- 2.5	9.1	98.9751/105.0397
♦	12.8	*Narirutin*	$C_{27}H_{32}O_{14}$	$[M-H]^-$	579.1712	579.1719	1.3	6.0	1159.3604
				$[M+H]^+$	581.1881	581.1865	- 1.6	36.9	273.0739
♦	13.3	*Naringin*	$C_{27}H_{32}O_{14}$	$[M-H]^-$	579.1737	579.1719	- 3.1	9.0	1159.3604
				$[M+H]^+$	581.1871	581.1865	- 1.0	64.8	273.0744
▬	13.5	*Quercetin-3-O-glucose-6´´-acetate*	$C_{23}H_{22}O_{13}$	$[M-H]^-$	505.0986	505.0988	0.4	5.1	–
				$[M+H]^+$	507.1133	507.1133	0.0	36.0	303.0499
▬	14.1	*Myricetin*	$C_{15}H_{10}O_8$	$[M-H]^-$	317.0302	317.0303	0.4	3.6	–
				$[M+H]^+$	319.0444	319.0448	1.3	4.2	273.0668
●	14.2	*Neohesperidin*	$C_{28}H_{34}O_{15}$	$[M-H]^-$	609.1817	609.1825	1.3	10.2	–
				$[M+H]^+$	611.1973	611.1970	- 0.4	54.7	–
▬	16.8	*Kaempferol*	$C_{15}H_{10}O_6$	$[M-H]^-$	285.0400	285.0405	1.6	1.2	–
				$[M+H]^+$	287.0550	287.0550	- 0.1	9.1	–

Table 4.1 (continued)

	t_r (min)	Compound	Formula	Selected ion	m/z experimental	m/z theoretical	Error (ppm)	mSigma Value	Fragments in source
▬	16.8	*Quercetin*	$C_{15}H_{10}O_7$	$[M-H]^-$	301.0348	301.0354	2.0	9.2	–
				$[M+H]^+$	303.0495	303.0499	- 0.4	17.7	–
♣	16.9	*trans-cinnamic acid*	$C_9H_8O_2$	$[M-H]^-$	147.0440	147.0452	2.7	3.1	–
				$[M+H]^+$	149.0596	149.0597	0.1	5.8	103.0543
	17.1	*Laricitrin*	$C_{16}H_{12}O_8$	$[M-H]^-$	331.0468	331.0459	- 2.6	11.6	–
				$[M+H]^+$	333.0608	333.0605	- 0.9	18.2	–
♦	17.2	*Poncirin*	$C_{28}H_{34}O_{14}$	$[M-H]^-$	593.1902	593.1876	- 4.4	8.1	–
				$[M+H]^+$	595.2026	595.2021	- 0.7	38.6	–
♦	18.7	*Naringenin*	$C_{15}H_{12}O_5$	$[M-H]^-$	271.0616	271.0612	1.6	3.1	–
				$[M+H]^+$	273.0753	273.0758	1.7	18.0	–
	18.9	*Apigenin*	$C_{15}H_{10}O_5$	$[M-H]^-$	269.0458	269.0455	- 0.8	6.9	–
				$[M+H]^+$	271.0596	271.0601	2.0	12.9	–
	19.3	*Luteolin*	$C_{15}H_{10}O_6$	$[M-H]^-$	285.0401	285.0405	1.3	2.3	–
				$[M+H]^+$	287.0552	287.0550	- 0.5	6.8	–
▬	19.9	*Isorhamnetin*	$C_{16}H_{12}O_7$	$[M-H]^-$	315.0512	315.0510	- 0.5	3.3	300.0277
				$[M+H]^+$	317.0657	317.0656	- 0.3	11.6	287.0551
	24.2	*Chrysin*	$C_{15}H_{10}O_4$	$[M-H]^-$	253.0508	253.0506	- 0.8	1.8	–
				$[M+H]^+$	255.0654	255.0652	- 0.9	2.5	–
♦	24.7	*Pinocembrin*	$C_{15}H_{12}O_4$	$[M-H]^-$	255.0675	255.0663	- 4.8	6.3	–
				$[M+H]^+$	257.0798	257.0808	4.1	96.2	–
	25.0	*Galangin*	$C_{15}H_{10}O_5$	$[M-H]^-$	269.0445	269.0455	3.8	4.9	–
				$[M+H]^+$	271.0601	271.0601	- 0.1	14.4	–
▬	25.3	*Kaempferide*	$C_{16}H_{12}O_6$	$[M-H]^-$	299.0556	299.0561	1.5	2.3	284.0310
				$[M+H]^+$	301.0707	301.0707	0.1	4.5	–
♦	30.9	*Pinocembrin-7-methyleter*	$C_{16}H_{14}O_4$	$[M-H]^-$	269.0816	269.0819	1.2	4.1	–
				$[M+H]^+$	271.0965	271.0965	0.1	4.9	167.0343

♣ Phenolic acids and related compounds. ▬ Flavonol. ♦ Flavanone. Flavone. ▲ Flavanol. ▪ Dihydroflavonol. ● Dihydrochalcone.

4.2.2. Avocado samples used in this study

We used three varieties of avocado: 'Hass', 'Lamb Hass' and 'Rugoro'. For each variety, we used the pulp of 3-4 pieces of fruit, which were frozen to be further freeze-dried. Many studies advise starting from samples freeze-dried, because it is easier their subsequent conservation [19, 22, 27].

4.2.3. Extraction of phenolic compounds from avocado

The preliminary studies led us to use AcOH, MeOH, EtOH and EtOAc, as the most proper solvents for the extraction of the secondary metabolites under study from avocado samples.

We prepared 4 extracts starting with 4 g of the freeze-dried (and homogenized) sample and using 40 mL of pure MeOH, EtOH, AcOH and EtOAc. We put the sample and the solvent inside a falcon tube and they were shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 4500 rpm for 10 min. After that, the supernatants were evaporated to dryness and redissolved in 1 mL of MeOH. Finally, the extracts were pre-concentrated to 100 µL of MeOH

Our aim was to determinate which one of the 4 selected solvents was able to extract more compounds in a higher concentration, using the same amount of sample and the same volume of solvent. The three varieties of avocado under studied were extracted by using the different solvents.

4.2.4. HPLC analysis

An Agilent 1200-RRLC 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. Phenolic compounds were separated by using a reverse phase C_{18} analytical column (ZORBAX Eclipse Plus, 4.6x150 mm, 1.8 µm particle size). Mobile phases A and B consist of water with 0.5% acetic acid, and ACN, respectively. Several gradients (A, B, C and D) were used in order to achieve the best separation among the 39 compounds belonging to our standard mix; the gradients changed according to the conditions described in ***Table 4.2***.

The flow rate used was 1.60 mL/min. The room temperature was kept at 20ºC. A volume of 10 µL of the avocado extracts or stock standard solutions was injected. The compounds separated were monitored in sequence, first with DAD over the range of 190-600 nm to achieve spectral data, and then with ESI-TOF MS detector. Peak identification was done bearing in mind migration time, spectral data and ESI-TOF MS information obtained from real samples and standards, and also with spiked real samples at different concentration levels.

Tabla 4.2. Different gradients used during the optimization of the chromatographic method.

Method	Minute	Gradient	
		Phase A	Phase B
(A)	0	99%	1%
	55	0%	100%
	57.5	99%	1%
	60	99%	1%
(B)	0	99%	1%
	55	40%	60%
	57	0%	100%
	60	99%	1%
(C)	0	99%	1%
	20	90%	10%
	55	40%	60%
	57	0%	100%
	60	99%	1%
(D)	0	99%	1%
	20	90%	10%
	50	60%	40%
	55	40%	60%
	57	0%	100%
	60	99%	1%

4.2.5. Mass spectrometry

The HPLC system was coupled to a MicroTOF (Bruker Daltonik, Bremen, Germany) using an orthogonal ESI (model G1607A from Agilent Technologies, Palo Alto, CA, USA). TOF analyzer provides greatly improved mass resolution (10000–15000 at 300 *m/z*) and significantly higher sensitivity and accuracy when acquiring full-fragment spectra compared with traditional instruments.

As mentioned before, the TOF mass spectrometer was equipped with an ESI operating in negative and positive ion polarity (sequential analyses) using a capillary

voltage of ±4.5 kV. The flow rate used in the chromatographic method, 1.6 mL/min, was too high for achieving stable ESI (maximum flow rate is around 1 mL/min), therefore it was necessary to use a flow divisor 1:6. In that way, the flow delivered into the mass spectrometer was reduced to 0.3 mL/min, low enough to avoid the introduction of humidity in the system. The other optimum values of the ESI-MS parameters, according to this flow, were: drying gas temperature, 190ºC; drying gas flow, 9 L/min; and nebulizer gas pressure, 2 bar.

The polarity of ESI and all of the parameters of MS detector were optimized using the height of the MS signal for the 39 phenolic compounds included in our standard mix as analytical parameter.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ Editor tool. The SmartFormula™ Editor uses a CHNO 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 theorical with the measured isotope pattern (Sigma Value™) for increased confidence in the suggested molecular formula. The smaller the sigma value and the error the better the fit, therefore for routine screening an error of 5 ppm and a threshold sigma value of 0.05 are generally considered appropriate. Even with very high mass accuracy (<1 ppm), many chemically possible formulas are obtained depending on the mass regions considered. So, high mass accuracy (<1 ppm) alone is not enough to exclude enough candidates with complex elemental compositions. The use of isotopic abundance patterns removes >95% of false candidates.

External calibration was performed using sodium formate cluster by switching the sheath liquid to a solution containing 5 mM NaOH in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior carrying out the phenol identification.

4.3. Results and discussion

4.3.1. HPLC-ESI-TOF MS method optimization

The solution containing the 39 phenolic compounds included in ***Table 4.1*** was used to optimize both the chromatographic and MS conditions. Initially, the chromatographic conditions were optimized according to the following criteria: chromatographic behavior (retention time which depends on the polarity of the compounds under study), sensitivity, analysis time and peak shape.

The quality of any chromatographic separation depends on the characteristics of the compounds to be separated and their interactions with the column and the solvent. A profiling method for phenolic compounds requires uniform separation across the range of polarities; from the most polar (hydroxybenzoic and hydroxycinnamic acids) to the least polar (aglycones and polymethoxylated flavonoids) compounds.

For the chromatographic separation we must select the proper chromatographic parameters (mobile phases, temperature, column type, chromatographic modes, etc.) to get the best response, with the best possible resolution among the analytes to be separated. Considering the literature previously published about the determination of this kind of compounds from different fruits and plants, our own experience, and the results we got during the preliminary studies, water + acetic acid (0.5%) and ACN were used as mobile phases [2, 14, 15]. The different profiles obtained at 280 nm by using the gradients A, B, C and D are shown in ***Figure 4.1***. In ***Figure 4.1(A)***, we can see that the method which provided the fastest separation was gradient A, since the composition of the mobile phase (regarding percentage of B (ACN)) is increasing more drastically. At that moment, we wanted to make sure whether gradient A was able to give us resolution enough, or perhaps one of the other methods could provide better resolution. The most critical area of the chromatogram is the one that the reader can see more in detail in ***Figure 4.1(B)***. The red asterisk marks the same compound in every case to facilitate the comparison. Gradient D gave us the longest analysis time without giving better resolution. Gradient B and C gave similar results, although gradient C is the method which provides better resolution in the critical area; however, the efficiency of the separation and the number of theoretical plates between 5-20 min were not very satisfactory. Considering the fact that detection was not only made by DAD, but also with

the powerful TOF MS analyzer, we considered as a compromise solution to use gradient A as optimum, since it provided reasonably good resolution, excellent peak shape, short analysis time and was a potent "profiling method" able to separate and detect as many of the components as possible in a single extract of a food material (changing the polarity of the mobile phase in the whole possible range, 1-100% B).

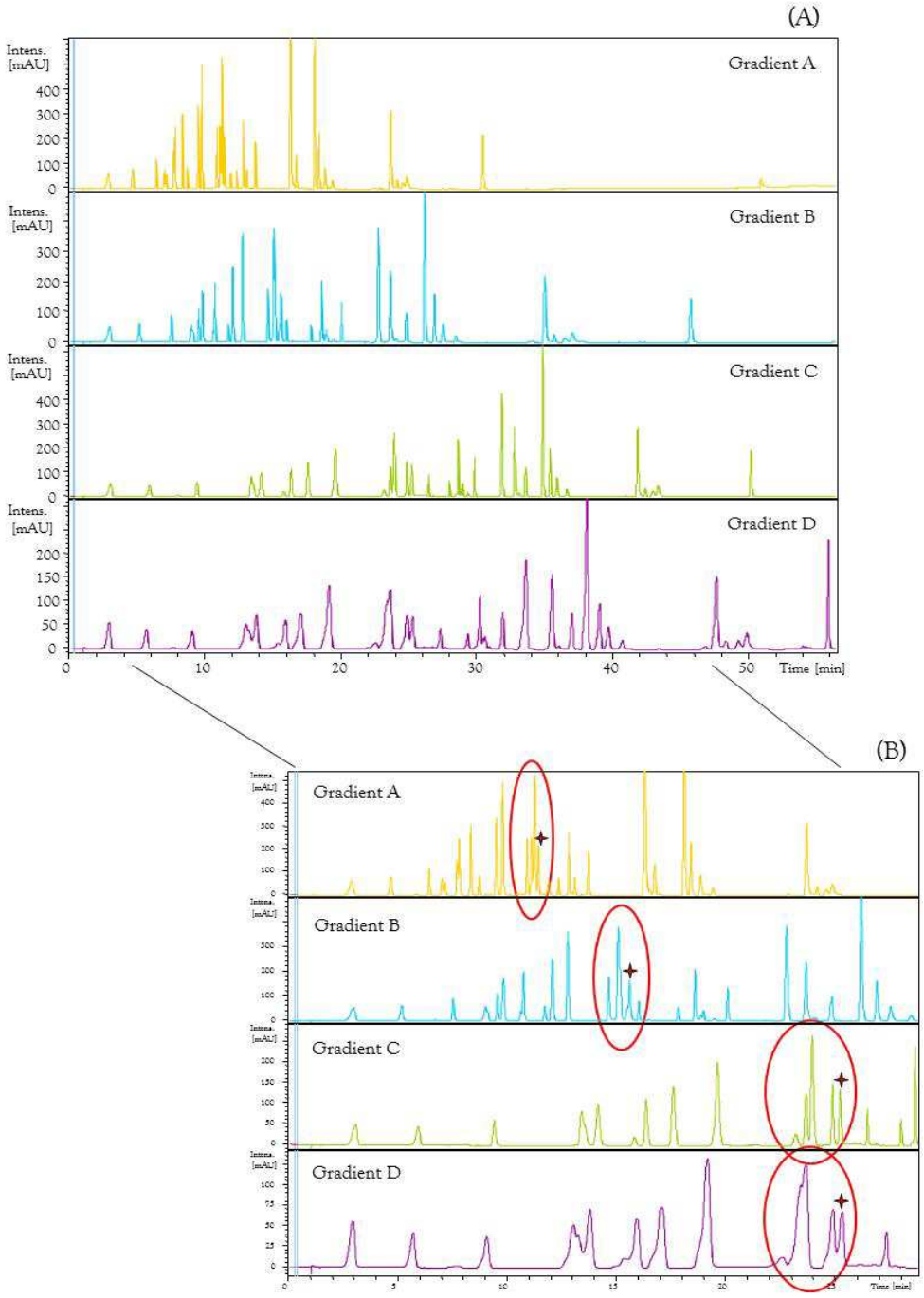

Figure 4.1. (A) Different profiles obtained for the standard mix containing 39 phenolic compounds at 280 nm by using several gradients for the method optimization. (B) Detail of the separation achieved between 0-28 min when gradients A, B, C and D were used. The most critical area is highlighted and the asterisk identifies the same compound in all cases.

The optimization of ESI-TOF MS conditions was made by direct infusion experiments of the standard mixture containing 39 phenolic compounds; we optimized source, transfer and detection parameters looking for the maximum sensitivity with the highest possible resolution. All the optimum parameters have been mentioned previously (see section 4.2.5).

Figure 4.2 shows the UV profiles at 240 and 280 nm and the EICs which compose the BPC (in negative polarity) of the standard mixture. We indicate as well in ***Figure 4.2*** the different areas of elution depending on the family of phenol (phenolic acids and related compounds, flavones, flavanols, etc.).

We made the analyses in negative and positive ion mode, because we could obtain additional information using both polarities and we could corroborate/confirm the identity of the compounds under study. ***Figure 4.3*** shows a comparison of the BPC obtained for the standard mix in positive and negative polarity. We can see that in negative polarity, the response factor of some compounds (phenolic acids and flavons, for example) is higher than positive mode, because not all the compounds are equally ionized in both polarities. For instance, gallic, protocatechuic and gentisic acids could not be detected properly in positive polarity (although the concentration was exactly the same), whilst happened the opposite for some other compounds. That is why we decided to carry out the detection in both polarities in every case, to have complementary information; this could be particularly important in the analysis of real avocado samples.

Table 4.1 summarizes information in positive and negative polarity of the standard mix containing 39 phenolic compounds, including their formula, selected ion, *m/z* experimental and calculated, error (in ppm), mSigma value, migration time, and fragments in source or other signals in MS.

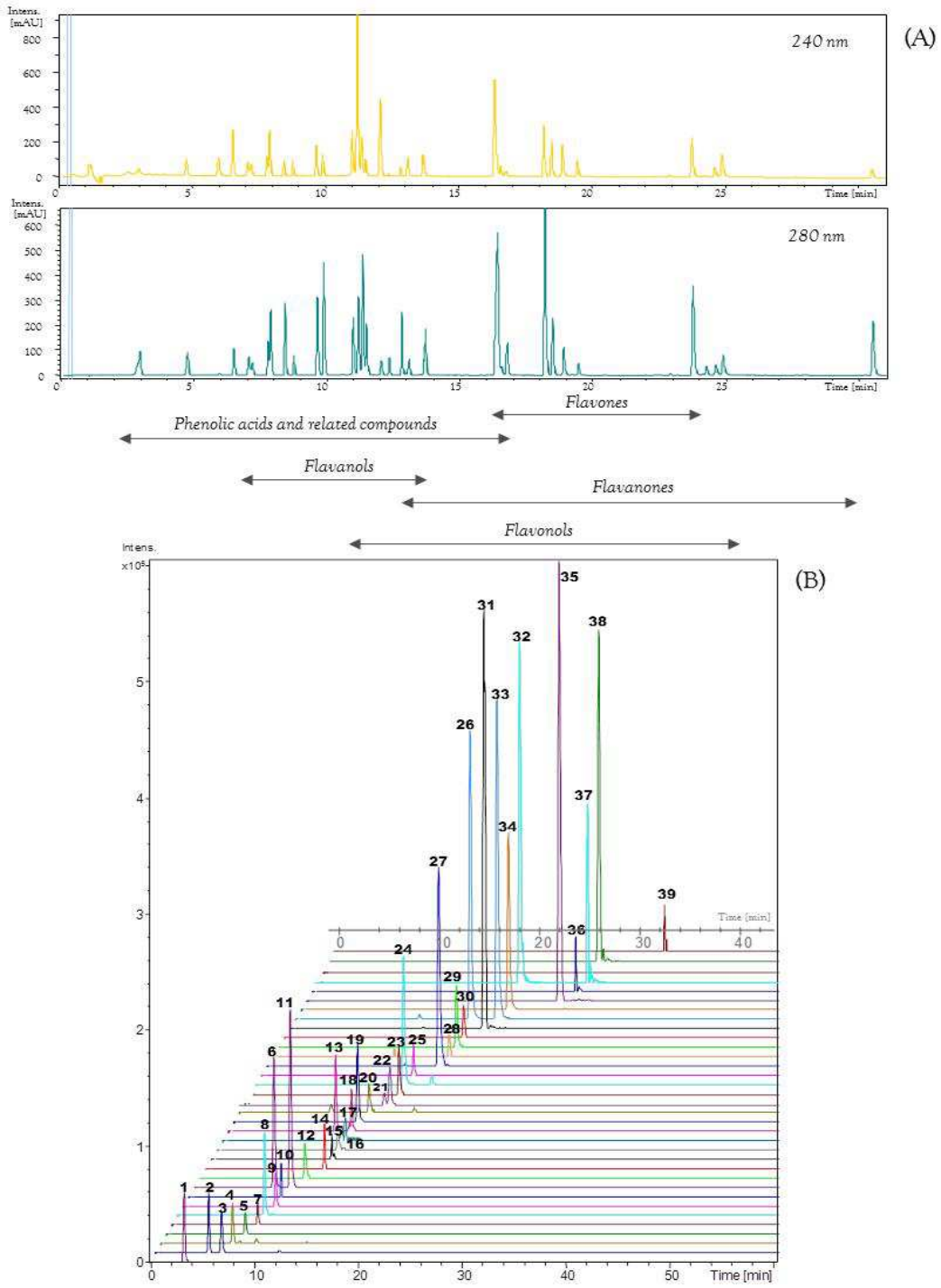

Figure 4.2. (A) UV profiles (240 and 280 nm) of the standard mixture of 39 phenolic compounds. Areas of elution of phenolic acids and related compounds, flavones, flavanols, flavanones and flavonols are defined. (B) EICs obtained when the optimum parameters for the separation and detection were used. Peaks identification: **1**, gallic acid; **2**, protocatechuic acid; **3**, gentisic acid; **4**, 4-hydroxybenzoic acid; **5**, chlorogenic acid; **6**, catechin; **7**, vanillic acid; **8**, caffeic acid; **9**, syringic acid; **10**, homovanillic acid; **11**, epicatechin; **12**, vanillin; **13**, *p*-coumaric acid; **14**, ferulic acid; **15**, ellagic acid; **16**, sinapinic acid; **17**, rutin; **18**, 3-hydroxycinnamic acid; **19**, taxifolin; **20**, benzoic acid; **21**, narirutin; **22**, naringin; **23**, quercetin-3-*O*-gluc-6´´-acet; **24**, myricetin; **25**, neohesperidin; **26**, *trans*-cinnamic acid; **27**, quercetin; **28**, kaempferol; **29**, laricitrin; **30**, poncirin; **31**, naringenin; **32**, apigenin; **33**, luteolin; **34**, isorhamnetin; **35**, chrysin; **36**, pinocembrin; **37**, galangin; **38**, kaempferide; **39**, pinocembrin-7-methyleter.

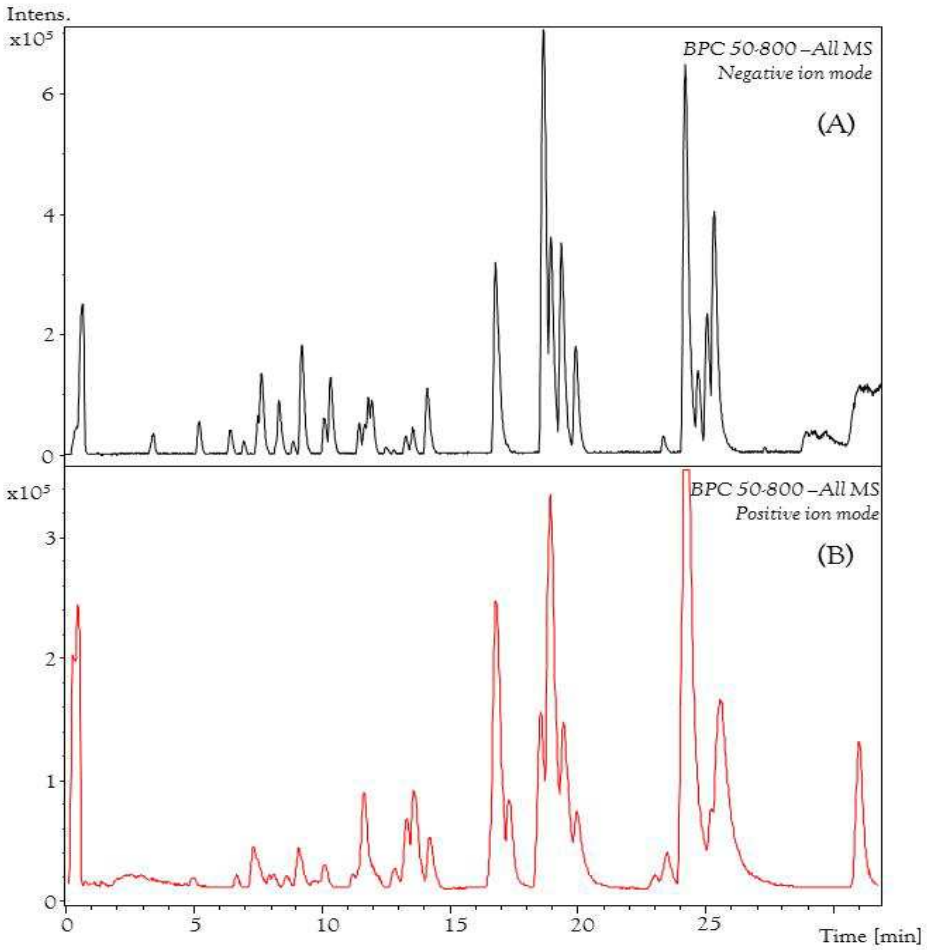

Figure 4.3. BPCs obtained in negative (A) and positive (B) ion polarity for the standard mixture.

4.3.2. Extraction of the compounds under study from avocado matrix

The initial extraction procedure is generally aimed at maximizing the amount and concentration of the compounds of interest. For that reason, we could say that the extraction can be considered as a very important step in this kind of study.

To carry out the optimization of the extraction protocol, we kept in mind that the solubility of phenols is controlled by the polarity of the solvent used, degree of polymerization of phenols, as well as the interaction of phenolic compounds with other plant-fruit constituents, such as carbohydrate and proteins [2, 28]. The solvents which are used more often in this kind of studies are MeOH, EtOH, AcOH, water, EtOAc and, to a lesser extent, propanol, dimethyl formamide, dimethyl sulfoxide and their combinations [29]. The preliminary studies led us to use AcOH, MeOH, EtOH and EtOAc as solvents for the extraction, since their polarities are quite similar (around 4.4-5.2).

In ***Figure 4.4*** we can see the profiles (BPC in negative polarity) of the 4 extracts obtained with the different solvents used (AcOH, MeOH, EtOH and EtOAc). The comparison was made taking into account the total integrated area, the number of peaks and the number of molecular features found by the software Data Analysis in each case by using exactly the same parameters as far as S/N threshold, area threshold, intensity threshold correlation coefficient threshold and minimum compound length are concerned. MeOH is the solvent which gave the richest profile in terms of number of peaks extracted and their concentration; the difference can be seen clearly between 2-25 min. The extracts obtained with AcOH and EtOH were quite similar, although the AcOHe extracted a greater number of compounds in comparison with EtOH. Although the 4 solvents used, were able to extract the phenolic compounds, MeOH extracts presented the best recovery. Moreover, MeOH gave us very clean extracts, which could be pre-concentrated more easily. For this reason, we will only show the profiles for the extracts of avocado obtained with MeOH.

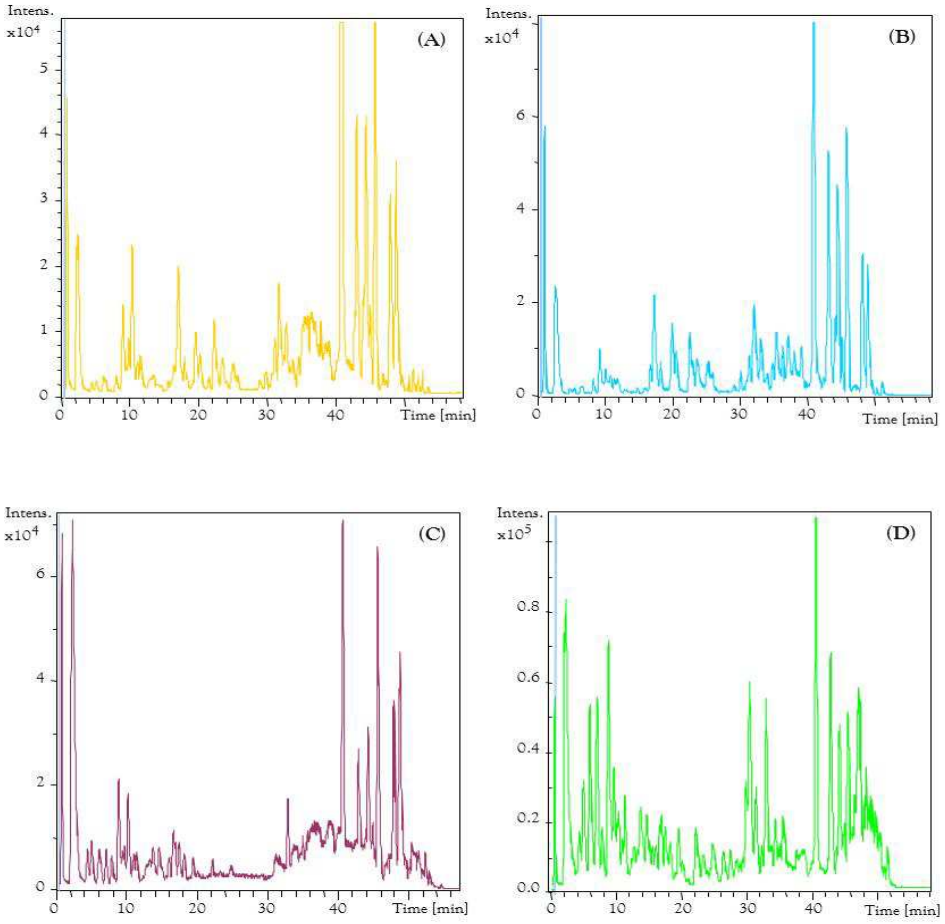

Figure 4.4. Comparison of the BPCs of extracts from 'Hass' variety avocado fruit prepared by using (A) AcOH, (B) EtOAc, (C) EtOH and (D) MeOH as extractant agent.

4.3.3. Potential of the described method

BPC of the methanolic extract from avocado was already shown in ***Figure 4.4(D)*** ('Hass' variety), but we think that it is very interesting to show and compare the UV-Vis profiles at 240, 280, 320, 440, 520 and 640 nm (see ***Figure 4.5***). The mentioned wavelengths were specially selected, since for instance, 240 and 280 nm have been widely used to determine different families of phenolic compounds, being 280 nm particularly useful for determining phenolic acids. 320 nm (approx.) is a very suitable wavelength when different subclasses of flavonoids have to be determined. 440 and 520 nm (approx.) are recommended when determination of anthocyanins wants to be achieved. We decided to include the channel of 640 nm as well to facilitate the possible detection of pigments, which were supposed to be extracted by MeOH, since the extracts were strongly colored.

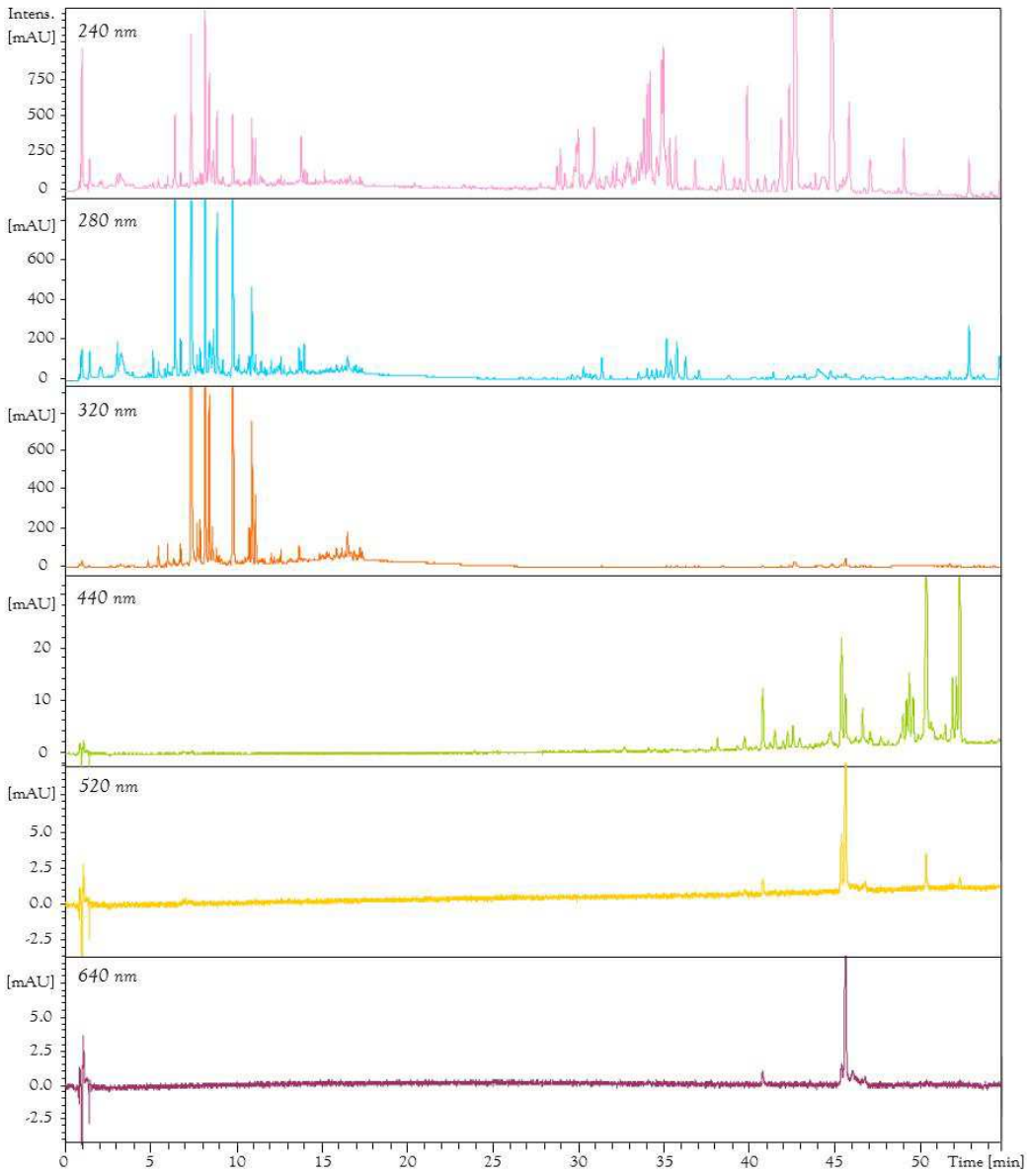

Figure 4.5. UV-Vis profiles at different wavelengths of a methanolic extract from a 'Hass' avocado sample.

DAD together with MS contributes to the whole picture of the sample with structural information (phenolic compound family). Taking together the information provided by both detectors represents a flexible tool for explorative studies and could be very useful in any metabolic profiling study.

The aim of this section is to stand out that, apart from the phenols we were interested in, some other constituents of avocado fruit could be separated and determined by our method. Taking advantage of the saved information, we could enlarge our knowledge (quite limited so far) about the composition of this important fruit.

4.3.4. Application of the method to the analysis of avocado samples

We proceeded to analyze 3 different varieties of avocado. ***Figure 4.6*** shows the BPC in negative polarity of the methanolic extracts obtained for the 3 varieties of avocado under study.

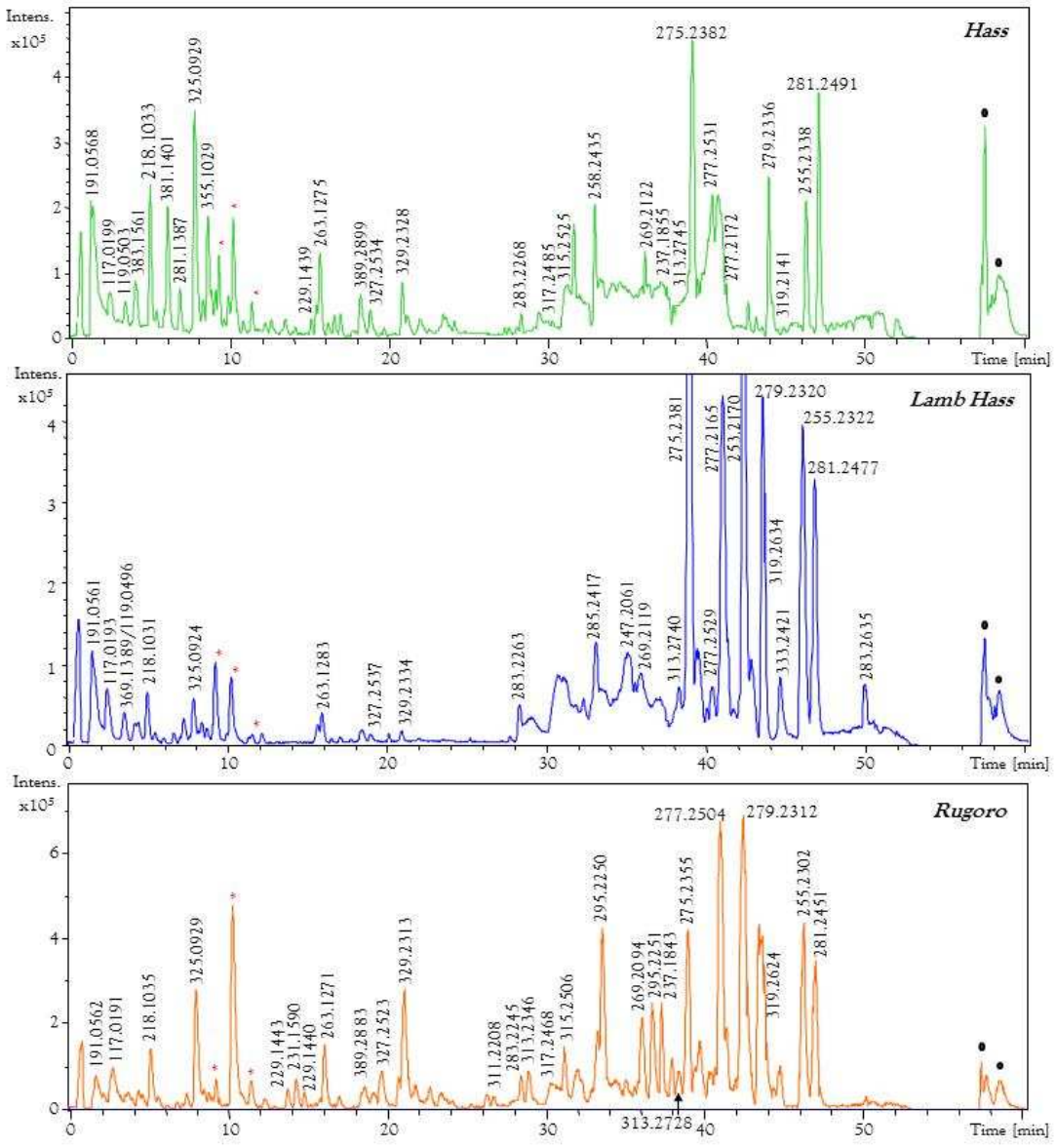

Figure 4.6. BPCs of the methanolic extract obtained from 'Hass', 'Lamb Hass' and 'Rugoro' varieties analyzed under optima conditions. (Only peaks with relevant intensity are shown in the figure). * Compounds whose identity was corroborated with standards. ● Peaks which appeared when blanks were analyzed (do not consider).

'Rugoro' variety contained the largest number of compounds whose identity was confirmed with standards, as it is demonstrated in ***Table 4.3***. This table includes information concerning the molecular formula (the compounds are in elution order), *m/z* experimental, *m/z* theoretical and retention time. We indicate in the table as well in

which varieties the compounds were found. Considering the 39 phenolic compounds included in the standard mixture used for the optimization of the method, we identified 17 phenols in our avocado samples. The 17 compounds were not found in the 3 varieties of avocado; for instance, in 'Rugoro' variety we could identify unequivocally 16 phenols, namely: protocatechuic acid, gentisic acid, 4-hydroxybenzoic acid, chlorogenic acid, catechin, caffeic acid, epicatechin, vanillin, *p*-coumaric acid, ferulic acid, sinapic acid, *trans*-cinnamic acid, laricitrin, naringenin, chrysin, and kaempferide.

Tabla 4.3. Phenolic secondary metabolites found in the methanolic extracts obtained from the 3 different varieties of avocado under study, (organized by upward retention time) whose identity was corroborated by standards. Error (ppm) and mSigma values were lower than 3.8 and 17.1 in every case.

Compound	Formula	m/z experimental	m/z theoretical	t_r (min)	Variety of avocado fruit		
					Rugoro	Lamb Hass	Hass
Potocatechuic acid	$C_7H_6O_4$	153.0196	153.0193	5.3	x	x	x
Gentisic acid	$C_7H_6O_4$	153.0201	153.0193	6.4	x	x	–
4-hydroxybenzoic acid	$C_7H_6O_3$	137.0242	137.0244	6.9	x	–	–
Chlorogenic acid	$C_{16}H_{18}O_9$	353.0879	353.0878	7.5	x	–	–
Catechin	$C_{15}H_{14}O_6$	289.0701	289.0718	7.7	x	x	x
Caffeic acid	$C_9H_8O_4$	179.0353	179.0350	8.3	x	x	–
Epicatechin	$C_{15}H_{14}O_6$	289.0718	289.0718	9.3	x	x	x
Vanillin	$C_8H_8O_4$	151.0396	151.0401	9.9	x	–	–
p-coumaric acid	$C_9H_8O_3$	163.0400	163.0401	10.3	x	x	x
Ferulic acid	$C_{10}H_{10}O_4$	193.0494	193.0506	11.4	x	x	x
Sinapinic acid	$C_{11}H_{12}O_5$	223.0616	223.0612	11.6	x	x	x
Benzoic acid	$C_7H_6O_2$	121.0295	121.0295	12.5	-	–	x
trans-cinnamic acid	$C_9H_8O_2$	147.0454	147.0452	16.8	x	x	x
Laricitrin	$C_{16}H_{12}O_8$	271.0615	271.0612	17.1	x	–	–
Naringenin	$C_{15}H_{12}O_5$	271.0618	271.0612	18.7	x	–	–
Chrysin	$C_{15}H_{10}O_4$	253.0493	253.0506	24.3	x	–	–
Kaempferide	$C_{16}H_{12}O_6$	299.0543	299.0561	25.3	x	–	–

x means that the compound was present in the avocado sample.

- means that the compound was not present in the avocado sample.

Apart from the compounds that we could identify with the help of commercial standards, there were others constituents of avocado that we could detect in the chromatograms. Using the potential of the analyzer that we were using, we could achieve a tentative identification, determining the *m/z* experimental, the possible molecular formula and even the name/s of possible candidate/s. ***Figure 4.6*** shows the *m/z* experimental signal found for some of the most intense compounds of the BPC, and ***Table 4.4*** summarizes that information, including the list of possible molecular formula achieved by SmartEditor (upward order of Sigma), a list of possible compounds and MS fragments-in source for 'Rugoro', 'Hass' and 'Lamb Hass' varieties, respectively.

Table 4.4. Tentative identification of compounds found in the methanolic extracts from hass, lamb-hass and rugoro avocado sample with considerable intensity in the BPC (in some cases, in positive polarity, the in-source fragments were most intense than $[M+H]^+$, they are underlined). Error (ppm) and msigma values were lower than 4.7 and 25.1 in every case.

NEGATIVE ION POLARITY								POSITIVE ION POLARITY	
m/z **experimental**	**t_r** (min)	**List of possibilities in Smart Editor-Molecular formula** (upward order of Sigma)	**Possible compounds** (for the first formula)	**Variety**			**MS Fragments in-source**	**m/z experimental**	**MS Fragments in-source**
				Hass	**Lamb Hass**	**Rugoro**			
191.0561	1.5	$C_7H_{12}O_6$	Quinic acid	X	X	X	–	193.0716	–
117.0193	2.5	$C_4H_6O_4$	Succinic acid Methylmalonic acid	X	X	X	–	119.0398	–
119.0496	3.5	$C_8H_8O_1$	Isocoumaran	X	X	–	–	121.0692	95.0498
383.1561	4.0	$C_{15}H_{28}O_{11}$ $C_{14}H_{22}N_7O_6$	–	X	–	–	–	–	–
218.1031	5.0	$C_9H_{17}N_1O_5$ $C_8H_{10}N_8$	Pantothenic acid (vitamin B_5)	X	X	X	146.0814	220.1174	98.9770 160.0822
281.1387	6.8	$C_{15}H_{22}O_5$ $C_{13}H_{20}N_3O_4$	Octyl gallate Isoctyl gallate	X	-	-	175.0606	283.1532	98.9738 160.0737
325.0929	7.9	$C_{15}H_{18}O_8$ $C_{13}H_{16}N_3O_7$	4-O-beta-D-Glucosyl-4-hydroxycinnamate *p*-Coumaroyl-D-glucose	X	X	X	145.0297	327.1074	<u>147.0445</u>
355.1029	8.6	$C_{16}H_{20}O_9$ $C_{14}H_{18}N_3O_8$	Gentiopicrin	X	–	–	175.0398	–	–
229.1443	13.7	$C_{12}H_{22}O_4$ $C_{10}H_{20}N_3O_3$	Dodecanoic acid Decanedioic acid 1,10-dimethyl ester Adipic acid di-n-propyl ester	–	–	X	187.0983	–	–
231.1590	14.2	$C_{12}H_{24}O_4$ $C_{10}H_{22}N_3O_3$	Methyl 9,9-dimethoxynonanoate (2S,3R)-2,3-dihydroxy-ethyl ester decanoic acid	–	–	X	-	233.1795	98.9746
229.1440	15.0	$C_{12}H_{22}O_4$ $C_{10}H_{20}N_3O_3$	Dodecanoic acid Decanedioic acid 1,10-dimethyl ester Adipic acid di-n-propyl ester	X	–	X	96.9582	–	–
263.1283	16.0	$C_{15}H_{20}O_4$	Abscisic acid	X	X	X	153.0922	265.1416	135.0809 201.1252 <u>247.1297</u>

Table 4.4 (continued)

m/z **experimental**	t_r (min)	**List of possibilities in Smart Editor-Molecular formula** (upward order of Sigma)	**Possible compounds** (for the first formula)	**Variety**			**MS Fragments in-source**	**m/z experimental**	**MS Fragments in-source**
				Hass	Lamb Hass	Rugoro			
389.2902	18.2	$C_{21}H_{42}O_6$	Pentadecyl D-glucoside 9,10,12,13-tetrahydroxyhenicosanoic acid	X	–	X	–	–	–
327.2538	18.8	$C_{19}H_{36}O_4$	(2S, 4S)-2,4-dihydroxyheptadec-16-enyl acetate	X	X	X	227.1631	–	–
329.2333	20.9	$C_{18}H_{34}O_5$ $C_{16}H_{32}N_3O_4$	9,10,13-trihydroxyoctadec-11-enoic acid	X	X	X	–	–	–
311.2208	26.8	$C_{18}H_{32}O_4$	(2S, 4S)-2,4-dihydroxyheptadec-16-ynyl acetate 4-acetoxy-1,2-dihydroxyheptadec-16-yne	–	–	X	116.9278 209.1148	–	–
283.2275	28.4	$C_{17}H_{32}O_3$ $C_{15}H_{30}N_3O_2$	2-methoxy-5Z-hexadecenoic acid 9-ceto-heptadecilic acid	X	X	X	145.0618	285.2420	95.0811 135.1108 249.2085
313.2346	28.9	$C_{18}H_{34}O_4$ $C_{16}H_{32}N_3O_3$	(9Z)-(7S, 8S)-dihydroxyoctadecenoic acid 18-hydroxy-9R,10S-epoxi-estearic acid	–	–	X	–	–	–
317.2468	30.3	$C_{21}H_{34}O_2$	(2E, 5E, 12Z, 15Z)-1-hydroxyheneicosa-2,5,12,15-tetraen-4-one	X	–	X	265.1456	319.2633	284.2455
315.2506	31.1	$C_{18}H_{36}O_4$ $C_{10}H_{38}N_1O_9$	9,10-dihydroxystearic acid (9R,10R)-dihydroxyoctadecanoic acid	X	–	X	–	317.2475	284.2451
285.2434	33.1	$C_{17}H_{34}O_3$ $C_{15}H_{32}N_3O_2$	3-hydroxypalmitic acid methyl ester 2-methoxyhexadecanoic acid	X	X	-	–	–	–
295.2250	33.5	$C_{18}H_{32}O_3$ $C_{16}H_{30}N_3O_2$	8-hydroxylinoleic acid (13S)-hydroxyoctadecadienoic acid	–	–	X	–	–	–
269.2122	35.8	$C_{16}H_{30}O_3$	1,2,4-trihydroxyheptadec-16-yne	X	X	X	116.9290	271.2268	231.1993
295.2251	36.6	$C_{18}H_{32}O_3$ $C_{16}H_{30}N_3O_2$	8-hydroxylinoleic acid (13S)-hydroxyoctadecadienoic acid	–	–	X	–	–	–
237.1850	37.3	$C_{15}H_{26}O_2$ $C_{13}H_{24}N_3O_1$	Centarol Daucol	X	–	X	–	–	–
313.2747	38.2	$C_{19}H_{38}O_3$	1,2,4-trihydroxynonadecane	X	X	X	225.1853 279.1969 293.2083	–	–
275.2381	38.9	$C_{19}H_{32}O_1$ $C_{14}H_{32}N_2O_3$	2-(1-pentadecenyl)-furan = Avocadofuran	X	X	X	–	277.2511	113.0607 267.2297
277.2535	40.4	$C_{19}H_{34}O_1$	Pentadecylfuran	X	X	X	–	279.2611	95.0847 263.2357

Table 4.4 (continued)

m/z **experimental**	t_r (min)	**List of possibilities in Smart Editor-Molecular formula** (upward order of Sigma)	**Possible compounds** (for the first formula)	**Variety**			**MS Fragments in-source**	**m/z experimental**	**MS Fragments in-source**
				Hass	Lamb Hass	Rugoro			
277.2167	41.1	$C_{18}H_{30}O_2$ $C_{16}H_{28}N_3O_1$	Linolenic acid 4-dodecylresorcinol	X	X	X	251.2344	279.2470	95.0850 233.2250
253.2170	42.3	$C_{16}H_{30}O_2$ $C_{14}H_{28}N_3O_1$	Palmitoleic acid 3-hexadecenoic acid	–	X	–	–	–	–
279.2336	43.5	$C_{18}H_{32}O_2$ $C_{16}H_{30}N_3O_1$	Linoleic acid (9Z, 11E)-octadecadienoic acid	X	X	X	–	–	–
319.2638	44.1	$C_{21}H_{36}O_2$	(2E,12Z,15Z)-1-hydroxyheneicosa-2,5,12,15-tetraen-4-one	X	X	X	279.2311	321.2798	277.2521 303.2644
333.2425	44.7	$C_{21}H_{34}O_3$ $C_{19}H_{30}N_3O_1$	4-tetradecoxybenzoic acid Tetradecyl 4-hydroxybenzoate Tridecyl 3-methoxybenzoate	–	X	–	251.2367 279.2313	335.2581	277.2561 295.2639
255.2322	46.1	$C_{16}H_{32}O_2$ $C_{14}H_{30}N_3O_1$	Palmitic acid Myristyl acetate	X	X	X	–	257.2374	219.2111
281.2491	47.0	$C_{18}H_{34}O_2$ $C_{16}H_{32}N_3O_1$	Oleic acid Elaidic acid	X	X	X	–	–	–
283.2635	49.9	$C_{18}H_{36}O_2$ $C_{16}H_{34}N_3O_1$	Stearic acid Ethyl palmitate Isooctadecanoic acid	–	X	X	–	–	–

X means that the compound was present in the avocado sample.

– means that the compound was not present in the avocado sample.

We tried to achieve a reliable identification of every compound detected within the chromatographic run using negative and positive ion polarities. Indeed, using this information (together with the fragmentation in-source), we could corroborate the tentative identification.

A large number of compounds were identified in negative polarity in all samples of avocado, specifically 29 in 'Rugoro', 27 in 'Hass' and 22 in 'Lamb Hass' variety. Positive mode made possible to corroborate the identity of 13 compounds in Rugoro, 14 compounds in 'Hass' and other 12 in 'Lamb Hass' samples. Those compounds belong to alkanols (1,2,4-trihydroxynonadecane, (2S, 4S)-2,4-dihydroxyheptadec-16-enyl acetate, etc.), furan-ring containing derivatives (2-(1-pentadecenyl)-furan and pentadecylfuran), carboxylic acids (quinic acid, succinic acid and methylmalonic acid) , saturated and unsaturated fatty acids (2-methoxy-5Z-hexadecenoic acid, 8-hydroxylinoleic acid, palmitic acid, oleic acid, dodecanoic acid, etc.), isoprenoids plant hormones (abscisic acid), and sesquiterpenoids (centarol, daucol), among others.

The three avocado´s varieties under study contained common compounds, like quinic acid, succinic acid, pantothenic acid, *p*-coumaroyl-*D*-glucose, abscisic acid, pentadecylfuran, avocado furan, oleic acid, etc. However, some compounds were only present in one of the varieties analyzed, being potential varietal markers. It is necessary to continue the current study increasing the number of varieties analyzed to be able to assure that a particular compound is a varietal marker; MS^n analyses would be advisable as well.

4.3.5. Repeatability study

Repeatability was studied by performing a series of separations using the optimized method on one of the samples the same day (intraday precision, n=12) and in three consecutive days (interday precision, n=36). The relative standard deviations (RSDs) of peak areas/retention time were determined by assaying seven of the compounds present in the extracts (protocatechuic acid, chlorogenic acid, epicatechin, ferulic acid, kaempferide and compounds with *m/z* 315.2525 and 295.2264).

The intraday repeatabilities on the total peak area/retention time (expressed as RSD) were between 1.7 and 3.1%, whereas the interday repeatabilities on total peak area/retention time were found in the range from 2.5 to 4.6%.

4.4. Conclusions

The separation by an effective HPLC profiling method with on-line detection by DAD and ESI-TOF-MS was successfully applied to the analysis of the secondary metabolites (phenolic compounds and some other components) extracted from avocado samples. Our LC-DAD/ESI-TOF MS method can determine more than 40 analytes in each run, 17 of them were unequivocally identify by using standards, and for the rest, we achieved a tentative identification considering the information provided by the powerful TOF, the chemical information that we can obtain from the chromatographic separation (polarity and size of the compounds) and the information previously published.

Significant differences were found when the analyses of the three varieties used in this study were compared. In our lab there are already some studies ongoing for studying more varieties of avocado fruit, making a proper deep comparison by using statistical tools looking for potential varietal markers.

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Chapter 5

UHPLC-TOF MS for analysis of avocado fruit metabolites: Method evaluation and applicability to the analysis of ripening degrees

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Abstract

We have developed an analytical method using UHPLC-UV/ESI-TOF MS for the comprehensive profiling of the metabolites found in the methanolic extracts of 13 different varieties of avocado at two different ripening degrees. Both chromatographic and detection parameters were optimized in order to maximize the number of compounds detected and the sensitivity. After achieving the optimum conditions, we performed a complete analytical validation of the method with respect to its linearity, sensitivity, precision, accuracy and possible matrix effects. The LODs ranged from 1.64 to 730.54 ppb (in negative polarity) for benzoic acid and chrysin, respectively, whilst they were found within the range from 0.51-310.23 ppb in positive polarity. The RSDs for repeatability test did not exceed 7.01% and the accuracy ranged from 97.2% to 102.0%. Our method was then applied to the analysis of real avocado samples and advanced data processing and multivariate statistical analysis (PCA, PLS-DA) were carried out to discriminate/classify the examined avocado varieties.

About 200 compounds belonging to various structural classes were tentatively identified; we are certain about the identity of around 60 compounds, 20 of which have been quantified in terms of their own commercially available standard.

Keywords: Ultra high performance liquid chromatography / Ultra high-resolution-time of flight mass spectrometry / Avocado (*Persea americana)* / Plant metabolites / Chemometrics

5.1. Introduction

The complexity of the plant metabolome cannot be overestimated. The endless variety of organic compounds present in fruits and vegetables is the product of primary and secondary plant metabolism [1]. The division on the “primary” and “secondary” metabolites is typical for plant-based metabolomics. The primary metabolites are directly involved in the normal growth, development, and reproduction (such as carbohydrates, amino acids, fatty acids, and organic acids), whilst those plant specific substances which determine the color of a fruit and vegetables, act as the signal molecules under stress conditions, protect plants against herbivores and microorganisms, attract pollinators and seed-dispersing animals, etc., are described as the secondary metabolites [2, 3]. In general,

plant secondary metabolites can be divided into 4 major groups: phenolic and polyphenolic compounds, terpenoids, alkaloids, and sulfur-containing compounds [4]. Among these four families, phenolic and polyphenolic compounds are attracting of the most attention, due to their role in the plant´s inmune response [5], their influence in the oxidative stability and organoleptic characteristics of foods [6], and the wide variety of health-promoting effects attributed to them [7-10].

The chemical diversity of plant specific substances in combination with time dependent mechanisms of their production and storage makes routine targeted methods of analysis sub-optimal [11]. Consequently, non-biased, exploratory type of analysis –which has its origin in the emerging field of metabolomics– appears to be a more appropriate approach for studying plant specific metabolites and their dynamic changes. A growing interest in metabolomics, especially in applicability of this approach to a wide range of biological targets related to human nutrition proves our point [12-15]. As a scientific discipline, metabolomics takes advantage of many technologies, their pros and cons have been reviewed extensively [11, 16, 17], but regardless of the technology used in any particular study, metabolomics offers the possibility to gain deeper insights into the fundamental biochemistry of the things we eat, a better understanding of the pathways responsible for the biosynthesis of nutritionally relevant metabolites and the last, but not the least, identifying biochemical basis of the health-beneficial effects of fruits and vegetables.

The avocado fruits (*Persea americana*) are a good example. They are valued not only for their unique flavor, texture, and nutritional profile, but also for their pharmacological properties [18-20]. It has been shown, for instance, that avocado unsaponifiables in combination with soybeans unsaponifiables demonstrate significant symptom modifying effect in a treatment of knee osteoarthritis [21]. Another important medicinal property of avocado fruit is its cancer-preventive effect. In an agreement with existing reports on phytochemicals based medicines, the cancer-preventive action of avocado fruit cannot be assigned to one particular compound but to a variety/combination of nutrients and phytochemicals (alkanols, terpenoid glycosides, various furan ring-containing derivatives, flavonoids and coumarins) [19]. As far as phenolic composition of this fruit is concerned,

there are several very interesting reports [22-29], however, it can be stated that the phenolic fraction of avocado is still quite unknown.

Here, we present an overview of applicability of UHPLC-UV/ESI-TOF MS platform for analysis of avocado fruit metabolites. We describe the analytical parameters of the method, provide structural assignment for a number of metabolites and finally we demonstrate the method applicability for analysis of ripening degrees of thirteen avocado fruit varieties.

5.2. Materials and methods

1.3.1. Chemicals and standards

The solvent used for the sample extraction was MeOH, which was purchased from Panreac (Barcelona, Spain). For preparing the mobile phases, ACN and formic acid were used; they were purchased from Lab-Scan (Dublin, Ireland) and Sigma-Aldrich (St. Louis, EEUU), respectively. All the solvents used were HPLC grade and they were used as received. Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q system (Millipore, Bedford, MA, USA).

Standards of quinic acid (cyclic polyol) and succinic acid (dicarboxilic acid) were purchased from Fluka (St. Louis, EEUU). Pantothenic acid (vitamin B5), abscisic acid (phytohormone), several hydroxybenzoic acids (2,4-dihydroxybenzoic, 3,5-dihydroxybenzoic, 2,6-dihydroxybenzoic, 2,3-dihydroxybenzoic, 4-hydroxybenzoic, 3-hydroxy-4-methoxybenzoic, benzoic, gentisic, syringic, protocatechuic, vanillic and gallic acids), some hydroxycinnamic acids (*o*-, *m*- and *p*-coumaric acids, caffeic, *trans*-cinnamic, ferulic, chlorogenic and sinapinic acids), two phenolic acids-related compounds (homovanillic acid and vanillin), and two flavanols (catechin and epicatechin) were from Sigma-Aldrich (St. Louis, EEUU). Laricitrin and kaempferide (O-methylated flavonols), chrysin (flavone) and naringenin (flavanone) were supplied by Extrasynthese (Lyon, France). This group of 32 standards was selected after the preliminary studies to optimize the method, since they could likely be found in the avocado samples we were working with; 27 of these 32 compounds (excluding 3,5- dihydroxybenzoic acid (α-resorcylic acid), protocatechuic (3,4-dihydroxybenzoic) acid, 2,6-dihydroxybenzoic acid (γ-resorcylic acid),

2,4-dihydroxybenzoic acid (β-resorcylic acid), and 2,3-dihydroxybenzoic acid (*o*-pyrocatechuic acid)) were used in the validation studies.

Taxifolin was used as IS to evaluate the reproducibility of the extraction system and the chromatographic runs; it was purchased from Extrasynthese (Lyon, France).

All the stock solutions, samples, solvents and reagents were filtered with a 5 µm membrane filter (Millipore, Bedford, MA, USA) before separation or injection in the instrument.

5.2.2. Samples

Fruits of 13 varieties of avocado (*Persea americana*) at two different ripening degrees were used: 'Hass', 'Lamb Hass', 'Gem', 'Marvel', 'Jiménez 1', 'Jiménez 2', 'Pinkerton', 'Colin V 33', 'Sir Prize', 'Tacambaro', 'Nobel', 'Harvest', and 'Hass Motril'. All the varieties were grown under identical environmental conditions (soil, rain, light, etc.). The samples used in this study were part of the collection from IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). For each variety, the pulp of 3-4 pieces of fruit, which were frozen to be further freeze-dried, was used.

A mixture of fruits from the 13 avocado varieties under study at the second ripening degree (mixing an equivalent volume of fruits of each variety) was used as analytical QC sample. For validation purposes, the mentioned QC samples and the standard mix composed by the 27 compounds previously mentioned in the Chemicals and standards section were used. Both samples were injected every 6 samples throughout the batch. Data derived from these samples were used to assess system stability.

5.2.3. Extraction procedure

Extracts were prepared according to Hurtado-Fernández et al. [29]; briefly, 4 g of the freeze-dried (and homogenized) sample were put inside a falcon tube adding 40 mL of pure MEOH. The tubes were shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 3000 rpm for 10 min. After that, the supernatants were evaporated to dryness and redissolved in 1 mL of MeOH.

5.2.4. UHPLC-MS instrument

UHPLC was conducted using a Dionex Ultimate system (Ultimate 3000 RS tandem LC system, Dionex, Amsterdam, The Netherlands), which was equipped with a pre-column (Acclaim 120 C18, 5 µm, 120 Å, 2 x 10 mm,) and two analytical columns (Acclaim RSLC 120 C18, 1.8 µm, 120 Å, 2.1 x 100 mm) working alternatively to speed up the acquisition series. Mobile phases A and B consisted of water with 0.1% of formic acid (v/v), and ACN with 0.1% of formic acid (v/v), respectively. The best separation was achieved using the following gradient elution program in the two columns executed in parallel mode: 0 min, 100% A and 0% B; 20 min, 92% A and 8% B; 40 min, 60% A and 40% B; 45 min 30% A and 70% B; 45.5 min, 0% A and 100% B; coming back to the initial conditions and being equilibrated. The flow rate used was 0.60 mL/min and the temperature was set at 50ºC. The injection volume was1 µL.

The compounds separated by UHPLC were monitored in sequence first with a UV (214, 240, 254 and 280 nm) and then with a MS detector. The UHPLC system was coupled to an ultra high resolution TOF MS maXis (Bruker Daltonik, Bremen, Germany). The MS was operated in negative and positive ionization modes and acquired data in the mass range from *m/z* 50 to 1000 with a spectra rate of 1 Hz. In negative mode, the capillary was set at +4000 V, the End Plate offset at –500 V, the Nebulizer gas at 2 bar and the Dry Gas at 8 L/min at 200°C. The polarity of ESI and all of the parameters of MS detector were optimized using the height of the MS signal for the compounds included in our standard mix and some other compounds found in the avocado QC samples.

Peak identification was done bearing in mind retention time, spectral data and ESI-UHR-TOF MS information obtained from real samples and standards, and also with spiked real samples at different concentration levels.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provided a list of possible elemental formulas by using the SmartFormula™ Editor tool.

Internal calibration was performed using sodium formate cluster by using a solution containing 5 mM NaOH in the sheath liquid of 0.2% formic acid in water/isopropanol

1:1 v/v. The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior carrying out the compound identification.

When the identity of an analyte could not be confirmed by using commercial available standards, experimental accurate MS data was manually searched for in on-line public databases such as Pubchem (http://pubchem.ncbi.nlm.nih.gov/), KNApSAcK (http://kanaya.naist.jp/knapsack_jsp/top.html), Metlin (http://metlin.scripps.edu/), KeggLigand (http://www.genome.jp/kegg/ligand.html), or ChemSpider (http://www.chemspider.com) to get a tentative identification.

5.2.5. Statistical studies

The UHPLC-MS data files were exported as mzXML files and aligned by using in-house developed alignment algorithm msalign2 tool (http://www.ms-utils.org/msalign2/); peak picking was performed using XCMS package (The Scripps Research Institute, La Jolla,USA) using the default settings except for bandwidth (bw parameter) for grouping of features set to 10.

The generated data matrix was imported to SIMCA-P 12.0 software package (Umetrics, Umeå, Sweden). The data were mean centred and unit variance-scaled prior to statistical analysis. The validity and the degree of overfitting of the PLS-DA models were checked using a 200 permutations test.

To identify metabolites of interest, rational chemical formulas were generated based on internally calibrated monoisotopic masses within 10 mDa mass error, using the SmartFormula tool within the DataAnalysis™ software package (Bruker Daltonik).

5.2.6. Validation studies

Linearity and sensitivity

Linearity of the detectors response (UV and TOF MS) was verified with solutions containing 27 standards at 6 different concentration levels over the range from the quantification limit to 66, 100 or 200 ppm, depending on each analyte. Calibration curves were prepared daily and estimates of the amount of the analytes in samples were interpolated from these graphs. Each point of the calibration graph corresponded to the mean value from three independent injections.

When UV detection was used, an evaluation of the sensitivity was made by determining the LOD values, which were calculated from the peak area on the basis of $3\sigma/b$, σ being the standard deviation of the blank (largest deviation of detector signal from baseline measured in a section of about 50-100 points in the absence of analyte) and b the intercept of every calibration equation.

When MS detection was employed, the LODs and the LOQs of the individual analytes in standard solutions were calculated according to the IUPAC method [30]. The sensitivity of the method in that case was studied by defining the LOD and LOQ for individual compounds in standard solutions. The LOD was considered S/N=3, whilst the LOQ was S/N=10. The LODs obtained using this approach were compared with those achieved by using the same calculation method as in UV and the results were in good agreement.

Precision and accuracy

The precision of the analytical procedure described was measured as repeatability and evaluated over the linear dynamic range at three different concentration levels (low (LOQ), medium (intermediate concentration value of the linear calibration range), high (higher concentration value of the linear calibration range)). The precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatabilities in the peak areas were determined as the RSD obtained for six consecutive injections of each standard at each concentration value, carried out within the same day and on three different days, respectively. The same was done when the intra- and inter-day repeatabilities (column-to-column) were checked; although in this case we obviously compared the results achieved by different columns.

Accuracy was evaluated with separately prepared individual primary stock solutions, mixtures and working solutions of all standards. It was calculated over the linear dynamic range at three different concentration levels (the same as for the precision); spiked QC samples were tested in 3 replicates per concentration and calculated with calibration curves obtained daily. The analyte concentrations were calculated and the accuracy was calculated by the ratio of this calculated concentration versus the theoretical (spiked) concentration.

Ion suppression and matrix effects

The strategy described by Nelson and Dolan [31] was used for systematic investigation of ion suppression in HPLC-MS analysis, producing a continuous flow of QC avocado solution resulting in a constant but elevated baseline in MS detection. This flow was then continuously mixed with eluate from an LC-separated blank sample.

To evaluate the matrix effect, we compared the UV and MS responses of the analytes under study spiked post-extraction with those in a neat solution (in triplicate), calculating the RF (which is considered to be the ratio between the peak area and the concentration of the analyte) when the analytes were in presence of the avocado matrix and in a neat solution. Whether significant differences between both values could be found was checked using ANOVA.

5.3. Results and discussion

5.3.1. UHPLC-UV/ESI-TOF MS method optimization

The solution containing the 32 selected standards and the QC avocado sample were used to optimize both the chromatographic and MS conditions. The UHPLC-ESI-TOF MS conditions were checked taking into account the chromatographic behavior of the analytes present in the samples under study, analysis time, sensitivity, peak shape, etc. In a first attempt, to take advantage of using an UHPLC system, the following gradient was tried with the mobile phases described before (12.5 min approx. analysis time) and a flow rate of 0.4 mL/min: 1 min 0 % phase B, then in 1 min to 10% phase B, held for 1 min at 10% phase B, and subsequently in 6.5 min to 100% phase B and held for 3 min at 100% phase B. The complete separation of the 32 analytes included in the mix was not achieved (proper separation of 3,5-dihydroxybenzoic, 2,6-dihydroxybenzoic and protocatechuic acids; gentisic and 4-hydroxybenzoic acids; chlorogenic and homovanillic acids; and sinapic and *o*-coumaric acids was not possible), but the separation of the compounds present in the QC avocado extract sample was particularly disappointing. We started then trying different linear gradients with the following gradient steepness: 10, 7.5, 5, 2.5 and 1.5 % ACN/min, which correspond with gradient times of 10, 13, 20, 40 and 65 minutes (approx), respectively. The best results were obtained with the last two gradients; therefore the conditions were optimized considering this information and using not only linear

gradients, but also step-gradients. Ten different gradients were checked, although to summarize the results achieved in this optimization we show 3 of the most representative (***Figure 5.1. Supporting information***.). The achieved separation for the standard mix by using gradient C was the best in terms of efficiency, peak resolution and precision; moreover, when a QC avocado sample was analyzed by this gradient, the peak distribution along the chromatogram was smoother or more uniform.

The influence of the flow rate was also studied. Higher flow rates than 0.7 mL/min originated tailed broad peaks and a rapid increase of the backpressure. A flow rate of 0.6 mL/min resulted in a little decrease in run time producing acceptable resolution. Although baseline separation was not achieved for 5 phenolic acids (3,5-dihydroxybenzoic, 2,6-dihydroxybenzoic and protocatechuic, and 2,4-dihydroxybenzoic and 2,3-dihydroxybenzoic), the optimum UHPLC conditions were the result of a compromise between the resolution of all the compounds present in the real avocado extracts and analysis time.

The ESI-TOF MS operating conditions were optimized by adjusting the needle-counter electrode distance, drying gas flow, drying gas temperature, nebulizer gas flow rate, and applied electrospray potentials while the standard mix and the QC sample were injected by direct infusion experiments. The source, transfer and detection parameters were optimized looking for the maximum sensitivity with the highest possible resolution. All the optimum parameters have been mentioned previously (Materials and Methods section).

Since several of the compounds included in our test mixture have never been studied in detail by ESI-TOF MS, we provide an overview of their signals in both positive and negative modes in ***Table 5.1. Supporting information***.

5.3.2. Analytical parameters of the method

Linearity and sensitivity

Calibration curves with high linearity ($r^2 > 0.98$) were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from UHPLC-UV or UHPLC-ESI-TOF MS analyses. The parameters of the regression equations, LOD, LOQ, calibration range, precision (expressed as repeatability within the

same column and the two columns working in-parallel), accuracy, and RF (to evaluate the possible matrix effect) are summarized in ***Table 5.1***.

Quinic, succinic and pantothenic acids could not be determined without using MS. The calibration curves for such compounds as gentisic acid, 4-hydroxybenzoic acid, catechin, isovanillic, chlorogenic, homovanillic acid, sinapic and *o*-coumaric acids were calculated only with MS detector due to their overlap with other analytes in the UV profile. Some compounds such as, for example, quinic acid and vitamin B_5 (so-called pantothenate) can be determined both in negative and positive polarity, although LOD and lineal range were better in negative mode. On the contrary, compounds like succinic acid should be measured in negative polarity, since its ionization in positive polarity is quite inefficient. Hydroxybenzoic acids can be detected in negative and positive polarities, with exception of gentisic and 4-hydroxybenzoic acids. Vanillic, syringic and benzoic acids could be perfectly detected at 254 and 280 nm as well (no overlapping signals). Our method had better analytical figures for all the hydroxycinnamic acids included in the mix in the negative polarity, although again, both polarities could be used except for *trans*-cinnamic acid. The calibration curves of *p*-coumaric, *m*-coumaric, ferulic and *trans*-cinnamic acids showed detection limits ranging from 6.26 to 24.54 ppb at 254 nm and from 2.89 to 6.33 ppb at 280 nm. The two flavanols under study had similar LODs in positive and negative polarities, but for the rest of the flavonoids evaluated, the analysis in the positive mode led to much better results. Abscisic acid content of an avocado sample could be properly determined by MS (in both polarities) and at 254 and 280 nm, although linearity was slightly better in positive polarity and UV detection.

Precision and accuracy

The precision of the method described was measured as repeatability. The intra- and inter-day repeatabilities in the peak areas were determined as the RSD obtained for six consecutive injections of each analyte at an intermediate concentration value of the calibration curve, carried out within the same day and on three different days. The same was made to calculate the column-to-column repeatability (intra- and inter-day).

Table 5.1 shows that acceptable levels of precision were obtained for the developed method in terms of repeatability since in all cases RSDs calculated were lower than 7.01%. It should be noted that although they are not specified, RSDs for migration times

are always lower than the ones for peak areas. The accuracy ranged from 97.22% to 102.03%.

Ion suppression and matrix effect

The results obtained while a continuous flow of analyte solution was continuously mixed with the eluate from an UHPLC-separated blank sample did not show any drop in the baseline over the complete LC run, which means that the ion suppressions from co-eluting compounds were negligible.

A post-extraction spike method was used to assess matrix effect. This approach compares the response factor (RF) of an analyte in neat solution to the RF of the analyte spiked into a blank matrix sample that has undergone through the sample preparation process.

Standards of the analytes dissolved in neat methanol were analyzed directly at three different concentration levels: low (3.3 to 6.6 ppm), medium (13.3 to 66 ppm) and high (66 to 200 ppm). The exact concentrations within a level were adjusted according to analytes properties. Then avocado QC sample was extracted and spiked after extraction at the same concentration levels. No statistical differences in peak area and RFs were observed for any of the analytes under study at any of the assayed concentrations (results for the intermediate value of concentration for each analyte are shown in ***Table 5.1***).

The results summarized in this validation section highlight that the method is a reliable approach for the analyses of this kind of compounds in avocado samples.

Table 5.1. Analytical parameters of the developed UHPLC-UV/ESI-TOF MS method.

Compound	Considered signal	Calibration curve	r^2	LOD (ppb)	LOQ (ppb)	Lineal range (ppm)	Repeat.[a]		Repeat. column-to-column[a]		Accuracy[b]	RF (neat solution)[c]	RF (with matrix)[c]
							Intra-day	Inter-day	Intra-day	Inter-day			
Quinic acid	$[M-H]^-$	y = 1087.3x + 3852.7	0.998	8.93	29.76	LOQ–200	0.73	3.25	2.98	4.03	97.71	1102.8	1056.9
	$[M+H]^+$	y = 366x – 866.4	0.983	30.19	100.64	LOQ–66							
Succinic acid	$[M-H]^-$	y = 18.822x + 13.02	0.982	515.83	1719.43	LOQ–100	0.89	4.25	2.25	4.18	101.13	19.1	18.2
Gallic acid	$[M-H]^-$	y = 650.42x + 532.03	0.999	14.93	49.76	LOQ–200	0.98	3.51	3.03	5.25	102.03	666.1	635.4
	$[M+H]^+$	y = 219.23x – 564.07	0.976	50.41	168.02	LOQ–66							
	UV 254nm	y = 2.8939x – 5.3543	0.990	8.95	29.82	LOQ–100							
	UV 280nm	y = 4.1139x – 7.2184	0.990	6.55	21.82								
Pantothenic acid	$[M-H]^-$	y = 1301.01x – 2811	0.998	7.46	24.88	LOQ–200	1.53	3.32	3.15	5.11	97.42	1289.1	1287.7
	$[M+H]^+$	y = 4019.7x – 1844	0.995	2.75	9.16	LOQ–66							
Gentisic acid	$[M-H]^-$	y = 568.85x – 1243.9	0.994	17.07	56.89	LOQ–200	1.61	3.51	3.45	5.03	96.65	570.3	576.4
4-Hydroxybenzoic acid	$[M-H]^-$	y = 50.58x + 236.4	0.990	191.95	639.84	LOQ–200	1.77	4.25	3.67	4.98	98.75	51.1	50.3
Vanillic acid	$[M-H]^-$	y = 93.49x – 528.3	0.991	103.85	346.17	LOQ–200	0.92	4.71	3.83	4.75	99.25	94.1	95.4
	$[M+H]^+$	y = 127.55x – 135.58	0.988	86.64	288.79	LOQ–66							
	UV 254nm	y = 4.2903x – 5.4019	0.991	6.03	20.11	LOQ–100							
	UV 280nm	y = 2.1347x – 3.1089	0.992	12.62	42.06								
Caffeic acid	$[M-H]^-$	y = 1021x – 3434	0.993	9.51	31.70	LOQ–200	1.98	4.11	4.01	4.44	98.36	1018.2	1099.9
	$[M+H]^+$	y = 72.601x – 361.46	0.992	152.21	507.36	LOQ–100							
	UV 254nm	y = 2.208x – 4.2687	0.989	11.73	39.08								
Catechin	$[M-H]^-$	y = 1522.4x + 13496	0.991	6.38	21.26	LOQ–200	2.03	3.53	4.10	5.72	97.45	1513.4	1465.1
	$[M+H]^+$	y = 1955.2x + 26028	0.987	5.65	18.84	LOQ–100							
Isovanillic acid	$[M-H]^-$	y = 58.146x – 505.99	0.981	166.98	556.58	LOQ–200	0.78	2.98	3.18	5.88	98.27	58.1	56.8
	$[M+H]^+$	y = 197.93x + 26.181	0.993	55.83	186.10	LOQ–100							
Chlorogenic acid	$[M-H]^-$	y = 1103.9x – 2265.2	0.991	8.80	29.32	LOQ–100	1.25	3.11	2.32	6.25	98.31	1099.9	1045.2
	$[M+H]^+$	y = 1387.6x + 6402.8	0.987	7.96	26.55	LOQ–66							
Homovanillic acid	$[M-H]^-$	y = 145.3x – 992.5	0.987	66.82	222.73	LOQ–200	1.32	3.34	2.40	4.81	99.22	146.5	1.43.3
	$[M+H]^+$	y = 35.62x – 103.1	0.994	310.23	1034.11	LOQ–100							
Vanillin	$[M-H]^-$	y = 40.474x – 89.268	0.994	239.88	799.60	LOQ–100	2.15	4.05	2.45	7.01	99.54	40.2	39.7
	$[M+H]^+$	y = 91.321x + 691.39	0.991	121.01	403.36								
	UV 254nm	y = 0.894x + 2.9121	0.998	28.96	96.53								
	UV 280nm	y = 3.333x + 11.644	0.997	8.08	26.94								

Table 5.1 (continued)

Compound	Considered signal	Calibration curve	r^2	LOD (ppb)	LOQ (ppb)	Lineal range (ppm)	Repeat.[a]		Repeat. column-to-column[a]		Accuracy[b]	RF (neat solution)[c]	RF (with matrix)[c]
							Intra-day	Inter-day	Intra-day	Inter-day			
Syringic acid	$[M-H]^-$	y = 207.68x – 1376.3	0.993	46.75	155.83	LOQ–200	1.89	3.98	2.66	5.45	98.98	208.1	200.0
	$[M+H]^+$	y = 206.34x – 103.31	0.979	53.56	178.52	LOQ–100							
	UV 254nm	y = 1.6619x – 2.7303	0.990	15.58	51.93								
	UV 280nm	y = 3.1954x – 5.0066	0.989	8.43	28.10								
p-coumaric acid	$[M-H]^-$	y = 481.12x – 1511.3	0.995	20.18	67.27	LOQ–200	1.11	3.66	2.76	5.15	99.35	479.6	456.6
	$[M+H]^+$	y = 73.419x + 156.32	0.978	150.51	501.71	LOQ–100							
	UV 254nm	y = 1.0549x – 1.8649	0.991	24.54	81.81								
	UV 280nm	y = 5.9811x – 9.3464	0.990	4.50	15.01								
Benzoic acid	$[M-H]^-$	y = 13.29x – 99.538	0.985	730.54	2435.14	LOQ–100	1.09	3.52	3.09	5.01	99.05	13.1	12.3
	$[M+H]^+$	y = 422.81x + 4802.8	0.992	26.14	87.12								
	UV 254nm	y = 0.7802x – 3.4450	0.991	33.18	110.61	LOQ–200							
	UV 280nm	y = 0.7033x – 2.5367	0.989	38.30	127.65								
Epicatechin	$[M-H]^-$	y = 1653.1x + 10099	0.993	5.87	19.58	LOQ–200	1.23	3.09	3.71	4.66	97.22	1601.4	1533.3
	$[M+H]^+$	y = 2435.7x + 30861	0.976	4.537	15.12	LOQ–100							
	UV 280nm	y = 0.6939x + 1.6339	0.999	38.82	129.38	LOQ–200							
m-coumaric acid	$[M-H]^-$	y = 811.17x – 1942.8	0.996	11.97	39.90	LOQ–200	1.57	3.22	3.31	4.89	98.26	809.2	788.4
	$[M+H]^+$	y = 63.371x – 89.128	0.976	174.38	581.26	LOQ–100							
	UV 254nm	y = 3.9251x – 5.7353	0.990	6.60	21.99								
	UV 280nm	y = 9.3222x – 14.517	0.989	2.89	9.63								
Ferulic acid	$[M-H]^-$	y = 531.41x – 128.86	0.998	18.27	60.90	LOQ–100	2.03	4.02	2.41	4.89	97.98	525.7	501.2
	$[M+H]^+$	y = 139.76x + 82.382	0.982	79.07	263.56								
	UV 254nm	y = 2.8036x – 18.515	0.989	9.23	30.78	LOQ–200							
	UV 280nm	y = 4.2531x – 7.3956	0.990	6.33	21.11	LOQ–100							
Sinapic acid	$[M-H]^-$	y = 685.74x + 481.35	0.999	14.16	47.19	LOQ–200	1.66	4.25	2.73	5.44	97.76	687.0	652.3
	$[M+H]^+$	y = 211.64x – 207.73	0.982	52.21	174.05	LOQ–100							
o-coumaric acid	$[M-H]^-$	y = 207.29x - 537.33	0.997	46.84	156.13	LOQ–200	1.88	4.12	2.85	5.25	99.03	205.4	195.8
	$[M+H]^+$	y = 64.719x + 89.233	0.977	170.75	569.15	LOQ–100							
trans-cinnamic acid	$[M-H]^-$	y = 32.985x - 347.18	0.984	294.34	981.15	LOQ–200	1.45	3.11	3.53	4.83	101.16	31.6	30.0
	UV 254nm	y = 4.1226x – 4.0496	0.991	6.28	20.93	LOQ–100							
	UV 280nm	y = 8.6274x – 8.0797	0.991	3.12	10.41								

Table 5.1 (continued)

Compound	Considered signal	Calibration curve	r^2	LOD (ppb)	LOQ (ppb)	Lineal range (ppm)	Repeat.[a]		Repeat. column-to-column[a]		Accuracy[b]	RF (neat solution)[c]	RF (with matrix)[c]
							Intra-day	Inter-day	Intra-day	Inter-day			
Abscisic acid	$[M-H]^-$	y = 736.33x + 88.016	0.998	13.19	43.95	LOQ–66	1.53	2.98	4.21	5.26	100.78	744.9	710.2
	$[M+H]^+$	y = 2889.7x - 28291	0.979	3.82	12.75	LOQ–100							
	UV 254nm	y = 5.3665x – 8.448	0.990	4.82	16.08								
	UV 280nm	y = 4.4438x – 7.2174	0.990	6.06	20.20								
Laricitrin	$[M-H]^-$	y = 4100.3x + 10338	0.998	2.37	7.89	LOQ–100	1.71	4.25	4.00	5.56	100.12	4187.2	3988.4
	$[M+H]^+$	y = 12883x + 55835	0.976	0.86	2.86								
	UV 254nm	y = 4.757x – 10.711	0.990	5.44	18.14								
	UV 280nm	y = 1.7488x – 3.9312	0.988	15.40	51.38								
Naringenin	$[M-H]^-$	y = 4213.3x + 7132.4	0.999	2.30	7.68	LOQ–200	1.88	4.74	3.73	5.61	98.05	4177.9	3972.4
	$[M+H]^+$	y = 10671x + 25887	0.994	1.04	3.45	LOQ–100							
	UV 254nm	y = 0.7799x – 5.0205	0.987	33.20	110.65	LOQ–200							
	UV 280nm	y = 3.8320x – 6.4597	0.990	7.03	23.43	LOQ–100							
Chrysin	$[M-H]^-$	y = 5906.1x + 41992	0.997	1.64	5.48	LOQ–200	2.03	3.76	3.38	6.03	98.87	5911.5	5633.6
	$[M+H]^+$	y = 21621x + 29178	0.982	0.51	1.70	LOQ–100							
	UV 254nm	y = 6.2286x – 39.018	0.988	4.16	13.86	LOQ–200							
	UV 280nm	y = 5.0399x – 6.8170	0.989	5.34	17.81	LOQ–100							
Kaempferide	$[M-H]^-$	y = 5350.8x + 29674	0.994	1.82	6.05	LOQ–200	1.51	3.67	2.99	6.25	99.11	5345.2	5100.6
	$[M+H]^+$	y = 19515x + 83845	0.988	0.57	1.89	LOQ–100							
	UV 254nm	y = 4.3294x – 28.654	0.989	5.98	19.93	LOQ–200							
	UV 280nm	y = 2.2766x – 13.794	0.989	11.83	39.44								

[a] RSD values (%) for peak areas corresponding to each analyte; measured from six consecutive injections of each analyte within the same day (intra-) and on three different days (inter-).

[b] The accuracy of the assay is the closeness of the test value obtained to the nominal value. It is calculated by determining trueness and precision. (%RSD).

[c] Response factors were calculated to evaluate the possible matrix effect.

[a, b, c] For calculating the figures included in this tables, MS detection signals (in negative polarity) were used.

5.3.3. Application of the method to the analysis of avocado extracts

Compound identification

Once the method was optimized and validated, its feasibility analyzing avocado extracts of 13 varieties at two ripening degrees was tested. ***Figure 5.1*** shows the typical chromatographic profile of 'Colin V 33' –one of the richest varieties (BPC in positive and negative polarity and the UV signals at 254 and 280 nm).

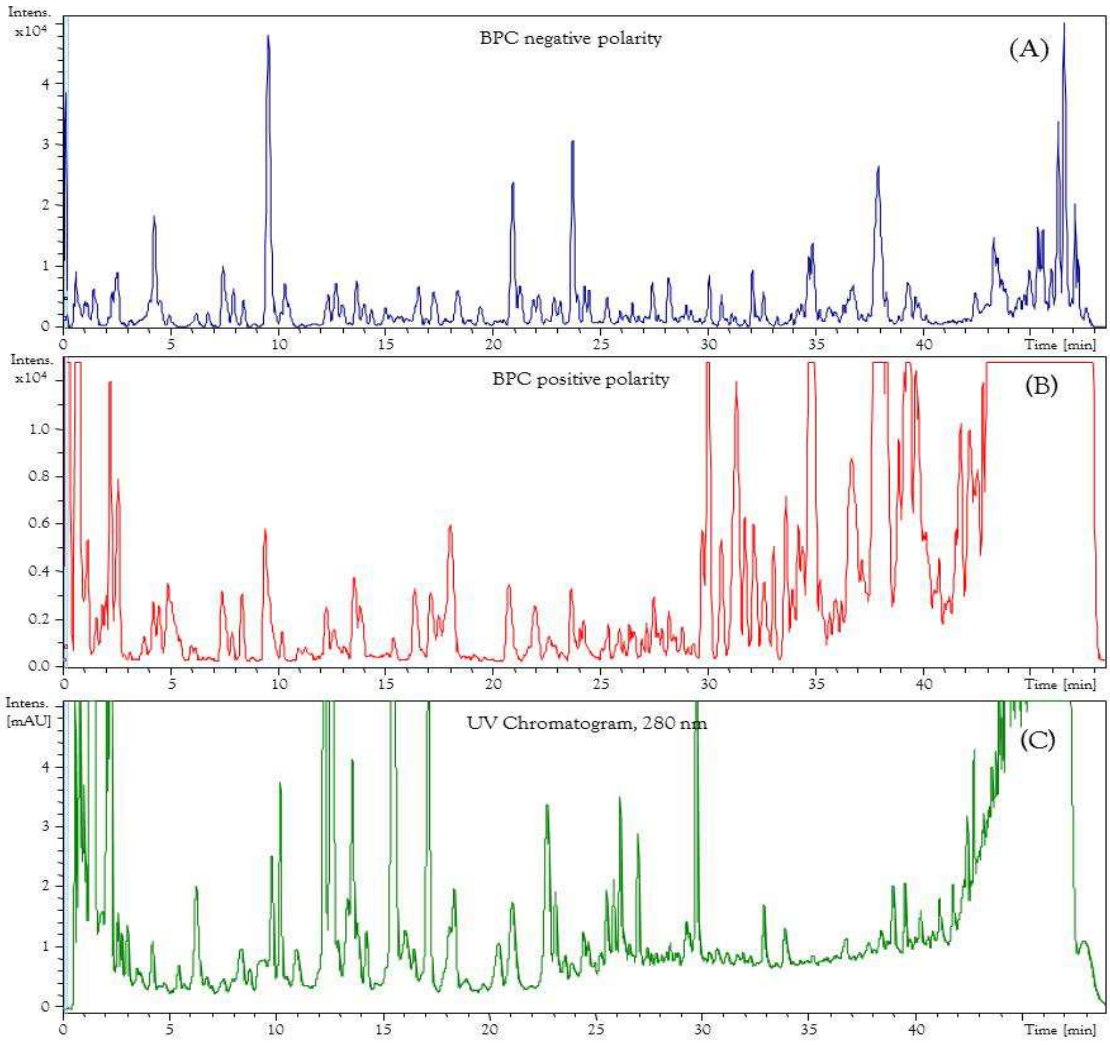

Figure 5.1. Profile obtained for analysis of 'Colin V 33' avocado sample at the second ripening degree under the optimum conditions. In (A) we see the BPC of the sample in negative polarity, (B) BPC in positive polarity MS detection, and (C) at 280 nm.

More than 200 different *m/z* signals have been studied in the methanol extracts of avocado using ESI-TOF MS both in positive and negative ionization modes. A combination of accurate mass and isotopic distribution was used for calculation of theoretical elemental formula of detected metabolites. At this stage, it was observed that

the analyzed avocado extract contained a number of metabolites belonging to different chemical classes: organic acids, sugars, nucleosides, vitamins, isoprenoids, phenolic acids and their glucoside-derivatives, amino acids, flavonoids, fatty acids, plant hormones, etc. We have verified the identity of some compounds using the commercial standards available in-house. For some other metabolites, however, we only provide a tentative identification based on a combination of experimental data, the information previously described in literature [19, 22-29], and open source on-line databases (see material and methods). ***Table 5.2*** shows data on 59 compounds, identity of which has been corroborated most accurately. ***Table 5.2*** in supporting information summarizes compounds with only tentative identity (***Table 5.2. Supporting information***).

It is important to emphasize that substantial variation in metabolic composition of avocado extracts has been observed: some compounds are present in all the varieties under study and some are specific for a particular variety or their ripening degrees. For example, among the 200 *m/z* features studied, 75 were present in all varieties in both ripening degrees (*e.g.* perseitol, quinic acid, succinic acid, uridine, phenylalanine, pantothenic acid, N-acetylphenylalanine, epicatechin, sinapic acid hexose or abscisic acid), whereas sinapic and gallic acid were only present in the variety 'Sir Prize' at its second ripening degree.

Table 5.2. Peak assignment of the metabolites contained in the different avocado samples using UHPLC-ESI-TOF MS.

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
0.56	–	211.0817 $[M-H]^-$	$C_7H_{16}O_7$	2.8	44.5	*Perseitol*	ALL
0.61	193.0699 $[M+H]^+$ 215.0222 $[M+Na]^+$ 231.0617 $[M+K]^+$	191.0551 $[M-H]^-$	$C_7H_{12}O_6$	4.1	8.7	*Quinic acid**	ALL
0.62	–	341.1096 $[M-H]^-$	$C_{12}H_{22}O_{11}$	3.7	36.0	*Sucrose*	ALL
0.64	–	179.0557 $[M-H]^-$	$C_6H_{12}O_6$	2.0	23.7	*Glucose / fructose*	ALL
1.06	193.0649 $[M+H]^+$ 215.1195 $[M+Na]^+$ 230.9716 $[M+K]^+$	191.0191 $[M-H]^-$ 111.0099 $[M-H-44-18-18]^-$	$C_6H_8O_7$	3.5	9.2	*Citric acid / isocitric acid*	G1, G2, HT1, HM1, HM2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, N1, N2, P1, P2, SP1, T1, T2
1.30	119.0440 $[M+H]^+$,	117.0195 $[M-H]^-$	$C_4H_6O_4$	- 1.8	25.4	*Succinic acid**	ALL
1.43	182.0798 $[M+H]^+$ 165.0536 $[M+H-17]^+$	180.0667 $[M-H]^-$ 163.0399 $[M-H-17]^-$	$C_9H_{11}NO_2$	- 0.6	6.9	*Tyrosine*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM2, N1, N2, P1, P2, SP2, T1, T2
1.55	245.0754 $[M+H]^+$ 267.0571 $[M+Na]^+$ 113.0342 $[M+H-132]^+$	243.0610 $[M-H]^-$ 200.0558 $[M-H-43]^-$	$C_9H_{12}N_2O_6$	4.0	13.8	*Uridine*	ALL
1.97	171.0285 $[M+H]^+$ 153.0168 $[M+H-18]^+$	169.0138 $[M-H]^-$	$C_7H_6O_5$	2.4	10.5	*Gallic acid**	SP2
2.12	268.1032 $[M+H]^+$ 136.0612 $[M+H-132]^+$	266.0888 $[M-H]^-$ 533.1779 $[2M-H]^-$ 312.0848 $[M-H+46]^-$ 134.0504 $[M-H-132]^-$	$C_{10}H_{13}N_5O_4$	2.4	25.2	*Adenosine*	ALL
2.69	166.0843 $[M+H]^+$	164.0720 $[M-H]^-$ 147.0469 $[M-H-17]^-$	$C_9H_{11}NO_2$	- 1.3	12.8	*Phenylalanine*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T2
4.46	339.0719 $[M+Na]^+$ 355.0597 $[M+K]^+$	315.0740 $[M-H]^-$	$C_{13}H_{16}O_9$	- 5.7	22.7	*Dihydroxybenzoic acid hexose*	ALL

Table 5.2 (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
4.94	220.1177 $[M+H]^+$ 242.0981 $[M+Na]^+$ 258.0679 $[M+K]^+$ 202.1063 $[M+H-18]^+$ 184.0950 $[M+H-18-18]^+$ 116.0337 $[M+H-18-18-68]^+$ 90.0557 $[M+H-18-18-94]^+$	218.1035 $[M-H]^-$ 146.0825 $[M-H-28-44]^-$	$C_9H_{17}NO_5$	- 0.5	11.1	*Pantothenic acid**	ALL
6.30	205.0969 $[M+H]^+$ 188.0680 $[M+H-17]^+$ 146.0541 $[M+H-18-42]^+$	203.0825 $[M-H]^-$	$C_{11}H_{12}N_2O_2$	0.5	33.3	*Tryptophan*	C1, C2, G1, G2, HT2, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM2, N1, N2, P2, SP2, T1, T2
9.68	455.1515 $[M+Na]^+$ 471.1245 $[M+K]^+$	431.1562 $[M-H]^-$ 477.1599 $[M-H+46]^-$	$C_{19}H_{28}O_{11}$	3.4	2.8	*Benzyl alcohol dihexose I*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, N1, N2, P1, P2, SP1, SP2, T1, T2
9.90	169.0440 $[M+H]^+$ 151.0374 $[M+H-18]^+$	167.0348 $[M-H]^-$	$C_8H_8O_4$	1.3	35.9	*Vanillic acid**	SP2
10.27	629.2398 $[M+Na]^+$ 6452167 $[M+K]^+$	605.2458 $[M-H]^-$ 651.2495 $[M-H+46]^-$ 443.1903 $[M-H-162]^-$	$C_{27}H_{42}O_{15}$	- 1.1	38.2	*Dehydrophaseic acid dihexose*	C1, C2, G1, G2, HM1, HM2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, N1, N2, P1, P2, SP1, SP2, T1, T2
11.11	181.0498 $[M+H]^+$ 163.0376 $[M+H-18]^+$	179.0345 $[M-H]^-$	$C_9H_8O_4$	3.0	56.7	*Caffeic acid**	HT2, LH1, LH2, N1, P2, SP1, SP2, T2
11.45	291.0844 $[M+H]^+$ 313.2735 $[M+Na]^+$ 329.0399 $[M+K]^+$ 165.0528 $[M+H-126]^+$ 139.0374 $^{1,3}A^+$	289.0718 $[M-H]^-$	$C_{15}H_{14}O_6$	0.0	28.1	*Catechin**	G1, C2, HM1, HM2, HT1, HS1, J1-1, J1-2, J2-1, LH1, LH2, MA1, MA2, P1, P2, SP1, SP2, T1, T2
12.32	467.1870 $[M+Na]^+$ 483.1616 $[M+K]^+$ 265.1412 $[M+H-162-18]^+$	443.1914 $[M-H]^-$ 489.1965 $[M-H+46]^-$ 281.1374 $[M-H-162]^-$	$C_{21}H_{32}O_{10}$	2.1	4.9	*Dehydrophaseic acid hexose I*	C1, C2, G1, G2, HM2, HT2, J1-1, J1-2, LH2, MA1, N1, N2, P2, SP2, T2
12.69	349.0878 $[M+Na]^+$ 365.0542 $[M+K]^+$	325.0930 $[M-H]^-$ 651.1945 $[2M-H]^-$ 163.0407 $[M-H-162]^-$ 145.0305 $[M-H-162-18]^-$ 117.0361 $[M-H-162-18-28]^-$	$C_{15}H_{18}O_8$	- 0.2	5.8	*Coumaroyl-hexose / coumaric acid hexose II*	C1, C2, G2, HM1, HM2, HT2, HS2, J1-1, J1-2, LH2, MA1, MA2, N2, P2, SP1, SP2, T2

Table 5.2 (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
12.71	355.1019 $[M+H]^+$ 377.0824 $[M+Na]^+$ 393.0578 $[M+K]^+$ 163.0377 $[M+H-192]^+$	353.0885 $[M-H]^-$ 191.0340 $[M-H-162^f]^-$	$C_{16}H_{18}O_9$	- 1.8	41.7	*Chlorogenic acid**	C2, G2, HS1, J1-1, J2-1, LH1, LH2, P1, P2, SP1, SP2, T1
13.06	153.0464 $[M+H]^+$ 125.0575 $[M+H-18]^+$	151.0399 $[M-H]^-$	$C_8H_8O_4$	1.0	50.1	*Vanillin**	C2, SP2
13.65	467.1863 $[M+Na]^+$ 4831562 $[M+K]^+$ 265.1420 $[M+H-162-18]^+$	443.1915 $[M-H]^-$	$C_{21}H_{32}O_{10}$	1.8	3.7	*Dehydrophaseic acid hexose II*	ALL
13.87	349.0803 $[M+Na]^+$ 365.0596 $[M+K]^+$	325.0939 $[M-H]^-$ 145.0305 $[M-H-162-18]^-$	$C_{15}H_{18}O_8$	- 3.2	16.9	*Coumaroyl-hexose / coumaric acid hexose II*	C1, C2, G2, HM1, HM2, HT2, HS2, J1-1, J1-2, LH2, MA2, N2, P2, SP2, T2
13.88	199.0536 $[M+H]^+$ 221.0442 $[M+Na]^+$ 237.0041 $[M+K]^+$ 181.0480 $[M+H-18]^+$ 155.0663 $[M+H-44]^+$	197.0460 $[M-H]^-$ 182.1967 $[M-13]^-$	$C_9H_{10}O_5$	- 2.2	46.8	*Syringic acid**	C2, N1, N2, SP2
14.00	455.1501 $[M+Na]^+$ 471.1248 $[M+K]^+$	431.1555 $[M-H]^-$	$C_{19}H_{28}O_{11}$	1.7	6.4	*Benzyl alcohol dihexose II*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, N1, N2, P1, P2, SP1, SP2, T1, T2
14.32	451.1878 $[M+H]^+$ 473.1091 $[M+Na]^+$	449.1109 $[M-H]^-$	$C_{21}H_{22}O_{11}$	- 4.3	23.1	*Eriodictyol hexose / Macrocarposide / Okanin hexose ...*	C1, C2, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, N1, N2, P2, SP1, SP2, T1, T2
15.53	165.0478 $[M+H]^+$ 187.1623 $[M+Na]^+$ 203.0073 $[M+K]^+$ 147.0514 $[M+H-18]^+$ 119.0440 $[M+H-18-28]^+$	163.0401 $[M-H]^-$ 119.0529 $[M-H-44]^-$	$C_9H_8O_3$	0.4	6.0	*p-Coumaric acid**	C1, C2, G2, HM2,HT2, HS2, J1-2, J2-2, LH2, MA2, N2, P2, SP2, T2
16.10	379.1032 $[M+Na]^+$ 395.0710 $[M+K]^+$ 195.0669 $[M+H-162]^+$ 177.0474 $[M+H-162-18]^+$	355.1029 $[M-H]^-$	$C_{16}H_{20}O_9$	1.4	19.1	*Ferulic acid hexose I*	C1, C2, G2, HM2, HT2, HS2, J1-1, J1-2, J2-2, LH2, MA2, N2, P2, SP1, SP2, T2

Table 5.2 (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
16.14	409.1102 $[M+Na]^+$ 425.0838 $[M+K]^+$	385.1128 $[M-H]^-$	$C_{17}H_{22}O_{10}$	1.2	19.0	*Sinapoyl-hexose / sinapic acid hexose I*	C1, C2, G1, G2, HT1, J1-2, LH2, MA1, MA2, N1, N2, P2, SP1, SP2, T1
16.37	120.0798 $[M+H-88]^+$	206.0821 $[M-H]^-$ 164.0719 $[M-H-42]^-$ 147.0495 $[M-H-42-17]^-$	$C_{11}H_{13}NO_3$	0.7	9.7	*N-acetylphenylalanine*	ALL
16.89	425.1405 $[M+Na]^+$ 441.1135 $[M+K]^+$	401.1453 $[M-H]^-$ 447.1534 $[M-H+46]^-$	$C_{18}H_{26}O_{10}$	0.2	23.8	*Benzyl alcohol hexose-pentose*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA2, N1, N2, P1, P2, SP1, SP2, T1, T2
17.21	305.1293 $[M+Na]^+$ 265.1405 $[M+H-18]^+$	281.1392 $[M-H]^-$ 237.1489 $[M-H-44]^-$	$C_{15}H_{22}O_5$	0.7	15.0	*Dehydrophaseic acid*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS2, J1-1, J1-2, J2-1, J2-2, LH1, MA1, N1, N2, P1, P2, SP1, SP2, T1, T2
17.28	379.0988 $[M+Na]^+$ 395.0742 $[M+K]^+$ 195.0622 $[M+H-162]^+$ 177.540 $[M+H-162-18]^+$	355.1048 $[M-H]^-$	$C_{16}H_{20}O_9$	0.9	18.9	*Ferulic acid hexose II*	C1, C2, G2, HM2, HT2, HS2, J1-1, J1-2, J2-2, LH2, MA2, N2, P2, SP2, T2
17.63	123.0441 $[M+H]^+$	121.0291 $[M-H]^-$	$C_7H_6O_2$	3.2	43.9	*Benzoic acid**	C1, C2, G1, HM1, HM2, HT1, HS1, HS2, J1-1, J1-2, J2-1, LH1, LH2, N1, P1, P2, SP1, SP2, T1, T2
17.70	291.0850 $[M+H]^+$ 313.0669 $[M+Na]^+$ 329.0386 $[M+K]^+$ 165.0565 $[M+H-126]^+$ 139.0374 $^{1,3}A^+$	289.0714 $[M-H]^-$	$C_{15}H_{14}O_6$	3.4	6.0	*Epicatechin**	ALL
17.93	495.1401 $[M+Na]^+$ 511.1279 $[M+K]^+$ 147.0677 $[M+H-308-18]^+$	471.1514 $[M-H]^-$ 943.4374 $[2M-H]^-$	$C_{21}H_{28}O_{12}$	- 1.3	33.6	*o-Coumaric acid rhamnosyl-hexose / o-coumaric acid coumaroyl-hexose I*	C1, C2, G2, HM1, HM2, HT1, HT2, HS2, J1-1, J1-2, J2-2, LH2, MA1, MA2, N1, N2, P2, SP1, SP2, T1, T2
18.35	409.1677 $[M+Na]^+$ 425.1314 $[M+K]^+$	385.1145 $[M-H]^-$	$C_{17}H_{22}O_{10}$	- 1.4	18.7	*Sinapoyl-hexose / sinapic acid hexose II*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, P2, SP1, SP2, T1, T2

Table 5.2 (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
18.40	473.9953 $[M+H]^+$ 495.1450 $[M+Na]^+$ 511.1250 $[M+K]^+$ 165.0505 $[M+H-308]^+$ 147.0431 $[M+H-308-18]^+$	471.1496 $[M-H]^-$ 943.3266 $[2M-H]^-$ 163.0371 $[M-H-308]^-$	$C_{21}H_{28}O_{12}$	2.6	8.9	*o-Coumaric acid rhamnosyl-hexose / o-coumaric acid coumaroyl-hexose II*	C1, C2, G1, G2, HT1, HT2, HM2, HS2, J1-2, J2-1, J2-2, LH2, MA2, N2, P2, SP2, T2
19.38	409.1114 $[M+Na]^+$ 425.0780 $[M+K]^+$	385.1131 $[M-H]^-$	$C_{17}H_{22}O_{10}$	3.7	11.4	*Sinapoyl-hexose / sinapic acid hexose III*	ALL
20.61	195.0943 $[M-H]^+$ 233.0067 $[M+K]^+$ 177.0532 $[M+H-18]^+$ 145.0247 $[M+H-50]^+$	193.0499 $[M-H]^-$ 178.0231 $[M-H-15]^-$	$C_{10}H_{10}O_4$	3.6	32.9	*Ferulic acid**	C1, G2, HM2, HT2, HS2, J1-1, J1-2, J2-2, LH2, MA2, N2, P2, SP2, T2
22.98	413.1155 $[M+H]^+$ 435.0879 $[M+Na]^+$ 451.0594 $[M+K]^+$ 395.0952 $[M+H-18]^+$ 165.0535 $[M+H-162-86]^+$ 147.0445 $[M+H-86-162-18]^+$	411.0922 $[M-H]^-$ 823.1929 $[2M-H]^-$ 367.1029 $[M-H-44]^-$ 205.0470 $[M-H-44-162]^-$ 163.0390 $[M-H-162-86]^-$	$C_{18}H_{20}O_{11}$	2.7	25.0	*Coumaric acid malonyl-hexose I*	G1, G2, LH2, N1, N2, P2
23.03	435.0883 $[M+H]^+$	433.0779 $[M-H]^-$	$C_{20}H_{18}O_{11}$	- 0.7	44.1	*Qercetin pentose / hydroxyluteolin pentose /herbacetin pentose…*	G1, G2, HM1, HM2, HT1, HT2, HS1, J1-1, J2-1, MA1, MA2, N1, N2, P1, P2, SP1, SP2, T1, T2
23.31	225.1450 $[M+H]^+$ 207.0645 $[M+H-18-32]^+$ 147.0469 $[M+H-18-60]^+$ 119.0470 $[M+H-78-28]^+$	223.0608 $[M-H]^-$	$C_{11}H_{12}O_5$	1.8	29.4	*Sinapic acid**	G2, HM2, SP2
24.07	435.0881 $[M+Na]^+$ 451.0622 $[M+K]^+$ 395.0952 $[M+H-18]^+$	411.0911 $[M-H]^-$ 823.2973 $[2M-H]^-$ 367.1023 $[M-H-44]^-$	$C_{18}H_{20}O_{11}$	5.3	21.5	*Coumaric acid malonyl-hexose II*	C2, G2, HM2, HT2, HS2, J1-2, J2-2, LH2, MA2, N2, SP2, T2
25.87	449.1767 $[M+Na]^+$	425.2253 $[M-H]^-$ 263.1362 $[M-H-162]^-$	$C_{21}H_{30}O_9$	- 2.6	36.3	*Abscisic acid hexose ester*	C1, C2, G1, G2, HT1, HT2, HS2, J1-1, J1-2, J2-2, LH1, LH2, MA1, MA2, N2, P1, P2, SP1, SP2, T2
26.39	–	287.0556 $[M-H]^-$	$C_{15}H_{12}O_6$	1.8	39.3	*Eriodictyol / dihydrokaempferol / tetrahydroxyflavanone*	ALL
29.37	201.1263 $[M+Na]^+$	177.0549 $[M-H]^-$ 533.2239 $[3M-H]^-$	$C_{10}H_{10}O_3$	4.5	6.6	*Methoxycinnamic acid*	C1, C2, G2, HT2, HM1, HM2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA2, N2, SP2, T2

Table 5.2 (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		Assigment[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
29.75	265.1403 $[M+H]^+$ 287.1235 $[M+Na]^+$ 303.0952 $[M+K]^+$ 247.1306 $[M+H-18]^+$ 229.1199 $[M+H-18-18]^+$ 201.1271 $[247-46]^+$ 187.1048 $[247-60]^+$ 163.0769 $[M+H-102]^+$ 135.0875 $[16G28]^+$	263.1286 $[M-H]^-$ 219.1409 $[M-H-44]^-$ 153.0931 $[M-H-44-66]^-$	$C_{15}H_{20}O_4$	1.0	38.4	*Abscisic acid**	ALL
31.34	333.0587 $[M+H]^+$	331.0452 $[M-H]^-$	$C_{16}H_{12}O_8$	2.2	19.0	*Laricitrin**	C2, P2, SP2
32.08	273.0745 $[M+H]^+$ 153.0194 $^{1,3}A^+$	271.0602 $[M-H]^-$	$C_{15}H_{12}O_5$	3.8	21.1	*Naringenin**	C2, P2, SP2
36.25	327.2686 $[M+Na]^+$ 343.1723 $[M+K]^+$	303.2178 $[M-H]^-$	$C_{15}H_{12}O_7$	- 0.3	16.2	*Trihydroxypalmitic acid*	C1, C2, G1, G2, HM1, HM2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, N1, N2, P1, P2, SP1, SP2, T1, T2
36.44	353.2268 $[M+Na]^+$	329.2322 $[M-H]^-$	$C_{18}H_{34}O_5$	3.4	15.0	*Trihydroxyoctadecenoic acid*	ALL
38.37	255.0634 $[M+H]^+$	253.0499 $[M-H]^-$	$C_{15}H_{10}O_4$	2.8	18.9	*Chrysin**	C2, P2, SP2
39.78	301.0688 $[M+H]^+$	299.0553 $[M-H]^-$	$C_{16}H_{12}O_6$	2.7	26.6	*Kaempferide**	C2, P2, SP2
45.39	–	295.2272 $[M-H]^-$	$C_{18}H_{32}O_3$	2.4	10.1	*Hydroxylinoleic acid / hydroxyoctadecadienoic acid I...*	ALL
46.49	–	295.2275 $[M-H]^-$	$C_{18}H_{32}O_3$	1.3	25.3	*Hydroxylinoleic acid / hydroxyoctadecadienoic acid II...*	C1, C2, G1, G2, HM2, HT2, HS1, HS2, J1-1, J1-2, J2-1,LH1, LH2, MA1, MA2, N2, P1, P2, SP1, SP2, T2
46.94	277.2454 $[M+H]^+$ 299.2779 $[M+Na]^+$ 315.2838 $[M+K]^+$	275.2373 $[M-H]^-$	$C_{19}H_{32}O$	2.8	12.2	*2-(Pentadecyl)-furan*	C1, C2, G1, G2, HM1, HM2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, N1, N2, P1, P2, SP1, SP2, T1, T2
47.14	–	277.2173 $[M-H]^-$	$C_{18}H_{30}O_2$	- 4.7	4.1	*Linolenic acid*	G2, MA2

[a] Average value. RSD of 7 % for the retention time.

[b] For many compounds, different *m/z* values rather than $[M+H]^+$ /$[M-H]^-$ have been detected in the MS spectra; when those ions were more intense than the corresponding $[M+H]^+$ /$[M-H]^-$, they have been underlined. The mentioned different *m/z* values mainly correspond to in-source fragments (typical losses detected have been -17 (NH_3), -18 (H_2O), -28 (CO), -42 (C_2H_2O), -43 (CHNO), -44 (CO_2), -132 (pentose), -162 (hexose)) and to sodium $[M+23]^+$ and potassium $[M+49]^+$ or formic acid $[M-H+46]^-$ adducts, in the positive and negative polarities, respectively.

[c] Accepted values of error and mSigma have been in general 5 ppm and 50, respectively, with the exception of some compounds with very low intensities or coeluting with 1 or 2 a.m.u. of difference.

[d] (I, II) different isomers; (*) identification confirmed by comparison with authentic standards.

[e] C: Colin V 33, G: Gem, HM: Hass Motril, HT: Harvest, HS: Hass, J1: Jiménez1, J2: Jiménez 2, LH: Lamb Hass, MA: Marvel, N: Nobel, P: Pinkerton, SP: Sir Prize, T: Tacambaro. Numbers 1 and 2 correspond to the 1st or 2nd ripening degree, respectively.

[f] In this case, loss of 162 does not correspond to an hexose, but to the caffeic acid moiety ($M-H_2O$).

Quantification of avocado metabolites

The quantitative results are presented in ***Table 5.3***. One of the objectives that we had was to compare the quantitative content (in terms of phenolic acids and related compounds, quinic acid, succinic acid, pantothenic acid, abscisic acid, and flavonoids) of 13 different avocado varieties just after being harvested. This fact can be very interesting since the varieties included in this study are quite unique (most of them are not available commercially and 5 of them are reported mutants of 'Hass' [32]) and the quantitative available information about this fraction of the avocado fruit is still very limited. Our second aim was to evaluate the quantitative evolution of the mentioned compounds and to quantify them in fruits ripe enough for consumption. Fruit maturity and harvesting time are determined according to external markers (color and size) or by measuring dry matter and oil content in the flesh [33]. Moreover, it is well-known that the ripeness degree of climacteric fruits (fruits that have high respiration rate during fruit ripening), such as avocado, can be correlated with rheological properties. The phenolic content of fruits is also affected by the degree of maturity. However, very little is known about the postharvest changes that the avocado fruit suffers and their relationship with the composition in terms of pseudo-polar metabolites; indeed, there is only a similar study focused on fatty acids [20].

The quantitative results, when possible, were obtained for MS in negative and positive polarity and UV detection (at 254 and 280 nm) to compare the results. In general, good agreement was found among the determined content using the four different signals (no statistical differences were found using ANOVA). ***Table 5.3***, unless it is specified, only shows the results achieved in negative polarity (to contain the size of the table).

Quinic acid was found in all the samples studied, although its concentration was higher in fruits from 'Colin V 33' and 'Pinkerton'. No trend related with the ripening degree was observed regarding the concentration of this analyte. Succinic acid was another of the metabolites detected in every sample, although its concentration was always higher at the second ripening degree regardless the cultivar. Again fruits of 'Colin V 33' had the highest amount of this compound. Gallic and vanillic acids were only found in Sir Prize samples at the second ripening degree, suggesting that these two

compounds are synthesized during ripening after harvest. Something similar was observed for vanillin and syringic acid, although in this case, they were present not only in fruits of Sir Prize, but also in fruits of 'Colin V 33' (at the second ripening degree); syringic acid was also determined in fruits of 'Nobel', where it was present at both ripening degrees. Caffeic acid was absent in fruits of 7 of the varieties under study, whilst catechin was only absent in fruits of 'Nobel'. Chlorogenic acid could not be detected (in ripe or unripe stages) in fruits of 'Harvest', 'Marvel', 'Hass Motril', and 'Nobel'. The cases of *p*-coumaric and ferulic acids were quite interesting, since it was possible to see a clear trend in their concentration related to ripening degree. In most cases, they were not detected at the first ripening degree (when the samples were just handpicked from the trees), however, after some days (when the fruits were ripe enough to be eaten), their concentration had increased considerably. The only case which did not follow this behaviour was 'Colin V 33'. Epicatechin was found in all the analyzed samples, although its concentration was higher for fruits of 'Sir Prize'. Abscisic acid and pantothenic acid -some of the analytes included in this study which have been never quantified before in avocado- were found in every sample. A possible correlation can be found between the amount of abscisic acid in the fruit and the ripening degree. The two *O*-methylated flavonols (laricitrin and kaempferide), the flavone (chrysin) and the flavanone (naringenin) were only detected in fruits of 'Colin V 33', 'Pinkerton' and 'Sir Prize' at the second ripening degree. Fruits of 'Sir Prize' at the second ripening degree were the richest in terms of these compounds, with amounts between 8.61 mg/kg for laricitrin and 9.15 mg/kg for kaempferide. ***Figure 5.2*** illustrates the differences found for one of the cultivars analyzed regarding the ripening degree. 'Hass' was chosen because in this variety the differences between the two ripening degrees were easily observed.

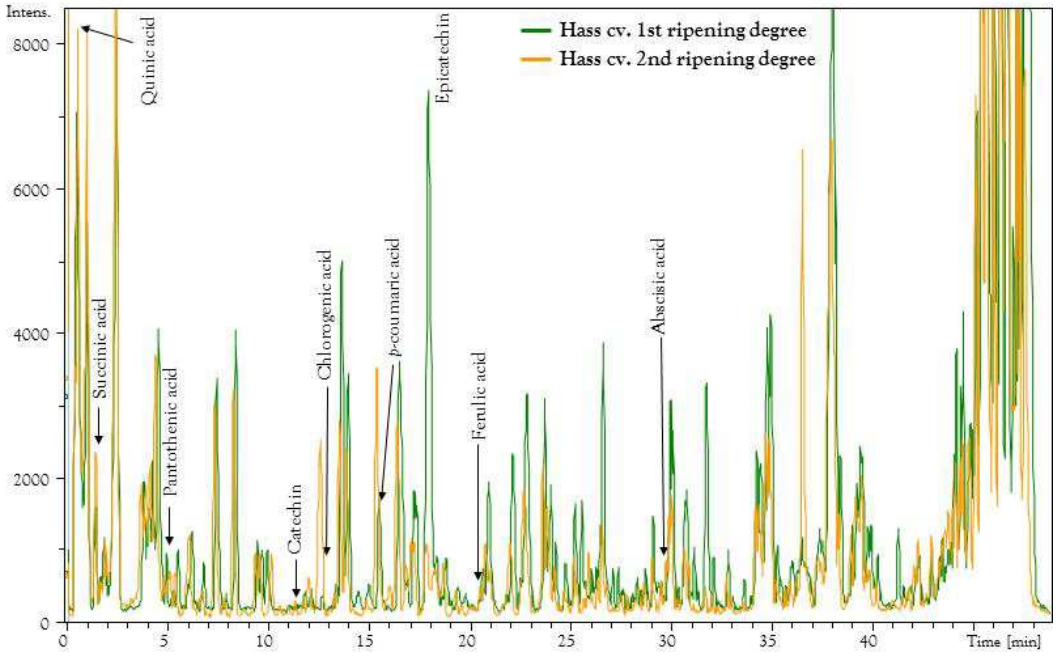

Figure 5.2. Comparison of the UHPLC-ESI-TOF MS (in negative polarity) profiles achieved by using the optimum chromatographic and MS conditions for Hass avocado *cv.* at two different ripening degrees.

It is difficult to compare our results with those obtained in previous works such as that of Golukcu and co-workers [28] because they used three different avocado cultivars (only one of the cultivars that we studied was included in their study ('Hass')); apart from that, they are expressing the values considering the weight of the edible part of avocado fruit taken at the beginning of the extraction procedure (not the weight of the dry sample); moreover, it is not specified in terms of which standards the quantification was carried out.

Tabla 5.3. Quantitative results (mg of analyte/kg of dry sample) of the different kinds of avocado cultivars analyzed by UHPLC-ESI (–)-TOF MS method.

Compound	Colin V 33		Gem		Harvest		Hass		Jiménez 1		Jiménez 2		Lamb Hass	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Quinic acid	18.95	12.92	1.61	3.09	11.64	10.42	1.25	1.80	2.21	2.35	1.67	1.68	8.11	13.90
Succinic acid	171.09	176.88	37.06	60.83	24.31	161.21	25.42	47.29	16.18	57.60	45.17	77.20	29.87	97.34
Gallic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Pantothenic acid	4.00	4.87	3.16	3.59	2.51	3.74	2.33	1.90	3.34	4.37	4.56	3.59	2.29	3.60
Vanillic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Caffeic acid	n.d	n.d	n.d	n.d	n.d	0.46	n.d	n.d	n.d	n.d	n.d	n.d	0.88	0.54
Catechin	n.d	n.q	0.15	n.d	0.55	n.d	0.37	n.d	0.34	0.15	0.09	n.d	n.q	0.37
Chlorogenic acid	n.d	n.q	n.d	n.q	n.d	n.d	1.74	n.d	0.91	n.d	0.77	n.d	n.q	1.67
Vanillin	n.d	2.10	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Syringic acid	n.d	6.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
p-coumaric acid	6.58	5.42	n.d	33.58	n.d	33.78	n.d	8.39	n.d	7.61	n.d	7.86	n.d	15.48
Benzoic acid*	n.d	3.93	1.20	n.d	1.24	n.d	9.85	1.14	4.11	2.46	1.75	n.d	5.09	6.01
Epicatechin	0.86	n.q	2.60	0.28	0.95	0.60	16.55	1.85	8.80	16.99	4.05	0.39	8.81	15.35
Ferulic acid	n.q	n.d	n.d	4.02	n.d	3.52	n.d	1.67	n.d	1.37	n.d	0.57	n.d	2.36
Sinapic acid	n.d	n.d	n.d	0.67	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Abscisic acid	4.24	3.62	0.96	2.66	1.67	9.94	n.q	2.62	1.00	7.03	1.27	2.48	5.68	10.49
Laricitrin	n.d	3.73	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Naringenin	n.d	4.32	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Chrysin	n.d	4.52	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
Kaempferide	n.d	4.69	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d

Table 5.3 (continued)

Compound	Marvel		Hass Motril		Nobel		Pinkerton		Sir Prize		Tacambaro	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Quinic acid	3.25	0.75	0.70	1.35	1.25	1.10	13.71	17.11	2.73	1.58	2.44	2.00
Succinic acid	38.43	117.64	27.60	81.51	23.50	101.06	27.34	66.70	33.85	166.74	29.43	85.12
Gallic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	8.61	n.d	n.d
Pantothenic acid	2.06	2.82	2.35	2.95	3.62	4.59	1.66	2.68	4.08	6.14	2.61	3.45
Vanillic acid	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	6.80	n.d	n.d
Caffeic acid	n.d	n.d	n.d	n.d	n.q	n.d	n.d	0.58	0.55	0.57	n.d	0.27
Catechin	n.q	n.q	0.12	0.02	n.d	n.d	0.79	0.43	0.62	0.21	0.40	0.11
Chlorogenic acid	n.d	n.d	n.d	n.d	n.d	n.d	2.20	1.25	1.17	0.88	n.q	n.d
Vanillin	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	6.28	n.d	n.d
Syringic acid	n.d	n.d	n.d	n.d	3.84	3.12	n.d	n.d	n.d	12.17	n.d	n.d
p-coumaric acid	n.d	22.95	n.d	8.12	n.d	8.76	n.d	5.39	n.d	27.76	n.d	6.61
Benzoic acid*	n.d	n.d	1.48	0.61	0.84	n.d	6.66	3.96	15.78	8.31	8.83	4.34
Epicatechin	0.43	0.20	3.95	4.59	0.23	n.d	10.90	7.56	26.72	1.84	15.34	9.49
Ferulic acid	n.d	4.29	n.d	1.39	n.d	0.53	n.d	0.85	n.d	4.85	n.d	1.01
Sinapic acid	n.d	n.d	n.d	0.30	n.d	n.d	n.d	n.d	n.d	0.75	n.d	n.d
Abscisic acid	0.56	4.94	0.47	2.25	n.q	2.90	0.92	2.00	2.81	6.74	0.63	3.09
Laricitrin	n.d	n.d	n.d	n.d	n.d	n.d	n.d	2.99	n.d	8.61	n.d	n.d
Naringenin	n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.25	n.d	8.66	n.d	n.d
Chrysin	n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.18	n.d	8.93	n.d	n.d
Kaempferide	n.d	n.d	n.d	n.d	n.d	n.d	n.d	3.32	n.d	9.15	n.d	n.d

Exploratory analysis of avocado extracts

Above we have tried to address the changes in the metabolic composition of avocado fruits associated with a ripening process using in depth analysis of the chromatograms, structural assignment of the metabolites and followed by the targeted quantitative analysis. We have shown that concentration of some metabolites demonstrates significant fluctuations depending on the ripening stage. However, to get insight into a ripening process and obtain an overview of metabolites changing most significantly between ripening degrees we had to use a different approach, namely multivariate analysis. To this end, we have applied an unsupervised multivariate method – PCA–. ***Figure 5.3(A)*** shows a score plot of PCA model. It is evident that the difference in the ripening degree is the most important source of the variability in the data. The second component reflects the changes between avocado fruits varieties. It is noteworthy to mention that the varieties 'Jiménez 1', 'Jiménez 2', 'Tacambaro' and 'Hass Motril' are clustered in the same area of the plot. All of them are as the mutants of Hass variety [32], which is also quite close to them. On the other hand, 'Sir Prize' and 'Colin V 33' –two varieties with the most differences in the metabolic composition of the fruit extract between first and the second ripening degrees –demonstrate the largest degree of separation in the first principle component.

To corroborate further on the metabolic changes associated with ripening, we have built a two class PLS-DA model using the degree of ripening as class characteristic. ***Figure 5.3(B)*** represents a cross-validated score plot of PLS-DA model (Q2 (cum) 0.803, R2Y (cum) 0.935). The features, most influential for the model, can be selected on the basis of VIP scores. ***Table 5.4*** summarizes all discriminative features with VIP values >1.5. Taking advantage of the intrinsic properties of UHR-TOF instrument as high mass accuracy, a precise isotopic distribution, using occasionally in-source or/and in-funnel fragmentations, we have provided a tentative assignment of those features as, for instance, adenosine; benzyl alcohol dihexose; 3,6-dihydroxy-3-methyl-6-(1-methylethyl)cyclohexane-1,2-diyl diacetate; and butyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside. The experimental work required for the final confirmation of the structures is currently ongoing in our laboratory.

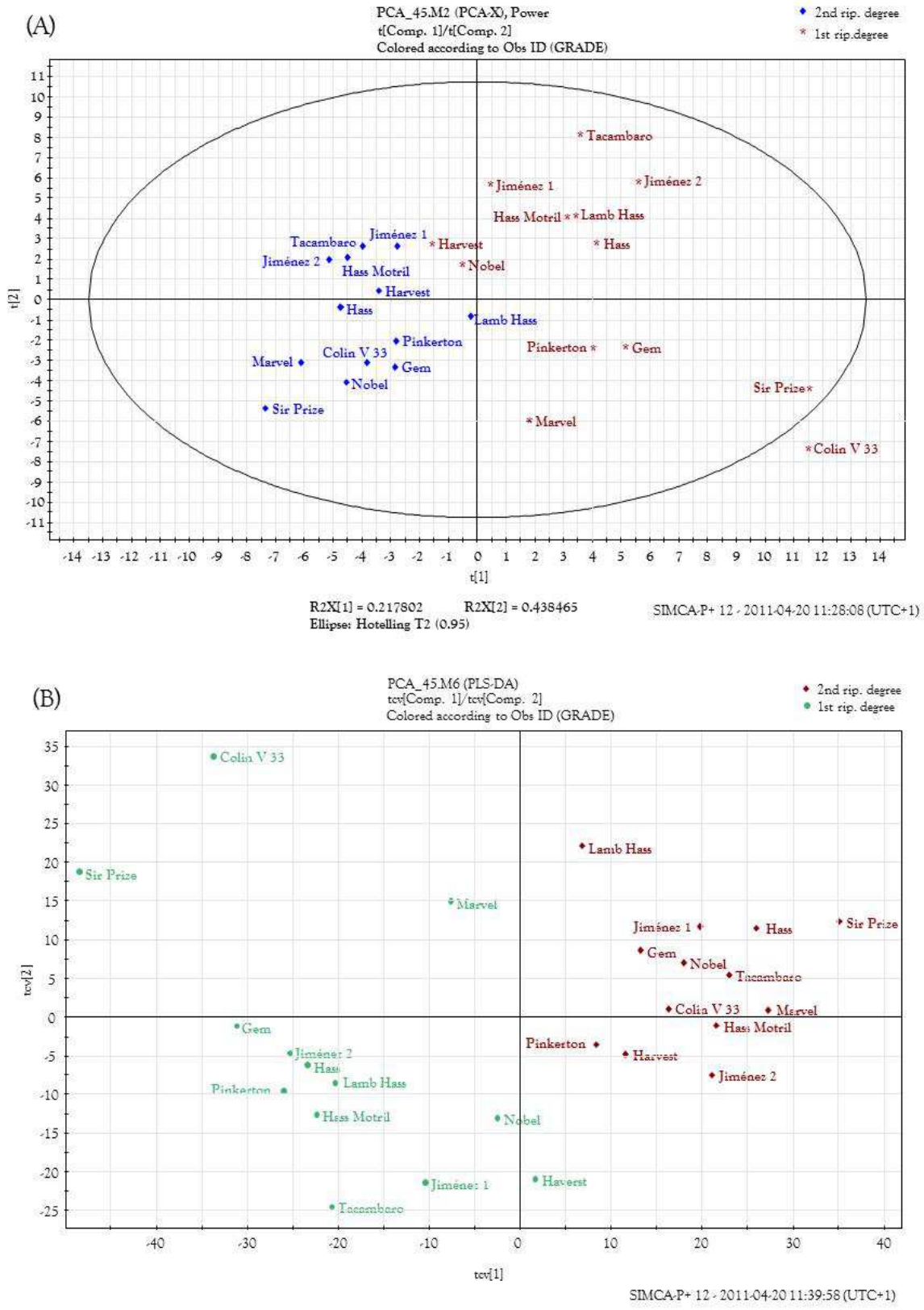

Figure 5.3. (A) PCA and (B) PLS-DA modelling of UHPLC-ESI-TOF MS data. Samples are colored according to ripening degree.

Tabla 5.4. *m/z* values and retention times of the possible classifiers (decreasing order of VIP score) of avocado variety and ripening degree obtained by statistical analysis (PLS-DA).

PLS-DA classifiers						
t_r (min)	**Classifiers signal in (+)[a]**		**Formula**	**Assignment**	**Classifiers signal in (–)[a]**	
	$[M-H]^+$	**Other signals in (+)**			**$[M-H]^-$**	**Other signals in (–)**
29.68	<u>311.1449</u> $[M+Na]^+$	211 $[M+H-18-60]^+$ 427 $[M+K]^+$ 193 $[M+H-78-18]^+$ 175 $[M+H-78-18-18]^+$ 157 $[M+H-78-18-18-18]^+$ 111 $[157-46]^+$	$C_{14}H_{24}O_6$	*3,6-dihydroxy-3-methyl-6-(1-methylethyl)cyclohexane-1,2-diyl diacetate / Bis(2-(hydroxymethyl)butyl) 2-butenedioate / D-Galactono-1,4-lactone, 5,6-O-octylidene*	287.1493	269 $[M-H-18]^-$ 227 $[M-H-60\ (18+42)]^-$ 209 $[M-H-60-18]^-$ 185 $[M-H-60-42]^-$
7.47	437.1404 $[M+K]^+$	<u>421</u> $[M+Na]^+$ 399 $[M+H]^+$	$C_{16}H_{30}O_{11}$	*butyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside / tert-butyl 4-O-β-D-galactopyranosyl-β-D-glucopyranoside / 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioic acid*	397.1715	<u>443</u> $[M-H+46]^-$
17.48	457.1471 $[M+K]^+$	419 $[M+H]^+$ <u>441</u> $[M+Na]^+$	$C_{19}H_{30}O_{10}$	*9-[3-(3,6,6a-trihydroxy-5-oxo-3,3a-dihydro-2H-furo[3,2-b]furan-6-yl)-5-hydroxyoxolan-2-yl]nonanoic acid*	417.1787	<u>463</u> $[M-H+46]^-$
1.91	268.1040 $[M+H]^+$	<u>136</u> $[268-132]^+$	$C_{10}H_{13}N_5O_4$	*Adenosine*	266.0888	533 $[2M-H]^-$ 312 $[M-H+46]^-$ <u>134</u> $[M-H-132]^-$
13.23	471.1263 $[M+K]^+$	<u>455</u> $[M+Na]^+$ 427 441 450	$C_{19}H_{28}O_{11}$	*Benzyl alcohol dihexose*	431.1555	–
24.81	<u>269.1359</u> $[M+Na]^+$	309, 285 $[M+K]^+$ 211 $[M+H-18-18]^+$ 193 $[M+H-18-18-18]^+$	$C_{12}H_{22}O_5$	*(1S)-3-ethyl-3,4,5-trihydroxy-2,2-dimethylcyclohexyl acetate / Hydroxydodecanedioic acid / dimethyl 3-hydroxydecanedioate / N-ethoxyacetylglycine ...*	245.1388	227 $[M-H-18]^-$ 209 $[M-H-18-18]^-$ 185 $[M-H-18-42]^-$
15.96	480.2028	<u>485</u> $[M+Na]^+$ 501 $[M+K]^+$	$C_{20}H_{30}O_{12}$	*2-(3,4-dihydroxyphenyl)ethyl 3-O-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside / 4-[(α-D-mannopyranosyloxy)methyl]benzyl α-D-mannopyranoside*	461.1653	439 393 283 207

5.4. Conclusions

An UHPLC-UV/ESI-TOF MS has been developed for the qualitative and quantitative determination of the metabolites present in the methanolic avocado extracts. A complete validation of the method was carried with respect to the linearity, sensitivity, precision, accuracy and matrix effects. The method was then applied to the analysis of 13 varieties of avocado at two different ripening degrees, and a number of compounds have been identified and quantified with available analytical standards. Moreover, using an exploratory approach based on a combination of unsupervised and supervised methods of multivariate statistics, we have pinpointed a set of compounds differentially regulated during the ripening process. Considering the encouraging results obtained in this study, the application of UHPLC-ESI-TOF MS in food metabolomics for classification of avocado samples and other purposes seems to be a very promising approach.

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Supporting information

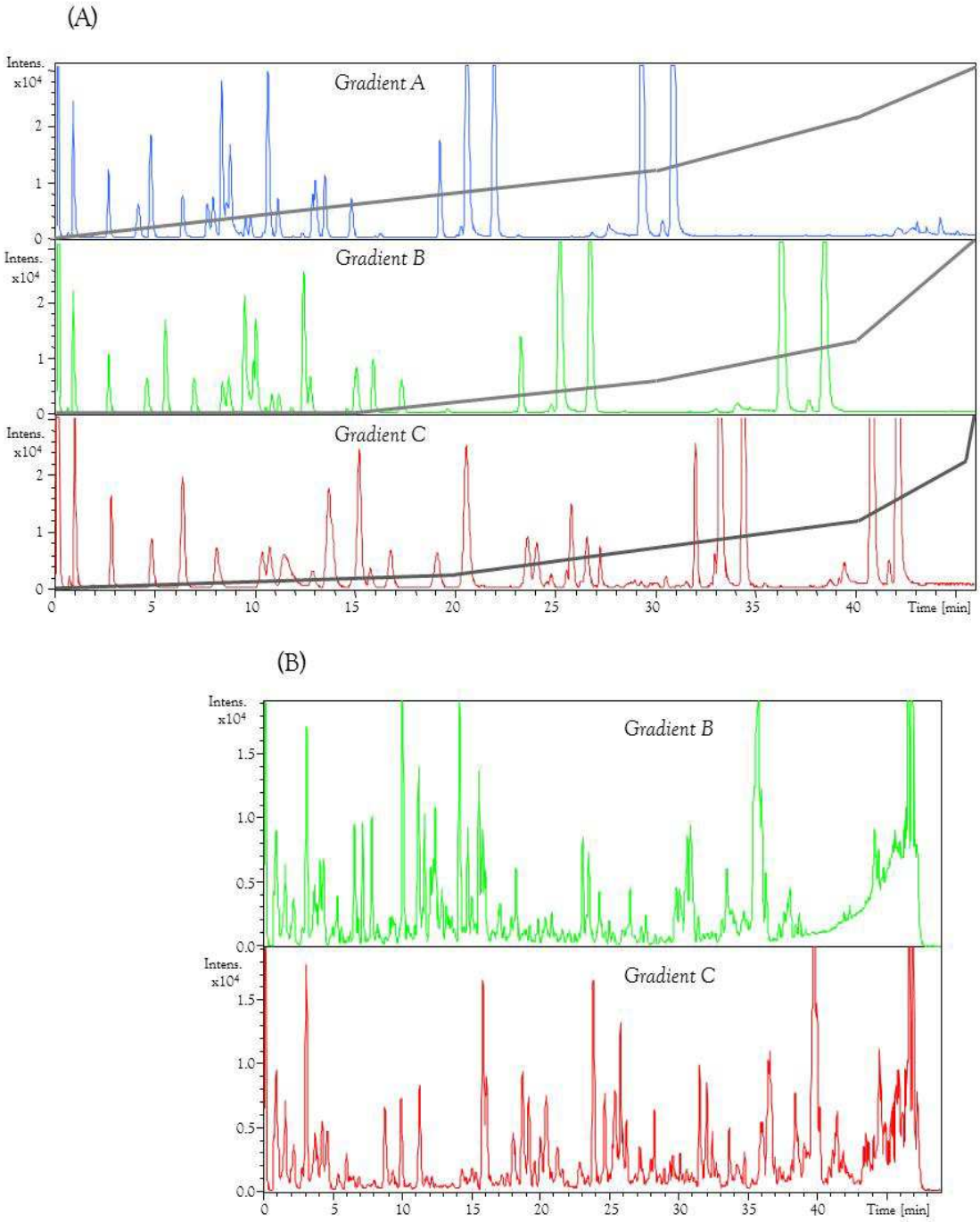

Figure 5.1. Supporting information. (A) Chromatographic separation achieved for 32 standard mix by using gradients A (at 0 min 0% ACN, at 15 min 20% ACN, at 30 min 40% ACN, at 40 min 70% ACN, at 45.5 min 100% ACN), B (at 0 min 0% ACN, at 20 min 20% ACN, at 40 min 40% ACN, at 45.5 min 100% ACN) and C (at 0 min 0% ACN, at 20 min 8% ACN, at 40 min 40% ACN, at 40 min 40% ACN, at 45.5 min 100% ACN). Flow rate 0.4 ml/min. MS detection in negative polarity. (B) Avocado QC sample (negative polarity).

Tabla 5.1. Supporting information. MS signal observed for the 32 compounds included in the standard mixture in negative and positive ion polarity, together with retention time, error and MS fragments in-source. MS fragments underlined means that they are more intense than the molecular ion.

Compound	Pseudo-molecular Ion	Molecular formula	m/z experimental	m/z theoretical	t_r (min)	Error (ppm)	mSigma value	MS fragments in-source
Quinic acid	$[M-H]^-$	$C_7H_{12}O_6$	191.0560	191.0561	0.64	0.4	8.3	–
	$[M+H]^+$		193.0703	193.0707		1.8	5.2	147.0643 $[M+H-H_2O-CO_2]^+$ 215.0511 $[M+Na]^+$ 231.0175 $[M+K]^+$
Succinic acid	$[M-H]^-$	$C_4H_6O_4$	117.0195	117.0193	1.31	- 1.8	25.4	–
	$[M+H]^+$		119.0354	119.0339		- 12.4	25.4	141.0151 $[M+Na]^+$
Gallic acid	$[M-H]^-$	$C_7H_6O_5$	169.0138	169.0142	1.98	2.4	6.1	125.0244 $[M-H-CO_2]^-$
	$[M+H]^+$		171.0285	171.0288		1.6	44.1	127.0404 $[M+H-CO_2]^+$ 153.0185 $[M+H-H_2O]^+$ 193.0075 $[M+Na]^+$
3,5-Dihydroxybenzoic acid (α-resorcylic acid)	$[M-H]^-$	$C_7H_6O_4$	153.0186	153.0193	3.55	4.9	5.4	109.0303 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0337	155.0339		1.1	44.0	93.0352 $[M+H-H_2O-CO_2]^+$ 111.0454 $[M+H-CO_2]^+$ 137.0242 $[M+H-H_2O]^+$ 177.0150 $[M+Na]^+$
Protocatechuic acid	$[M-H]^-$	$C_7H_6O_4$	153.0194	153.0193	3.55	- 0.5	5.7	109.0297 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0337	155.0339		1.1	44.0	93.0352 $[M+H-H_2O-CO_2]^+$ 111.0454 $[M+H-CO_2]^+$ 137.0242 $[M+H-H_2O]^+$ 177.0150 $[M+Na]^+$
2,6-Dihydroxybenzoic acid (γ-resorcylic acid)	$[M-H]^-$	$C_7H_6O_4$	153.0188	153.0193	3.55	3.5	6.9	109.0303 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0337	155.0339		1.1	44.0	93.0352 $[M+H-H_2O-CO_2]^+$ 111.0454 $[M+H-CO_2]^+$ 137.0242 $[M+H-H_2O]^+$ 177.0150 $[M+Na]^+$
Pantothenic acid	$[M-H]^-$	$C_9H_{17}NO_5$	218.1028	218.1034	5.01	2.9	11.7	88.0417 $[M-H-130]^-$ 146.0820 $[M-H-CO-CO_2]^-$
	$[M+H]^+$		220.1175	220.1179		2.0	4.0	90.0572 $[M+H-130]^+$ 184.0955 $[M+H-H_2O]^+$ 202.1070 $[M+H-H_2O]^+$ 242.0967 $[M+Na]^+$ 258.0721 $[M+K]^+$

Table 5.1. **Supporting information** (continued)

Compound	Pseudo-molecular Ion	Molecular formula	m/z experimental	m/z theoretical	t_r (min)	Error (ppm)	mSigma value	MS fragments in-source
Gentisic acid	$[M-H]^-$	$C_7H_6O_4$	153.0193	153.0193	6.00	0.2	16.0	109.0313 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0338	155.0339		0.8	44.0	137.0237 $[M+H-H_2O]^+$
4-Hydroxybenzoic acid	$[M-H]^-$	$C_7H_6O_3$	137.0246	137.0244	6.14	- 1.2	3.6	93.0344 $[M-H-CO_2]^-$
	$[M+H]^+$		139.0388	139.0390		1.1	43.9	95.0486 $[M+H-CO_2]^+$ 121.0312 $[M+H-H_2O]^+$
2,4-Dihydroxybenzoic acid (β-resorcylic acid)	$[M-H]^-$	$C_7H_6O_4$	153.0191	153.0193	7.82	1.4	8.5	109.0302 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0335	155.0339		2.5	44.0	137.0242 $[M+H-H_2O]^+$ 177.0139 $[M+Na]^+$
2,3-Dihydroxybenzoic acid (o-pyrocatechuic acid)	$[M-H]^-$	$C_7H_6O_4$	153.0190	153.0193	8.10	2.1	4.8	109.0296 $[M-H-CO_2]^-$
	$[M+H]^+$		155.0341	155.0339		- 1.2	44.0	137.0234 $[M+H-H_2O]^+$
Vanillic acid	$[M-H]^-$	$C_8H_8O_3$	167.0348	167.0350	10.09	1.3	50.2	123.0452 $[M-H-CO_2]^-$ 152.0104 $[M-H-CH_3]^-$
	$[M+H]^+$		169.0494	169.0495		0.7	50.2	93.0351 $[M+H-76]^+$ 125.0616 $[M+H-CO_2]^+$ 151.0377 $[M+H-H_2O]^+$ 191.0286 $[M+Na]^+$ 207.0021 $[M+K]^+$
Caffeic acid	$[M-H]^-$	$C_9H_8O_4$	179.0345	179.0350	10.98	3.0	11.7	135.0448 $[M-H-CO_2]^-$
	$[M+H]^+$		181.0498	181.0495		1.2	56.7	135.0448 $[M+H-H_2O-CO_2]^+$ 163.0395 $[M+H-H_2O]^+$ 203.0349 $[M+Na]^+$ 219.0031 $[M+K]^+$
Catechin	$[M-H]^-$	$C_{15}H_{14}O_6$	289.0715	289.0718	11.29	0.8	12.8	245.0829 $[M-H-CO_2]^-$
	$[M+H]^+$		291.0867	291.0863		- 1.3	5.1	139.0401 $[M+H-152]^+$ 165.0522 $[M+H-126]^+$ 313.0727 $[M+Na]^+$ 329.0376 $[M+K]^+$
Isovanillic acid	$[M-H]^-$	$C_8H_8O_4$	167.0338	167.0350	11.85	6.9	50.2	123.0450 $[M-H-CO_2]^-$ 152.0060 $[M-H-CH_3]^-$
	$[M+H]^+$		169.0495	169.0495		0.4	50.2	93.0373 $[M+H-76]^+$ 125.0616 $[M+H-CO_2]^+$ 151.0414 $[M+H-H_2O]^+$ 191.0325 $[M+Na]^+$ 207.0051 $[M+K]^+$

Table 5.1. **Supporting information** (continued)

Compound	Pseudo-molecular Ion	Molecular formula	m/z experimental	m/z theoretical	t_r (min)	Error (ppm)	mSigma value	MS fragments in-source
Chlorogenic acid	$[M-H]^-$	$C_{16}H_{18}O_9$	353.0876	353.0878	12.58	0.5	2.5	191.0553 $[M-H\text{-caffeic}]^-$
	$[M+H]^+$		355.1027	355.1024		- 0.9	10.2	163.0398 $[M+H\text{-quinic}]^+$ 377.0885 $[M+Na]^+$ 393.0615 $[M+K]^+$
Homovanillic acid	$[M-H]^-$	$C_9H_{10}O_4$	181.0505	181.0506	12.82	0.9	56.7	137.0584 $[M-H-CO_2]^-$
	$[M+H]^+$		183.0647	183.0652		2.4	56.7	205.0487 $[M+Na]^+$ 221.0176 $[M+K]^+$
Vanillin	$[M-H]^-$	$C_8H_8O_4$	151.0399	151.0401	13.33	1.0	50.1	136.0161 $[M-H-CH_3]^-$
	$[M+H]^+$		153.0549	153.0546		- 1.7	50.1	93.0361 $[M+H-60]^+$ 125.0587 $[M+H-CO]^+$
Syringic acid	$[M-H]^-$	$C_9H_{10}O_5$	197.0450	197.0455	13.69	2.9	8.7	153.0552 $[M-H-CO_2]^-$ 182.0224 $[M-H-CH_3]^-$
	$[M+H]^+$		199.0608	199.0601		3.7	56.8	140.0475 $[M+H-CO_2-CH_3]^+$ 155.0666 $[M+H-CO_2]^+$ 181.0460 $[M+H-H_2O]^+$ 221.0451 $[M+Na]^+$ 237.0128 $[M+K]^+$
p-Coumaric acid	$[M-H]^-$	$C_9H_8O_3$	163.0401	163.0401	15.49	-0.5	8.0	119.0507 $[M-H-CO_2]^-$
	$[M+H]^+$		165.0547	165.0546		- 0.5	56.5	91.0570 $[M+H-74]^+$ 119.0503 $[M+H-H_2O-CO_2]^+$ 147.0449 $[M+H-H_2O]^+$ 187.0404 $[M+Na]^+$ 203.0118 $[M+K]^+$
Benzoic acid	$[M-H]^-$	$C_7H_6O_2$	121.0291	121.0295	16.95	3.2	43.8	–
	$[M+H]^+$		123.0454	123.0441		- 11.1	5.1	–
Epicatechin	$[M-H]^-$	$C_{15}H_{14}O_6$	289.0710	289.0718	17.77	2.7	2.2	245.0817 $[M-H-CO_2]^-$
	$[M+H]^+$		291.0873	291.0863		- 3.3	10.1	139.0403 $[M+H-152]^+$ 165.0522 $[M+H-126]^+$ 313.0740 $[M+Na]^+$ 329.0490 $[M+K]^+$

Table 5.1. **Supporting information** (continued)

Compound	Pseudo-molecular Ion	Molecular formula	m/z experimental	m/z theoretical	t_r (min)	Error (ppm)	mSigma value	MS fragments in-source
m-coumaric acid	$[M-H]^-$	$C_9H_8O_3$	163.0397	163.0401	19.57	2.4	14.3	119.0504 $[M-H-CO_2]^-$
	$[M+H]^+$		165.0555	165.0546		- 5.5	56.5	91.0572 $[M+H-H_2O-CO- CO_2]^+$ 119.0512 $[M+H-H_2O-CO_2]^+$ 147.0458 $[M+H-H_2O]^+$ 187.0419 $[M+Na]^+$ 203.0110 $[M+K]^+$
Ferulic acid	$[M-H]^-$	$C_{10}H_{10}O_4$	193.0502	193.0506	20.41	2.2	12.1	149.0611 $[M-H-CO_2]^-$ 178.0261 $[M-H-CH_3]^-$
	$[M+H]^+$		195.0650	195.0652		0.8	62.9	117.0359 $[M+H-78]^+$ 145.0299 $[M+H-50]^+$ 177.0557 $[M+H-H_2O]^+$ 217.0505 $[M+Na]^+$ 233.0243 $[M+K]^+$
Sinapic acid	$[M-H]^-$	$C_{11}H_{12}O_5$	223.0600	223.0612	23.13	5.4	9.7	208.0372 $[M-H-CH_3]^-$
	$[M+H]^+$		225.0762	225.0757		- 2.7	39.7	119.0514 $[M+H-106]^+$ 147.0461 $[M+H-78]^+$ 175.0400 $[M+H-50]^+$ 207.0663 $[M+H-H_2O]^+$ 247.0637 $[M+Na]^+$ 263.0422 $[M+K]^+$
o-coumaric acid	$[M-H]^-$	$C_9H_8O_3$	163.0397	163.0401	23.43	2.1	60.7	119.0501 $[M-H-CO_2]^-$
	$[M+H]^+$		165.0542	165.0546		2.4	56.5	103.0571 $[M+H-62]^+$ 123.0458 $[M+H-42]^+$ 147.0457 $[M+H-H_2O]^+$ 187.0416 $[M+Na]^+$ 203.0136 $[M+K]^+$
trans-cinnamic acid	$[M-H]^-$	$C_9H_8O_2$	147.0447	147.0452	28.64	3.3	56.4	103.0550 $[M-H-CO_2]^-$
	$[M+H]^+$		–	–		–	–	–
Abscisic acid	$[M-H]^-$	$C_{15}H_{20}O_4$	263.1283	263.1289	29.71	2.2	10.8	153.0914 $[M-H-110]^-$ 219.1371 $[M-H-CO_2]^-$
	$[M+H]^+$		265.1452	265.1434		- 6.5	2.4	229.1254 $[M+H-2H_2O]^+$ 247.1348 $[M+H-H_2O]^+$ 287.1342 $[M+Na]^+$ 303.1070 $[M+K]^+$

Table 5.1. **Supporting information** (continued)

Compound	Pseudo-molecular Ion	Molecular formula	m/z experimental	m/z theoretical	t_r (min)	Error (ppm)	mSigma value	MS fragments in-source
Laricitrin	$[M-H]^-$	$C_{16}H_{12}O_8$	331.0452	331.0459	31.19	2.2	2.6	–
	$[M+H]^+$		333.0630	333.0605		- 7.5	8.9	355.0532 $[M+Na]^+$ 371.0245 $[M+K]^+$
Naringenin	$[M-H]^-$	$C_{15}H_{12}O_5$	271.0602	271.0612	32.11	3.8	4.7	–
	$[M+H]^+$		273.0787	273.0757		- 10.4	n.a.	295.0667 $[M+Na]^+$ 311.0367 $[M+K]^+$
Chrysin	$[M-H]^-$	$C_{15}H_{10}O_4$	253.0499	253.0506	38.32	2.8	1.6	–
	$[M+H]^+$		255.0684	255.0652		- 12.7	5.0	277.0565 $[M+Na]^+$ 293.0245 $[M+K]^+$
Kaempferide	$[M-H]^-$	$C_{16}H_{12}O_6$	299.0553	299.0561	39.68	2.7	6.8	284.0311 $[M-H-CH_3]^-$
	$[M+H]^+$		301.0745	301.0707		- 12.8	5.1	323.0643 $[M+Na]^+$ 339.0324 $[M+K]^+$

Tabla 5.2. Supporting information. Tentative identification of other *m/z* signals found in the different avocado samples using UHPLC-ESI-TOF MS.

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
0.74	607 $[M+H]^+$ 629 $[M+Na]^+$ 645 $[M+K]^+$	605.1942 $[M-H]^-$	$C_{22}H_{38}O_{19}$	-1.3	33.5	*Glucosyl-xylosyl-xylosyl-glucose / xylosyl-glucosyl-glucosyl-xylose I*	ALL
1.03	348 $[M+H]^+$	346.0546 $[M-H]^-$	$C_{12}H_9N_7O_6$	- 0.9	27.0	*N-amino-2,4-dinitro-N-[(2-nitrophenyl)diazenyl]aniline / ethyl 3-nitro-6-(4-nitropyrazol-1-yl)pyrazolo[1,5-a]pyrimidine-2-carboxylate / 1-(4-amino-1,2,5-oxadiazol-3-yl)-5-(4-methoxy-3-nitrophenyl)triazole-4-carboxylic acid …*	ALL
1.15	607 $[M+H]^+$ 629 $[M+Na]^+$ 645 $[M+K]^+$	605.1915 $[M-H]^-$	$C_{22}H_{38}O_{19}$	2.4	5.9	*Glucosyl-xylosyl-xylosyl-glucose / xylosyl-glucosyl-glucosyl-xylose II*	ALL
1.43	–	399.0960 $[M-H]^-$	$C_{18}H_{16}N_4O_7$	- 3.9	43.0	*2-(6-amino-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)-2-oxoethyl (1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)acetate / 2-(1,3-benzodioxol-5-yl)-2-oxoethyl (1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)acetate …*	C1, C2, G2, HT1, J1-1, J1-2, LH2, N1, P2, SP2
1.99	391 $[M+Na]^+$ 407 $[M+K]^+$	367.1251 $[M-H]^-$	$C_{14}H_{24}O_{11}$	- 1.3	51.9	*3-Hydroxy-2-(2-methylpropyl)-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-butanedioic acid / 4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol / [(2,3,4,5,6)-5-acetyloxy-3-hydroxy-2-(hydroxymethyl)-6-[(2,3)-2,3,4-trihydroxybutoxy]oxan-4-yl] acetate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
2.27	–	647.2031 $[M-H]^-$	$C_{24}H_{40}O_{20}$	1.4	5.4	*2-(2-Hydroxy-3-{[(1,2)-1-(hydroxymethyl)-3-oxo-2-{[(3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}propyl]oxy}-3-{[(2,3,4,5,6)-4,5,6-trihydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl]oxy}propyl)pentanedioic acid / glucopyranosyl-2,3-anhydro-mannopyranosyl-glucopyranosyl-glucopyranose I…*	C1, C2, HT2, N2, P2, SP2, T2
2.54	371 $[M+H]^+$ 393 $[M+Na]^+$ 409 $[M+K]^+$	369.1398 $[M-H]^-$ 415 $[M-H+46]^-$	$C_{14}H_{26}O_{11}$	1.2	9.2	*Methyl 4-(6-deoxymannoheptopyranosyl) galactopyranoside / 6-deoxyglucoheptopyranosyl 6-deoxyglucoheptopyranoside / methyl 4-(6-deoxymannoheptopyranosyl)-galactopyranoside …*	ALL
2.56	649 $[M+H]^+$ 671 $[M+Na]^+$ 687 $[M+K]^+$ 631 $[M+H-18]^+$ 459 $[M+H-190]^+$	647.2039 $[M-H]^-$	$C_{24}H_{40}O_{20}$	0.1	7.8	*2-(2-Hydroxy-3-{[(1,2)-1-(hydroxymethyl)-3-oxo-2-{[(3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}propyl]oxy}-3-{[(2,3,4,5,6)-4,5,6-trihydroxy-2-(hydroxymethyl)tet rahydro-2H-pyran-3-yl]oxy}propyl)pentanedioic acid / glucopyranosyl-2,3-anhydro-mannopyranosyl-glucopyranosyl-glucopyranose II…*	ALL

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
2.83	341 $[M+H]^+$ 363 $[M+Na]^+$ 379 $[M+K]^+$	339.1294 $[M-H]^-$ 385 $[M-H+46]^-$	$C_{13}H_{24}O_{10}$	0.7	19.6	*Methyl-2-fucopyranosyl-galactoside / pseudo-laminarabiose / pseudo-cellobiose …*	C1, C2, G1, G2, HT1, HT2, HS2, J1-2, J2-2, LH1, LH2, HM2, N1, SP1, SP2, T1, T2
3.09	325 $[M+Na]^+$ 371 $[M+K]^+$	301.0932 $[M-H]^-$	$C_{13}H_{18}O_8$	- 1.1	27.2	*2,3,4,5,6-Pentahydroxyhexyl 2-hydroxybenzoate / 4,4-bis(ethoxycarbonyl)heptanedioate / (5-acetyl-4,6-diacetyloxyoxan-3-yl) acetate…*	ALL
3.51	385 $[M+H]^+$ 407 $[M+Na]^+$ 423 $[M+K]^+$	383.1568 $[M-H]^-$ 429 $[M-H+46]^-$	$C_{15}H_{28}O_{11}$	- 2.5	55.1	*Propyl 4-hexopyranosylhexopyranoside /methyl 4-galactopyranosyl-2,3-dimethyl-galactopyranoside / propan-2-yl 4-galactopyranosyl-mannopyranoside I…*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
4.04	649 $[M+H]^+$ 671 $[M+Na]^+$ 687 $[M+K]^+$ 631 $[M+H-18]^+$ 459 $[M+H-190]^+$	647.2036 $[M-H]^-$	$C_{24}H_{40}O_{20}$	2.7	12.1	*2-(2-Hydroxy-3-{[(1,2)-1-(hydroxymethyl)-3-oxo-2-{[(3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}propyl]oxy}-3-{[(2,3,4,5,6)-4,5,6-trihydroxy-2-(hydroxymethyl)tet rahydro-2H-pyran-3-yl]oxy}propyl)pentanedioic acid / glucopyranosyl-2,3-anhydro-mannopyranosyl-glucopyranosyl-glucopyranose III…*	ALL
4.24	385 $[M+H]^+$ 407 $[M+Na]^+$ 423 $[M+K]^+$	383.1552 $[M-H]^-$ 429 $[M-H+46]^-$	$C_{15}H_{28}O_{11}$	1.7	4.1	*Propyl 4-hexopyranosylhexopyranoside /methyl 4-galactopyranosyl-2,3-dimethyl-galactopyranoside / propan-2-yl 4-galactopyranosyl-mannopyranoside II…*	ALL
4.52	385 $[M+H]^+$ 407 $[M+Na]^+$ 423 $[M+K]^+$	383.1557 $[M-H]^-$ 429 $[M-H+46]^-$	$C_{15}H_{28}O_{12}$	0.5	18.1	*Propyl 4-hexopyranosylhexopyranoside /methyl 4-galactopyranosyl-2,3-dimethyl-galactopyranoside / propan-2-yl 4-galactopyranosyl-mannopyranoside III…*	ALL
4.66	781 $[M+H]^+$ 803 $[M+Na]^+$ 819 $[M+K]^+$ 649 $[M+H-132]^+$	779.2454 $[M-H]^-$	$C_{29}H_{48}O_{24}$	1.1	14.0	–	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2
5.09	385 $[M+H]^+$ 407 $[M+Na]^+$ 423 $[M+K]^+$	383.1550 $[M-H]^-$ 429 $[M-H+46]^-$	$C_{15}H_{28}O_{11}$	2.2	19.7	*Propyl 4-hexopyranosylhexopyranoside /methyl 4-galactopyranosyl-2,3-dimethyl-galactopyranoside / propan-2-yl 4-galactopyranosyl-mannopyranoside IV…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N2, P2, SP1, SP2, T1, T2
5.63	419 $[M+Na]^+$ 435 $[M+K]^+$	395.1535 $[M-H]^-$ 441 $[M-H+46]^-$	$C_{16}H_{28}O_{11}$	1.8	50.6	*Hex-2-ulofuranosyl 3-C-but-3-en-1-ylhexopyranoside / fructofuranosyl (3)-3-C-but-3-en-1-yl-ribo-hexopyranoside I …*	G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
6.00	385 $[M+H]^+$ 407 $[M+Na]^+$ 423 $[M+K]^+$	383.1564 $[M-H]^-$ 429 $[M-H+46]^-$	$C_{15}H_{28}O_{11}$	- 1.2	17.9	*Propyl 4-hexopyranosylhexopyranoside /methyl 4-galactopyranosyl-2,3-dimethyl-galactopyranoside / propan-2-yl 4-galactopyranosyl-mannopyranoside V…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N2, P2, SP1, SP2, T1, T2
6.07	361 $[M+Na]^+$ 377 $[M+K]^+$	337.1137 $[M-H]^-$	$C_{13}H_{22}O_{10}$	- 2.9	29.5	*3-(Hexopyranosyloxy)propane-1,2-diyl diacetate / 3-(galactopyranosyloxy)propane-1,2-diyl diacetate / dimethyl 3-(glucopyranosyloxy)pentanedioate …*	G2, J1-2, LH2, HM2, P2, SP2
6.19	781 $[M+H]^+$ 803 $[M+Na]^+$ 819 $[M+K]^+$ 649 $[M+H-132]^+$	779.2473 $[M-H]^-$	$C_{29}H_{48}O_{24}$	- 1.3	11.6	–	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2
7.20	515 $[M+Na]^+$ 531 $[M+K]^+$	491.1757 $[M-H]^-$	$C_{21}H_{32}O_{13}$	2.7	41.3	*4-(1,3-dihydroxypropyl)-2-methoxyphenyl 2-[(2,3,4)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]-glucopyranoside / (1,4,5,7)-5-[(6-deoxy-mannopyranosyl)oxy]-7-(hydroxymethyl)-1,4,5,7-tetrahydrocyclopenta[c]pyran-1-yl-glucopyranoside / 2,4-dimethoxybenzyl 6-glucopyranosyl-glucopyranoside …*	G1, G2, HT1
7.43	399 $[M+H]^+$ 421 $[M+Na]^+$ 437 $[M+K]^+$	397.1715 $[M-H]^-$ 443 $[M-H+46]^-$	$C_{16}H_{30}O_{11}$	0.1	8.1	*Butyl 4-galactopyranosyl-glucopyranoside / tert-butyl 4-galactopyranosyl-glucopyranoside / 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioic acid I…*	ALL
7.90	399 $[M+H]^+$ 421 $[M+Na]^+$ 437 $[M+K]^+$	397.1699 $[M-H]^-$ 443 $[M-H+46]^-$	$C_{16}H_{30}O_{11}$	4.2	19.1	*Butyl 4-galactopyranosyl-glucopyranoside / tert-butyl 4-galactopyranosyl-glucopyranoside / 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioic acid II…*	C1, C2, G1, G2, HT1, HT2, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
7.98	333 $[M+H]^+$ 355 $[M+Na]^+$ 371 $[M+K]^+$	331.1036 $[M-H]^-$ 169 $[M-H-162]^-$	$C_{14}H_{20}O_9$	- 0.5	19.4	*3,4-Dihydroxyphenyl glycol hexose / 3,4-bis(methylene)-Hexanedioic acid hexose / cyclohexene-1,2-dicarboxylic acid hexose …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-2, LH1, LH2, MA1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
8.37	399 $[M+H]^+$ 421 $[M+Na]^+$ 437 $[M+K]^+$	397.1698 $[M-H]^-$ 443 $[M-H+46]^-$	$C_{16}H_{30}O_{11}$	4.4	19.1	*Butyl 4-galactopyranosyl-glucopyranoside / tert-butyl 4-galactopyranosyl-glucopyranoside / 3,6,9,12,15,18,21-heptaoxatricosane-1,23-dioic acid III…*	ALL
9.25	415 $[M+Na]^+$ 431 $[M+K]^+$	391.1236 $[M-H]^-$ 437 $[M-H+46]^-$	$C_{16}H_{24}O_{11}$	2.5	18.8	*Shanzhiside / caryoptosidic acid/ [(2,3,4,5)-3,4,5-triacetyloxy-6-(1-hydroxyethoxy)oxan-2-yl]methyl acetate …*	C1, C2, G1, G2, HT2, HS1, J1-1,J1-2, LH1, LH2, MA1, N1, N2, P1, P2, SP1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
9.33	279 $[M+H]^+$ 301 $[M+Na]^+$ 317 $[M+K]^+$	277.2173 $[M-H]^-$	$C_8H_{14}N_4O_7$	- 14.3	59.2	*[1,4-Diamino-3-(2-aminoacetyl)peroxy-1,4-dioxobutan-2-yl] 2-aminoacetate*	ALL
9.53	383 $[M+H]^+$ 405 $[M+Na]^+$ 421 $[M+K]^+$ 365 $[M+H-18]^+$ 365 $[M+H-76]^+$ 163 $[M+H-120]^+$ 145 $[163-18]^+$ 127 $[163-18-18]^+$	381.1401 $[M-H]^-$ 763 $[2M-H]^-$	$C_{15}H_{26}O_{11}$	0.4	1.8	*Sucrose monoallyl ether I*	C1, C2, MA1, HM2, P1, P2, T2
9.85	–	375.1283 $[M-H]^-$	$C_{16}H_{24}O_{10}$	3.6	3.1	*Loganic acid / 7-epiloganic acid*	C1, C2, G1, G2, HT1, HT2, J1-2, J2-1, MA1, MA2, HM1, N1, N2, P1, SP1, SP2, T1, T2
10.12	413 $[M+H]^+$ 435 $[M+Na]^+$ 451 $[M+K]^+$	411.1855 $[M-H]^-$ 457 $[M-H+46]^-$	$C_{17}H_{32}O_{11}$	4.2	21.4	*Methyl 2,3,6-trimethyl-4-(4-methylhexopyranosyl)hexopyranoside / 3-methylbutyl 2-glucopyranosyl-glucopyranoside / methyl 4-(2-[2-(2-glucopyranosyloxyethoxy)ethoxy]ethoxy)butanoate I…*	C1, C2, G2, HS1, J2-2, MA2, N2, P2, SP1, T1
10.32	383 $[M+H]^+$ 405 $[M+Na]^+$ 421 $[M+K]^+$	381.1397 $[M-H]^-$ 763 $[2M-H]^-$	$C_{15}H_{26}O_{11}$	1.5	6.1	*Sucrose monoallyl ether II*	C1, C2, J2-2, LH1, LH2, MA1, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T2
10.49	361 $[M+Na]^+$	337.1510 $[M-H]^-$ 675 $[2M-H]^-$	$C_{14}H_{26}O_9$	- 1.8	18.6	*1,3-Dihydroxypropan-2-yl 6-pentanoyl-galactopyranoside / (2)-2-(galactopyranosyloxy)-3-hydroxypropyl pentanoate …*	C1, C2, G1, G2, MA1, MA2
10.57	379 $[M+Na]^+$	351.1308 $[M-H]^-$	$C_{14}H_{24}O_{10}$	- 3.3	18.4	*Methyl 4-(6,7-dideoxy-galacto-hept-6-enopyranosyl)-galactopyranoside / methyl 4-[(1,4,5,6)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-en-1-yl]-glucopyranoside I…*	C1, C2, J1-2, J2-2, HM2, P2
11.28	447 $[M+H]^+$ 469 $[M+Na]^+$ 485 $[M+K]^+$ 224 $[M+H-223]^+$ 165 $[M+H-282]^+$	445.1338 $[M-H]^-$ 491 $[M-H+46]^-$ 222 $[M-223]^-$ 163 $[M-H-282]^-$ 119 $[163-44]^-$	$C_{19}H_{26}O_{12}$	3.1	38.8	*Violutoside / bungeiside D / canthoside A …*	G2, HT2, HS2, J2-2, LH2, HM2, N1, N2, SP1, SP2, T2
11.63	247 $[M+H]^+$	245.0123 $[M-H]^-$	$C_{12}H_6O_6$	12.8	23.9	*3-Glyoxyloyl-6-carboxycoumarin / 9-hydroxy-7-oxofuro[3,2-g]chromene-6-carboxylic acid …*	G1, G2, HT1, HT2, P1, P2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
12.10	–	401.1092 $[M-H]^-$	$C_{17}H_{22}O_{11}$	- 0.6	23.7	*10-Methylixoside / apodanthoside*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA2, HM1, HM2, P1, P2, SP1, SP2, T1, T2
12.12	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.0978 $[M-H]^-$	$C_{14}H_{16}N_2O_5$	2.8	12.7	*4-[3-(3,4-Dimethoxyphenyl)-1,2,4-oxadiazol-5-yl]butanoic acid / 2,4,5-trimethoxy-(5-methyl-1,2-oxazol-3-yl)benzamide / ethyl 5-(2-hydrazinyl-2-oxoethoxy)-2-methyl-1-benzofuran-3-carboxylate …*	G2, HT2, J2-2, MA2, N2, P2, SP2, T2
12.20	407 $[M+H]^+$ 429 $[M+Na]^+$ 445 $[M+K]^+$	405.1386 $[M-H]^-$ 451 $[M-H+46]^-$	$C_{17}H_{28}O_8$	4.0	19.8	*Ipolamiide / 10-hydroxyloganin / morroside …*	C1, C2, G1, G2, HT1, HT2, LH2, MA1, MA2, N1, N2, P1, P2, T2
12.35	435 $[M+Na]^+$ 451 $[M+K]^+$	411.1852 $[M-H]^-$ 457 $[M-H+46]^-$	$C_{17}H_{32}O_{11}$	4.5	5.7	*Methyl 2,3,6-trimethyl-4-(4-methylhexopyranosyl)hexopyranoside / 3-methylbutyl 2-glucopyranosyl-glucopyranoside / methyl 4-(2-[2-(2-glucopyranosyloxyethoxy)ethoxy]ethoxy)butanoate II…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, P1, P2, SP1, SP2, T1, T2
12.67	485 $[M+Na]^+$ 501 $[M+K]^+$ 480 $[M+H+17]$	461.1662 $[M-H]^-$ 507 $[M-H+46]^-$	$C_{20}H_{30}O_{12}$	0.4	36.0	*2-(3,4-Dihydroxyphenyl)ethyl 3-(6-deoxy-mannopyranosyl)-glucopyranoside / 4-[(mannopyranosyloxy)methyl]benzyl mannopyranoside II …*	C1, C2, MA2, N1, P1, P2
12.98	485 $[M+Na]^+$ 501 $[M+K]^+$	461.1658 $[M-H]^-$ 507 $[M-H+46]^-$	$C_{20}H_{30}O_{12}$	0.5	24.1	*2-(3,4-Dihydroxyphenyl)ethyl 3-(6-deoxy-mannopyranosyl)-glucopyranoside / 4-[(mannopyranosyloxy)methyl]benzyl mannopyranoside III …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
13.23	471 $[M+Na]^+$ 487 $[M+K]^+$	447.1488 $[M-H]^-$ 493 $[M-H+46]^-$	$C_{19}H_{28}O_2$	4.4	24.2	*2,5-Dimethylphenyl undec-10-enoate / 4-(glucopyranosyl-glucopyranosyloxy)benzylalcohol…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
13.47	403 $[M+Na]^+$ 419 $[M+K]^+$	379.1575 $[M-H]^-$ 425 $[M-H+46]^-$	$C_{16}H_{28}O_{10}$	2.3	24.0	*3-(4-Acetyl-2,3-dimethyl-fucopyranosyl)-rhamnopyranose / methyl 6-deoxy-4-galactofuranosyl-2,3-(1-methylethylidene)-mannopyranoside I …*	C1, C2, G1, G2, HT1, HS1, HS2, J1-1, J1-2, J2-1, LH1, LH2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2
14.34	513 $[M+Na]^+$ 529 $[M+K]^+$	489.1596 $[M-H]^-$	$C_{21}H_{30}O_{13}$	3.6	12.2	*4-Acetyl-2-methoxyphenyl 6-allopyranosyl-allopyranoside / methyl (1,4a,5,7,7a)-5,7-bis(acetyloxy)-1-(glucopyranosyloxy)-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate…*	C1, C2, G2, HT1, HT2, HS2, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM2, N2, P2, SP2, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
15.01	403 $[M+Na]^+$ 419 $[M+K]^+$	379.1601 $[M-H]^-$ 425 $[M-H+46]^-$	$C_{16}H_{28}O_{10}$	3.3	18.4	*3-(4-Acetyl-2,3-dimethyl-fucopyranosyl)-rhamnopyranose / methyl 6-deoxy-4-galactofuranosyl-2,3-(1-methylethylidene)-mannopyranoside II ...*	ALL
15.15	–	471.2075 $[M-H]^-$	$C_{20}H_{32}N_4O_9$	4.6	16.2	*(2)-2-[[(2)-1-[(2)-2-[[(2,3)-2-amino-3-methylpentanoyl]amino]-4-carboxybutanoyl]pyrrolidine-2-carbonyl]amino]butanedioic acid / 1-[2-[[2-[(2-amino-3-carboxypropanoyl)amino]-4-carboxybutanoyl]amino]-3-methylpentanoyl]pyrrolidine-2-carboxylic acid ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
15.21	–	451.2173$[M-H]^-$	$C_{20}H_{36}O_{11}$	2.5	18.2	*2-Cyclohexylethyl 4-glucopyranosyl-glucopyranoside / methyl 9-[(2-arabinofuranosyl-arabinofuranosyl)oxy]nonanoate / (1)-1,5-anhydro-4-glucopyranosyl-1-(2-oxooctyl)-glucitol ...*	ALL
15.56	403 $[M+Na]^+$ 419 $[M+K]^+$	379.1616 $[M-H]^-$ 425 $[M-H+46]^-$	$C_{16}H_{28}O_{10}$	4.6	56.2	*3-(4-Acetyl-2,3-dimethyl-fucopyranosyl)-rhamnopyranose / methyl 6-deoxy-4-galactofuranosyl-2,3-(1-methylethylidene)-mannopyranoside III ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
15.83	379 $[M+Na]^+$	351.1282 $[M-H]^-$	$C_{14}H_{24}O_{10}$	4.2	18.7	*Methyl 4-(6,7-dideoxy-galacto-hept-6-enopyranosyl)-galactopyranoside / methyl 4-[(1,4,5,6)-4,5,6-trihydroxy-3-(hydroxymethyl)cyclohex-2-en-1-yl]-glucopyranoside II...*	ALL
15.95	403 $[M+Na]^+$ 419 $[M+K]^+$	379.1613 $[M-H]^-$	$C_{16}H_{28}O_{10}$	2.3	29.9	*3-(4-Acetyl-2,3-dimethyl-fucopyranosyl)-rhamnopyranose / methyl 6-deoxy-4-galactofuranosyl-2,3-(1-methylethylidene)-mannopyranoside IV ...*	C1, C2, HT1, HT2, J1-1, LH1, LH2, P1, P2, SP1, SP2
16.00	–	417.1048 $[M-H]^-$	$C_{17}H_{22}O_{12}$	- 2.3	21.0	*[2,3-Diacetyloxy-3-(3,4-diacetyloxy-5-oxooxolan-2-yl)propyl] acetate / (2,3,4,5-tetraacetyloxy-6,7-dioxoheptyl) acetate ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP2, T1, T2
16.52	485 $[M+Na]^+$ 501 $[M+K]^+$	461.1663 $[M-H]^-$	$C_{20}H_{30}O_{12}$	1.5	4.1	*2-(3,4-Dihydroxyphenyl)ethyl 3-(6-deoxy-mannopyranosyl)-glucopyranoside / 4-[(mannopyranosyloxy)methyl]benzyl mannopyranoside I...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N2, P1, P2, SP1, SP2, T1, T2
16.62	417 $[M+Na]^+$ 433 $[M+K]^+$	393.1741 $[M-H]^-$ 439 $[M-H+46]^-$	$C_{17}H_{30}O_{10}$	6.4	19.7	*3-Hex-3-en-1-yl 6-xylopyranosyl-glucopyranoside / 2-glucopyranosyloxypropane-1,3-diyl dibutanoate I...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, LH1, LH2, MA1, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
17.21	–	431.1530 $[M-H]^-$	$C_{20}H_{32}O_{10}$	3.6	84.9	*Deaminotri-O-isopropylidene tunicamine / (5R,6R,7R,8S)-5,6,7,8-tetraacetyloxydodecanoic acid / ethyl 2,3,4,6-tetra-O-propanoyl-D-glucopyranoside I…*	ALL
17.35	–	433.2060 $[M-H]^-$	$C_{20}H_{34}O_{10}$	4.5	10.9	*[2-Acetyloxy-3-[6-(2,3-diacetyloxypropoxy)hexoxy]propyl] acetate / 2-[5-[2-(3,4,5-trihydroxy-6-methyloxan-2-yl)oxycyclohexyl]oxypentyl]propanedioic acid I*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
17.92	–	433.2084 $[M-H]^-$	$C_{20}H_{34}O_{10}$	- 1.1	22.6	*[2-Acetyloxy-3-[6-(2,3-diacetyloxypropoxy)hexoxy]propyl] acetate / 2-[5-[2-(3,4,5-trihydroxy-6-methyloxan-2-yl)oxycyclohexyl]oxypentyl]propanedioic acid II*	ALL
18.05	395 $[M+Na]^+$ 411 $[M+K]^+$	371.0975 $[M-H]^-$	$C_{16}H_{20}O_{10}$	2.2	17.7	*[2-acetyl-3,5-dihydroxy-6-[(2,3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]phenyl] acetate / methyl (3,4,5,6)-3,4,5,6-tetraacetyloxycyclohexene-1-carboxylate …*	ALL
18.15	465 $[M+H]^+$	463.1821 $[M-H]^-$	$C_{20}H_{32}O_{12}$	1.7	18.5	*2-2-[2-3,5-dioxo-4-{[(2,3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}tetrahydrofuran-2-yl]-2-hydroxyethyl octanoate / 2-2-[(2)-3,5-dioxo-4-{[(2,3,4,5,6)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl]oxy}tetrahydrofuran-2-yl]-2-hydroxyethyl 2-(propan-2-yl)pentanoate / …*	ALL
18.26	419 $[M+H]^+$ 441 $[M+Na]^+$ 457 $[M+K]^+$	417.1787 $[M-H]^-$ 463 $[M-H+46]^-$	$C_{19}H_{30}O_{10}$	0.4	33.6	*9-[3-(3,6,6-trihydroxy-5-oxo-3,3-dihydro-2H-furo[3,2-b]furan-6-yl)-5-hydroxyoxolan-2-yl]nonanoic acid*	G1, G2, HS1, J1-2, J2-1, LH1, LH2, MA1, MA2, HM1, P1, SP1, SP2, T1
18.34	–	431.1907 $[M-H]^-$	$C_{20}H_{32}O_{10}$	2.5	7.8	*Deaminotri-O-isopropylidene tunicamine / (5R,6R,7R,8S)-5,6,7,8-tetraacetyloxydodecanoic acid / ethyl 2,3,4,6-tetra-O-propanoyl-D-glucopyranoside II …*	ALL
18.79	417 $[M+Na]^+$ 433 $[M+K]^+$	393.1726 $[M-H]^-$ 439 $[M-H+46]^-$	$C_{17}H_{30}O_{10}$	1.8	55.7	*3-Hex-3-en-1-yl 6-xylopyranosyl-glucopyranoside / 2-glucopyranosyloxypropane-1,3-diyl dibutanoate II…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
19.85	405 $[M+Na]^+$ 421 $[M+K]^+$	381.1756 $[M-H]^-$ 427 $[M-H+46]^-$	$C_{16}H_{30}O_{10}$	2.6	21.8	*4-[(2-Deoxylyxo-hexopyranosyl)oxy]butyl 2-deoxylyxo-hexopyranoside I*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
20.08	439 $[M+Na]^+$ 455 $[M+K]^+$	415.1623 $[M-H]^-$ 461 $[M-H+46]^-$	$C_{19}H_{28}O_{10}$	- 1.5	21.0	*Phenethyl alcohol xylopyranosyl-glucopyranoside I*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
20.30	–	443.1942 $[M-H]^-$	$C_{20}H_{28}O_{11}$	- 1.1	28.1	*Sibiricaphenone / methyl 2,3,4-triacetyl-1-(cyclohexylcarbonyl)hexopyranuronate / methyl 4-(4,6-benzylidene-gulopyranosyl)-glucopyranoside I…*	C1, C2, HT1, LH1, LH2, P1, P2
20.50	–	433.2072 $[M-H]^-$	$C_{20}H_{34}O_{10}$	1.8	14.7	*[2-Acetyloxy-3-[6-(2,3-diacetyloxypropoxy)hexoxy]propyl] acetate / 2-[5-[2-(3,4,5-trihydroxy-6-methyloxan-2-yl)oxycyclohexyl]oxypentyl]propanedioic acid III*	C1, C2, HT2, HS2, J1-2, J2-1, J2-2, LH2, MA1, MA2, HM1, HM2, P1, P2, T1, T2
20.89	405 $[M+Na]^+$ 421 $[M+K]^+$	381.1758 $[M-H]^-$ 427 $[M-H+46]^-$	$C_{16}H_{30}O_{10}$	2.1	4.2	*4-[(2-Deoxylyxo-hexopyranosyl)oxy]butyl 2-deoxylyxo-hexopyranoside II*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
20.98	525 $[M+Na]^+$ 541 $[M+K]^+$	501.1588 $[M-H]^-$	$C_{17}H_{28}O_7$	4.4	5.9	*Sibirioside B / wahlenoside A / cistanoside F…*	C2, G2, J1-2, HT2, HS2, MA2, HM2, P2, SP2
21.23	331 $[M+Na]^+$	307.1390 $[M-H]^-$ 615 $[2M-H]^-$	$C_{13}H_{24}O_8$	2.9	11.0	*Butyl 6-O-[(2)-2-hydroxypropanoyl]-glucopyranoside / 2,6-dideoxy-4-(6-deoxy-3-methyl-glucopyranosyl)hexose …*	C1, C2, J1-2, MA1, MA2, N1
21.85	405 $[M+Na]^+$ 421 $[M+K]^+$	381.1771 $[M-H]^-$ 427 $[M-H+46]^-$	$C_{16}H_{30}O_{10}$	- 1.3	7.4	*4-[(2-Deoxylyxo-hexopyranosyl)oxy]butyl 2-deoxylyxo-hexopyranoside III*	C1, C2, G2, MA1, P1, P2, SP1, SP2
22.08	439 $[M+Na]^+$ 455 $[M+K]^+$	415.1594 $[M-H]^-$ 461 $[M-H+46]^-$	$C_{19}H_{28}O_{10}$	3.9	2.6	*Phenethyl alcohol xylopyranosyl-glucopyranoside II*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
22.71	–	395.1938 $[M-H]^-$ 441 $[M-H+46]^-$	$C_{17}H_{32}O_{10}$	1.5	97.5	*Hex-2-ulofuranosyl 3-C-but-3-en-1-ylhexopyranoside / fructofuranosyl (3)-3-C-but-3-en-1-yl-ribo-hexopyranoside II…*	C1, C2, G1, G2, HT1, HT2, J2-2, LH2, MA1, N2, P1, P2, SP1, SP2, T1, T2
22.81	463 $[M+Na]^+$	439.1824 $[M-H]^-$	$C_{21}H_{30}O_{12}$	- 0.6	53.8	*2-Hydroxy-3-methoxy-5-prop-2-en-1-ylphenyl 6-O-[(2,3,4)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]-glucopyranoside / (2)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-yl 6-[(2,3,4)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]-glucopyranoside / 4-acetyl-2-methoxyphenyl 6-(6-deoxymannopyranosyl)-glucopyranoside I…*	C1, C2, J1-1, MA1

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
22.82	439 $[M+Na]^+$ 455 $[M+K]^+$	415.1624 $[M-H]^-$ 461 $[M-H+46]^-$	$C_{19}H_{28}O_{10}$	- 3.5	22.0	*Phenethyl alcohol xylopyranosyl-glucopyranoside III*	C1, C2, HT2, MA1, MA2, HM1, P1, P2
22.99	419 $[M+Na]^+$ 435 $[M+K]^+$	395.1938 $[M-H]^-$ 441 $[M-H+46]^-$	$C_{17}H_{32}O_{10}$	- 3.8	27.4	*Hex-2-ulofuranosyl 3-C-but-3-en-1-ylhexopyranoside / fructofuranosyl (3)-3-C-but-3-en-1-yl-ribo-hexopyranoside III...*	ALL
23.11	–	443.1554 $[M-H]^-$	$C_{20}H_{28}O_{11}$	1.2	6.2	*Sibiricaphenone / methyl 2,3,4-triacetyl-1-(cyclohexylcarbonyl)hexopyranuronate / methyl 4-(4,6-benzylidene-gulopyranosyl)-glucopyranoside II...*	C1, C2, G1, G2, HS2, J1-2, J2-2, LH2, MA1, MA2, HM1, HM2, N2, P1, P2, SP1, SP2, T2
23.38	–	275.1513 $[M-H]^-$ 257 $[M-H-18]^-$	$C_{13}H_{24}O_6$	- 4.5	95.3	*Bis(2-hydroxyethyl) nonanedioate / dibutyl 2-hydroperoxy-2-methylbutanedioate I ...*	C1, C2, HS1, J1-1, J2-1, J2-2, MA1, HM2, N1, P1, SP2
23.65	415 $[M+Na]^+$ 431 $[M+K]^+$	391.1619 $[M-H]^-$	$C_{17}H_{28}O_{10}$	- 0.6	23.3	*Bis(2-methoxyethyl) 2,4-diacetyl-2,4-bis(hydroxymethyl)pentanedioate / 2-(hydroxymethyl)-5-[(2,2,7,7-tetramethyltetrahydro-3aH-bis[1,3]dioxolo[4,5-b:4',5'-d]pyran-5-yl)methoxy]tetrahydrofuran-3,4-diol ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2
23.66	419 $[M+Na]^+$ 435 $[M+K]^+$	395.1916 $[M-H]^-$ 441 $[M-H+46]^-$	$C_{17}H_{32}O_{10}$	1.7	5.7	*Hex-2-ulofuranosyl 3-C-but-3-en-1-ylhexopyranoside / fructofuranosyl (3)-3-C-but-3-en-1-yl-ribo-hexopyranoside IV...*	ALL
23.78	–	275.1492 $[M-H]^-$ 257 $[M-H-18]^-$	$C_{13}H_{24}O_6$	2.9	14.6	*Bis(2-hydroxyethyl) nonanedioate / dibutyl 2-hydroperoxy-2-methylbutanedioate II...*	C1, C2, MA1, P1, P2, SP1, SP2, T1
23.90	429 $[M+H]^+$ 451 $[M+Na]^+$	427.1955 $[M-H]^-$	$C_{21}H_{32}O_9$	4.4	7.5	*2,3-Dideoxy-1-(2,2-dimethylpropanoyl)-2-[-(6-methoxy-1,3-benzodioxol-5-yl)(methoxymethoxy)methyl]-erythro-pentitol / (1)-1-[(2,3,4,5,7,8,8a)-8-(benzyloxy)-2,3,7-trimethoxy-2,3-dimethylhexahydro-5H-pyrano[3,4-b][1,4]dioxin-5-yl]ethane-1,2-diol I ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA1, HM1, HM2, N2, SP1, SP2, T1, T2
24.27	453 $[M+Na]^+$ 469 $[M+K]^+$	429.1838 $[M-H]^-$ 475 $[M-H+46]^-$	$C_{20}H_{30}O_{10}$	- 0.9	36.7	*2-Phenylethyl 6-(6-deoxymannopyranosyl)-glucopyranoside / 3-hex-1-en-3-yl 2,3,4,6-tetra-acetyl-glucopyranoside / 3-hex-3-en-1-yl 2,3,4,6-tetra-acetyl-glucopyranoside ...*	ALL
24.44	497 $[M+Na]^+$	473.1661 $[M-H]^-$	$C_{21}H_{30}O_{12}$	0.7	9.7	*2-Hydroxy-3-methoxy-5-prop-2-en-1-ylphenyl 6-O-[(2,3,4)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]-glucopyranoside / (2)-3-(4-hydroxy-3-methoxyphenyl)prop-2-en-1-yl 6-[(2,3,4)-3,4-dihydroxy-4-(hydroxymethyl)tetrahydrofuran-2-yl]-glucopyranoside / 4-acetyl-2-methoxyphenyl 6-(6-deoxymannopyranosyl)-glucopyranoside II...*	C1, C2, G2, J1-2, J2-2, MA1, MA2, HM2, P1, P2, SP1, SP2
24.71	449 $[M+Na]^+$	425.2039 $[M-H]^-$	$C_{18}H_{34}O_{11}$	- 2.5	26.3	*Hexyl 2-allopyranosyl-gulopyranoside / hexyl 6-allopyranosyl-gulopyranoside / 2,3,4-trimethyl-glucopyranosyl 2,3,4-trimethyl-glucopyranoside ...*	C1, C2, LH1, LH2, MA1, HM2, N1, N2, SP1, SP2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
25.18	271 $[M+Na]^+$ 287 $[M+K]^+$	247.1545 $[M-H]^-$	$C_{12}H_{24}O_5$	2.3	13.4	*3-Hydroxy-2,2-bis(hydroxymethyl)propyl heptanoate / hexyl 3-deoxy-xylo-hexopyranoside /2-[2-(2-butoxyethoxy)ethoxy]ethyl acetate…*	ALL
25.21	–	427.1964 $[M-H]^-$	$C_{21}H_{32}O_9$	2.3	43.7	*2,3-Dideoxy-1-(2,2-dimethylpropanoyl)-2-[-(6-methoxy-1,3-benzodioxol-5-yl)(methoxymethoxy)methyl]-erythro-pentitol / (1)-1-[(2,3,4,5,7,8,8a)-8-(benzyloxy)-2,3,7-trimethoxy-2,3-dimethylhexahydro-5H-pyrano[3,4-b][1,4]dioxin-5-yl]ethane-1,2-diol II …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J2-1, J2-2,MA1, MA2, HM2, N2, SP1, SP2
25.26	463 $[M+Na]^+$	439.2173 $[M-H]^-$ 485 $[M-H+46]^-$	$C_{19}H_{36}O_{11}$	2.7	27.1	*(8)-2,6-Anhydro-7,9,10,11,12,13-hexadeoxy-3-glucopyranosyl-glycero-gulo-tridecitol / methyl 2,3-dimethyl-4-(2,3,4,6-tetra-methyl-idopyranosyl)-glucopyranoside I…*	C1, C2, G1, G2, LH1, N1, N2, P1, P2, SP1, SP2
25.31	269 $[M+Na]^+$ 285 $[M+K]^+$ 211 $[M+H-18-18]^+$ 193 $[M+H-18-18-18]^+$	245.1388 $[M-H]^-$ 227 $[M-H-18]^-$ 209 $[M-H-18-18]^-$ 185 $[M-H-18-42]^-$	$C_{12}H_{22}O_5$	2.5	60.4	*1-3-Ethyl-3,4,5-trihydroxy-2,2-dimethylcyclohexyl acetate / Hydroxydodecanedioic acid / dimethyl 3-hydroxydecanedioate / N-ethoxyacetylglycine …*	ALL
25.38	493 $[M+Na]^+$, 509 $[M+K]^+$, 453 $[M+H-18]^+$	469.1339 $[M-H]^-$ 939 $[2M-H]^-$	$C_{21}H_{26}O_{12}$	2.7	13.0	*4,8-Dihydroxynaphthalen-1-yl 6-arabinofuranosyl-glucopyranoside / 4-methyl-2-oxo-2H-chromen-7-yl 4-galactopyranosyl-xylopyranoside I*	G2, HS2, J1-2, J2-2, LH2, MA2, HM2, P2, SP2, T2
25.87	463 $[M+Na]^+$ 479 $[M+K]^+$	439.2143 $[M-H]^-$ 485 $[M-H+46]^-$	$C_{19}H_{36}O_{11}$	9.5	26.7	*(8)-2,6-Anhydro-7,9,10,11,12,13-hexadeoxy-3-glucopyranosyl-glycero-gulo-tridecitol / methyl 2,3-dimethyl-4-(2,3,4,6-tetra-methyl-idopyranosyl)-glucopyranoside II…*	C1, C2, G1, G2, HS2, LH1, N2, P2, SP1, SP2
25.99	493 $[M+Na]^+$ 509 $[M+K]^+$ 453 $[M+H-18]^+$	469.1363$[M-H]^-$	$C_{21}H_{26}O_{12}$	- 2.4	26.0	*4,8-Dihydroxynaphthalen-1-yl 6-arabinofuranosyl-glucopyranoside / 4-methyl-2-oxo-2H-chromen-7-yl 4-galactopyranosyl-xylopyranoside II*	G2, HS2, J1-2, J2-2, LH2, MA2, HM2, P2, T2
26.07	463 $[M+Na]^+$	439.2202 $[M-H]^-$ 485 $[M-H+46]^-$	$C_{19}H_{36}O_{11}$	- 3.8	35.4	*(8)-2,6-Anhydro-7,9,10,11,12,13-hexadeoxy-3-glucopyranosyl-glycero-gulo-tridecitol / methyl 2,3-dimethyl-4-(2,3,4,6-tetra-methyl-idopyranosyl)-glucopyranoside III…*	C1, C2, G2, HS1, HS2, J1-2, J2-1, LH1, LH2, N2, P2, SP2
26.15	595 $[M+Na]^+$	571.2384 $[M-H]^-$ 617 $[M-H+46]^-$	$C_{27}H_{40}O_{13}$	2.2	37.4	*(1,2)-2-[(6-Deoxy-galactopyranosyl)oxy]cyclohexyl 3-[(1)-1-carboxy-2-phenylethyl]-galactopyranoside / (2)-8-({[(2,3,4)-3-ethylidene-2-(glucopyranosyloxy)-5-(methoxycarbonyl)-3,4-dihydro-2H-pyran-4-yl]acetyl}oxy)-2,6-dimethyloct-2-enoic acid …*	C1, C2, G2, HT2, MA1, P1, P2, SP2
26.55	469 $[M+H]^+$ 491 $[M+Na]^+$ 451 $[M+H-18]^+$ 403 $[M+H-66]^+$	467.1674 $[M-H]^-$	$C_{26}H_{28}O_8$	4.5	29.2	*Artoindonesianin B / 5,7,2'5'-tetrahydroxy-4'-methoxy-8-prenyl-3-(9-hydroxy)prenylflavone / varixanthone …*	C1, C2, G1, G2, HT1, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
26.57	589 $[M+Na]^+$ 605 $[M+K]^+$	565.2198 $[M-H]^-$	$C_{24}H_{38}O_{15}$	- 1.7	35.1	–	ALL
26.72	–	465.2013 $[M-H]^-$ 511 $[M-H+46]^-$	$C_{21}H_{30}N_4O_8$	- 4.8	24.8	*Ustiloxin F / methyl 2-[[2-[[4-amino-2-[(2-methylpropan-2-yl)oxycarbonylamino]-4-oxobutanoyl]-methylamino]-2-(2-hydroxyphenyl)acetyl]amino]acetate ....*	C1, C2, MA1, SP1
27.03	479 $[M+H]^+$, 501$[M+Na]^+$	477.2668 $[M-H]^-$ 523 $[M-H+46]^-$	$C_{30}H_{38}O_5$	- 3.5	46.0	*3-Geranyl-4,2',4',6'-tetrahydroxy-5-prenyldihydrochalcone / methyl 3,10-dihydroxy-2,4a,6,6a,9,14a-hexamethyl-11-oxo-3,4,5,13,14,14b-hexahydro-1H-picene-2-carboxylate / [5-hydroxy-17-(1-methoxy-1-oxopropan-2-yl)-10,13-dimethyl-1,2,3,4,6,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-3-yl] benzoate I...*	C1, C2, G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, HM1, HM2, N1, N2, SP1, T1, T2
27.21	479 $[M+H]^+$, 501$[M+Na]^+$	477.2662 $[M-H]^-$ 523 $[M-H+46]^-$	$C_{30}H_{38}O_5$	- 3.3	44.3	*3-Geranyl-4,2',4',6'-tetrahydroxy-5-prenyldihydrochalcone / methyl 3,10-dihydroxy-2,4a,6,6a,9,14a-hexamethyl-11-oxo-3,4,5,13,14,14b-hexahydro-1H-picene-2-carboxylate / [5-hydroxy-17-(1-methoxy-1-oxopropan-2-yl)-10,13-dimethyl-1,2,3,4,6,12,14,15,16,17-decahydrocyclopenta[a]phenanthren-3-yl] benzoate II...*	C1, C2, G1, HS1, J1-1, J2-1, J2-2, HM1, T1, T2
27.38	–	453.1968 $[M-H]^-$ 321 $[M-H-132]^-$	$C_{19}H_{34}O_{12}$	2.1	19.4	*Butyl (3)-3-[(6-lyxopyranosyl-gulopyranosyl)oxy]butanoate / methyl 6-[[2-(6-deoxy-galactopyranosyl)-galactopyranosyl]oxy}hexanoate ...*	C1, C2, MA1
27.60	433 $[M+Na]^+$	409.2033 $[M-H]^-$	$C_{25}H_{30}O_5$	- 4.2	26.3	*2-Geranyl-3,4,2',4'-tetrahydroxydihydrochalcone / 4,2',4',6'-Tetrahydroxy-3,5-diprenyldihydrochalcone / Kanzonol Y ...*	C1, C2, G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA2, HM1, HM2, N2, P1, P2, SP1, SP2, T2
27.65	–	393.1761 $[M-H]^-$	$C_{17}H_{30}O_{10}$	1.2	17.6	*3-Hex-3-en-1-yl 6-xylopyranosyl-glucopyranoside / 2-glucopyranosyloxypropane-1,3-diyl dibutanoate III...*	C1, C2, HT2, HS2, P1, P2, SP1
27.83	565 $[M+H]^+$ 587 $[M+Na]^+$	563.2721 $[M-H]^-$ 519 $[M-H-44]^-$	$C_{26}H_{44}O_{13}$	- 2.1	48.5	–	C1, C2, G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA1, HM1, HM2, T1, T2
28.10	565 $[M+H]^+$ 587 $[M+Na]^+$	563.2710 $[M-H]^-$ 519 $[M-H-44]^-$	$C_{26}H_{44}O_{13}$	- 0.2	7.3	–	C1, C2, G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH2, MA1, HM1, HM2, N1, N2, T1, T2
28.15	471 $[M+Na]^+$ 487 $[M+K]^+$	447.1265 $[M-H]^-$	$C_{29}H_{20}O_5$	- 5.9	45.2	*[3-(3-Methylphenoxy)-4-oxochromen-7-yl] 4-phenylbenzoate / 1-(2-methylphenyl)dodecane-1,2-dione...*	C1, C2, 2-2, HS2, J1-2, J2-2, LH2, MA2, HM2, SP2, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
28.34	475 $[M+Na]^+$ 491 $[M+K]^+$	451.1624 $[M-H]^-$	$C_{22}H_{28}O_{10}$	- 6.0	34.2	*3,6-Dioxocyclohexa-1,4-diene-1,2,4,5-tetrayl tetrabutanoate / bis(2-methoxyethyl) 2-(1,3-benzodioxol-5-yl)-4-hydroxy-4-methyl-6-oxocyclohexane-1,3-dicarboxylate / methyl (1,4a,6,7,7a)-6-({[(2,3,4)-3-formyl-4-(2-methoxy-2-oxoethyl)-2-methyl-3,4-dihydro-2H-pyran-5-yl]carbonyl}oxy)-1-hydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[c]pyran-4-carboxylate / ...*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
28.41	471 $[M+Na]^+$ 487 $[M+K]^+$	447.1274 $[M-H]^-$	$C_{22}H_{24}O_{10}$	5.0	24.6	*Sakuranin / androechin / agehoustin C...*	G2, J1-2, LH1, LH2, MA2, HM2, N2, P2, T2
28.69	–	439.1051 $[M-H]^-$	$C_{23}H_{20}O_9$	- 3.7	31.2	*Pongamoside A / propyl 4-{[5,7-bis(acetyloxy)-4-oxo-4H-chromen-3-yl]oxy}benzoate / methyl 4-[(acetyloxy)methyl]-2,5-bis(1,3-benzodioxol-5-yl)-4,5-dihydrofuran-3-carboxylate ...*	HT1, HT2, P2
28.94	565 $[M+H]^+$ 587 $[M+Na]^+$	563.2704 $[M-H]^-$ 519 $[M-H-44]^-$	$C_{26}H_{44}O_{13}$	1.1	59.0	–	C1, C2, J2-1, LH1, LH2, MA1, MA2, HM1
29.89	589 $[M+Na]^+$ 605 $[M+K]^+$	565.2263 $[M-H]^-$	$C_{28}H_{38}O_{12}$	4.8	14.4	–	G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
30.02	311 $[M+Na]^+$ 427 $[M+K]^+$ 211 [M+H-18-60 (18+42)]$^+$ 193 [M+H-18-60-18]$^+$ 175 [M+H-18-60-18-18]$^+$ 157 [M+H-18-60-18-18-18]$^+$ 111 [157-46]$^+$	287.1493 $[M-H]^-$ 269 $[M-H-18]^-$ 227 $[M-H-18-42]^-$ 209 $[M-H-60-18]^-$ 185 $[M-H-60-42]^-$	$C_{14}H_{24}O_6$	2.4	7.4	*(1,2,3,6)-3,6-Dihydroxy-3-methyl-6-(1-methylethyl)cyclohexane-1,2-diyl diacetate/ Bis(2-(hydroxymethyl)butyl) 2-butenedioate / D-Galactono-1,4-lactone, 5,6-O-octylidene*	ALL
30.59	367 $[M+Na]^+$ 383 $[M+K]^+$	343.1764 $[M-H]^-$	$C_{17}H_{28}O_7$	- 0.6	9.7	*4-Hydroxymonic acid / diethyl 2,4-diacetyl-3-(4-hydroxybutyl)pentanedioate / [6-oxo-6-(2-prop-2-enoyloxyethoxy)hexyl] 6-hydroxyhexanoate I...*	ALL
30.68	–	283.1544 $[M-H]^-$	$C_{15}H_{24}O_5$	2.4	13.8	*Dihydroartemisinin / dihydrohymenovin ...*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
30.84	<u>515</u> $[M-H]^+$ 537 $[M+Na]^+$ 553 $[M+K]^+$ 497 $[M+H-18]^+$ 461 $[M+H-18-18-18]^+$	513.2342 $[M-H]^-$	$C_{25}H_{38}O_{11}$	- 0.2	27.9	*[(1,3,4a,5,9a)-1-[(2)-2-acetyloxypropanoyl]oxy-4a-methyl-5-[(2-methylpropan-2-yl)oxy]-9-oxo-1,3,4,5,6,7,8,9a-octahydrocyclohepta[c]pyran-3-yl] (2)-2-acetyloxypropanoate / 5-[(2,3,4,5,6)-3,5-dihydroxy-2-(hydroxymethyl)-6-[1-methyl-3-(2,6,6-trimethyl-4-oxo-cyclohex-2-en-1-yl)allyloxy]tetrahydropyran-4-yl]oxy-3-hydroxy-3-methyl-5-oxo-pentanoic acid …*	C1, C2, G1, G2,HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM2, N1, N2, P1, P2, SP1, SP2, T2
31.63	<u>367</u> $[M+Na]^+$ 383 $[M+K]^+$	343.1756 $[M-H]^-$	$C_{17}H_{28}O_7$	1.7	18.5	*4-Hydroxymonic acid / diethyl 2,4-diacetyl-3-(4-hydroxybutyl)pentanedioate / [6-oxo-6-(2-prop-2-enoyloxyethoxy)hexyl] 6-hydroxyhexanoate II …*	C1, C2, G1, HS1, HS2, J1-2, J2-2, LH1, LH2, MA1, HM1, N1, N2, P1, P2, SP1, SP2, T1
32.03	235 $[M+H]^+$	233.0432 $[M-H]^-$ 218 $[M-H-15]^-$	$C_{12}H_{10}O_5$	4.2	9.5	*7-Methoxycoumarin-4-acetic acid / methyl 2-(7-hydroxy-2-oxochromen-4-yl)acetate …*	G1, G2, HT1, HS1, HS2, J1-1, J2-1, LH1, MA1, MA2, HM1, N1, SP1, T1, T2
32.03	<u>549</u> $[M+H]^+$ 571 $[M+Na]^+$ 587 $[M+K]^+$ 531 $[M+H-18]^+$	547.2767 $[M-H]^-$ <u>503</u> $[M-H-44]^-$	$C_{26}H_{44}O_{12}$	2.8	20.7	*[4,5-Dicarboxyoxy-2-(6-methylheptoxycarbonyloxy)cyclohexyl]6-methylheptyl carbonate / [4,5-dicarboxyoxy-2-(2-ethylhexoxycarbonyloxy)cyclohexyl] 2-ethylhexyl carbonate I*	C1, C2, G1, G2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, MA1, HM1, HM2, N1, P1, SP1, T1,
32.54	<u>549</u> $[M+H]^+$ 571 $[M+Na]^+$ 587 $[M+K]^+$ 531 $[M+H-18]^+$	547.2748 $[M-H]^-$ <u>503</u> $[M-H-44]^-$	$C_{26}H_{44}O_{12}$	2.1	10.2	*[4,5-Dicarboxyoxy-2-(6-methylheptoxycarbonyloxy)cyclohexyl]6-methylheptyl carbonate / [4,5-dicarboxyoxy-2-(2-ethylhexoxycarbonyloxy)cyclohexyl] 2-ethylhexyl carbonate II*	C1, C2, G1, G2, MA1, HM1, SP1, T1
32.72	365 $[M+Na]^+$	341.1574 $[M-H]^-$	$C_{24}H_{22}O_2$	- 4.5	34.5	*1,3,6-Triphenylhexane-1,5-dione / (3-methyl-4,4-diphenylbut-3-enyl) benzoate / 3-methyl-1,2,5-triphenylpentane-1,5-dione …*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, N1, N2, P1, P2, SP1, SP2, T1, T2
33.82	313 $[M+Na]^+$	289.2018 $[M-H]^-$	$C_{15}H_{30}O_5$	0.9	18.6	*2,3-Dihydroxypropyl 5-hydroxydodecanoate / 4-(8-ethoxyoctoxy)-2-hydroxy-2-methylbutanoate / 13-hydroxy-12,12-bis(hydroxymethyl)tridecanoate …*	C1, C2, G1, G2, HS1, J1-1, J1-2, J2-1, J2-2, MA1, HM1, HM2, SP1, SP2, T1, T2
34.13	<u>413</u> $[M+Na]^+$ 429 $[M+K]^+$	389.2898 $[M-H]^-$ <u>435</u> $[M-H+46]^-$	$C_{21}H_{42}O_6$	2.7	3.8	*2-[2-[2-(2-Methoxyethoxy)ethoxy]ethoxy]ethyl dodecanoate / (5-tert-butylperoxy-2,5-dimethylhexan-2-yl)oxy 2-ethylhexyl carbonate / pentadecyl glucoside I…*	ALL
34.30	<u>413</u> $[M+Na]^+$ 429 $[M+K]^+$	389.2886 $[M-H]^-$ <u>435</u> $[M-H+46]^-$	$C_{21}H_{42}O_6$	6.4	9.2	*2-[2-[2-(2-Methoxyethoxy)ethoxy]ethoxy]ethyl dodecanoate / (5-tert-butylperoxy-2,5-dimethylhexan-2-yl)oxy 2-ethylhexyl carbonate / pentadecyl glucoside II…*	ALL

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
34.64	<u>411</u> $[M+Na]^+$ 427 $[M+K]^+$	387.2733 $[M-H]^-$ <u>433</u> $[M-H+46]^-$ 369 $[M-H-18]^-$ 351 $[M-H-18-18]^-$ 327 $[M-H-18-42]^-$	$C_{21}H_{40}O_6$	4.9	71.1	*[2,2-Bis(hydroxymethyl)-3-octanoyloxypropyl] octanoate / 2,3-dihydroxypropanoyl 2-hydroxyoctadecanoate / 10-hydroxydecanoyloxymethyl 10-hydroxydecanoate I...*	ALL
34.83	<u>411</u> $[M+Na]^+$ 427 $[M+K]^+$	387.2739 $[M-H]^-$ <u>433</u> $[M-H+46]^-$ 369 $[M-H-18]^-$ 351 $[M-H-18-18]^-$ 327 $[M-H-18-42]^-$	$C_{21}H_{40}O_6$	3.5	34.6	*[2,2-Bis(hydroxymethyl)-3-octanoyloxypropyl] octanoate / 2,3-dihydroxypropanoyl 2-hydroxyoctadecanoate / 10-hydroxydecanoyloxymethyl 10-hydroxydecanoate II...*	ALL
35.60	–	470.3103 $[M-H]^-$	$C_{21}H_{41}N_7O_5$	4.4	9.3	*Isoleucylisoleucylarginylalanine / alanyl-lysyl-lysyl-[(1)-1-methyl-2-oxoethyl]-alaninamide / alanyl-N'5'-(diaminomethylidene)-ornithyl-leucyl-isoleucine ...*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
36.71	<u>451</u> $[M+Na]^+$ 467 $[M+K]^+$	427.2685 $[M-H]^-$ <u>473</u> $[M-H+46]^-$	$C_{23}H_{40}O_7$	3.9	23.7	*[2-Hydroxy-2-(5-hydroxy-3-oxo-4-pentadecoxyfuran-2-yl)ethyl] acetate / 1,3-dihydroxypropan-2-yl 7-[(1,2,3)-3,5-dihydroxy-2-[(3)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoate / (2,5)-6-[(1,2,4a,6,8,8a)-8-(2,2-dimethylbutanoyloxy)-6-hydroxy-2-methyl-1,2,3,4,4a,5,6,7,8,8a-ecahydronaphthalen-1-yl]-2,5-dihydroxyhexanoic acid I...*	C1, C2, G1, G2, HT2, HS1, HS2, J1-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1
37.82	317 $[M+H]^+$	315.2170$[M-H]^-$	$C_{17}H_{32}O_5$	2.1	19.9	*(13-Hydroxytridecyl)(methyl)propanedioic acid / 7-hydroxy-2,2,12,12-tetramethyltridecanedioic acid / 2-3-hydroxy-2-[(3-methylbutanoyl)oxy]propyl 4-methyl-3-(propan-2-yl)pentanoate ....*	C1, C2, G1, G2, J2-1, MA1, P2
37.88	<u>453</u> $[M+Na]^+$ 469 $[M+K]^+$ 317 $[M+H-114]^+$	429.2846 $[M-H]^-$ <u>475</u> $[M-H+46]^-$ 369 $[M-H-60]^-$ 351 $[M-H-60-18]^-$	$C_{23}H_{42}O_7$	2.3	22.8	–	ALL
38.25	<u>453</u> $[M+Na]^+$ 469 $[M+K]^+$ 317 $[M+H-114]^+$	429.2823 $[M-H]^-$ <u>475</u> $[M-H+46]^-$ 369 $[M-H-60]^-$	$C_{23}H_{42}O_7$	- 3.4	54.8	–	C1, C2, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
38.84	469 $[M+Na]^+$ 485 $[M+K]^+$	445.2783 $[M-H]^-$ 491 $[M-H+46]^-$	$C_{19}H_{38}N_6O_6$	1.9	37.3	*Glutaminyl-[(2)-6-amino-1-(carboxyoxy)hexan-2-yl]-2'-(5-aminopentyl)glycinamide / alanyllysyllysylthreonine / 7,15,15a-trimethyl-11-(1-methylethyl)-3-(2-methylpropyl)-5,9,13-trioxo-14-oxa-3,4,7,8,11,12-hexaazahexadecan-1-oic acid I…*	C1, C2, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
39.06	469 $[M+Na]^+$ 485 $[M+K]^+$	445.2781 $[M-H]^-$ 491 $[M-H+46]^-$	$C_{19}H_{38}N_6O_6$	0.9	47.5	*Glutaminyl-[(2)-6-amino-1-(carboxyoxy)hexan-2-yl]-2'-(5-aminopentyl)glycinamide / alanyllysyllysylthreonine / 7,15,15a-trimethyl-11-(1-methylethyl)-3-(2-methylpropyl)-5,9,13-trioxo-14-oxa-3,4,7,8,11,12-hexaazahexadecan-1-oic acid II…*	ALL
39.33	452 $[M+Na]^+$ 467 $[M+K]^+$	427.2681 $[M-H]^-$ 473 $[M-H+46]^-$	$C_{23}H_{40}O_7$	4.8	49.0	*[2-Hydroxy-2-(5-hydroxy-3-oxo-4-pentadecoxyfuran-2-yl)ethyl] acetate / 1,3-dihydroxypropan-2-yl 7-[(1,2,3)-3,5-dihydroxy-2-[(3)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoate / (2,5)-6-[(1,2,4,6,8,8a)-8-(2,2-dimethylbutanoyloxy)-6-hydroxy-2-methyl-1,2,3,4,4a,5,6,7,8,8a-ecahydronaphthalen-1-yl]-2,5-dihydroxyhexanoic acid II…*	C1, C2, G1, G2, HT1, HT2, HS1, J1-2, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P2, SP1, SP2, T1, T2
39.34	469 $[M+Na]^+$, 485 $[M+K]^+$	445.2783 $[M-H]^-$ 491 $[M-H+46]^-$	$C_{19}H_{38}N_6O_6$	- 0.1	18.2	*Glutaminyl-[(2)-6-amino-1-(carboxyoxy)hexan-2-yl]-2'-(5-aminopentyl)glycinamide / alanyllysyllysylthreonine / 7,15,15a-trimethyl-11-(1-methylethyl)-3-(2-methylpropyl)-5,9,13-trioxo-14-oxa-3,4,7,8,11,12-hexaazahexadecan-1-oic acid III…*	ALL
39.81	369 $[M+Na]^+$ 385 $[M+K]^+$	345.2274 $[M-H]^-$	$C_{18}H_{34}O_6$	2.6	15.0	*Hydroperoxy-dihydroxy-octadecenoic acid / Dihydroxy-octadecanedioic acid / Bis(2-ethoxyethyl)sebacate I…*	C1, C2, G1, G2,HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
40.08	369 $[M+Na]^+$ 385 $[M+K]^+$	345.2268 $[M-H]^-$	$C_{18}H_{34}O_6$	4.3	31.3	*Hydroperoxy-dihydroxy-octadecenoic acid / Dihydroxy-octadecanedioic acid / Bis(2-ethoxyethyl)sebacate II…*	C1, C2, G1, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
40.55	367 $[M+Na]^+$	343.2127 $[M-H]^-$	$C_{18}H_{32}O_6$	- 0.3	22.9	*Triisovalerin / trivalerin / 10,11-dihydroxy-9,12-dioxooctadecanoic acid …*	C1, C2, P2, SP1, T1
41.87	658 $[M+H]^+$	656.3189$[M-H]^-$	$C_{39}H_{47}NO_8$	- 2.4	85.1	*{3-[(1)-1-[({(2)-1-[(2)-2-Cyclohexyl-2-phenylacetyl]piperidin-2-yl}carbonyl)oxy]-3-(3,4-dimethoxyphenyl)propyl]phenoxy}acetic acid / benzyl (2,5,8a)-2,5-bis(3-{[(benzyloxy)carbonyl]oxy}propyl)octahydroquinoline-1(2H)-carboxylate I …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, SP1, SP2, T1, T2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
42.08	658 $[M+H]^+$	656.3204 $[M-H]^-$	$C_{39}H_{47}NO_8$	- 1.3	70.7	*{3-[(1)-1-[({[(2)-1-[(2)-2-Cyclohexyl-2-phenylacetyl]piperidin-2-yl}carbonyl)oxy]-3-(3,4-dimethoxyphenyl)propyl]phenoxy}acetic acid / benzyl (2,5,8a)-2,5-bis(3-{[(benzyloxy)carbonyl]oxy}propyl)octahydroquinoline-1(2H)-carboxylate II …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, SP1, SP2, T1, T2
42.39	440 $[M+H]^+$	438.3209 $[M-H]^-$	$C_{25}H_{45}NO_5$	3.7	10.1	*Ethyl 4,4-dimethyl-3,5-dioxo-1-(3-hydroxy-3-methylnonyl)-2-pyrrolidineheptanoate / [(2)-1-[(2,3)-3-(8-methylnonyl)-4-oxooxetan-2-yl]nonan-2-yl] 2-formamidoacetate / [(2,3)-2-acetamido-3-acetyloxynonadec-4-enyl] acetate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP2, T1, T2
42.72	658 $[M+H]^+$	656.3188 $[M-H]^-$	$C_{39}H_{47}NO_8$	- 2.8	75.2	*{3-[(1)-1-[({[(2)-1-[(2)-2-Cyclohexyl-2-phenylacetyl]piperidin-2-yl}carbonyl)oxy]-3-(3,4-dimethoxyphenyl)propyl]phenoxy}acetic acid / benzyl (2,5,8a)-2,5-bis(3-{[(benzyloxy)carbonyl]oxy}propyl) octahydroquinoline-1(2H)-carboxylate III …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, SP1, SP2, T1, T2
43.23	422 $[M+H]^+$	420.3110 $[M-H]^-$	$C_{25}H_{43}NO_4$	2.1	4.9	*4-Dodecyl-3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine / N-dodecyl-3,4,5-triethoxy-benzamide I…*	ALL
43.46	–	480.3319 $[M-H]^-$	$C_{27}H_{47}NO_7$	2.5	16.2	*2-Amino-5-pentadecylphenyl hexopyranoside / (3,5,14,20)-14-amino-20-hydroxypregnan-3-yl 6-deoxy-mannopyranoside…*	ALL
43.61	398 $[M+H]^+$	396.3111 $[M-H]^-$	$C_{23}H_{43}NO_4$	2.2	23.1	*Hexadec-2-enoyl carnitin / 4-hydroxy-1-octadecanoylpyrrolidine-2-carboxylic acid / heptadecyl 2-(prop-2-enoxycarbonylamino)acetate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP2, T1, T2
43.78	–	456.3313 $[M-H]^-$	$C_{25}H_{47}NO_6$	3.9	28.8	*Ethyl 3-[(3a,5,6,6a)-6-methoxy-2,2-dimethyl-3a,5,6,6a-tetrahydrofuro[2,3-d][1,3]dioxol-5-yl]-3-(dodecylamino)propanoate / (3a,5,6,6a)-3a-(dodecoxymethyl)-2,2-dimethyl-5-(morpholin-4-ylmethyl)-6,6a-dihydro-5H-furo[2,3-d][1,3]dioxol-6-ol / tert-butyl (4,5)-5-[3-hydroxy-9-(oxan-2-yloxy)nonyl]-2,2,4-trimethyl-1,3-oxazolidine-3-carboxylate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP2, T1, T2
44.22	317 $[M+H]^+$ 339 $[M+Na]^+$ 355 $[M+K]^+$	315.2538 $[M-H]^-$	$C_{18}H_{36}O_4$	0.8	76.3	*Dihydroxystearic acid / hydroxyethoxyethyl myristate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM2, N1, N2, P1, P2, SP1, SP2

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
44.29	439 $[M+K]^+$	399.2739 $[M-H]^-$	$C_{22}H_{40}O_6$	3.3	21.3	*9,10-Diacetoxyoctadecanoic acid / (2-acetyloxy-3-hydroxy-propyl)-16-methoxyhexadec-9-enoate / 2-(2-hexadecanoyloxypropanoyloxy)propanoic acid I…*	ALL
44.34	439 $[M+K]^+$	399.2682 $[M-H]^-$	$C_{22}H_{40}O_6$	3.4	34.7	*9,10-Diacetoxyoctadecanoic acid / (2-acetyloxy-3-hydroxy-propyl)-16-methoxyhexadec-9-enoate / 2-(2-hexadecanoyloxypropanoyloxy)propanoic acid II…*	ALL
44.42	451 $[M+Na]^+$ 467 $[M+K]^+$	427.2701 $[M-H]^-$ 473 $[M-H+46]^-$	$C_{23}H_{40}O_7$	0.1	26.8	*[2-Hydroxy-2-(5-hydroxy-3-oxo-4-pentadecoxyfuran-2-yl)ethyl] acetate / 1,3-dihydroxypropan-2-yl 7-[(1,2,3)-3,5-dihydroxy-2-[(3)-3-hydroxyoct-1-enyl]cyclopentyl]hept-5-enoate / (2,5)-6-[(1,2,4,6,8,8a)-8-(2,2-dimethylbutanoyloxy)-6-hydroxy-2-methyl-1,2,3,4,4a,5,6,7,8,8a-ecahydronaphthalen-1-yl]-2,5-dihydroxyhexanoic acid III…*	C1, C2, G1, G2, HT2, HS2, J1-2, J2-2, LH2, MA1, MA2, HM2, N2, P2, SP1, SP2, T2
44.59	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.2341 $[M-H]^-$	$C_{19}H_{32}O_2$	- 3.8	35.1	*Methyl linolenate / pinolenic acid methyl ester/ grevillol I …*	ALL
44.64	439 $[M+K]^+$	399.2736 $[M-H]^-$	$C_{29}H_{36}O$	- 4.6	16.9	*[Decoxy(diphenyl)methyl]benzene / 2-phenyl-1,3-bis(4-tert-butylphenyl)propan-2-ol I…*	ALL
44.65	417 $[M+H]^+$ 439 $[M+Na]^+$	415.2682 $[M-H]^-$	$C_{22}H_{40}O_7$	3.0	41.7	*Mono(lauryl-triethyleneglycolether)maleate / 3-Carboxy-2-hexadecyl-3-hydroxy-pentanedioic acid / 6-palmitoyl-ascorbate I*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
44.86	–	422.3258 $[M-H]^-$	$C_{25}H_{45}NO_4$	4.2	2.0	*Methyl (2,4)-4-[(2-oxohexadecanoyl)amino]oct-2-enoate / 2-hexadecyl 1-prop-2-en-1-yl pyrrolidine-1,2-dicarboxylate …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
44.97	417 $[M+H]^+$ 439 $[M+Na]^+$	415.2692 $[M-H]^-$	$C_{22}H_{40}O_7$	2.1	23.3	*Mono(lauryl-triethyleneglycolether)maleate / 3-Carboxy-2-hexadecyl-3-hydroxy-pentanedioic acid / 6-palmitoyl-ascorbate II*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
44.99	478 $[M+H]^+$ 500 $[M+Na]^+$ 516 $[M+K]^+$	476.2742 $[M-H]^-$	$C_{25}H_{39}N_3O_6$	- 2.8	43.0	*Methyl-(tert-butoxycarbonyl)-phenylalanyl-2-methylalanyl-leucinate / methyl (tert-butoxycarbonyl)-phenylalanyl-leucyl-2-methylalaninate I…*	ALL

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
45.02	421 $[M+Na]^+$ 437 $[M+K]^+$	397.2607 $[M-H]^-$	$C_{22}H_{38}O_6$	- 2.9	6.1	*7,16-Dioxodocosanedioic acid / bis[4-(tetrahydrofuran-2-yl)butan-2-yl] hexanedioate ...*	ALL
45.02	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.2311 $[M-H]^-$	$C_{19}H_{32}O_2$	6.3	26.8	*Methyl linolenate / pinolenic acid methyl ester/ grevillol II ...*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
45.23	520 $[M+H-46]^+$	564.3297 $[M-H]^-$	$C_{29}H_{47}N_3O_8$	- 1.2	17.8	*Ethyl 3-[[2-(2-hydroxy-3-methylphenyl)-2-[[3-hydroxy-2-[(2-methylpropan-2-yl)oxycarbonylamino]propanoyl]-(5-methylhexan-2-yl)amino]acetyl]amino]propanoate / ethyl 3-[[2-(4-hydroxy-3-methylphenyl)-2-[[3-hydroxy-2-[(2-methylpropan-2-yl)oxycarbonylamino]propanoyl]-(5-methylhexan-2-yl)amino]acetyl]amino]propanoate I...*	ALL
45.26	478 $[M+H]^+$ 500 $[M+Na]^+$ 516 $[M+K]^+$	476.2757 $[M-H]^-$	$C_{25}H_{39}N_3O_6$	- 2.4	9.8	*Methyl-(tert-butoxycarbonyl)-phenylalanyl-2-methylalanyl-leucinate / methyl (tert-butoxycarbonyl)-phenylalanyl-leucyl-2-methylalaninate II...*	ALL
45.50	520 $[M+H-18-28]^+$	564.3290 $[M-H]^-$	$C_{29}H_{47}N_3O_8$	- 3.1	14.4	*Ethyl 3-[[2-(2-hydroxy-3-methylphenyl)-2-[[3-hydroxy-2-[(2-methylpropan-2-yl)oxycarbonylamino]propanoyl]-(5-methylhexan-2-yl)amino]acetyl]amino]propanoate / ethyl 3-[[2-(4-hydroxy-3-methylphenyl)-2-[[3-hydroxy-2-[(2-methylpropan-2-yl)oxycarbonylamino]propanoyl]-(5-methylhexan-2-yl)amino]acetyl]amino]propanoate II...*	ALL
45.53	–	289.2174 $[M-H]^-$	$C_{19}H_{30}O_2$	- 0.2	36.8	*Benzyl laurate / dodecyl benzoate / stearidonic acid methyl ester II...*	ALL
45.86	435 $[M+Na]^+$ 451 $[M+K]^+$	411.2733 $[M-H]^-$ 457 $[M-H+46]^-$	$C_{23}H_{40}O_6$	4.8	25.2	*Bis[3-(oxolan-2-yl)propyl] nonanedioate / ethyl-G13-hydroxy-3-[hydroxy(methoxy)methylidene]-2-oxononadec-10-enoate...*	ALL
45.88	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.2318 $[M-H]^-$	$C_{19}H_{32}O_2$	3.8	29.1	*Methyl linolenate / pinolenic acid methyl ester/ grevillol III ...*	ALL
45.95	–	447.2510 $[M-H]^-$	$C_{24}H_{36}N_2O_6$	- 2.1	183.0	*1,1'-(Ethane-1,2-diyldiimino)bis[3-(2-ethoxyphenoxy)propan-2-ol] / 3-[(tert-butoxycarbonyl-tert-butyltyrosyl)amino]cyclopentanecarboxylic acid ...*	ALL

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
46.09	439 $[M+K]^+$	399.2717 $[M-H]^-$	$C_{29}H_{36}O$	-0.5	46.3	*[Decoxy(diphenyl)methyl]benzene / 2-phenyl-1,3-bis(4-tert-butylphenyl)propan-2-ol II…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
46.09	333 $[M+H]^+$ 355 $[M+Na]^+$ 371 $[M+K]^+$	331.2249 $[M-H]^-$	$C_{21}H_{32}O_3$	7.0	21.1	*Decyl 2-methoxy-4-prop-2-enylbenzoate / (4)-1-(4-hydroxy-3-methoxyphenyl)tetradec-4-en-3-one / 4-n-dodecyloxycinnamic acid I…*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
46.12	478 $[M+H]^+$ 500 $[M+Na]^+$ 516 $[M+K]^+$	476.2755 $[M-H]^-$	$C_{25}H_{39}N_3O_6$	-2.6	27.3	*Methyl-(tert-butoxycarbonyl)-phenylalanyl-2-methylalanyl-leucinate / methyl (tert-butoxycarbonyl)-phenylalanyl-leucyl-2-methylalaninate III…*	ALL
46.22	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.2319 $[M-H]^-$	$C_{19}H_{32}O_2$	3.5	24.9	*Methyl linolenate / pinolenic acid methyl ester/ grevillol IV …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
46.22	522 $[M+H-18-28]^+$	566.3455 $[M-H]^-$ 506 $[M-H-60]^-$	$C_{29}H_{49}N_3O_8$	-1.5	14.9	–	ALL
46.50	522 $[M+H-18-28]^+$	566.3458 $[M-H]^-$ 506 $[M-H-60]^-$	$C_{29}H_{49}N_3O_8$	-1.9	15.5	–	ALL
46.55	–	289.2164 $[M-H]^-$	$C_{19}H_{30}O_2$	3.2	52.0	*Benzyl laurate / dodecyl benzoate / stearidonic acid methyl ester I…*	ALL
46.55	334 $[M+H]^+$ 355 $[M+Na]^+$ 371 $[M+K]^+$	331.2244 $[M-H]^-$	$C_{21}H_{32}O_3$	3.7	108.3	*Decyl 2-methoxy-4-prop-2-enylbenzoate / (4)-1-(4-hydroxy-3-methoxyphenyl)tetradec-4-en-3-one / 4-n-dodecyloxycinnamic acid II…*	C1, C2, G1, G2, HT1, HT2, HS1, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
46.64	417 $[M+H]^+$ 439 $[M+Na]^+$	415.2679 $[M-H]^-$	$C_{22}H_{40}O_7$	5.3	33.7	*Mono(lauryl-triethyleneglycolether)maleate / 3-Carboxy-2-hexadecyl-3-hydroxy-pentanedioic acid / 6-O-Palmitoyl-ascorbate III*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
46.76	–	279.1978 $[M-H]^-$	$C_{17}H_{28}O_3$	-4.3	79.0	*Hydroxy-heptadecatrienoic acid / dodecyl furoate …*	ALL

Table 5.2. **Supporting information** (continued)

t_r (min)[a]	ESI(+) TOF MS[b]	ESI(-) TOF MS[b]	Molecular formula [M] generated	For experimental $[M-H]^-$		*Assignment*[d]	Avocado samples containing the compound[e]
				Error (ppm)[c]	mSigma value[c]		
46.77	–	225.1851 $[M-H]^-$	$C_{14}H_{26}O_2$	4.1	17.6	*Myristoleic acid / 5-methyl-2-tridecenoic acid / 2-tetradecenoic acid …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-1, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, SP2, T1, T2
46.82	281 $[M+Na]^+$ 297 $[M+K]^+$	257.2118 $[M-H]^-$	$C_{15}H_{30}O_3$	1.6	16.8	*Hydroxy-pentadecanoic acid / Hydroxypropyl dodecanoate / Methoxyethyl laurate…*	C1, C2, G1, G2, HT2, HS1, HS2, J1-1, J1-2, J2-1, LH1, LH2, MA1, MA2, HM1, HM2, P1, P2, SP1, SP2, T1, T2
46.86	335 $[M+H]^+$ 355 $[M+Na]^+$ 371 $[M+K]^+$	331.2256 $[M-H]^-$	$C_{21}H_{32}O_3$	2.5	115.0	*Decyl 2-methoxy-4-prop-2-enylbenzoate / (4)-1-(4-hydroxy-3-methoxyphenyl)tetradec-4-en-3-one / 4-n-dodecyloxycinnamic acid III…*	ALL
46.97	293 $[M+H]^+$ 315 $[M+Na]^+$ 331 $[M+K]^+$	291.2319 $[M-H]^-$	$C_{19}H_{32}O_2$	3.6	20.1	*Methyl linolenate / pinolenic acid methyl ester/ grevillol V …*	C1, C2, G1, G2, HT1, HT2, HS1, HS2, J1-1, J1-2, J2-2, LH1, LH2, MA1, MA2, HM1, HM2, N1, N2, P1, P2, SP1, T1, T2
46.99	422 $[M+H]^+$ 444 $[M+Na]^+$	420.3099 $[M-H]^-$	$C_{25}H_{43}NO_4$	4.9	6.8	*4-Dodecyl-3,5-diethoxycarbonyl-1,4-dihydro-2,6-dimethylpyridine / N-dodecyl-3,4,5-triethoxy-benzamide II…*	ALL
47.46	–	433.2354 $[M-H]^-$	$C_{28}H_{34}O_4$	4.5	29.5	*1,16-Diphenylhexadecane-3,5,12,14-tetrone / (2,4-dihydroxy-6-methoxy-3,5-dimethylphenyl)[(1,6)-4-(4-methylpent-3-en-1-yl)-6-phenylcyclohex-3-en-1-yl]methanone …*	ALL

A book chater has been published derived from this work:

- E. Hurtado-Fernández, M. Gómez-Romero, A. Carrasco-Pancorbo, A. Fernández-Gutiérrez. Determination of avocado fruit metabolites by UHPLC-MS: Complementarity with other analytical platforms, in; Ultra performance liquid chromatography-Mass spectrometry: Evaluation and applications in food analysis. X. X (Eds). CRC press, Taylor & Francis group, Boca ratón, FL, USA (2014) pp-pp.

Chapter 6

Avocado metabolic changes over the development and maturation: Preliminary results of a longitudinal study

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Abstract

Herewith, a very interesting study about the evolution of 4 avocado varieties collected at different time points over their harvest season is presented. This type of longitudinal approaches can give us, in a very simple way, worthy information, since a comprehensive metabolic profiling of avocado fruit is achieved. All the samples under study (around 175 avocado extracts) have been analyzed by a powerful HPLC-DAD/FL/ESI-IT MS method, and the obtained data has been subsequently treated with chemometrics tools to evaluate those changes produced in avocado composition depending on fruit harvesting date. It has been possible to determine around 200 metabolites in a single run (30 min), and five known compounds (*p*-coumaric, chlorogenic, quinic and abscisic acids, and epicatechin) have been quantified in terms of their pure standards. The information achieved by this interesting approach can offer a real alternative to farmers for an existing problem, because the specific and adequate avocado picking time tend to be established according to the oil content and dry matter in the avocado flesh. Besides, nowadays there are numerous evidences about the "positive" influence that some of the avocado compounds have on human health. Thus, being aware about how the ripening process affects the metabolites, it is possible to know the optimum time to harvest the fruits for getting the maximum benefits.

Keywords: Food metabolomics / *Persea americana* / Metabolic evolution (longitudinal study) / Chemometrics / Liquid chromatography / Mass spectrometry

6.1. Introduction

The avocado (*Persea americana*) is a berry fruit with a dark green leathery skin and a very large seed [1], which is grown on five continents and almost 50 countries around the world [2, 3]. During the last decades, the demand of this fruit has considerably grown in several markets, causing a remarkable increase of the volume of avocado that is intended for exporting [4]. This is, in part, caused by the increasing evidence of the health benefits associated with avocado consumption (apoptosis in human oral cancer cell lines, antibacterial activity, avocado´s unsaponifiables used as food supplements, anti-inflammatory effect, etc.) [5-8]. Based on their respiratory pattern and ethylene biosynthesis during ripening, avocado fruits are further classified as climacteric type, i.e.

harvested at full maturity, can be ripened off the parent plant [9]. These fruits may be retained on the tree for several months after reaching the physiological maturity, this means that horticultural maturity can be manipulated by simply delaying harvest and allows marketers to co-ordinate harvest when demand is strong [10]. However, it is quite evident that fruits picked early in the season will not have the same texture, flavour, dry matter percentage and oil content, firmness, possible internal defects (bruising), phytochemical profile, etc. as the fruits harvested at the end of the season. Reid [11] defined horticultural or commercial maturity as the developmental stage where harvested fruit will undergo normal ripening and provide good eating quality. This stage is very difficult to determine, since maturation does not produce changes in external appearance. This subject has been extensively discussed in literature [3, 12-16] and different strategies have been used to determine avocado fruit maturity, such as correlation between dry weight and oil content, fruit weight to length ratio, size, absent of defects, and firmness [17-20].

Advances in chromatography and electrophoresis, both coupled to MS have enabled to deal with the characterization of as many metabolites as possible in any biological system [21-24]. Indeed, the use of powerful tools and new approaches (such as those used in metabolomics) could help to accomplish a comprehensive characterization of avocado metabolome. So far, to determine the composition of avocado fruit, targeted approaches –where a particular kind of compounds is determined [1, 25-30]– have been used more extensively than global and exploratory analysis [31, 32]. However, it seems that the latter are more appropriate for studying avocado metabolites and their dynamic changes over the ripening, for instance.

Within this context it is important to stand out that in the field of food metabolomics numerous studies have been published investigating fruit composition and the relationship between fruit quality and factors such as fruit varieties, developmental stages, climatic conditions, degree of maturity, cultivation practices, biotic or abiotic stresses, etc. Metabolic profiling is considered to be a very promising tool to be used in these kinds of studies [31, 33-35]; however, something which has to be considered is the fact that biological metabolic profiles have a very complex nature, influenced by many external factors. In other words, the plant metabolic profiles are highly variable within a

system and may vary a lot even from day to day; metabolic profiles reflect not only belonging to one particular group (for instance, individuals coming from the same fruit variety), but they depend remarkably on several environmental factors as well as transportation and storage conditions. A way to overcome possible negative effects of this variability on the interpretation of metabolomics data is multiple sampling over time per individual in a longitudinal study design [36]. The main advantage of this approach is the possibility to get an insight into the biological processes, which are usually missed by a simple, static comparison of, for example, "ripe and unripe" samples.

The evolution of fatty acids, sugars, carbohydrates, or the content of some other analytes in avocado fruit has been checked during ripening or storage by several research groups [1, 26, 37-40], however, to the best of our knowledge there is no single study where the metabolic evolution of different varieties of this tropical fruit has been checked over the season by using a metabolic profiling LC-MS approach.

In principle, although the most widely used data analysis methods in metabolomics, such as PCA and PLS-DA, are applicable, they could not be considered as optimal [41] for such dynamic data, being necessary to make use of other methods that allow separating levels of variation. A number of statistical methods that can deal with longitudinal [42] or paired [36, 42] data exist. The longitudinal design offers possibilities for differential analysis; depending on the question of interest, one may focus on differences among the individuals, on variation within each individual class or identification of unique profiles for each subject.

The aim of this study was to determine the metabolic evolution that 4 different avocado varieties ('Hass', 'Reed', 'Bacon', 'Fuerte') undergo over the maturation by using a reverse phase-LC-MS approach. All the varieties were grown under identical conditions, fact which would facilitate the comparison of the metabolic profiles and their evolution. The LC-MS (DAD and FL detectors were used in parallel as well) determined more than 250 molecular features per run. Statistical tools (data pre-processing and data analysis) were used to achieve a more complete picture of the fundamental biochemistry of the avocado fruit, trying to facilitate the understanding of the pathways responsible for the biosynthesis of nutritionally relevant metabolites.

6.2. Materials and methods

6.2.1. Chemicals and reagents

All reagents were of analytical grade and used as received. ACN from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used for preparing mobile phases. Doubly deionized water with a conductivity of 18.2 MΩ was obtained by using a Milli-Q-system (Millipore, Bedford, MA, USA). Standards of quinic acid and succinic acid were purchased from Fluka (St. Louis, EEUU). Pantothenic, abscisic, *p*-coumaric, ferulic, citric and malic acids, as well as two flavanols (catechin and epicatechin) were from Sigma-Aldrich (St. Louis, EEUU). Standards of perseitol (from Carbosynth (Comptom, UK)) and chlorogenic acid from Extrasynthese (Lyon, France) were also used in the current study. β-estradiol, from Sigma-Aldrich (St. Louis, EEUU) was used as IS; it was useful to evaluate the reproducibility of the extraction system and the chromatographic runs. Some other pure standards of metabolite mentioned in previous chapters of this PhD work were used in the preliminary studies.

Stock solutions at concentration of 250 mg/L for each metabolite were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations (within the range 0.5-250 ppm). All solutions were stored in dark flask at -20 ºC. Before separation or injection in the instrument, all the stock solutions, samples, solvents and reagents were filtered with a 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

6.2.2. Samples

The samples selected in this study were part of the germplasm collection from the IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). Fruits grown under identical environmental conditions (soil, rain, light, etc.) of 4 varieties were studied ('Hass', 'Reed', 'Bacon' and 'Fuerte'). The samples were handpicked at different maturity stages (different periods depending on the variety) of 2011-2012 season. The aim was to evaluate the metabolic changes that occur in each variety during its main harvesting season in Southern Spain. ***Table 6.1*** summarizes the dates at which each sample was collected during their harvest seasons.

Tabla 6.1. Summary of the harvest dates corresponding to the different samples of each avocado variety that have been used in this study.

Year	Month	Avocado variety			
		'Bacon'	*'Fuerte'*	*'Hass'*	*'Reed'*
2011	*February*			22/02/2011	
	March			21/03/2011	
	April			18/04/2011	
	May			20/05/2011	
	June			27/06/2011	27/06/2011
	July			06/07/2011	06/07/2011 18/07/2011
	August			01/08/2011 31/08/2011	01/08/2011 17/08/2011 31/08/2011
	September	31/09/2011		16/09/2011 30/09/2011	16/09/2011 31/09/2011
	October	07/10/2011 17/10/2011 24/10/2011 31/10/2011	31/10/2011		17/10/2011
	November	07/11/2011 14/11/2011 21/11/2011 29/11/2011	14/11/2011 29/11/2011		
	December		12/12/2011		
2012	*January*		03/01/2012 17/01/2012 31/01/2012		
	February		16/02/2012		

Five different samples coming from the same batch were studied for each harvest date. For each variety, we had a total of 8-10 different time points over the season. The number of time points and the frequency in the sampling depended on how long the optimum harvesting time was for each variety. The pulp of 3-4 pieces of fruit, which were weighted, peeled, sliced, frozen to be further freeze-dried, was used. Fruits were processed when they reached the edible ripening stage.

A pooled sample of all the avocado extracts under study (e.g. mixing an equivalent volume of each one) was used as analytical QC sample. For validation purposes, the

mentioned QC samples and a standard mix composed by the 12 compounds previously mentioned in the Chemicals and standards section were used. QC samples were injected every 5 samples throughout the batch. Data derived from these samples were used to assess system stability. The standard mix was injected at the end of each sequence at different concentration levels (9 concentration levels between 0.5 and 200 ppm) to build the corresponding calibration curves.

6.2.3. Extraction procedure

Sample extracts were prepared according to the protocol previously described by Hurtado-Fernández et al. [29, 31]. Briefly, 4 g of the freeze-dried (and homogenized) sample were mixed with 40 mL of pure MeOH and 25 ppm of IS. A vortex was used for shaking the tubes during 30 min. The supernatants were taken and centrifuged at 3000 rpm for 10 min, and after that were evaporated to dryness and redissolved in 1 mL of MeOH.

6.2.4. LC-ESI-IT MS system

An Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a diode-array detector (DAD) and a FL was used. The avocado metabolites under study were separated by using a Zorbax C18 analytical column (4.6 x 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at room temperature and a flow rate of 0.8 mL/min. The mobile phases used were water with 0.5% of acetic acid (Phase A) and ACN (Phase B), and the linear solvent gradient changed according to the following conditions: 0 min, 95% A and 5% B; 30 min, 0% A and 100% B; at 31.5 min initial conditions were used again and afterwards the column re-equilibrated for 2.5 min. A volume of 10 μL of the methanolic extracts of avocado or standard mix was injected in each case. The compounds separated were monitored in parallel with a DAD (254, 280 and 330 nm), with a FL (excitation wavelength 280 nm and emission wavelength 339 nm) and with a MS.

The LC system was coupled to a Bruker Daltonics Esquire 2000TM IT (Bruker Daltonik, Bremen, Germany). The IT MS was running in negative ion mode and the capillary voltage was set at +3200 V. The scan range was 50-1000 m/z, and the optimum values of the ESI-MS parameters were: drying gas temperature, 300°C; drying gas flow, 9

L/min; and nebulizing gas pressure, 30 psi. Instrument control, data acquisition and processing were carried out using the software Esquire Control and Data Analysis 4.0 (Bruker Daltonik), respectively.

The polarity of ESI and all the detection parameters of IT (and TOF) MS were optimized using the height of the MS signal for the compounds included in our standard mix and some other compounds found in the avocado QC extracts. In order to achieve stable electrospray, a flow divisor 1:4 was used, reducing the flow rate used in the LC method to 0.2 mL/min, which is low enough to avoid the introduction of humidity in the system.

In ESI-IT MS, apart from the analyses carried out in *Full Scan*, we used MS/MS mode too (auto MS/MS). When the second approach was selected, different MS conditions, in particular, octopole, skimmers voltages and trap drive were tried to get the optimum fragmentation of the compounds under study.

For identification purposes, we used HPLC-ESI-TOF MS. The LC system was coupled to an ultra high resolution Q-TOF (Bruker Daltonik, Bremen, Germany). The LC method was exactly the same as described above. The MS operated in negative and positive ionization modes and acquired data in the mass range from m/z 50 to 1000 with a spectra rate of 1 Hz. In negative mode, the capillary was set at +4000V, the End Plate offset at −500V, the Nebulizer Gas at 2 Bar and the Dry Gas at 9 L/min at 250°C. The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltonik), which provides a list of possible elemental formulas by using the SmartFormula™ Editor tool.

Linearity of the MS response was verified with solutions containing 12 standards at 9 different concentration levels over the range from the quantification limit to 200 ppm. Each point of the calibration graph corresponded to the mean value from three independent injections. The LODs and LOQs of the individual analytes in standard solutions were calculated according to the IUPAC recommendations [43]. Furthermore, the precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatabilities in the peak areas were determined as the RSD from the area values of several metabolites detected when the QC samples were

injected obtained for 5-10 consecutive injections, carried out within the same day and on 7 different days, respectively.

6.2.5. Data analysis

The LC-MS data files were exported as mzXML files and aligned by using in-house developed alignment algorithm msalign2 tool (http://www.ms-utils.org/msalign2/); peak picking was performed using XCMS package (The Scripps Research Institute, La Jolla,USA) using the default settings except for bandwidth (bw parameter) for grouping of features set to 10.

The generated data matrix was imported to SIMCA-P 12.0 software package (Umetrics, Umeå, Sweden). The data were mean centred and unit variance-scaled prior to statistical analysis. The validity and the degree of overfitting of the PLS-DA models were checked using a 200 permutations test.

6.3. Results and discussion

6.3.1. Application of the LC-DAD-FL-MS method to the analysis of avocado extract

The QC avocado sample prepared containing all the avocado extracts under study was used to optimize both the chromatographic and MS conditions. The LC-ESI-IT MS conditions were checked taking into account the chromatographic behavior of the analytes present in the samples under study, analysis time, sensitivity, peak shape, etc.

The feasibility of the chosen conditions was also checked analyzing QC samples prepared specifically for each variety, including only the extracts of that particular avocado variety.

Figure 6.1 shows the chromatogram obtained for a QC sample using DAD (at 3 different wavelengths) and FL working in parallel. ***Figure 6.2*** includes an example of the BPC of the same sample obtained in LC-ESI-IT MS.

More than 200 different m/z signals were detected in the methanol extracts of avocado using ESI-IT MS both in negative ionization mode. The identification of an important number of metabolites present in the extracts was accomplished taking into account the results achieved in previous chapters and the use of commercially available standards.

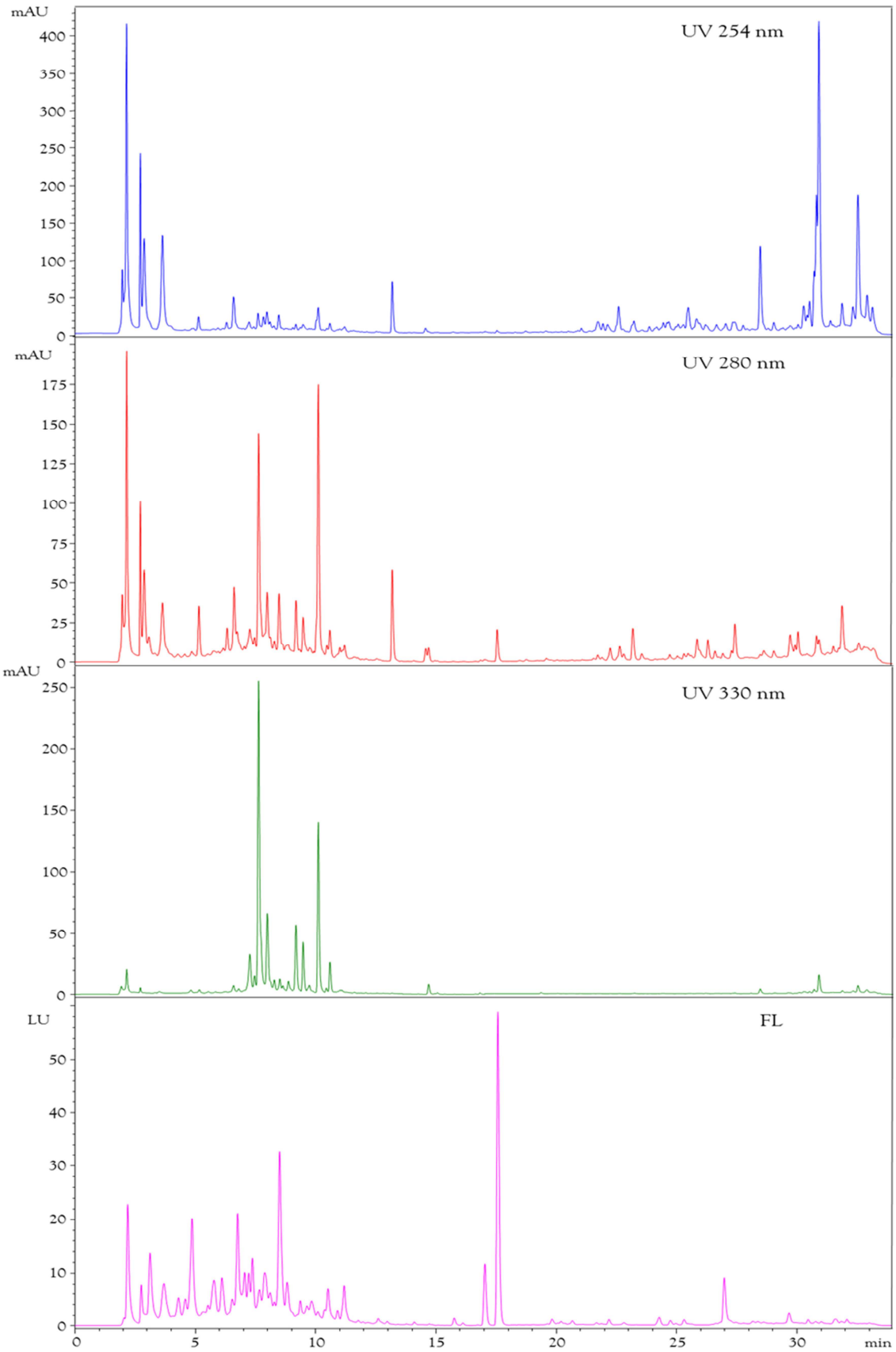

Figure 6.1. Chromatograms obtained for DAD (at 254, 280 and 330 nm) and FL (excitation wavelength 280 nm and emission wavelength 339 nm).

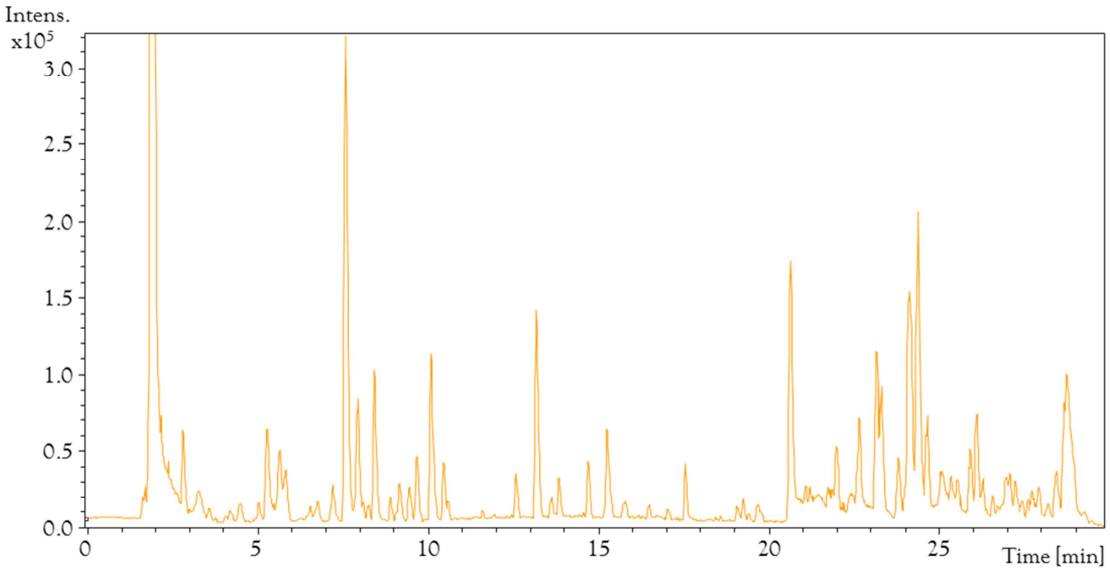

Figure 6.2. BPC obtained for a QC sample in LC-ESI-IT MS.

It was observed that the method was useful to determine in the analyzed avocado extracts a number of metabolites belonging to different chemical classes: organic acids, sugars, nucleosides, vitamins, phenolic acids and their glucoside-derivatives, amino acids, flavonoids, fatty acids, plant hormones, etc. So in approximately 30 min, information about an important number of compounds could be get. For some of the compounds, we had pure standards commercially available; one of the further steps which are going to be considered in the near future is to carry out their quantification.

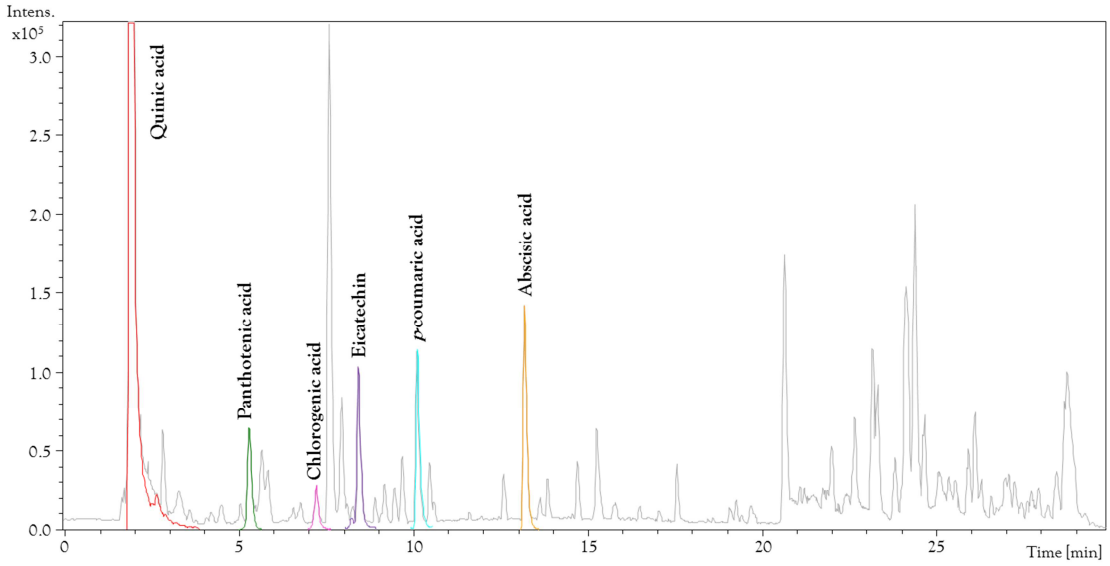

Figure 6.3. BPC obtained for a QC sample in LC-ESI-IT MS (grey chromatogram) and the EICs of some of the metabolites to be quantified in terms of their own pure standard.

Since the number of the samples to be analyzed within a sequence was quite high (almost 300 injections were made), it was very important to assure the repeatability (both intra- e interday). Samples were injected in a completely randomized order (meter más). The order of injection was the following one: every 5 injections of avocado extract, a blank and a QC sample were injected. Every day, at the end of the sequence a calibration curve (5 different concentration levels) was made. Before starting the subsequent sequence, the interface, spray shield and sprayer were cleaned; we used as well a cleaning procedure for the column.

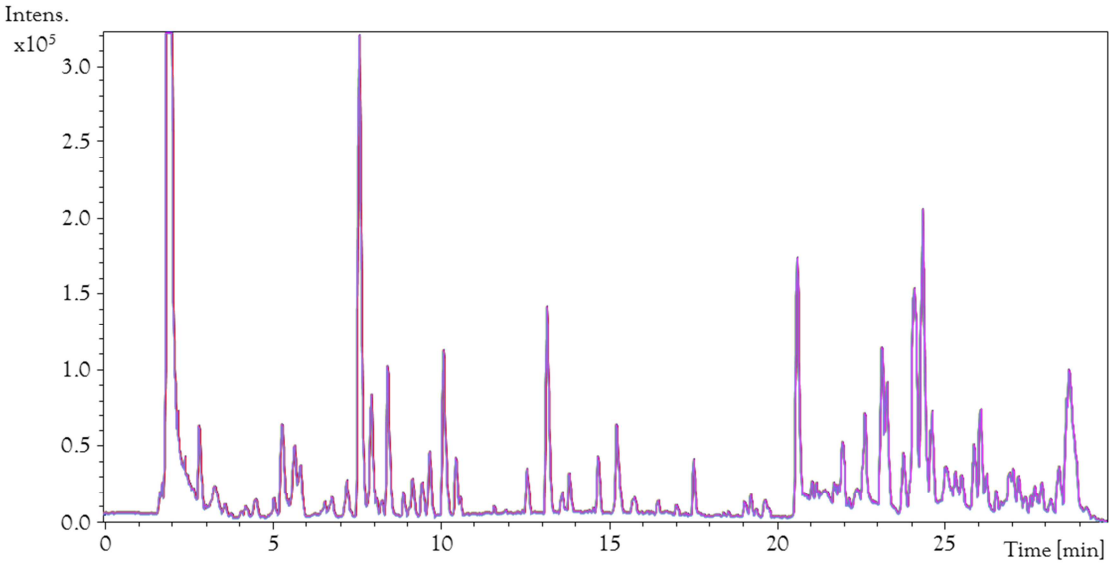

Figure 6.3. BPCs obtained for a QC sample in LC-ESI-IT MS from 10 different injections over the sequence to give an idea about the repeatability of the methodology.

As stated before, the precision of the analytical procedure was measured as repeatability (intra- and inter-day), which were expressed as the relative standard deviation (RSD) in the peak area. Repeatability (intra- and inter-day) were determined, respectively, by considering the areas of 5 metabolites of the QC sample in analyses carried out within the same day (repeatability, n=5) and on five consecutive days (intermediate precision, n=10). The results obtained for the precision, in terms of repeatability, were acceptable; being in all the cases lower than 5.25% for intra-day precision and 7.86% for inter-day precision, respectively.

6.3.2. Exploratory analysis of avocado extracts

When the comparison of a high number of samples containing a very large number of variables have to be carried out, one important task is to build an organized two-

dimensional data matrix consisting of metabolites and quantitative variables for a large amount of raw data sets. They undergo the pre-processing step which covers peak alignment, baseline correction, normalization, etc. Afterwards, multivariate analysis is a convenient way to extract the metabolites of interest from the data matrices created. PCA is a non-supervised approach very used in metabolomics, where the modeling is done without user intervention and solely on the explanatory variables. HCA is another non-supervised method widely used. In addition to the non-supervised approaches, the supervised multivariate techniques are used for identifying classifiers (relevant metabolites). The projection to latent structures (PLS) based approach, which includes PLS, PLS-DA, OPLS and OPLS-DA, can extract Y-correlated information from the X matrix.

In the current case, PCA or PLS-DA on all the samples using the different classes have been used. To detect up-regulated or down-regulated metabolites within each class, regression analysis on each group/class separately will be applied. PLS will use the harvesting time a y-variable, applying as well a univariate search of metabolites correlated with harvesting time.

Since PCA and PLS-DA could not be considered as optimal for the dynamic data obtained in a longitudinal type of study, other methods, separating levels of variation, should be also used. Multilevel component analysis (MCA) and individual recognition strategy are going to be evaluated.

In a first stage, MS data will be considered, however, in further stages of the study these data will be completed by using DAD and FL signals too.

6.3.3. Studying the evolution of some particular metabolites

In previous chapters of the current thesis, it has been observed that some metabolites changed their concentration very drastically over the ripening. We wanted to corroborate those results in the samples harvested over the whole season, checking whether the trend was the same in all the varieties under study.

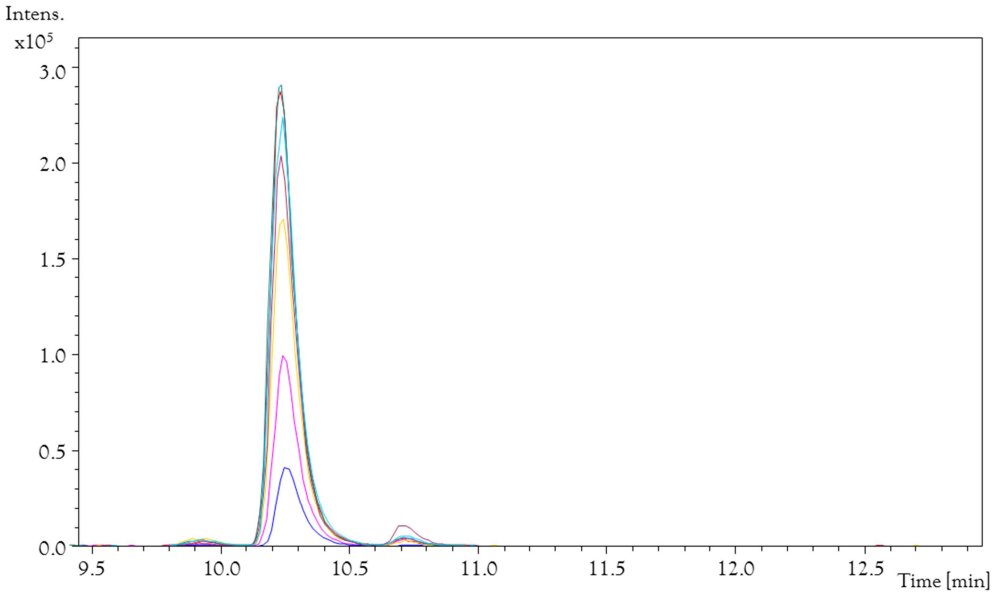

Figure 6.5. EICs of *m/z* 163 corresponding to *p*-coumaric acid in samples from different time points of *cv.* Reed.

In Reed variety, Contreras-Gutiérrez et al. [40] observed an increase in the concentration of *p*-coumaric acid during the ripening process. What is appreciated here is that the concentration level of this phenolic acid is increasing over the season. In samples harvested in September or October the amounts found are higher than in other collected at the beginning of the harvesting period. It is important to highlight that for each time point we had 5 replicates (for most of the cases); these replicates were not instrumental, but horticultural. They were composed by fruits harvested from different trees at the same date in order to cover the real variability that can be found in this kind of studies. The same was observed for *cv.* 'Bacon' and 'Hass', whilst it is difficult to claim that the same is true for 'Fuerte', where a clear trend of raising of the level of this compound is not observable.

The concentration of abscisic acid varied a lot during the harvesting period of *cv.* 'Reed', 'Hass' and 'Bacon', decreasing markedly its concentration as the fruits were harvested later. When samples from 'Fuerte' were compared, the fluctuations on the determined levels did not allow achieving any concluding results. This could be related to the fact that is the variety in which the global harvesting period was shorter, causing a more subtle metabolic evolution.

The concentration levels of some other compounds are being calculated at the moment; they will contribute to give a global vision about what happens over the development and maturation of these four varieties.

6.4. Conclusions

A new LC-DAD-FL-MS method has been developed for the qualitative and quantitative characterization of the metabolites present in methanolic avocado extracts, being able to determine more than 200 metabolites in single run (30 min). The method has been applied to analyze a very unique sample selection, which included avocado fruits (of 4 varieties ('Hass', 'Fuerte', 'Bacon' and 'Reed')) harvested at different time points over the season, with the aim to study the metabolic changes during fruit development and maturation. Exploratory analysis based on the combination of non-supervised and supervised methods are already being applied, trying to identify the metabolites differentially regulated during the maturation process. Other approaches such as multilevel component analysis are being also evaluated.

Acknowledgements

The authors are very grateful to Junta de Andalucía (Project P09-FQM-5469), to the International Campus of Excellence (CEI Granada 2009) and to University of Granada (Pre-doctoral grant) for financial assistance. They appreciate as well the support gave by J. González-Fernández, E. Guirado and J.M. Hermoso who provided the samples included in this study contributing with valuable scientific support.

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Section III: GAS CHROMATOGRAPHY

GC is a physical separation method introduced by James and Martin in 1952. The separation of the different compounds present in a sample primarily occurs on the basis of the differences in their volatilities and structures. Being a gas-phase separation method, it has been traditionally used for the analysis of volatile and thermally stable compounds; however, many metabolites are not suitable for GC analysis because of their physicochemical properties. Thus, occasionally an additional step in the sample preparation process, named derivatization reaction, is required to increase the volatility and thermal stability of the substances to be analyzed.

This analytical technique presents several important advantages: reasonable analysis time, sensitive, reliable, relatively simple, not as expensive as LC in general terms, highly accurate quantitative analysis, and efficient. All these characteristics together with the introduction of fused silica columns have turned GC into one of the most important and powerful method of analyzing complex mixtures, even more when it is coupled to MS.

GC could be considered as the first analytical separation technique applied to the performance of a metabolomics study, because the work developed by Linus Pauling in 1971 —where GC were use to analyze quantitatively urine vapour— is often considered the first metabolomics publication, even though this term was not coined till 29 years later. In 2000, the coupling GC-MS was chosen by Oliver Fiehn to study the metabolome of Arabidopsis thaliana, work were the concept of metabolomics was defined for the first time. Since that date, GC-MS has become a major tool in metabolomics researches owing to the high chromatographic resolution of GC and the high selectivity and sensitivity of MS. Capillary GC is today the most important and common analytical method, which is characterized by the use of capillary columns. Fused silica capillary columns are frequently the most used, because they are easy to handle, can be changed rapidly and are available in many high quality forms.

In this last section of the experimental part, results and discussion, it has been demonstrated the potential of the coupling of GC to two detection systems, such as FID and MS, to carry out different metabolomics approaches that allow enahancing the knowledge about avocado fruit. Besides, different interfaces have been used to combine GC and MS: the traditional EI and a novel ionization source for GC, such as APCI.

Although the utilization of GC-MS is nowadays very spread out, GC has been usually coupled to FID. ***Chapter 7*** encloses a comparison of GC-APCI-TOF MS and GC-FID for the quantitative study of 13 avocado varieties at two different ripening degrees. Both techniques have been validated and compared in terms of the analytical parameters and the quantitative results.

To date, APCI has not been a very common interface for GC-MS and its scarce use can be due to the lack of available databases that allow the compound identification. ***Chapter 8*** describes the development of a spectral database for GC-APCI-TOF MS, which includes an important number of metabolites that can be easily found in biofluids and food samples. This work has been carried out during a stay of 3 months in the Centre for Proteomics and Metabolomics of the Leiden University Medical Centre (Leiden, The Netherlands) in the research group of Prof. André M. Deelder.

Chapter 9 shows the very first application of GC-APCI-TOF MS to the analysis of avocado fruit, demonstrating the feasibility of the new database too. A detailed comparison between the new coupling and the traditional GC-EI-Q MS has been made in terms of compound identification ability. A comprehensive study of the metabolome of 3 avocado varieties is performed, being possible to identify around 100 metabolites.

Finally, in ***Chapters 10*** and ***11***, different chemometric tools have been applied to treat the data achieved by each platform optimized in chapter 9 (GC-APCI-TOF MS, GC-EI-Q MS). As in previous sections, the main objective of the use of multivariate analysis is to obtain valuable information that allow us a better understanding about the metabolic evolution of 13 avocado varieties at two different ripening stages. The identification of those compounds responsible of the differentiation between samples (varietal markers) and ripening stages (ripening markers) can be very useful information for food **scientists** and farmers.

Chapter 7

Quantitative characterization of important metabolites of avocado fruit by gas chromatography coupled to atmospheric pressure chemical ionization-time of flight mass spectrometry and flame ionization detectors

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Abstract

Herewith the development of a GC methodology, where two different detectors (FID and APCI-TOF MS) were used *on-line*, to determine quantitative changes in the metabolic profile of *Persea americana* is presented. The metabolic evolution of 13 avocado varieties was checked quantifying analytes belonging to several chemical families (organic acids, phytohormones, vitamins, flavonoids and phenolic acids). The method was fully validated and the analytical parameters of both detectors were compared, showing both of them acceptable analytical features. GC-FID showed better results in terms of precision (repeatability and intermediate precision), but LODs and LOQs were significantly higher (oscillating from 24.8 to 800 µg/L and from 82.7 µg/L to 2666.7 µg/L, respectively) than for MS (LODs were found within the range of 0.1-207.8 µg/L and LOQs of 0.5-692.5 µg/L).

A total of 27 compounds were quantified by GC-APCI-MS, whilst GC-FID allowed the proper quantification of 7 analytes. The concentration of organic acids, flavonoids and vitamins tend, in general, to decrease with the ripening process, whereas phenolic acids such as ferulic or *p*-coumaric acids usually increase their concentration as the fruit ripens. To corroborate further on the metabolic changes associated with the avocado varieties and/or ripening, we used principal component analysis, identifying quinic and *p*-coumaric acids, epicatechin and quercetin as some of the most influential compounds explaining the classification of the samples under study.

Keywords: Gas chromatography / Food metabolomics / Atmospheric pressure chemical ionization / *Persea americana* / Flame ionization detector / Principal component analysis

7.1. Introduction

High consumption of fruits and vegetables has been consistently correlated with lower incidence of certain types of cancer and cardiovascular diseases [1]. Their nutritional quality is closely related to the presence of fiber, vitamins, soluble sugars, amino acids and other phytochemicals. The latter represent a concept which is often used as synonym of bioactive compounds [2], which are essential and non-essential compounds that occur in nature, are part of the food chain, and can be shown to have an effect on

human health [3]. It is easy to understand that because of the important role of these compounds in maintaining food quality and nutritive value, fruit compositional analysis is of interest to food science, dietetics, agricultural production and related areas.

Despite the fact that for many decades, traditional medicine of South-American and African indigenous had used different parts of *Persea americana* plant to treat diverse diseases [3, 4], it has been only in the last few years, when the oleaginous fruit of avocado is increasingly consumed [5], in part because of the correlation between its phytochemical composition and its reported health-benefits [6-8]. Avocado fruit is a complex mixture of compounds whose concentration varies a lot; it contains proteins, sugars, vitamins, sterols, tannins, fatty acids, amino acids, alkanols, terpenoid glycosides, various furan ring-containing derivatives, phenolic compounds, coumarins, etc [5, 8-10]. This complexity makes the analysis of the avocado metabolome quite a challenging task, and so far there is no method that can give a complete qualitative and quantitative picture of it.

When metabolomics is applied to evaluate fruit development, great efforts have been made to investigate fruit composition and to explain the relationship between fruit quality and numerous factors, including, but not limited to, fruit variety, developmental stage, ripening degree, climatic conditions, etc. In the case of avocado, for instance, a unique feature is that the fruit mature on the tree, but only ripen after harvest; the fruit has a long harvesting period depending on cultivar and the identification of horticultural and commercial maturity is difficult due to invisible external changes [9]. The measurement of dry matter and oil content in the flesh, or the control of markers such as color and size are some of the most common strategies to determine fruit maturity and picking time [9, 11-13]. Although these strategies are widely used, fruit-to-fruit variability in oil content and varietal differences in accumulation rate, make the percent of oil an imperfect threshold for maturity. Compounds such as fatty acids [5, 8, 14-16], sugars [5, 17], carbohydrates [14], carotenoids and tocopherols [14], total protein [17], phenolic, ascorbic acid and glutathione contents [9], aroma volatiles [18], etc. have been also determined in an important number of studies where the variation in the composition of avocado fruit during ripening or storage was evaluated.

Metabolites of interest have been frequently determined in avocado samples by using LC, GC and CE coupled to different detection systems [10, 14, 18-23]. If particular attention is paid to GC, it is possible to stand out that among all different detectors that can be coupled to this separation technique, FID and MS have been the most extensively used. Harley et al. [24] and McWilliam and Dewar [25] were responsible in 1958 of the introduction of FID, which is considered as one of the most universal and popular detectors for GC as a consequence of its simplicity, reasonable sensitivity, low level of noise and excellent linear range [26]; it allows the detection of carbon-containing compounds giving a proportional response to the increase of carbon atoms. On the other hand, GC in combination with MS has been broadly exploited in plant metabolomics [27], since this coupling puts together the separation power of GC with the capabilities of the modern MS instruments. Most of the commercial GC-MS systems use vacuum stage ionization sources, being EI the most common one; however, those ion production systems that do not require vacuum conditions, as APCI, are recently gaining interest [28]. In the last few years, several researches focused on the systematic evaluation of the potential of GC-APCI-MS have been published [28-34]. These examples include the determination of metabolites in biological fluids [30], pesticides in fruits [34] and phenolic compounds in oils [31], problem solving and process understanding in pharmaceutical development [32], development and application of the first database for GC-APCI-MS [28], and the comprehensive evaluation of the performance of GC-APCI-MS and its comparison with other GC-MS platforms [33], etc. It has been already demonstrated that GC-APCI-TOF MS can be a real alternative to conventional GC-MS, enhancing the coverage of the metabolome of different biological systems; however more research is needed to evaluate its capabilities to carry out quantitative analysis.

The current study describes the development and fully validation of a GC methodology, where two different detectors (FID and APCI-TOF MS) were used *on-line*, to determine quantitative changes in the metabolic profile of a complex matrix, such as *Persea americana*. The growing evidence of the health benefits of the avocado fruit is both driving increased consumption and stimulating research on potential health effects; however, it is absolutely necessary to know in depth the composition of what we eat to go further understanding their physiological effects. To achieve this purpose the determination of 27 metabolites belonging to different chemical families (organic acids,

phenolic acids, flavonoids, phytohormones and vitamins) was carried out in 13 avocado cultivars (at two different ripening degree), comparing the quantitative results achieved by FID and APCI-MS, and applying statistical tools to study the influence that the quantified compounds had on the differentiation between varieties and ripening degrees. So far limited information is available on the evolution of relevant metabolites present in avocado from the time when the fruit is picked till the moment in which the consumer eats it.

7.2. Materials and methods

7.2.1. Chemicals and standards

Only analytical reagent grade chemicals were used for this study. Taking into account previous results [10, 22, 35], a group of 27 standards belonging to different chemical families was selected. Quinic acid (cyclic polyol) was acquired from Fluka (St. Louis, USA). Pantothenic acid (vitamin B_5), abscisic acid (phytohormone), benzoic acid (carboxylic acid), several hydroxybenzoic acids (gentisic, γ-resorcylic, o-pyrocatechuic, 4-hydroxybenzoic, isovanillic, syringic, vanillic and gallic acids), some hydroxycinnamic acids (o-, m- and p-coumaric acids, caffeic, trans-cinnamic, ferulic, and sinapic acids), 3 phenolic acids-related compounds (homovanillic and chlorogenic acids and vanillin), two flavanols (catechin and epicatechin), and a flavonol (quercetin) were from Sigma-Aldrich (St. Louis, USA). Kaempferide (O-methylated flavonol) and naringenin (flavanone) were supplied by Extrasynthese (Lyon, France), as well as taxifolin, which was used as IS to evaluate the reproducibility of the extraction system and the chromatographic runs. Standard solution containing the 27 compounds under study was prepared in MeOH at a concentration of 13.2 mg/L.

Freshly opened 1 ml bottles of BSTFA + 1% TMCS from Pierce (Rockford, IL, USA) were used as derivatization reagent. Fluka (St. Louis, USA) was the supplier of APCI tune mix and pyridine (99% ultra-pure GC grade).

MeOH was chosen as solvent to prepare sample extracts and it was purchased from Panreac (Barcelona, Spain).

7.2.2. Samples, extraction and derivatization reaction

The samples used in this study were part of the germplasm collection from the IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). Fruits of 13 varieties (at two ripening degrees (fruits just harvested and fruits ready for consumption)) were studied: 'ColinV 33', 'Gem', 'Harvest', 'Hass', 'Hass Motril', 'Jiménez 1', 'Jiménez 2', 'Lamb Hass', 'Marvel', 'Nobel', 'Pinkerton', 'Sir Prize' and 'Tacambaro', being all grown under identical environmental conditions (soil, rain, light, etc.).

Pulp of 2-3 pieces of fruit was homogenized, frozen and freeze-dried to prepare sample extracts in accordance with Hurtado-Fernández et al. [10, 22]. Briefly, 4 g of the freeze-dried (and homogenized) sample were mixed with 40 mL of pure MeOH and 50 ppm of IS. The tubes were shaken in a vortex during 30 min. The supernatants were taken and centrifuged at 3000 rpm for 10 min, and after that, were evaporated to dryness and redissolved in 5 mL of MeOH. Aliquots of 25 μL of this methanolic extract were taken, evaporated and redissolved in the proper volume of derivatization reagent.

A QC sample was used to assure the stability of the system and to evaluate different analytical parameters. This QC sample was made by mixing equivalent volumes of the extracts of each variety of avocado fruits at first ripening degree. For validation purposes, the mentioned QC sample and a standard mix composed by the 27 compounds previously mentioned in section 7.2.1 were used. Both samples were injected every 5 samples throughout the batch.

The derivatization reactions of the standard solutions and avocado extracts were carried out on-line with a programmable MultiPurpose Sampler by adding 50 μL of BSTFA + 1% TMCS to a dried aliquot of the sample and incubating at room temperature for 60 min.

7.2.3. Analytical equipment

An Agilent 7890A GC system (Agilent, Palo Alto, USA) equipped with a HP-5-MS column (30 m, 0.25 mm ID and 0.25 μm film thickness) was used to carry out the chromatographic separations. It was coupled to two detectors running in parallel (FID and MS), being required a column flow splitter with deactivated capillaries (0.6 m, 0.25 mm ID, 0.25 μm film thickness) to allow the simultaneous acquisition with both

detectors. Aliquot of the derivatized samples (1 µL) were used for the injection. Automated injection was done using a programmable MultiPurpose Sampler MPS2 (Gerstel, Mülheim an der Ruhr, Germany). Splitless injection mode was applied by a septumless CIS PTV injector. The purge time of the PTV injector was 60 s, using a purge flow of 3 mL/min and setting the temperature at 250°C. Sequential washing steps of the 10 µL syringe were included before and after each injection, as well as a step of sample pumping to remove possible small air bubbles. Helium was used as carrier gas at a constant flow rate of 1 mL/min.

The temperature gradient used in this study was the following: the initial column temperature was kept at 140°C for 5 min, then increase at a temperature rate of 3°C/min until 220°C, keeping that value for 1 min. Later on, the temperature raised from 220°C to 241°C at 10°C/min and it was held for 1 min; and finally, the temperature was increased from 241°C to 310°C at 3°C/min, maintaining that temperature for 5 min.

As it was mentioned above, two different detectors were used. One of them was a FID, operating by applying the following conditions: 300°C of front detector temperature, hydrogen flow of 40 mL/min and 370 mL/min for air flow. The second detector used was an ultra high resolution TOF MS MaXis (Bruker Daltonics, Bremen, Germany), that was coupled to the GC system by using a multipurpose source as APCI interface, which was equipped with GC transfer line [36]. The temperature of this transfer line to the mass spectrometer was kept at 300 °C. The APCI-MS operated in positive mode. The APCI vaporizer temperature was 300°C, the corona discharge needle worked at a voltage of +4000 nA and the nebulizer gas (nitrogen) pressure was set at 3.5 Bar. Dry gas (nitrogen) temperature and flow rate were 300°C and 2 L/min, respectively. Capillary voltage was set at +2000 V and the end-plate offset at −500 V. Spectra were acquired within the mass range between 50 and 1000 m/z with 1 Hz frequency. Both an external and internal calibrations were performed injecting an APCI tune mix solution and using cyclic-siloxanes, respectively [30].

For MS and FID, DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany) and ChemStation (Agilent Technologies, Palo Alto, USA) software were used for data processing, respectively.

7.2.4. Validation studies

A complete analytical validation of the method, considering linearity and sensitivity, repeatability, possible matrix effect and accuracy, was performed both for APCI-MS and FID. It was carried out by using the mix of the 27 standards and the QC samples.

The linearity and sensitivity of the proposed method was checked establishing external calibration curves. LODs and LOQs for each individual compound of the standard solution were calculated as a signal-to-noise ratio of 3 and 10 [37], respectively, for the MS data. For FID, detection (LOD=$3S_0/b$) and quantification limits (LOQ=10 S_0/b) were determined as well; these values were calculated taking into account the IUPAC recommendations [38], where S_0 is the standard deviation of the blank and b the slope of each calibration equation.

QC samples were spiked with 0.2, 0.85 and 3.3 mg/L of standard solution (standard-added calibration) to study the possible existence of matrix effect. It was evaluated by comparison of the RFs (calculated considering the peak area and the analyte concentration ratio) of the spiked QC samples with those achieved for the neat solution of standards (standard mix solution containing the 27 compounds) at the same concentration.

Repeatability, intermediate precision and trueness were the parameters used to evaluate the accuracy of the analytical procedure. Measures of the standard mix solution at an intermediate concentration of the linear range were carried out within the same day (n=5) and on three consecutive days (n=5) to determine repeatability and intermediate precision, respectively, both of them expressed as relative standard deviation (RSD). Trueness was expressed as recovery since there was not *Certified Reference Materials* (CRMs) available; the recovery was estimated by analyzing a sample before and after the addition of a known amount of analytes (by spiking or standard addition). In this case, the recovery is calculated from the difference between the results obtained before and after spiking as a fraction of the added amount. For that, a QC sample was spiked with different concentrations of the 27 metabolites under study.

7.2.5. Statistical analysis

The Unscrambler® v9.7 software (CAMO Software AS, Oslo, Norway) was used to perform PCA. This method is used to facilitate the identification of natural grouping tendencies within a dataset, carrying out an exploratory analysis of the data.

7.3. Results and discussion

7.3.1. Analytical parameters of the method

The analytical parameters of our GC-FID/APCI-MS method were evaluated before proceeding to the analysis of the 13 avocado varieties at two ripening degrees. ***Table 7.1*** summarizes the analytical parameters for FID and APCI-MS, it includes the retention time, calibration curves, determination coefficients, LOD and LOQ, linear range, and the figures related to matrix effect and accuracy.

External calibration curves were prepared using the standard mix of 27 metabolites at 9 different concentration levels (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.7, 3.3 and 6.6 mg/L). In MS, the calibration curves were prepared considering the Extracted Ion Chromatogram (EIC) of the most intense feature in the MS spectrum of each metabolite, although it is important to bear in mind that all the detected fragments in the MS spectra as well as their relative abundance were considered for carrying out the identification. The linearity was reasonably good for both detectors, being the determination coefficients (r^2) of the standard calibration curves higher than 0.981 for APCI-MS and higher than 0.988 in the case of FID.

Table 7.4. Analytical parameters of the GC-FID/APCI-TOF MS method.

t_r	Analyte	Detector	External calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Linear range (mg/L)	MATRIX EFFECT		ACCURACY		
								RF (neat solution)[a]	RF (with matrix)[a]	Repeatability[b]	Intermediate Precision[b]	Trueness[c]
3.68	Benzoic acid	MS	y= 257446x + 61421	0.994	1.4	4.8	LOQ-6.6	264023.7	249283.5	3.3	5.0	105.4
		FID	y= 3.8449x + 0.8828	0.990	25.0	83.3		3.9	3.8	0.3	2.1	103.7
9.60	Vanillin	MS	y= 586809x – 20851	0.988	0.5	1.6	LOQ-6.6	556986.6	560254.8	8.3	9.1	98.9
		FID	y= 1.5738x – 0.0329		95.5	318.2		1.7	1.7	4.6	6.1	108.2
9.78	*trans*-cinnamic acid	MS	y= 286795x – 7976	0.997	1.1	3.5	LOQ-6.6	260550.0	281479.3	2.6	9.2	104.9
		FID	y= 3.0412x – 0.2031		49.4	164.7		3.2	3.2	2.6	4.4	109.8
12.32	4-hydroxybenzoic acid	MS	y= 860736x + 5477	1.000	0.4	1.4	LOQ-6.6	863064.6	817208.9	7.6	8.3	93.7
		FID	y= 4.2469x – 0.1513	0.996	24.8	82.7		4.1	4.1	0.4	2.0	106.5
16.22	*o*-pyrocatechuic acid	MS	y= 2512017x – 56448	0.998	0.1	0.5	LOQ-1.7	2163829.2	2101752.6	6.2	6.3	93.8
		FID	y= 4.7289x – 0.1113		28.9	96.2	LOQ-6.6	4.8	4.7	1.4	2.7	104.5
16.74	Vanillic acid	MS	y= 692103x – 9363	0.992	0.5	1.7	LOQ-6.6	693220.3	649465.9	6.1	9.4	87.1
		FID	y= 3.9350x – 0.2304	0.999	38.2	127.3		3.8	3.7	0.6	6.9	83.3
16.92	Isovanillic acid	MS	y= 692133x + 67310	0.989	0.5	1.8	LOQ-6.6	701480.3	680811.2	6.7	8.7	98.5
		FID	–	–	–	–	–	–	–	–	–	–
16.97	γ-resorcylic acid	MS	y= 1783711x + 78072	0.990	0.2	0.7	LOQ-3.3	1951092.3	1986407.1	6.5	10.9	111.6
		FID	–	–	–	–	–	–	–	–	–	–
17.04	Homovanillic acid	MS	y= 2452289x – 60695	0.998	0.2	0.5	LOQ-1.7	2194284.7	2267956.9	2.3	6.1	93.7
		FID	y= 4.6951x – 0.3289		32.0	106.7	LOQ-6.6	4.6	4.4	0.5	4.3	98.4
17.34	Gentisic acid	MS	y= 2617877x – 54667	0.997	0.5	1.8	LOQ-1.7	2309072.5	2385076.2	1.7	6.9	84.5
		FID	y= 5.9914x + 0.2942	1.000	25.1	83.6	LOQ-6.6	6.1	6.1	1.3	1.5	106.3
18.08	*o*-coumaric acid	MS	y= 1378035x + 32567	0.989	0.3	0.9	LOQ-3.3	1385366.9	1409178.7	5.4	6.2	95.3
		FID	y= 3.4591x – 0.2044	0.999	43.4	144.8	LOQ-6.6	3.4	3.3	1.3	2.1	96.0
20.00	*m*-coumaric acid	MS	y= 1218161x – 41018	0.997	0.3	1.0	LOQ-1.7	1103382.8	1146727.0	8.8	9.2	101.1
		FID	y= 4.8234x – 0.2889	0.999	31.2	103.8	LOQ-6.6	4.7	4.5	0.5	8.5	103.9
20.67	Quinic acid	MS	y= 453107x – 18332	0.999	0.8	2.7	LOQ-6.6	424289.3	427895.2	8.0	11.9	101.2*
		FID	y= 2.0641x – 0.0083	0.997	72.8	242.6		2.2	2.2	0.4	1.0	99.0*
21.13	Syringic acid	MS	y= 589841x + 17728	0.998	0.6	2.1	LOQ-6.6	585790.1	585558.2	3.9	7.3	106.7
		FID	y= 2.7679x – 0.0424	0.999	50.0	166.7		2.9	2.7	0.2	0.7	89.6
22.12	*p*-coumaric acid	MS	y= 913618x + 58269	0.996	0.4	1.4	LOQ-6.6	1024639.4	961329.5	8.4	8.2	96.9*
		FID	y= 4.3447x – 0.3509	0.995	25.0	83.3		4.4	4.2	0.8	1.6	94.0*
23.31	Gallic acid	MS	y= 2078969x + 36884	0.987	0.2	0.6	LOQ-1.7	1960172.2	2093955.7	6.0	6.6	107.4
		FID	y= 6.0289x – 0.4604	0.998	24.9	83.1	LOQ-6.6	5.9	5.9	0.4	5.4	96.4

Table 7.5. (continued)

t_r	Analyte	Detector	External calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Linear range (mg/L)	MATRIX EFFECT		ACCURACY		
								RF (neat solution)[a]	RF (with matrix)[a]	Repeatability[b]	Intermediate Precision[b]	Trueness[c]
24.28	**Pantothenic acid**	MS	y= 1918819x – 23591	0.997	0.2	0.6	LOQ-1.7	1712951.2	1621585.4	8.3	8.8	109.9
		FID	y= 3.4244x – 0.1706	0.998	43.9	146.3	LOQ-6.6	3.8	3.8	0.4	1.9	94.2
26.84	**Ferulic acid**	MS	y= 1856838x – 62968	0.996	0.2	0.7	LOQ-1.7	1612439.3	1630342.9	6.9	7.8	91.1
		FID	y= 3.7852x + 0.1602	0.995	25.0	83.3	LOQ-6.6	4.0	4.0	0.7	2.8	112.2
28.28	**Caffeic acid**	MS	y= 1692769x – 46283	0.997	0.2	0.7	LOQ-1.7	1501465.1	1583049.6	7.7	9.0	110.1
		FID	y= 4.0506x + 0.0340	0.996	37.1	123.6	LOQ-6.6	4.1	4.1	0.5	4.1	93.1
29.70	**Abscisic acid**	MS	y= 605135x + 8027	0.999	0.6	2.0	LOQ-6.6	621356.8	614027.6	11.8	11.2	86.0
		FID	y= 1.7746x + 0.0632	0.990	84.7	282.2		1.9	1.9	0.5	4.4	104.6
31.21	**Sinapic acid**	MS	y= 1107680x + 193621	0.984	0.3	1.1	LOQ-6.6	1366698.3	1309075.1	6.0	7.5	109.6
		FID	y= 2.7612x – 0.0531	0.994	50.0	166.7		2.5	2.6	0.8	1.6	96.3
44.73	**Naringenin**	MS	y= 1011590x – 94235	0.998	0.4	1.2	LOQ-6.6	1012654.4	941879.3	8.2	8.6	97.0
		FID	y= 2.8839x – 0.6183	0.990	200.0	666.7		2.4	2.5	1.3	2.6	102.0
44.95	**Epicatechin**	MS	y= 604169x – 21003	0.995	0.6	2.0	LOQ-1.7	581723.4	587553.0	9.1	9.1	110.8
		FID	y= 3.1341x – 0.1898	0.991	47.9	159.8	LOQ-6.6	3.3	3.1	0.2	0.4	101.3
45.49	**Catechin**	MS	y= 484931x – 12997	0.990	0.8	2.5	LOQ-1.7	457179.8	469640.7	8.3	8.6	94.4
		FID	y= 3.4435x – 0.0871	0.996	43.6	145.4	LOQ-6.6	3.3	3.3	0.6	4.2	96.0
47.79	**Kaempferide**	MS	y= 641806x – 156028	0.981	0.5	1.6	LOQ-6.6	609140.5	626426.7	6.4	9.5	92.5
		FID	y= 1.7646x – 0.4389	0.992	800.0	2666.7		1.3	1.2	1.0	1.9	87.8
50.31	**Chlorogenic acid**	MS	y= 329719x – 26885	0.998	1.1	3.7	LOQ-6.6	311606.7	303055.7	9.6	10.2	106.7
		FID	y= 2.0286x – 0.1044	0.995	100.0	330.0		2.1	2.0	0.6	4.9	101.8
51.36	**Quercetin**	MS	y= 1782x – 2043	1.000	207.8	692.5	LOQ-3.3	1858.1	1716.8	7.0	7.3	105.3
		FID	–	–	–	–	–	–	–	–	–	–

[a] Response factors were calculated to evaluate the possible matrix effect at 3 concentration levels (0.2, 0.85 and 3.3 mg/L). Only the intermediate value (0.85 mg/L) of the neat solution and of the spiked avocado extract is showed; the other concentrations showed the same behaviour.

[b] RSD values (%) for peak areas corresponding to each compound; measured from 5 consecutive injections within the same day (repeatability or intra-day precision) and on three different days (intermediate or inter-day precision), by using the standard mix at an intermediate concentration level for each compound.

[c] The trueness of the assay is the closeness of the average test value obtained to the nominal value. It was measured by calculating the recovery (%), spiking a blank matrix with 0.2, 0.85 and 3.3 mg/L of each compound. When no indicated, the shown values correspond to the intermediate value of 0.85 mg/L. Values with asterisk (*) means that the concentration considered was 3.3 mg/L.

In general, detection and quantification limits were significantly lower for the APCI-MS detector. APCI-MS showed LODs ranging from 0.1 µg/L (*o*-pyrocatechuic acid) to 207.8 µg/L (quercetin), whereas for FID they were between 24.8 µg/L for 4-hydroxybenzoic acid and 800 µg/L for kaempferide. As far as LOQs are concerned, they were found between 0.5-692.5 µg/L and 82.7-2666.7 µg/L for APCI-MS and FID, respectively.

One of the most remarkable differences between both detectors has to do with the dynamic range. For FID it was observed that all the compounds included in our standard mix presented a linear behavior up to the highest concentration level studied; for MS did not always occur the same; the linear range was, in general, not as wide as for FID.

For the evaluation of matrix effect, known amounts of our standard mix solution were added to the QC sample, and the responses of these compounds were compared with those of the metabolites standards solution (neat solution) at the same concentration, showing no significant differences (when they were compared by ANOVA analysis). Therefore, it is possible to say that matrix effect was not observed.

Repeatability (intra- and inter-day) were determined, respectively, by analyzing the mix of 27 metabolites at an intermediate concentration level for each analyte, within the same day (repeatability, n=5) and on five consecutive days (intermediate precision, n=5). As shown in Table 1, the results obtained for the precision, in terms of repeatability, were acceptable for both detectors; being in all the cases lower than 12%. RSD values were lower for FID, ranging from 0.2% to 4.6%. In the case of APCI-MS, they ranged from 1.7% to 11.8%, respectively. A similar behavior was observed when the intermediate precision was checked; the values for FID (0.4-8.5%) were again lower than for APCI-MS (5.0%-11.9%). As expected, values of intermediate precision were higher than repeatability values (for both detection systems), except for abscisic acid, *p*-coumaric acid and epicatechin, for which the results were quite similar.

The obtained recoveries (%) for each individual compound proved the trueness of the developed method, since they varied within the range of 84.5-111.6% (APCI-MS) and 83.3-112.2% (FID). Considering the AOAC guidelines, which establish a good trueness from 80 % to 115 %, it is possible to claim that our method is truthful.

7.3.2. Application of the GC-FID/APCI-TOF MS method to characterize the metabolic profiles of 13 avocado varieties at two ripening degrees

Quantitative results

At this point, the optimized GC method coupled to two different detectors was applied to the analysis of 13 varieties of avocado at two different ripening degrees, trying to achieve several aims: to quantify as many metabolites as possible (of those 27 included in our mixture) in the avocado extracts under study; to compare the achieved results by GC-FID and GC-APCI-TOF MS, checking whether they were in good agreement; and to observe the evolution of the metabolic profile of the avocado varieties tested during ripening.

Figure 7.1(A) includes the FID chromatogram of a standard mix at a concentration of 0.8 mg/L and ***Figure 7.1(B)*** shows the EICs of the metabolites included in the standard mix revealed by APCI-MS at the same concentration level.

Although the elution windows for each chemical family are overlapping in some parts, some general surmises can be made. The organic acids elute at the beginning of the run, then we can observe the phenolic acids and vitamins, and at the end of the chromatogram the flavonoids and the phytohormone appear. The chromatographic resolution was very appropriate over the whole run, even in the area where vanillic, isovanillic, γ-resorcylic, homovanillic, gentisic and o-coumaric acids eluted very close to each other. Vanillin and trans-cinnamic acid represented a critical pair of compounds, which were separated with baseline resolution after the optimization. Epicatechin and catechin, at the end of the run, had very similar retention times as well, but the resolution between them was good enough.

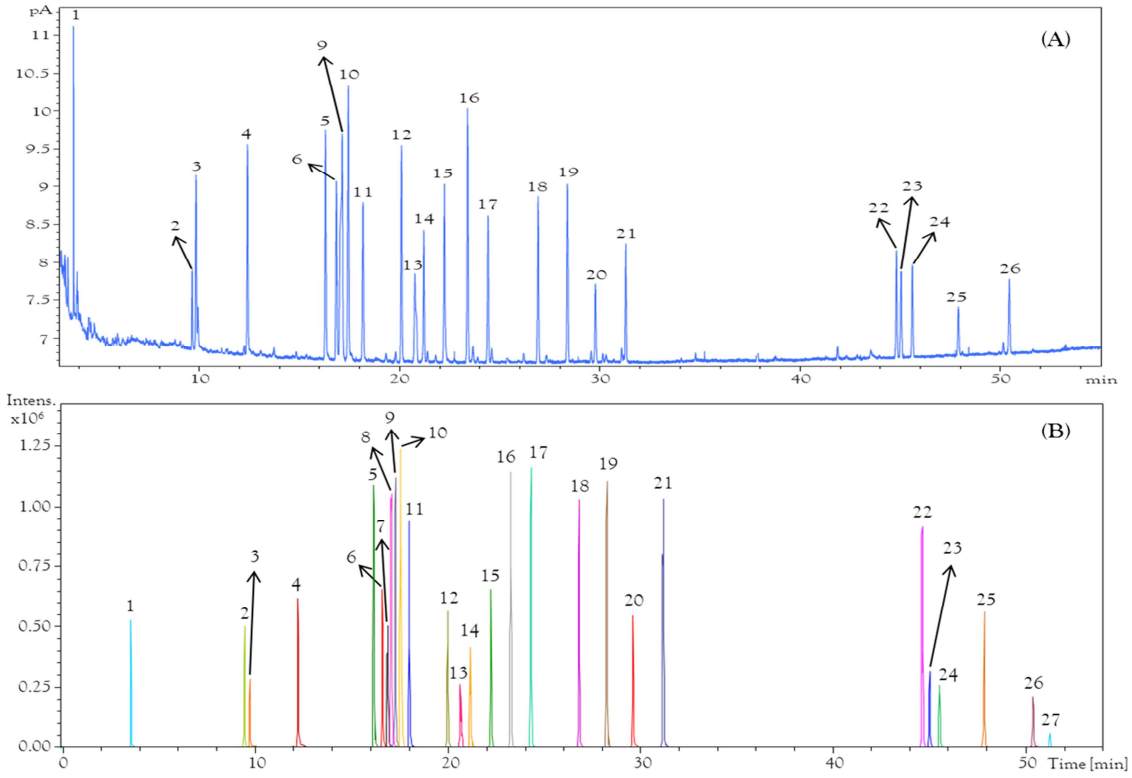

Figure 7.1. (A) FID chromatogram of a standard mix at a concentration of 0.85 mg/L and (B) shows the EICs of the metabolites included in the standard mix revealed by APCI-MS at the same concentration level. Peak identification: **1:** benzoic acid; **2:** vanillin; **3:** *trans*-cinnamic acid; **4:** 4-hydroxybenzoic acid; **5:** *o*-pyrocatechuic acid; **6:** vanillic acid; **7:** isovanillic acid; **8:** γ-resorcylic acid; **9:** homovanillic acid; **10:** gentisic acid; **11:** *o*-coumaric acid; **12:** *m*-coumaric acid; **13:** quinic acid; **14:** syringic acid; **15:** *p*-coumaric acid; **16:** gallic acid; **17:** pantothenic acid, **18:** ferulic acid; **19:** caffeic acid; **20:** abscisic acid; **21:** sinapic acid; **22:** naringenin; **23:** epicatechin; **24:** catechin; **25:** kaempferide; **26:** chlorogenic acid; **27:** quercetin.

Tables 7.2, 7.3 and ***7.4*** enclose the concentration levels -expressed as mg/kg- obtained for the compounds that have been quantified using GC-APCI-MS and GC-FID. The results are structured according to the different chemical categories of the determined metabolites: phenolic acids, flavonoids, and organic acids and vitamins. GC-APCI-MS allowed the quantification of 27 compounds, whereas by using GC-FID it was just possible to properly quantify 7 analytes. This fact could be explain bearing in mind the complexity of the sample under study (with high fat content together with an assortment of proteins, sugars, vitamins, sterols, tannins, coumarins, alkanols, phenolic compounds, etc.), which brings co-elution and overlapping problems that can not be solve by FID. Moreover, LODs and LODs in FID were much higher than in MS, complicating very much the determination of the compounds present at low concentration levels.

Table 7.2. Organic acids, phytohormone and vitamin quantitative results (mg/kg) obtained for the avocado samples under study by using GC-FID/APCI-TOF MS.

Avocado samples	ORGANIC ACIDS				PHYTOHORMONE		VITAMIN	
	Benzoic acid		*Quinic acid*		*Abscisic acid*		*Pantothenic acid*	
	MS	FID	MS	FID	MS	FID	MS	FID
Colin V 33	–	–	136.86	134.17	3.29	3.03	3.70	n.q
	4.03	n.q	63.08	63.10	2.18	2.31	4.20	n.q
Gem	1.15	n.q	7.52	7.11	0.64	n.q	2.92	n.q
	1.56	n.q	19.83	21.02	2.03	2.04	3.34	n.q
Harvest	1.54	n.q	45.55	45.91	1.15	1.18	1.96	n.q
	1.51	n.q	53.58	53.87	6.23	6.54	2.75	n.q
Hass	1.11	n.q	5.33	6.78	0.21	–	2.34	n.q
	1.30	n.q	3.50	4.08	2.44	2.95	1.38	n.q
Hass Motril	1.29	n.q	4.81	5.28	0.42	n.q	2.57	n.q
	0.73	n.q	2.12	2.39	1.82	1.68	1.47	n.q
Jiménez 1	4.17	n.q	17.55	17.89	0.84	0.95	3.19	n.q
	2.16	n.q	14.88	15.74	5.38	5.26	3.80	n.q
Jiménez 2	1.87	n.q	8.34	8.61	0.97	1.08	4.17	n.q
	0.92	n.q	17.24	17.51	1.87	1.99	3.63	n.q
Lamb Hass	1.70	n.q	24.96	25.32	3.77	3.62	1.78	n.q
	1.80	n.q	63.49	63.70	5.91	5.91	3.30	n.q
Marvel	–	–	13.95	14.17	0.46	n.q	2.04	n.q
	0.76	n.q	3.69	3.89	2.12	2.25	2.72	n.q
Nobel	0.81	n.q	6.75	7.07	0.08	–	3.67	n.q
	–	–	2.27	2.93	1.56	1.60	4.54	n.q
Pinkerton	1.40	n.q	86.27	87.09	0.99	0.934	1.52	n.q
	1.56	n.q	28.43	29.82	1.63	1.60	2.35	n.q
Sir Prize	2.35	n.q	7.11	7.32	1.93	1.87	2.83	n.q
	3.48	n.q	3.84	3.94	5.05	5.09	4.50	n.q
Tacambaro	1.87	n.q	10.62	10.64	0.65	n.q	2.33	n.q
	1.37	n.q	8.43	8.44	3.16	3.26	3.54	n.q

–: non detected

n.q: non quantifiable

The lines with the green shading correspond with the ripe samples. Those with white background color are unripe samples.

Table 7.3. Quantitative results (mg/kg) in terms of flavonoids obtained for the avocado samples under study by using GC-FID/APCI-TOF MS.

Avocado samples	FLAVONOIDS									
	Naringenin		*Epicatechin*		*Catechin*		*Kaempferide*		*Quercetin*	
	MS	FID	MS	FID	MS	FID	MS	FID	MS	FID
Colin V 33	–	–	0.37	n.q	–	–	–	–	1.97	–
	–	–	0.03	–	–	–	–	–	–	–
Gem	–	–	2.12	2.48	0.18	n.q	0.93	–	17.11	–
	–	–	0.28	n.q	–	–	–	–	3.06	–
Harvest	–	–	0.80	0.94	–	–	–	–	20.65	–
	–	–	0.46	0.57	–	–	0.31	–	3.76	–
Hass	0.10	–	14.86	15.15	0.34	n.q	–	–	13.25	–
	0.07	–	1.63	2.06	–	–	–	–	5.57	–
Hass Motril	0.21	–	3.84	3.95	0.13	n.q	0.29	-	8.47	–
	0.08	–	4.15	4.38	0.02	–	–	–	6.06	–
Jiménez 1	–	–	8.17	8.14	0.27	n.q	–	–	8.62	–
	–	–	16.37	16.26	0.14	n.q	–	–	6.04	–
Jiménez 2	0.07	–	4.44	4.50	0.08	–	–	–	7.20	–
	–	–	0.56	0.60	0.06	–	–	–	7.57	–
Lamb Hass	0.34	–	9.12	9.20	–	–	–	–	6.93	–
	–	–	11.09	11.25	–	–	–	–	4.45	–
Marvel	–	–	0.59	n.q	–	–	–	–	n.q	–
	–	–	0.30	n.q	–	–	–	–	0.06	–
Nobel	0.08	–	0.63	n.q	0.04	–	–	–	5.38	–
	0.25	–	0.18	n.q	0.04	–	0.35	–	–	–
Pinkerton	–	–	10.54	11.69	0.82	n.q	–	–	22.88	–
	–	–	7.35	8.48	0.35	n.q	–	–	3.64	–
Sir Prize	–	–	25.43	25.54	0.52	n.q	–	–	8.95	–
	–	–	1.46	1.48	0.13	n.q	–	–	2.85	–
Tacambaro	–	–	14.80	14.92	0.32	n.q	–	–	11.61	–
	0.29	–	9.23	9.27	0.11	–	1.02	–	4.73	–

–: non detected

n.q: non quantifiable

The lines with the green shading correspond with the ripe samples. Those with white background color are unripe samples.

Table 7.4. Phenolic acids quantitative results (mg/kg) obtained for the 13 avocado samples included in this study by using GC-FID/APCI-TOF MS.

PHENOLIC ACIDS		Avocado samples													
		Colin V 33		Gem		Harvest		Hass		Hass Motril		Jiménez 1		Jiménez 2	
Vanillin	MS	–	–	–	0.17	–	0.24	–	–	–	–	0.04	0.33	0.13	0.12
	FID	–	–	–	–	–	–	–	–	–	–	–	n.q	–	–
trans-cinnamic acid	MS	–	–	–	–	–	0.98	0.79	0.91	–	–	–	–	–	0.77
	FID	–	–	–	–	–	n.q	n.q	n.q	–	–	–	–	–	n.q
4-hydroxybenzoic acid	MS	0.13	0.17	0.09	0.64	0.11	0.47	0.04	0.05	0.07	0.17	0.07	0.20	0.12	0.11
	FID	n.q	n.q	–	n.q	n.q	n.q	–	–	–	n.q	–	n.q	n.q	n.q
o-pyrocatechuic acid	MS	–	–	–	–	–	–	–	–	–	–	–	0.15	–	–
	FID	–	–	–	–	–	–	–	–	–	–	–	n.q	–	–
Vanillic acid	MS	–	–	–	–	–	0.07	–	–	–	–	–	0.06	0.09	0.07
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isovanillic acid	MS	–	–	–	–	–	–	–	–	–	–	–	0.02	–	–
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
γ-resorcylic acid	MS	0.08	0.05	–	–	0.02	0.02	–	–	–	–	–	–	–	–
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Homovanillic acid	MS	–	–	0.04	–	0.01	0.06	–	0.02	–	–	–	–	–	–
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gentisic acid	MS	–	–	0.14	–	–	–	–	–	0.12	–	–	–	–	–
	FID	–	–	n.q	–	–	–	–	–	n.q	–	–	–	–	–
o-coumaric acid	MS	–	–	–	–	–	–	–	–	–	–	–	0.02	–	–
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
m-coumaric acid	MS	–	–	0.32	0.21	0.29	0.32	0.23	0.32	0.30	0.37	0.31	0.30	0.22	0.27
	FID	–	–	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q
Syringic acid	MS	0.23	0.18	0.23	0.21	0.20	0.32	0.12	0.12	0.12	0.12	0.12	0.09	0.24	0.11
	FID	n.q	n.q	n.q	n.q	n.q	n.q	–	–	–	–	–	–	n.q	–
p-coumaric acid	MS	4.19	2.96	0.75	30.86	0.53	29.89	0.69	8.06	0.66	7.38	0.61	7.71	1.79	7.84
	FID	4.13	3.08	0.80	31.33	0.80	32.20	0.67	7.67	0.72	7.77	0.71	7.94	1.94	7.92
Gallic acid	MS	0.02	0.01	–	–	0.01	0.03	–	–	0.02	–	0.01	0.01	0.01	–
	FID	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Ferulic acid	MS	0.49	0.76	0.14	3.72	0.36	3.35	0.11	1.52	0.14	1.57	0.17	1.47	0.26	0.63
	FID	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q	n.q
Caffeic acid	MS	–	–	–	–	–	0.45	–	–	0.11	0.76	0.07	0.46	0.44	0.68
	FID	–	–	–	–	–	0.47	–	–	n.q	0.79	–	0.47	0.52	0.73
Sinapic acid	MS	0.13	0.09	0.07	0.64	0.09	0.40	0.07	0.29	0.09	0.64	0.10	0.33	0.15	0.26
	FID	–	–	–	n.q	–	n.q	–	n.q	–	n.q	–	n.q	n.q	n.q
Chlorogenic acid	MS	0.12	0.10	0.21	0.56	0.24	0.10	1.69	0.23	0.20	–	0.91	0.59	0.70	0.46
	FID	–	–	n.q	n.q	n.q	–	1.73	n.q	–	–	1.02	n.q	0.73	n.q

Table 7.4. (continued)

PHENOLIC ACIDS		Avocado samples											
		Lamb Hass		Marvel		Nobel		Pinkerton		Sir Prize		Tacambaro	
Vanillin	MS	–	–	–	–	0.05	0.16	–	–	–	0.95	0.08	0.31
	FID	–	–	–	–	–	–	–	–	–	n.q	–	n.q
trans-cinnamic acid	MS	–	0.66	–	1.32	–	1.17	0.50	–	1.92	1.24	0.86	1.04
	FID	–	n.q	–	n.q	–	n.q	n.q	–	n.q	n.q	n.q	n.q
4-hydroxybenzoic acid	MS	0.06	0.41	0.06	0.15	0.04	0.17	0.04	0.30	0.08	0.23	0.03	0.18
	FID	–	n.q	–	n.q	–	n.q	–	n.q	–	n.q	–	n.q
o-pyrocatechuic acid	MS	–	0.12	–	–	–	–	–	–	–	–	–	0.11
	FID	–	n.q	–	–	–	–	–	–	–	–	–	n.q
Vanillic acid	MS	–	–	–	–	–	–	–	–	0.10	0.03	0.09	0.16
	FID	–	–	–	–	–	–	–	–	n.q	–	–	n.q
Isovanillic acid	MS	–	–	–	–	–	–	–	–	–	0.02	0.05	0.07
	FID	–	–	–	–	–	–	–	–	–	–	–	–
γ-resorcylic acid	MS	–	–	–	–	–	–	–	–	0.09	0.06	–	0.07
	FID	–	–	–	–	–	–	–	–	–	–	–	–
Homovanillic acid	MS	–	–	–	–	–	–	0.01	–	0.02	0.02	–	0.09
	FID	–	–	–	–	–	–	–	–	–	–	–	n.q
Gentisic acid	MS	0.12	0.14	0.10	–	–	0.03	–	–	0.01	0.03	–	0.09
	FID	n.q	n.q	n.q	–	–	–	–	–	–	–	–	n.q
o-coumaric acid	MS	–	–	–	–	–	0.03	–	–	0.02	0.07	–	0.06
	FID	–	–	–	–	–	–	–	–	–	–	–	–
m-coumaric acid	MS	–	–	–	–	–	–	–	–	0.24	0.40	0.29	0.38
	FID	–	–	–	–	–	–	–	–	n.q	n.q	n.q	n.q
Syringic acid	MS	0.14	0.31	0.14	0.11	3.54	3.11	0.10	0.18	0.02	0.22	0.10	0.14
	FID	n.q	n.q	–	–	3.54	3.07	–	n.q	–	n.q	–	n.q
p-coumaric acid	MS	0.42	14.94	0.45	15.65	0.97	7.77	0.49	4.70	1.16	19.94	1.14	7.22
	FID	0.41	14.66	0.47	16.62	1.19	7.85	0.50	4.92	1.20	20.10	1.18	7.37
Gallic acid	MS	0.02	0.02	0.01	0.01	0.01	0.05	0.03	0.02	0.01	0.01	–	0.08
	FID	–	–	–	–	–	–	–	–	–	–	–	n.q
Ferulic acid	MS	0.14	2.34	0.14	3.13	0.09	0.60	0.07	0.77	0.13	4.57	0.38	1.17
	FID	n.q	n.q	n.q	n.q	–	n.q	–	n.q	n.q	n.q	n.q	n.q
Caffeic acid	MS	0.54	0.59	0.21	0.25	0.16	0.31	0.17	0.70	0.43	0.46	0.14	0.30
	FID	0.62	0.67	n.q	n.q	n.q	0.32	n.q	0.78	0.45	0.50	n.q	n.q
Sinapic acid	MS	0.03	0.25	0.05	0.11	0.07	0.16	0.05	0.16	0.07	0.71	0.05	0.31
	FID	–	n.q	–	n.q	–	n.q	–	n.q	–	n.q	–	n.q
Chlorogenic acid	MS	0.42	1.04	0.36	0.58	0.42	0.11	2.09	1.30	1.17	0.86	0.55	0.14
	FID	n.q	1.08	n.q	n.q	n.q	–	2.21	1.51	1.25	0.88	n.q	–

–: non detected
n.q: non quantifiable
The columns with the green shading correspond with the ripe samples. Those with white background color include the values for the unripe samples.

Taking into account just the analytes quantified by using both detection systems (quinic, abscisic, syringic, *p*-coumaric, caffeic and chlorogenic acids, and epicatechin), it is noteworthy to mention that the results achieved by GC-FID and GC-APCI-MS are in accordance and they do not show significant differences.

From the 27 quantified compounds by APCI-MS, 10 metabolites were found in all the evaluated samples; they were 4-hydroxybenzoic, abscisic, chlorogenic, ferulic, pantothenic, *p*-coumaric, quinic, sinapic and syringic acids, and epicatechin. However, other metabolites seemed to be characteristic of some samples –as isovanillic acid ('Jiménez 1', 'Sir Prize' and 'Tacambaro')– or of a particular ripening stage (such as *o*-pyrocatechuic acid, that was detected just in some ripe avocado samples (('Jiménez 1', 'Lamb Hass' and 'Tacambaro')).

It is very difficult to establish a common behavior of a particular chemical family, but we will try to make some general statements. It could be said that the concentration of the flavonoids and organic acids tend to diminish as the fruit ripens. In the case of phytohormones, such as abscisic acid, the concentration decreases with the ripening process. When we talk about phenolic acids, this family can be divided into two big groups: hydroxycinnamic and hydroxybenzoic acids. Hydroxycinnamic acid derivatives usually present higher concentration when the fruit is ripe; however, we can not generalize for the group of hydroxybenzoic acids because of the high variability of the results.

The results summarized above are in good agreement with previously published reports [10, 23, 39] where similar trends where observed for some of the metabolites determined in the current study. In the cited studies, the authors found that quinic, benzoic and pantothenic acids, epicatechin and catechin, for instance, decreased during ripening, and, on the contrary, the concentration of ferulic and *p*-coumaric acids (hydroxycinnamic acids) increased.

Quinic acid was one of the metabolites found in higher concentration levels. It was determined by FID and APCI-MS, being the results very similar. For all the varieties, except from 'Gem', 'Harvest' and 'Jiménez 2', its concentration was lower for the ripe samples. Oms-Oliu et al. [40] described the same decrease of certain organic acids in other

climacteric fruits. Benzoic acid was found in almost every sample under study (when APCI-MS was used as detector). It was just not found in 'Colin V 33' at 1st ripening stage (unripe) and ripe Nobel. Abscisic acid is a plant hormone which is the major player in mediating the adaptation of the plants to stress. It functions in many plant developmental processes, including bud dormancy. It is degraded by the enzyme (+)-abscisic acid 8'-hydroxylase into phaseic acid. What we observed here is that the concentration of this compound was in all the cases higher when the ripe fruits were analyzed (the tendency was the opposite only for 'Colin V 33'). Pantothenic acid or vitamin B_5 was present in every sample and its content was, in general, higher for the ripe samples. Only for 'Hass', 'Hass Motril' and 'Jiménez 2', a slight decrease of its concentration was observed.

Belonging to the flavonoids family, we determined naringenin, epicatechin, catechin, kaempferide and quercetin. FID was only useful for carrying out the determination of epicatechin and catechin (although the latter was found at non quantifiable levels). Epicatechin and quercetin were the most abundant ones; they both were at higher concentration levels in the extracts prepared from the fruits just harvested. The same decreasing of flavonoids has been observed when the metabolic prolife of other fruits have been evaluated [41, 42]. The found levels of epicatechin ranged from 0.03 to 25.43 mg/kg for ripe 'Colin V 33'and unripe' Sir Prize', respectively, and those of quercetin were found between 0.06 and 22.88 mg/kg (for ripe 'Marvel' and unripe 'Pinkerton', respectively). Naringenin and kaempferide were found in very few samples.

As far as phenolic compounds are concerned, we can stand out that metabolites such as 4-hydroxybenzoic, syringic, *p*-coumaric, ferulic and sinapic acids were found in every variety (in fruits just harvested and in ripe ones), being *p*-coumaric acid by far the most abundant analyte. Its concentration increased remarkably when ripe samples were analyzed (reaching amounts of about 32 mg/kg), except for Colin V 33 *cv*. Ferulic acid increased as well its concentration, but not in such pronounced way. Several authors have explained that these two metabolites are related to browning reactions of fruits and have observed similar trends in other fruits [43, 44] and in avocado [35] during ripening. Vanillin, and *o*-pyrocatechuic, vanillic, isovanillic, *γ*-resorcylic, homovanillic, gentisic and *o*-coumaric acids were present in only few of the varieties under study. Chlorogenic acid concentrations were always lower for the extracts prepared from ripe fruits.

Figure 7.2 shows some examples where the changes in the concentration between unripe and ripe samples can be observed for quinic and pantothenic acids, epicatechin, *p*-coumaric, ferulic and chlorogenic acids.

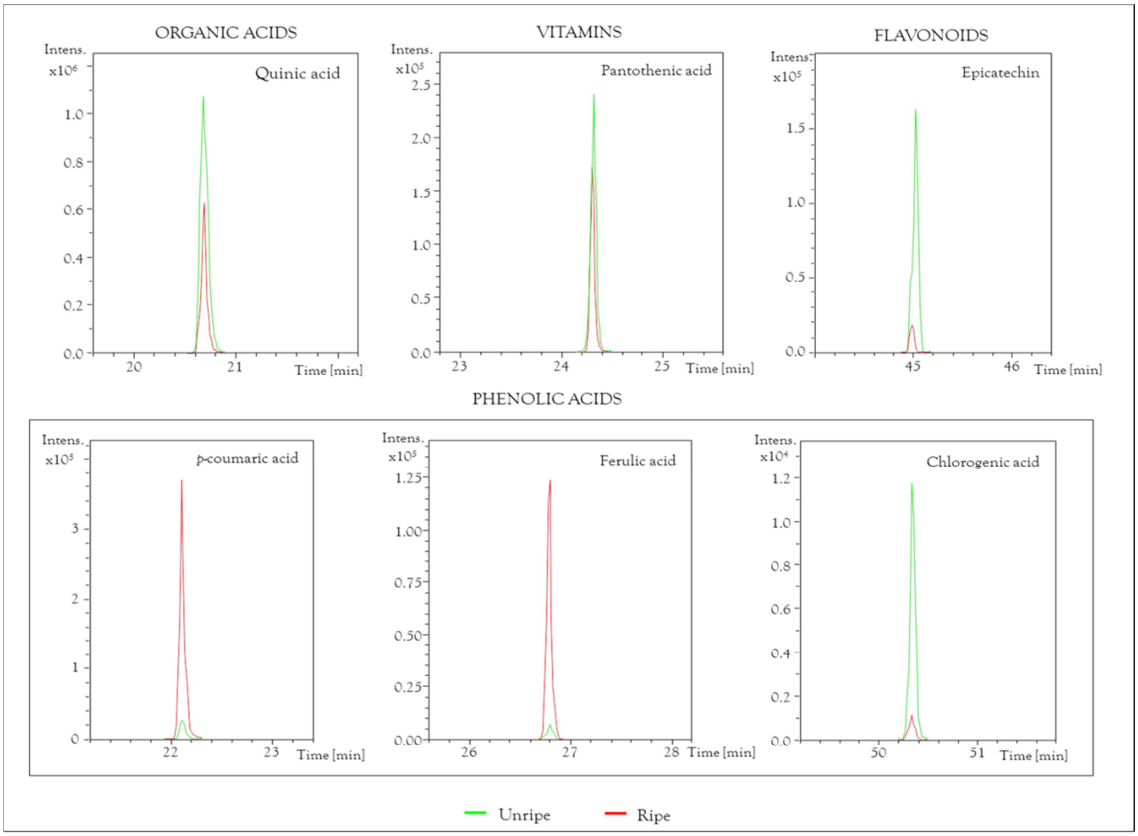

Figure 7.2. EICs of epicatechin, quinic, pantothenic acids, *p*-coumaric, ferulic and chlorogenic acids found in one unripe and ripe **'**Sir Prize**'** sample.

Exploratory data analysis: PCA results

An unsupervised multivariate method, such as PCA, has been used to evaluate the influence that metabolite fluctuations observed during the avocado fruit ripening could have on samples classification. Two PCA experiments were carried out considering the quantitative results obtained by GC-FID and GC-APCI-MS, respectively. The matrices used for the study were built including 78 samples (26 samples by triplicate) and 27 variables, in the case of GC-APCI-MS, and 7 variables for GC-FID.

The score and loading plots obtained for both matrices are shown in ***Figures 7.3*** and ***7.4***, and they represent PC2 *vs.* PC1; these first two components explained the 94.5% and the 96.5% of total variability of the data set for GC-APCI-MS and GC-FID, respectively.

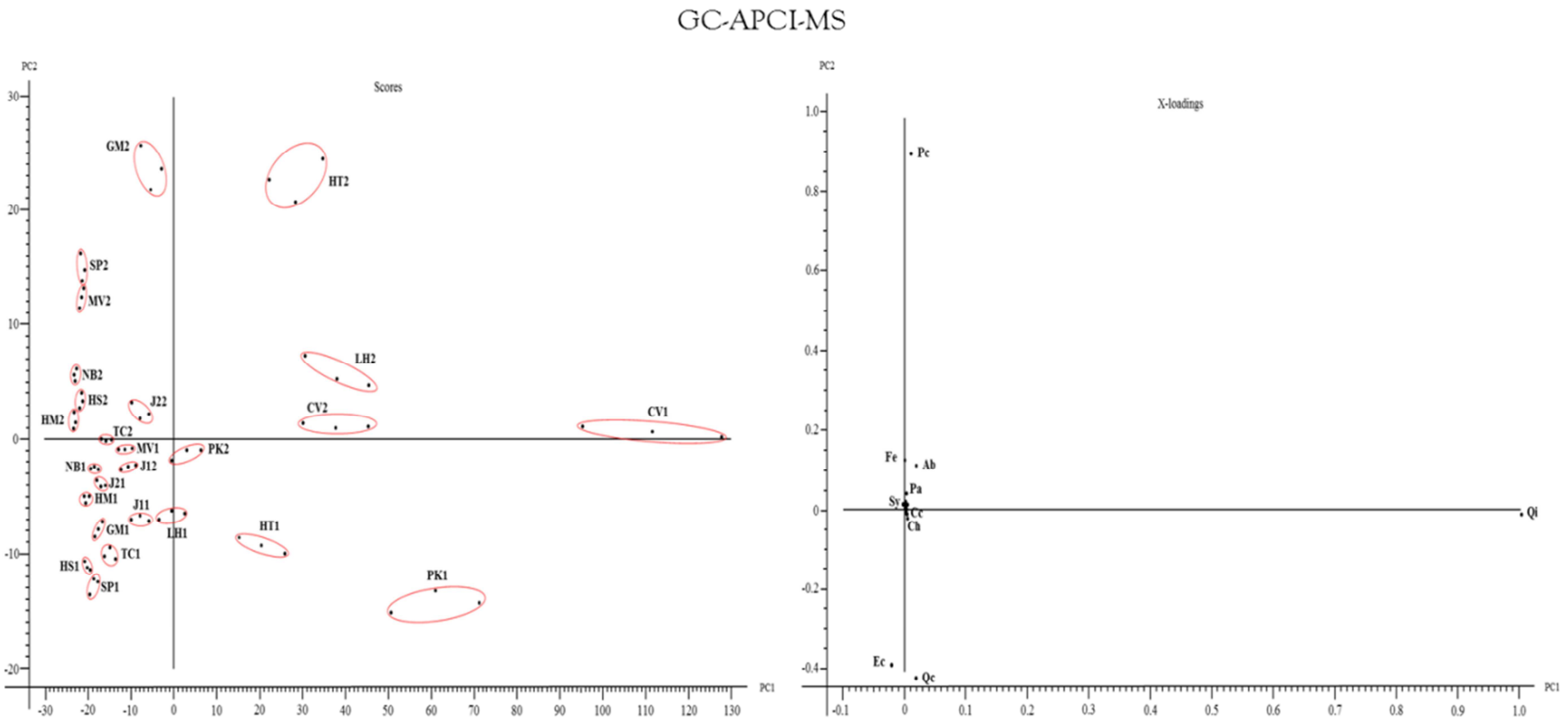

Figure 7.3. Score and loading plots of PCA modelling obtained for GC-APCI-TOF MS. Loading plot shows an area where different compounds overlap: Be, Vn, Tc, 4h, Op, Va, Iv, Gr, Hv, Ge, Oc, Mc, Ga, Ca, Si, Na and Ka.

CV: Colin V 33; **GM**: Gem; **HT**: Harvest; **HS**: Hass; **HM**: Hass Motril, **J1**: Jiménez 1; **J2**: Jiménez 2; **LH**: Lamb Hass; **MV**: Marvel; **NB**: Nobel; **PK**: Pinkerton; **SP**: Sir Prize; **TC**: Tacambaro. **1**: first ripening degree; **2:** second ripening degree. **Be**: benzoic acid; **Vn:** vanillin; **Tc**: *trans*-cinnamic acid; **4h**: 4-hydroxybenzoic acid; **Op**: *o*-pyrocatechuic acid; **Va**: vanillic acid; **Iv:** isovanillic acid; **Gr**: *γ*-resorcylic acid; **Hv:** homovanillic acid; **Ge**: gentisic acid; **Oc**: *o*-coumaric acid; **Mc**: *m*-coumaric acid; **Qi:** quinic acid; **Sy**: syringic acid; **Pc**: *p*-coumaric acid; **Ga**: gallic acid; **Pa:** pantothenic acid; **Fe**: ferulic acid; **Ca**: caffeic acid; **Ab:** abscisic acid; **Si**: sinapic acid; **Na**: naringenin; **Ec:** epicatechin; **Cc:** catechin; **Ka:** kaempferide; **Ch**: chlorogenic acid; **Qc**: quercetin.

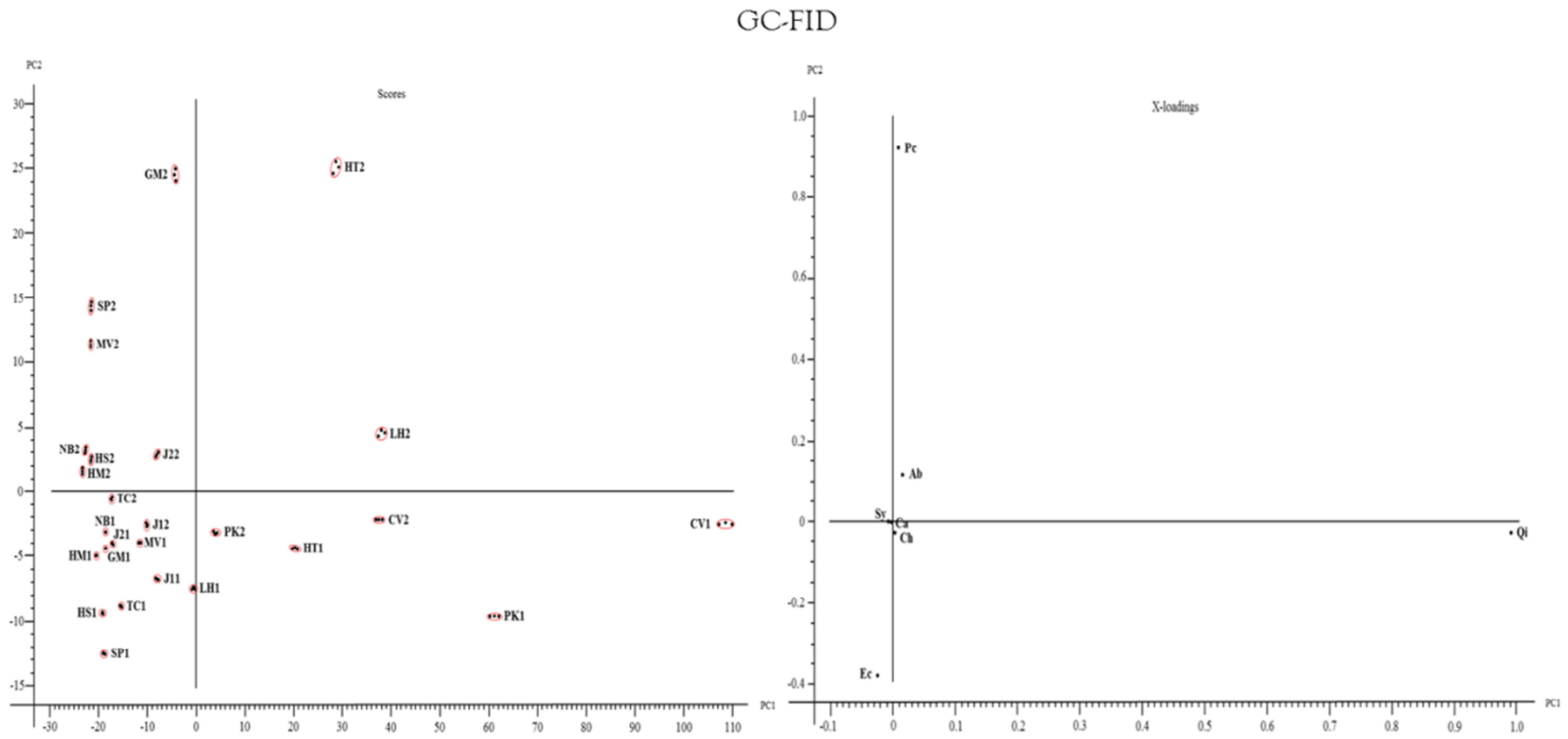

Figure 7.4. Score and loading plots of PCA modelling obtained for GC-FID. Identification legends of varieties and compounds as in ***Figure 7.3.***

Even though the number of metabolites determined by each platform differs quite a lot, it is remarkable the fact that both detection systems give very similar score and loading plots of PCA modelling. It turned out that the 7 analytes determined by FID were some of the most influential compounds in the discrimination. The first component (PC1) reflects the differences among avocado fruit varieties and it was principally built considering the influence of quinic acid. The higher the value of positive scores, the higher the amount of quinic acid; and as it can be seen in the figures, 'Colin V 33' at 1st ripening degree is the sample that shows the largest separation distance in the PC1.

When PC2 is taken into account, it is possible to appreciate a clear separation between samples of the 1st stage of ripeness and the 2nd one; hence, the PC2 is related to the ripening process, which is the most important source of data variability. Samples coming from 'Gem' and 'Harvest' cultivars are the most separated in the plot, whereas ripe and unripe samples of 'Colin V 33' are laying at the same level. In general, the majority of ripe samples are located in the positive score area of PC2 axis and that means higher amounts of *p*-coumaric and, to a lesser extent, of ferulic and abscisic acids. This entire means that these phenolic acids play an important role on the discrimination between ripe and unripe samples and its concentrations usually increase as the fruit ripens. On the contrary, the negative score area is related with higher concentrations of epicatechin and quercetin (just for MS PCA, since it was not determined by FID) and it is in this part of the plot where all the unripe samples are found, being Sir Prize and Pinkerton at the 1st stage of ripeness the richest ones.

Both ***Figure 7.3*** and ***Figure 7.4*** revealed that 'Colin V 33' and 'Harvest' at the two different ripening degrees, ripe 'Lamb Hass' and unripe 'Pinkerton' were the avocado samples showing the highest metabolic differences (when compared with the rest).

Although the classification achieved with both detectors are quite similar, there are some differences and the most evident one is the dispersion of the sample replicates, which is much lower for FID than for MS. This fact can be explained observing the repeatability and intermediate precision values shown in ***Table 7.1.***

7.4. Concluding remarks

To conduct clinical studies on the health benefits of avocado consumption, any variation in the composition of the avocado must be adequately considered. Avocados are picked when they are mature but unripe, that means that they can have different phytochemical composition at different time points during ripening. To control this could enlarge our knowledge about the metabolic evolution of this matrix.

The quantitative evaluation of the metabolic profile of 13 avocado varieties (at two ripening degrees) was carried out. To the best of our knowledge, this is the first time in which a GC-FID/APCI-TOF MS methodology is used for the quantitative analysis of relevant metabolites (organic acids, vitamins, flavonoids and phenolic acids) in *Persea americana*. The method was fully validated and the analytical performances of both detectors were compared. It was possible to quantify 27 compounds by GC-APCI-MS and just 7 using GC-FID. In general, the concentration of flavonoids and organic acids tended to decrease as the fruit ripens; observing an opposite tendency for hydroxycinnamic acid derivatives and vitamin B_5. The PCA classification achieved was quite similar for both detection systems, demonstrating that FID is still useful to determine some of the most influential metabolites of avocado fruit in the discrimination between ripe and unripe samples (*p*-coumaric acid and epicatechin) and/or cultivars (quinic acid).

Although the results achieved by GC-FID were quite good, it is evident that it is not possible to compete with GC-APCI-TOF MS, which in comparison allows an easier identification and a more proper quantification of the metabolites. Thus, GC-APCI-TOF MS is even more settled as a very useful and important technique in the metabolomics toolbox.

Acknowledgements

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Chapter 8

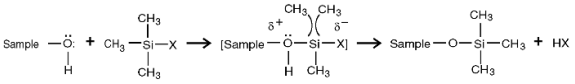
Development of a spectral database for gas chromatography-atmospheric pressure chemical ionization-time of flight ionization mass spectrometry

8.1. Introduction

Since the introduction of commercial systems of GC in 1954 and the first coupling with MS in 1957 [1], this technique has undergone to several changes and improvements that have turned it into one of the most used platforms in several analytical chemistry fields [2, 3]. It has become a routine technique for the analysis of different compounds in industrial and environment samples, for pharmaceutical, clinical, toxicological and forensic applications; and also in the area of food analysis, where it has been applied to the study of residues or contaminants and several food components [4]. Besides, GC-MS is also one of the preferred tools in metabolomics, even before the concept of metabolomics or metabolic profiling appeared [5] –despite the fact that this technique is not ideal considering the compound volatility required [6, 7]–, being used to the study of the metabolic profile of plants, microorganism, mammals and humans [8, 9].

As aforementioned, GC-MS presents an important characteristic, which could be a limitation, and it is that the compounds to be analyzed should be volatiles and thermally stables. However, some of the metabolites that naturally occur in plants are not volatile enough for GC separation and it is necessary that they become volatiles by means of a derivatization process, which increases and slightly complicates the sample preparation [7]. The derivatization reaction is a chemical modification of the analyte functionality that allows the chromatographic separation [10] and its aim is to increase the compound detectability, volatility, stability and sensitivity. Ideally, a derivatization method should be fast, reproducible and not very expensive; and it should quantitatively convert each metabolite to only derivative species. There are different reactions of derivatization that has been traditionally used and they are summarized in ***Table 8.1*** [10]. The sylilation reaction, by using BSTFA or MSTFA, is the most popular and widely applied because resulting derivatives are stable and present good reproducibility; but on the other hand it has some drawbacks: not easy to automate, slow, and the residual derivatization reagent remaining after the reaction causes a diminution on the lifetime of the column [8]. Frequently, before the sylilation, a previous step of methoxyamination is carried out to protect keto- and aldehyde groups, avoiding cyclic formation of carbohydrates and losses of carboxyl groups of those metabolites containing a keto- group [11].

Table 8.1. Types of derivatization reactions commonly used in GC-MS analysis.

Type	Reaction	Reagents
Alkylation	$RCOOH + PhCH_2X \rightarrow RCOOCH_2Ph + HX$	* Dialkylacetals * Diazoalkales * Pentafluorobenzyl bromide (PFBBr) * Benzylbromide * Boron trifluoride (BF_3) (in methanol or butanol) * Tetrabutylammonium hydroxyde (TBH)
Sylilation		* Hezamethyldisilzane (HMDS) * Trimethylchlorosilane (TMCS) * Trimethylsilylimidazole (TMSI) * Bistrimethylsilylacetamide (BSA) * Bistrimethylsilyltrifluoroacetamida (BSTFA) * N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) * Trimethylsilyldiethylamine (TMS-DEA) * N-methyl-N-t-butyldimethylsilyltrifluoroacetamide (MTBSTFA) * Halo-methylsilyl derivatization reagents
Acylation	$CH_3OCOCOCH_3 + HOR \xrightarrow{H^+(Catalyst)} CH_3OCOR' + HOCOCH_3$	* Fluoracylimidazoles * Fluorinated anhydrides * N-methyl-bis(trifluoroacetamide) (MBTFA) * Pentafluorobenzoyl chloride (PFBCI) * Pentafluoropropanol (PFPOH)

It is well-known that the coupling of GC to MS is normally made through EI or CI, interfaces that work under vacuum conditions. However, although it was developed in 1973 [12], since 2005 APCI –a typical LC-MS interface– is gaining interest as alternative source for GC-MS analysis, due to the improvements made by McEwen and McKay [13], and Schievek et al. [14]. To an adequate interfacing of GC and APCI, a transfer line is required, which should be located at the capillary column outlet and the MS inlet. It is especially important that the temperature of this transfer line is enough high to prevent compound condensation. The principal difficulties of this design can be found in the problem to position the transfer line in relation to the MS inlet in a reproducible way. As well as in the efficiency lost caused by the migration of the analytes through the transfer line.

Comparing APCI and the traditional ionization systems for GC (EI/CI), it produces a softer fragmentation of the analytes generating fewer fragments and preserving the parent ion, as well as it allows the ionization of compounds with higher molecular weights and polarities. Ionization in APCI occurs in the gas phase and it is based on ion-molecule reactions that take place at atmospheric pressure. The process is initiated by

means of electrons emitted from a corona discharge needle. These electrons ionize the nitrogen used as dry/nebulizer gas in the source and the ionized nitrogen reacts with the gas coming from the GC (containing our analytes). Both positive and negative ions could be formed; the first ones are usually obtained by proton transfer or charge exchange, whereas negative ions appear as a consequence of resonance/dissociative capture of thermal electrons or ion-molecule interactions [12, 13].

To date, the number of applications using this platform has experienced an increase, but GC-APCI-MS is still far from being a routine technique. It has been used to the analysis of biofluids [15, 16], pharmaceutical impurities [17], metabolome of *Escherichia coli* [18], sediments [19], food extracts [20], and pesticide residues [21-24]. All these applications for GC-APCI-MS are grouped in ***Figure 8.1***.

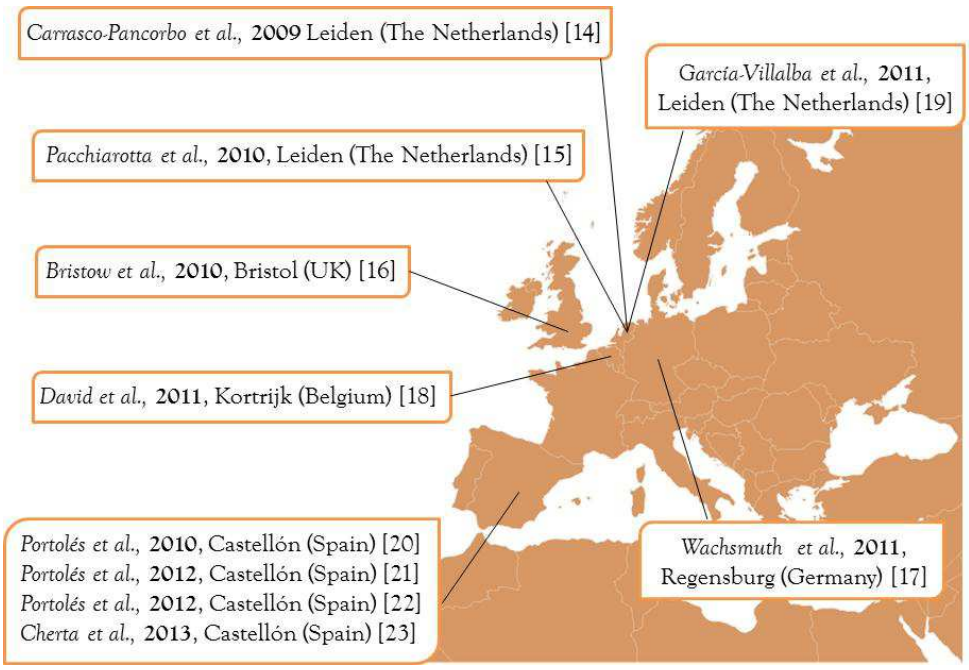


Figure 8.1. GC-APCI-MS applications described in literature and located by countries.

One of the main reasons of the scarce number of applications by using GC-APCI-MS could be the lack of available database that facilitates the identification of the compounds under study. Thus, the principal motivation of this work was to solve this problem by creating a new spectral library that includes a wide variety of metabolites that can be found both in biofluid and food extract samples.

8.2. Materials and methods

8.2.1. Chemicals and standards

Freshly opened 1 mL bottles of MSTFA + 1% TMCS and BSTFA + 1% TMCS were used as derivatization reagents (Pierce; Rockford, IL, USA). Methoxyamine hydrochloride and the mixes of saturated alkanes (C7-C30 and C7-C40) were purchased from Supelco (Zwijndrecht, Netherlands), whereas APCI tune mix and pyridine (99% ultra-pure GC grade) were acquired from Fluka (Zwijndrecht, Netherlands).

150 standards of compounds belonging to different chemical groups were supplied by Sigma-Aldrich (Zwijndrecht, Netherlands), Extrasynthese (Lyon, France), Fluka (Zwijndrecht, Netherlands) and Carbosynth (Comptom, United Kingdom), and all of them were analytical grade.

8.2.2. Standard solution preparation

Each standard was individually weighted and dissolved in a proper solvent to have a "stock solution" at a final concentration of 5000 ppm. Then, "working solutions" at 500 ppm were prepared by mixing the necessary volumes of "stock solutions" and MeOH. These "working solutions" were those used to prepare the final standard solution to be injected at concentrations of 10, 25 or 50 ppm, depending on the response of each analyte. The proper volume of each standard was taken and evaporated to dryness for the subsequent derivatization reaction.

8.2.3. Derivatization

The reaction of derivatization was made on-line and in two steps: methoxyamination (to block cyclic formation) and sylanization. Firstly, 40 µL of methoxyamine solution (20 mg/mL in pyridine) were added to the dried standard solution and then the mix was incubated at 40°C during 40 min. Afterwards, 50 µL of MSTFA were incorporated and incubated again for 30 min at 40°C. Some of the compounds were thermolabile, for that the methoxyamination step was performed at room temperature during 16 h with the following addition of 50 µL of MSTF and incubation during 1 h also at room temperature. Some others analytes, as flavonoids, does not need methoxyamination, so

this step could be removed. In the case of these substances, 40 µL of pyridine and 50 µL of BSTFA were added and the mix was incubated for 1 h at 60°C.

10 µL of hydrocarbons solution (C7-30 or C7-40) (25 ppm) were added to all the standard solutions, that allows the calculation of Kovats indices.

8.2.4. GC-FID/APCI-MS analysis

An Agilent 7890A GC (Agilent, Palo Alto, USA) equipped with a column HP-5-MS (30 m, 0.25 mm ID, 0.25 µm film thickness) were used coupled to two detectors running in parallel; reason for which a column flow splitter is required with deactivated capillaries (0.6 m. 0.25 mm ID. 0 µm film thickness) allowing the simultaneous acquisition with both FID and MS detectors. For sample injection a septumless PTV injector was used and 1 µL of the standard solutions were applied by splitless injection with a programmable MultiPurpose Sampler MPS2 (Gerstel, Mülheim an der Ruhr, Germany). For each analysis the purge time of the PTV injector was set to 60s at a purge flow rate of 20 mL/min and an equilibration time of 1 min. Helium was used as carrier gas at a constant flow rate of 1 mL/min through the column.

Compounds with the addition of hydrocarbons solution C7-C30 were injected using the following temperature program: 40°C held for 1 minute, 10°C/min till 310°C held for 2 minutes. However, those requiring the addition of the hydrocarbons solution C7-C40 (late elution compounds) were injected with the following temperature program: 40°C held for 1 minute, 7°C/min till 310°C held for 15 minutes.

The FID flows and temperature were set as follows: front detector 300 °C, hydrogen flow: 40 mL/min and air flow: 370 mL/min. This detector was used for the calculation of the Kovats indices relative to the specific column in use.

A maXis 4G (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated Qq-TOF MS, was used as mass analyzer. The coupling of GC to MS was made by using an APCI source provided with a transfer line to the MS which was kept at a temperature of 300°C. The APCI source operated in positive mode. The temperature and the flow rate of the dry gas (nitrogen) were 300°C and 2 L/min, respectively. The APCI vaporizer temperature was 300°C; the pressure of the nebulizer gas (nitrogen) was set to 3.5 bar and the voltage of the corona discharge needle was +2000 nA. Capillary voltage was set to

−1000V and the end-plate offset to −1000 V. Data were acquired in a mass range from *m/z* 100 to *m/z* 1000 with 1 Hz acquisition rate. Before each chromatographic run, a tune mix solution was used as external calibrant and injected in Flow Injection mode. To achieve the best possible fragmentation of the compounds under study, MS/MS experiments were carried out selecting the precursor ion and applying different collision energies for each analyte.

8.3. Results and discussion

The database development was carried out following the steps enclosed in ***Figure 8.2***.

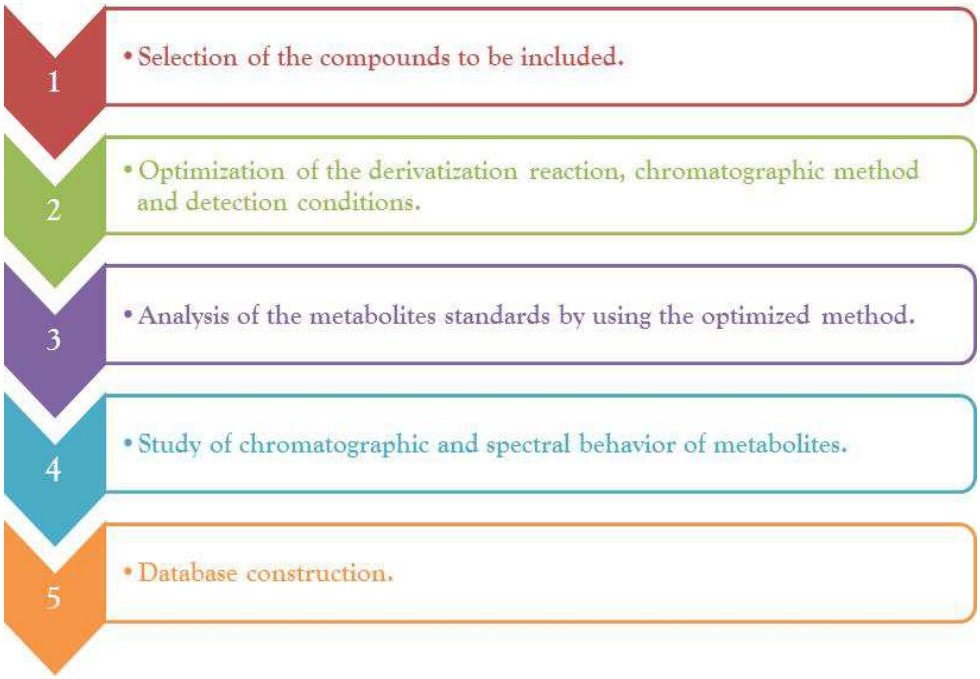


Figure 8.2. Followed steps for the spectral library creation.

8.3.1. Selection of the compounds

In a first attempt, the creation of a GC-APCI-MS database was intended to facilitate the identification of metabolites from food extracts and human biofluids. For this reason, the selection of the compounds to be included was made taking into account which compounds could be found in these types of matrices. ***Figure 8.3*** shows the different chemical families of compounds that were selected to be part of the library.

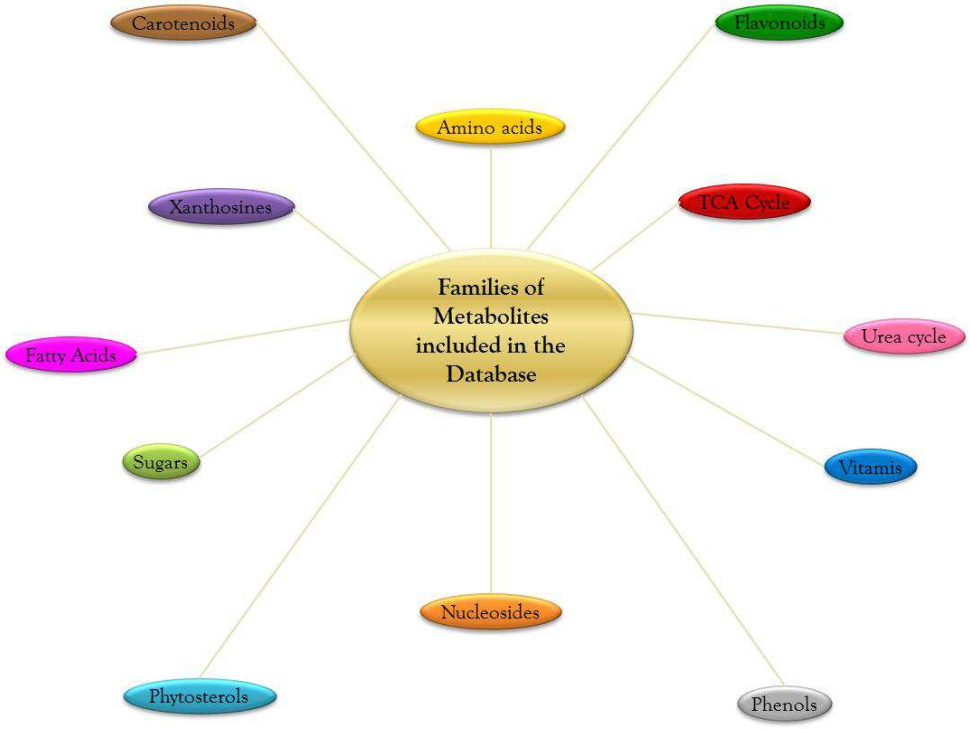


Figure 8.3. Chemical groups of the different standards included in the spectral database for GC-APCI-MS.

8.3.2. Optimization of the derivatization reaction, chromatographic method and detection conditions

The optimization of all the variables involves in the analysis of the metabolites plays a very important role for the development of the library, because of the wide variety of substances that were analyzed.

As it has been mentioned in the Introduction of this chapter, the derivatization reaction is one of the most characteristic step of the analytical process when GC is used as separation technique. Thus, the optimization of all the variables that can affect this chemical reaction is important. Factors that should be studied and optimized are temperature and time of reaction, amount (volume) of derivatization reagent, solvent used for the reaction, and efficiency of the reaction.

Once the most convenient derivatization reaction is chosen, the chromatographic method must be exhaustively optimized pursuing the highest speed and the best analysis resolution. The principal variables to be optimized are the following: carrier gas and its flow rate, injection mode (split, splitless, PTV), volume of injection and temperature gradient. In this work, two different temperature programs were required to cover the

wide variety of substances to be analyzed; one of 30 min for samples with hydrocarbons solution C7-30, and another one of 55 min for the mixtures with hydrocarbons solution C7-40.

Two different detection systems were used, being necessary an optimization of both of them. FID is characterized by its operation simplicity and there are only a few variables to consider: hydrogen and air flows, and front detector temperature. The flows of hydrogen and air are the key in the operation of the detector, because to keep the flame lit they should be in a ratio between 8% and 12%. The temperature is also relevant to prevent solute condensation, thus FID usually operates from 250°C to 300°C.

In relation to MS, APCI interface and MS analyzer conditions were optimized to provide strong mass signals for all the compounds and their fragments. The parameters that we should consider were transfer line temperature, flow rate and temperature of the dry gas, APCI vaporizer temperature, pressure of the nebulizer gas, voltages and all the parameters related to the lenses and the transmission. Among them, APCI vaporizer temperature could be the most important one because of the influence that it has on signal intensity.

To perform MS/MS analysis the selected precursor ion was the most abundant detected in the MS spectra. Both Auto MS and MRM modes were proved and collision energies were selected to accomplish the best possible fragmentation depending on the intensity of the precursor ion.

8.3.3. Optimized method application to the analysis of metabolites

When all the variables were optimized, the analytical method was then applied to the analysis of the 150 metabolites selected to be part of the first GC-APCI-MS database.

Before and after the injection of each derivatized standard solution, the syringe used was washed to prevent possible contaminations.

At the beginning of the day, the instrument was calibrated both external and internally. The external calibration was carried out to provide consistent mass values for a complete experimental sequence, because of the compensation of temperature drift in the mass spectrometer. To perform it an APCI calibration tune mix was used. On the other hand, the internal calibration was performed using cyclic-siloxanes, which are a typical background in GC-MS.

The MS and MS/MS spectra were checked paying attention to the intensity values, exact mass and isotopic distribution. If the spectra fulfilled the requirements, they could be included in the library; if not, the analysis should be repeated at different concentration values till reach the adequate one.

8.3.4. Study of chromatographic and spectral behavior of metabolites

Mass spectra interpretation

Despite the fact that each metabolite generates specific MS and MS/MS spectra, it is possible to observe common losses (see ***Table 8.2***). Some of these losses are internal molecule fragments and others are related to the presence of the derivatization reagent. Some of the common losses (CH_3, O, CO) are rather non-specific, whereas other can provide structural information. The neutral loss TMSOH (mass 90.0501 Da) is characteristic for the hydroxyl group –the most reactive towards silylation functional group– and consecutive losses of TMSOH can give an indication of the number of OH groups presents in the molecules. The loss of 118.0450 Da (TMSOH+CO) is characteristic for the carboxylic groups. The presence of an amino group can be corroborated by the release of the TMS group with theoretical mass of 72.0395 Da. Occasionally, we observe a radical molecular ion M^+ with the consequent loss of TMSO (89.0423 Da) and additional fragments related to the TMS group with theoretical mass of 73.0474 or 74.0551 Da, depending on the stability of the molecule.

The analysis of the spectral behavior of each individual compound would require a wide number of pages. To contain the size of the text MS interpretation has been carried out for each chemical category, assuming that all the metabolites that belong to the same group present similar characteristics and as a consequence they could have comparable behaviors.

Table 8.2. Common neutral losses observed in MS and MS/MS spectra of GC-APCI-MS.

Neutral loss	Theoretical mass
CH_3	15.0235
O	15.9949
CH_4	16.0313
NH_3	17.0265
H_2O	18.0106
C_2H_2	26.0157
CHN	27.0109
CO	27.9949
CH_2O	30.0106
CH_4O	32.0262
C_3H_2	38.0157
C_2H_2O	42.0106
CO_2	43.9898
C_2H_4O	44.0262
CH_2O_2	46.0055
C_3H_8Si (TMS)	72.0395
C_3H_9Si	73.0474
$C_3H_9SiCH_3$	88.0708
$C_3H_9SiNH_2$	89.0661
C_3H_9SiOH (TMSOH)	90.0501
C_3H_9SiOH+CO (TMSOH+CO)	118.0450

➢ Phenolic acids

It is possible to distinguish between two groups of phenolic acids depending on its origin: derivatives of hydroxycinnamic and hydroxybenzoic acids. Belonging to the first group, database includes *o*-, m- and *p*-coumaric acids, ferulic, sinapic and chlorogenic acids, etc. The MS pattern for these metabolites is characterized by an intense signal that corresponds to the complete silylated form of the compound, and the spectra also show the loss of CH_4 and the loss of TMSOH.

Gallic, vanillic, protocatechuic, gentisic, 4-hydroxybenzoic and γ-, α-, and β-resorcylic acids are some of the compounds derived from hydroxybenzoic acid. As in the previous group, in general, the predominant MS signal is again the form completely silylated+H and the fragmentation pattern is characterized by the losses of CH_4 and TMSOH as well. Besides, the loss of CO_2 is observable in a big part of the hydroxybenzoic acids spectra.

Homovanillic acid is an exception within this group of phenolic acids, because it gives a major ion that result from the loss of 118.0450 Da (TMSOH+CO).

Regardless of the type of phenolic acid, the ions appreciated in the MS spectra are the same in the case of isomers, but they differ in terms of their relative intensities. The difference found considering the signal intensity could be related with the stability of the compounds and this fact has to be with the position of the functional groups. MS/MS analysis allows unequivocally confirming the identification of the different structural isomers.

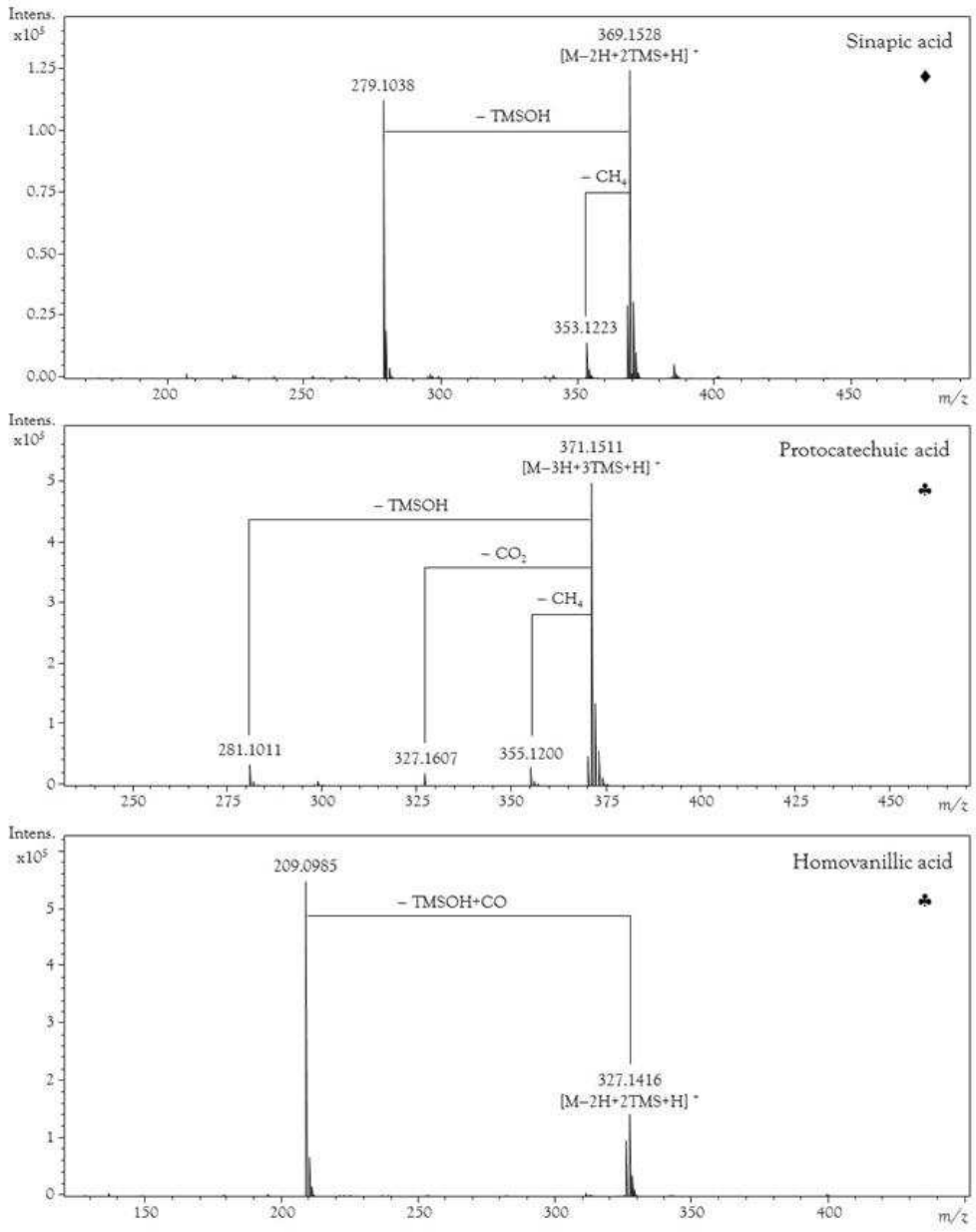


Figure 8.4. Example of several MS spectra that belong to each different group of phenolic acids: ♦ derived from hydroxycinnamic acid, and ♣ derived from hydroxybenzoic acid.

➢ Flavonoids

Metabolites from different families of flavonoids (flavones, flavonols and flavanones) have been included in the spectral library for GC-APCI-MS, and they are characterized for losing a CH_4 and additional fragments related to the TMS group with mass 73.0474 or 74.0552 Da according to the molecule stability. Catechin and epicatechin are two flavonols where the loss of TMSOH is more easily appreciated than for other flavonoids.

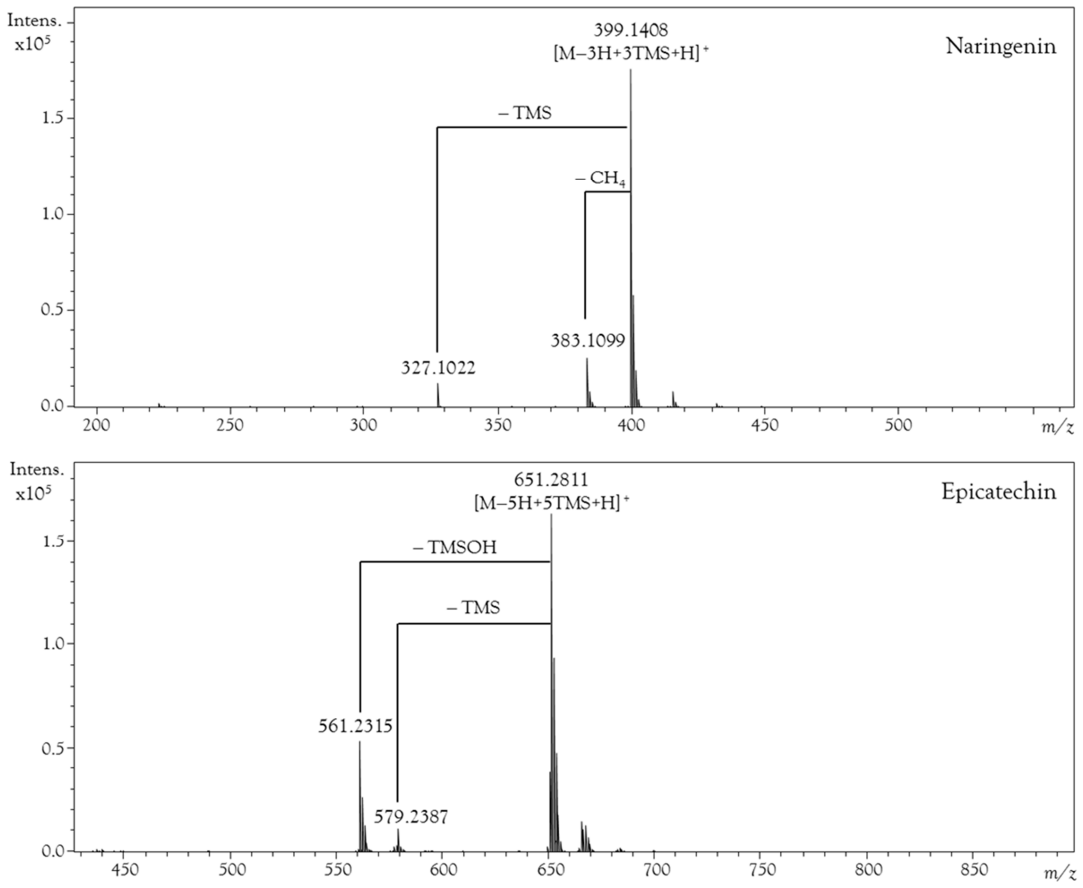


Figure 8.5. MS spectra of two different types of flavonoids.

➢ Organic acids

Organic acids are a group formed by a wide number of compounds. These molecules are relatively small and it is not very usual that contain nitrogen atoms in their structure. For this reason, these kinds of substances show the loss of TMS group as the only constant feature of their spectra. Nevertheless, benzoic or hippuric acids present an additional fragment ion $C_7H_5O^+$ (105.0335 Da) that corresponds to the benzoyl ion stabilized by the aromatic ring (see ***Figure 8.6***).

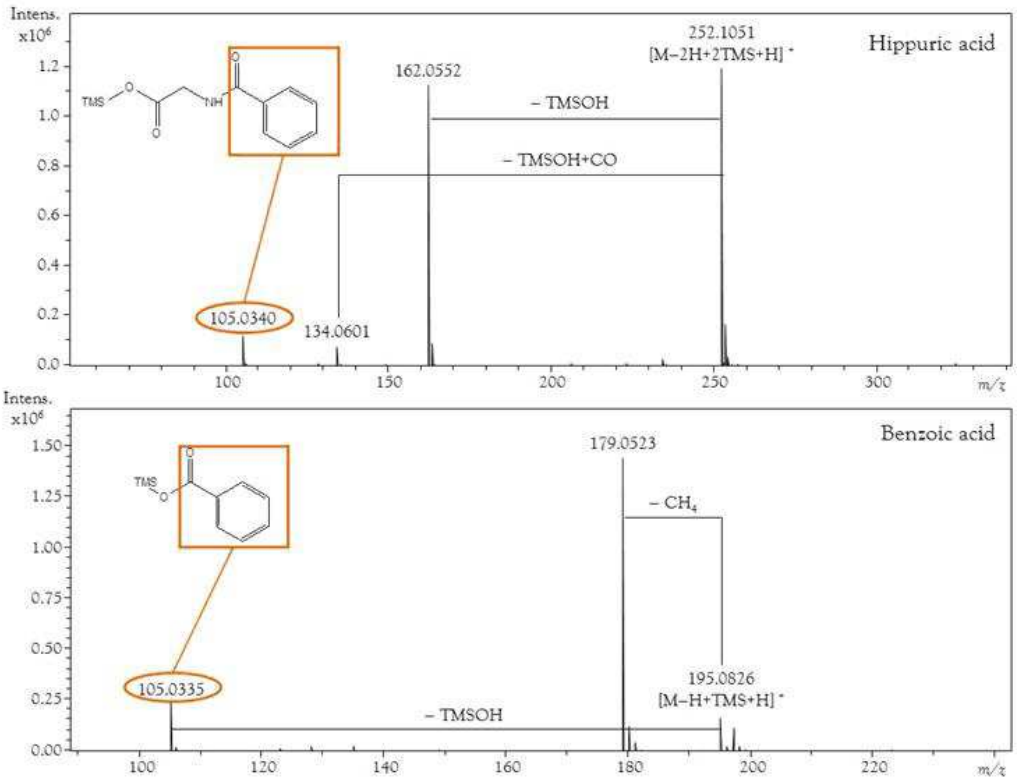


Figure 8.6. MS spectra of two organic acid examples.

➢ Fatty acids

Looking at the MS spectra of fatty acids, not much information is obtained, because the main feature is the expected one (the molecular ion corresponding to the complete derivatized form of the carboxylic group $[M-H+TMS+H]^+$), although in this group of compounds it is also appreciable a signal related to adduct-in source $[M-H+TMS+72]^+$. However, if we pay attention to the MS^2 spectra, some peculiarities are revealed. In addition to the classical losses of TMSOH and the cluster series with consecutive losses of CH_2, we have observed two additional fragments corresponding to the loss of CH_4 from the protonated molecular ion and its substitution with a H_2O molecule.

Regardless of the unsaturation factor of the fatty acids, we could detect the radical fragment corresponding to the TMS backbone after alpha-cleavage ($C_5H_{11}O_2Si^+$) with the theoretical *m/z* 131.0523. Moreover, in both mono- and polyunsaturated series of C16 and C18, the fragment $C_{10}H_{15}^+$ with theoretical *m/z* 135.1168 indicates the presence of a double bond. Unfortunately, at the moment we can not provide a strategy for the location of the position of the double bond.

➢ Amino acids

Amino acids are formed by two different functional groups, each one with a different reactivity towards the silylation reagent: the carboxylic group is more reactive than the amino group. Thus, it is usual that the chromatogram of these metabolites shows more than one peak.

The most intense signal tends to be the complete derivatized form and the spectra always show the losses corresponding to the carboxylic group (TMSOH and TMSOH+CO) as well as the loss of TMS from the amino group. To illustrate what happens with one of the compounds belonging to this group, L-proline has been taken as representative example ***(Figure 8.7)***.

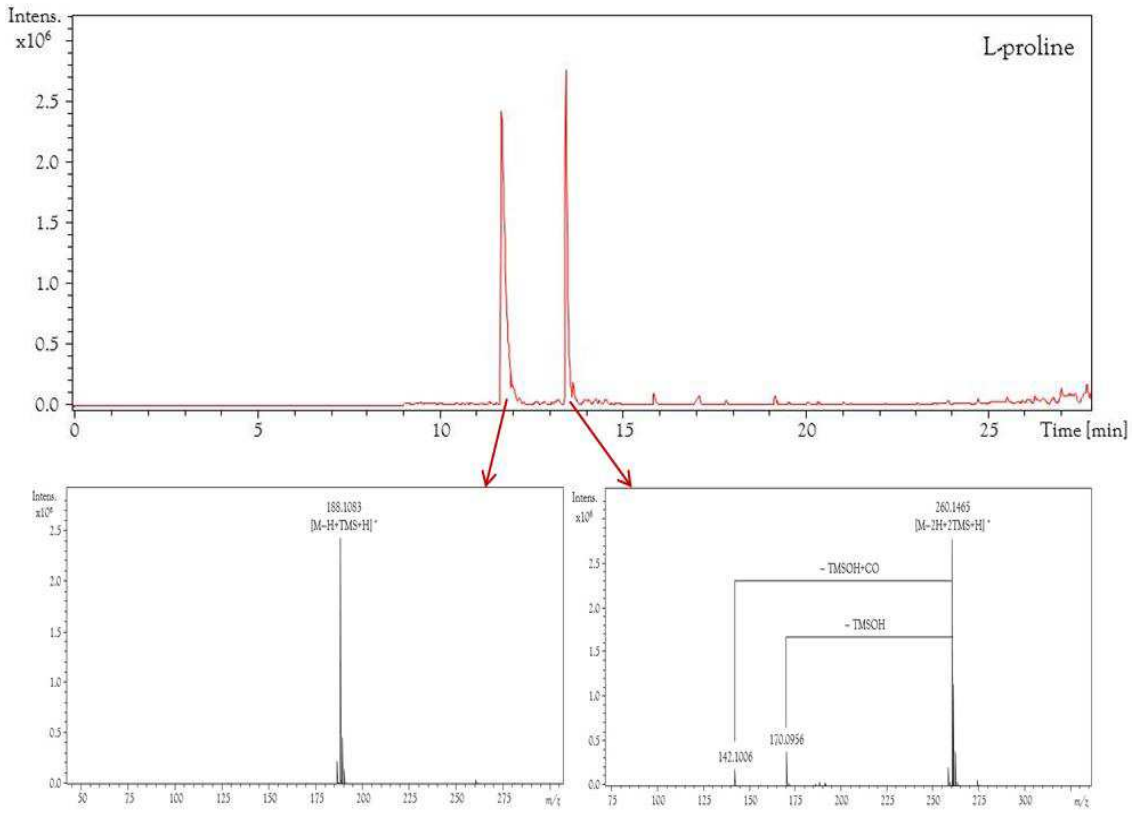


Figure 8.7. MS spectra of L-proline as illustration of the APCI behavior of amino acids.

➢ Vitamins

Different vitamins such as nicotinic acid, pantothenic acid, pyridoxine, ascorbic acid, etc. were included in the GC-APCI-MS database as well. Their analysis revealed that all the compounds showed the complete silylation of their active hydrogen, being the

molecular ion $[M-XH+XTMS+H]^+$ the predominant signal in the MS spectra. It is interesting to highlight that, in general, this chemical family present a very high resistance to the in-source fragmentation process, although in every case we could observe losses of TMSOH.

➢ Sugars

As commented in *section 8.2.3*, the derivatization reaction implied two different steps: methoxyamination and silylation. The first one is a classical derivatization strategy for sugars, because the oximation of keto group is blocked and the formation of enolic forms is inhibited, preventing the cyclization. This reduces the possible isomeric forms of a single sugar to just alpha and beta anomers of the linear form of the sugars. Hence, all the sugars are represented as molecular ions (molecular mass with the addition of TMS groups on the active hydrogens) and the CH_3N group on the carboxylic oxygen.

It is well-known that among these compounds we can find monosaccharides (aldohexoses and ketohexoses) and disaccharides. Some notable differences have been appreciated studying the MS spectra of the diverse metabolites belonging to this chemical family included in the database. Glucose (aldohexose) and fructose (ketohexose) have been chosen to illustrate what occurs in the case of monosaccharides. Both compounds show *m/z* 570 as the molecular ion of the MS spectra. However, the biggest difference between them is the relative abundance of the *m/z* 480 fragment corresponding to the $[M-TMSOH+H]^+$, which is 98.6% for glucose and 18.9% for fructose. MS^2 spectra also contain signals characteristic for aldo- and ketohexoses. In the case of aldohexoses (glucose), there are two specific fragments at *m/z* 307.1599 ($C_{12}H_{31}O_3Si_3^+$) and 217.1090 ($C_9H_{21}O_2Si_2^+$); whereas for ketohexoses (fructose) the most representative fragments are *m/z* 319.1558 ($C_{13}H_{31}O_3Si_3^+$) and 205.1065 ($C_8H_{21}O_2Si_2^+$) (see ***Figure 8.8***).

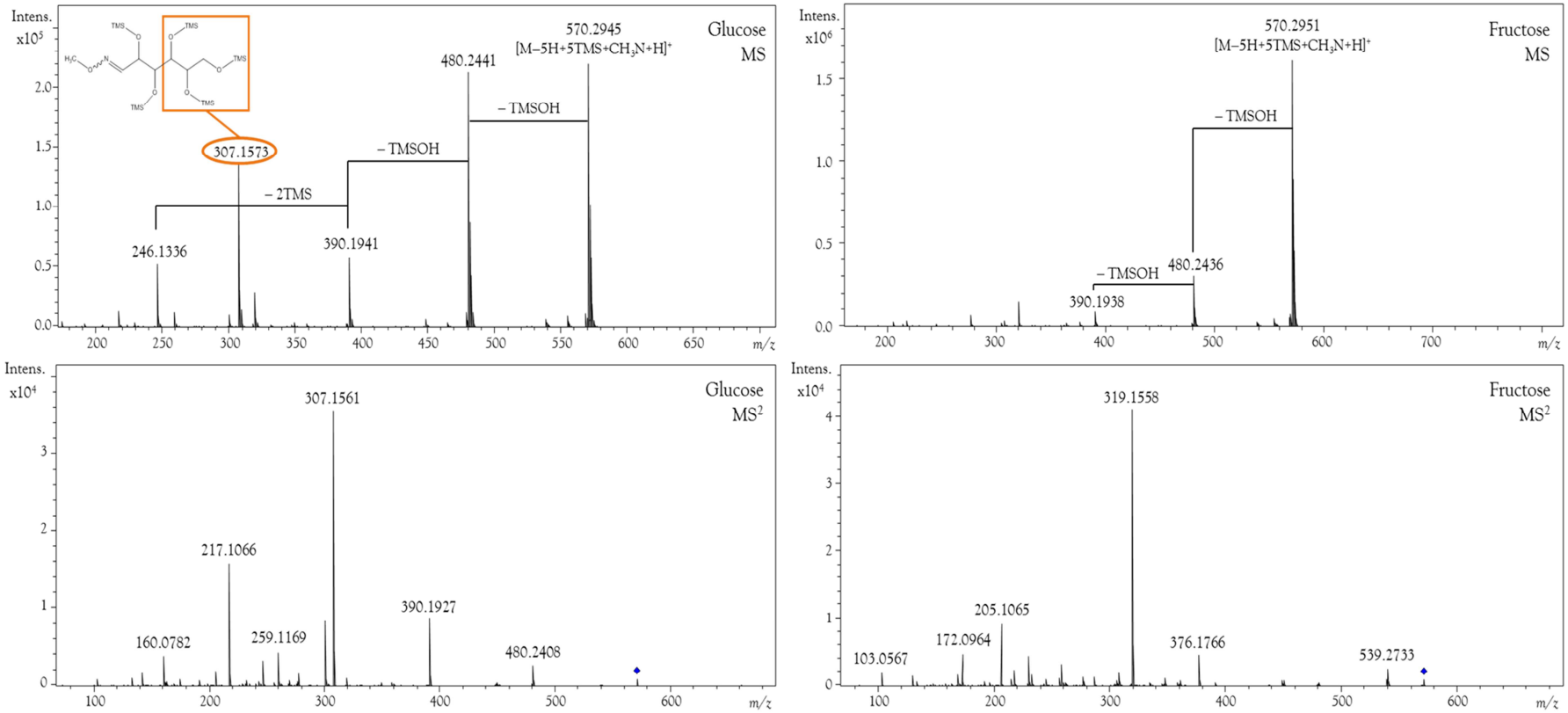


Figure 8.8. Example of the MS and MS^2 spectra of different monosaccharides.

Database also includes spectral information about some disaccharides. This family of compounds can be divided in two different groups: reducing (e.g. lactose) and non-reducing sugars (e.g. sucrose), which present significant differences that have been provides by the analysis of the metabolites. The first differences are noticeable in the chromatographic behavior of the compounds: lactose, for instance, shows two peaks corresponding to the two anomers, while sucrose –which has no open chain form– is represented by a single peak. MS spectra of these compounds are apparently different. The spectrum of lactose is characterized by the presence of the molecular ion at *m/z* 948.4639 $[M–8H+8TMS+CH_3N+H]^+$, the first glucose moiety ion at *m/z* 570 –which is the most intense signal of the spectra– and the second glucose moiety after the glycosidic break and the subsequent loss of a TMSOH group at *m/z* 361.1665. A distinct feature of non-reducing sugar (sucrose) spectra is a dominant fragment at *m/z* 361.1688 and low intensity signal of the single moiety at *m/z* 539.2525.

Other type of compounds studied from this chemical family are the six carbons alcohol sugars, which do not have oxime forms. For that, the fragmentation resulted in subsequential losses of TMSOH giving always at least one of these ions *m/z* 307.1576 ($C_{12}H_{31}O_3Si_3^+$), 217.1075 ($C_9H_{21}O_2Si_2^+$) or 205.1075 ($C_8H_{21}O_2Si_2^+$).

FID spectrum and calculation of Kovats index

The data obtained from FID was used to calculate the Kovats index of each metabolite, which "expresses the number of carbon atoms (multiplied by 100) of a hypothetical normal alkene which would have an adjusted retention time identical to that of the peak of interest when analyzed under identical conditions" [25]. The retention index of an unknown compound, measured on several different columns, is useful for identifying the unknown by comparing it with tabulated retention indices.

Kovats indices of the metabolites under study were calculated with the following equation:

$$I = 100\left[n + (N-n)\frac{\log\left(t'_{r(unknown)}\right) - \log\left(t'_{r(n)}\right)}{\log\left(t'_{r(N)}\right) - \log\left(t'_{r(n)}\right)}\right]$$

Where: I=Kovats retention index

n=number of carbon atoms eluting before the peak of interest

N=number of carbon atoms eluting after the peak of interest

t'_r=the adjusted retention time

The next figure (***Figure 8.9***) illustrates the FID spectrum for caffeine standard with the mix of hydrocarbons solution C7-30. Applying the previous equation, the Kovats index achieved for this metabolite by using our method was 1870, whereas the value from NIST library was fairly similar, 1840.

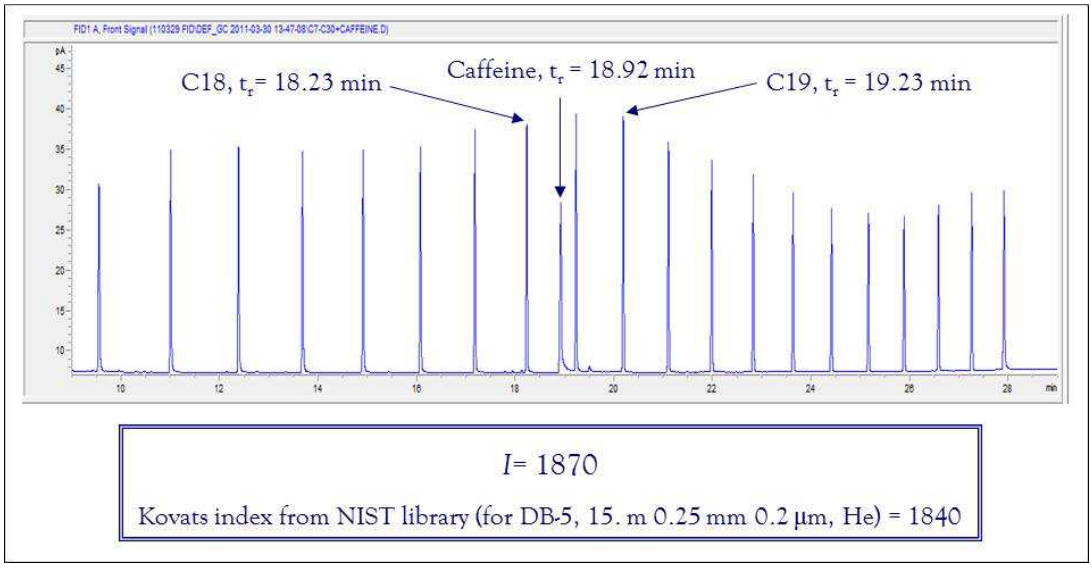


Figure 8.9. Kovats index calculation for the pure standard of the caffeine.

8.3.5. Database construction

The Metabolite Database is designed to be freely accessible for the public over the internet. It is built exclusively from open source software, or software that will soon enter the open domain. Debian GNU/Linux 6.0.1 is used as operating system running an Apache web server (version 5.3.3) with PHP 5.2 as scripting language for retrieving the information from a MySQL (version 5.1.49-3) database.

The architecture of the database consists of several tables containing the information about the metabolite, MS spectra and MS^2 spectra.

It is accessible through a website (http://metams.lumc.nl/), which has an interface that facilitates several search options, such as name, molecular formula or KEGG number. Moreover, a specific MS spectra search is also allowed using the measured *m/z*

value with a set tolerance (default: 0.05). In general, this search is performed solely on the molecular ion information which is often the *m/z* value of the metabolite derivatized form. Some metabolites have multiple derivatization forms and, for each of them, a separate MS spectrum is stored. The MS spectrum selected in the search is highlighted. Furthermore, in order to identify unequivocally the compounds of interest, the theoretical isotopic distribution of the molecular ion is also provided. The MS mode includes fragments related to the in-source or in-funnel fragmentation: a search option for these fragments (with the same tolerance) is available. The output results are sorted by *m/z* error and they are shown separately. When a result is selected, all the information and MS spectrum of the metabolite are displayed.

An MS^2 search can be performed on multiple fragments with a set mass tolerance threshold (default: 0.05) and the molecular ion information can be included for a more accurate search. The output results are sorted by *m/z* error and the MS^2 spectrum displays relative intensities of the fragments. If multiple MS^2 spectra are given for a metabolite, the selected MS^2 spectrum from the search is highlighted.

Each compound is also correlated with the retention indices calculated for the used column, and, since the database is GC-oriented, the Kovats index of each metabolite is stored and searchable.

8.4. Conclusions

This chapter summarizes the performed work to generate a web-based GC-APCI-TOF-MS database. It includes a number of spectra of compounds from the most common chemical families (amino acids, sugars, fatty acids, organic acids, phenols, vitamins, etc.) found in food extracts and human biofluids. All data included in the library were acquired using a modern UHR-TOF instrument, which ensures the high quality of the generated MS and MS/MS spectra. The database is fully searchable and includes retention indices and all possible silylation forms for a given compound.

The practical value of this spectral library will certainly improve if it incorporates data obtained with instruments from different vendors, by using different mass analyzers for data acquisition, as well as including other types of derivatization reactions. In addition, an increase of the number of metabolites of the database would be necessary, because the

150 compounds included so far (it is being updated) only represent a fraction of all the metabolites that might be encountered when metabolomics studies are performed.

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Derived from this work it is possible to find a publication in an international journal:

- T. Pacchiarotta, R. J. E. Derks, E. Hurtado-Fernández, P. van Bezooijen, A. Henneman, R. Schiewek, A. Fernández-Gutiérrez, A. Carrasco-Pancorbo, A. M. Deelder, O. A. Mayboroda. Online spectral library for GC-atmospheric pressure chemical ionization-TOF MS. *Bioanalysis* 5 (2013) 1515-1525.

Chapter 9

Evaluation of gas chromatography-atmospheric pressure chemical ionization-mass spectrometry as an alternative to gas chromatography-electron ionization-mass spectrometry: Avocado fruit as example

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Abstract

Although GC-APCI-MS was developed more than 40 years ago this coupling is still far from being a routine technique. One of the reasons explaining the limited use of GC-APCI so far is the lack of spectral database which facilitates the identification of the compounds under study.

The first application of a very recently developed GC-APCI database to identify as many compounds as possible in a complex matrix such as avocado fruit is presented here. The results achieved by using this database has been checked against those obtained using traditional GC-EI-MS and a comparison of the MS signals observed in both ionization sources has been carried out. 100 compounds belonging to different chemical families were identified in the matrix under study.

Considering the results of this study, the wide range of application (in terms of polarity and size of analytes) and the robustness of APCI as interface, the high quality of TOF spectra, and our database as a publicly available resource, GC-APCI-TOF MS is definitively a valuable addition to the "metabolomics toolbox".

Keywords: Atmospheric pressure chemical ionization / Avocado / Electron ionization / Gas chromatography / Mass spectrometry / Metabolomics

9.1. Introduction

GC-MS is one of the most important and widely used techniques in analytical chemistry. A combination of the unsurpassed separation power of GC with the capabilities of the modern MS instruments makes it a method of choice for the analysis of any type of complex chemical and biological samples [1]. With the development of metabolomics as an independent research field, GC-MS quickly turned into an essential tool of the new discipline [2-8], being the most extensively exploited in plant metabolomics [9-14].

EI and CI are the most common ionization techniques used with GC; both of them imply a hyphenation of GC and MS under vacuum conditions. The strongest asset of the EI is a highly reproducible and characteristic fragmentation of the substances [15] and, as a consequence, the possibility of using open source or/and commercial 70eV databases.

The strength of the EI may, under certain circumstances, turn into a weakness as the fragmentation of the compounds is often so extensive that is becoming detrimental to a structural significance of the parent ion. In this context, ionization systems producing a less abundant fragmentation started gaining more interest. CI could be an alternative to EI, but since the ionization is produced using a reagent gas and the fragmentation obtained is dependent on several parameters (such as nature and pressure of this gas, electron energy and temperature of the source, etc. [16]), the robustness of the MS data is not considered as its strongest capability. All this, in addition to the fact that CI is not as universal as EI, having a more restricted application range (selectivity is very dependent on different CI conditions), do not make of CI a very favorable alternative.

API techniques made the hyphenation of LC and MS possible and are probably responsible for the successful embedment of MS into bioanalytical sciences. The applications of the atmospheric pressure with GC were till recently very limited, even if they could overcome some of the limitations of EI and CI. The first experimental solution for coupling of GC and MS under atmospheric pressure was proposed by Horning et al. [17] more than four decades ago; however, due to several technical issues GC-APCI remained an exotic technique. Only in the first decade of the twenty-first century McEwen and McKay in 2005 [18] and Schiewek et al. in 2008 [19] finally proposed source designs which led to the commercially available GC-APCI instruments. This, in turn, stimulated systematic evaluation of the potential of GC-APCI-MS and the comparison of its performance with standard GC-MS platforms [20-28]. A chronological description of some of these applications could include, for instance, Östman et al. [20] who developed an APCI microchip for the quantitative analysis of volatiles and semi-volatiles compounds. The miniaturized source was used later to determine polychlorinated biphenyls in environmental samples [21]. In 2009, Carrasco-Pancorbo et al. [22] presented a detailed analytical evaluation of the performance of GC-APCI in combination with a TOF mass spectrometer, demonstrating the applicability of this coupling for metabolic profiling of cerebrospinal fluid. A year later, the same research group evaluated an on-line coupling of GC-APCI-MS and flame ionization detector (FID) as a way of cross-detector analysis [23]. In 2010, Portolés et al. [24], evaluated the potential of APCI source in GC-QTOF MS for pesticide residue analysis, studying around 100 analytes. Bristow et al. [25], in a very interesting article, established the first

simple comparison of this new platform with the traditional ones (using EI or CI), making evident the applicability of GC-APCI-MS for problem solving and process understanding in pharmaceutical development. Wachsmuth and co-workers [26] were pioneers providing a comprehensive evaluation of GC-APCI-TOF MS and comparing 5 different GC platforms in terms of reproducibility, dynamic range, and limits of detection and quantification using 43 metabolites and 12 stable isotope labelled standards. Using a similar approach, Portolés et al. [27, 28], in two different manuscripts, compared EI *vs.* APCI, highlighting some of the advantages that APCI ion source presents, facilitating, for instance, the application of tandem MS.

Despite the good results summarized in all these works and the advantages that APCI shows *vs.* EI or CI, this coupling is still far from being a routine technique. The lack of the spectral libraries allowing quick interpretation of the data is one of the factors limiting the development of APCI based applications. Only recently Pacchiarotta et al. [29] presented a web-based spectral library for GC-APCI-TOF MS (http://metams.lumc.nl/), which includes MS, MS^2 spectra and retention indices of a wide number of compounds belonging to different chemical groups (amino acids, fatty acids, sterols, phenolic compounds, vitamins, sugars, organic acids, etc.).

In the current study, we have applied GC-APCI-MS to the analysis of avocado fruit (*Persea americana*). Avocado fruits are famous for their high nutrition profile and beneficial health effects, however detailed analysis of this matrix presents number of challenges due to the chemical complexity (high fat content together with an assortment of proteins, sugars, vitamins, sterols, tannins, coumarins, alkanols, phenolic compounds, etc.) -affected by genetic, environmental factors and storage conditions. Some of these metabolites have been previously determined by GC, such as the volatile fraction, lipids, protein and amino acids, and carbohydrates of the oil [30, 31], pulp [32-35] and honey [36] of this fruit. However, avocado fruit is still a quite unknown matrix; any comprehensive characterization of this fruit has been carried out by traditional GC-MS, even less by using GC-APCI. Indeed, there are just two applications of this technique in food analysis, one for olive oil [37] and the other one for transgenic lettuce [38]; both publications were focused on the analysis of two specific groups of chemicals (phenolic compounds and volatile organic compounds, respectively).

The aim of this work was to demonstrate that GC-APCI-TOF MS can be a real alternative to conventional GC-MS, enhancing the coverage of the metabolome of different biological systems, producing less fragmentation in MS and facilitating the identification of the compounds under study taking into account the information provided by a new APCI library. To achieve this purpose we have compared the potential of GC-APCI-TOF MS *vs.* GC-EI-Q MS for the comprehensive metabolic characterization of avocado fruit, contributing to enlarge the knowledge about this interesting matrix.

9.2. Materials and methods

9.2.1. Chemicals and standards

All chemicals used in this study were analytical grade. Sample extracts were prepared with MeOH from Panreac (Barcelona, Spain). Sigma-Aldrich (St. Louis, USA), Extrasynthese (Lyon, France), Fluka (St. Louis, EEUU), and Carbosynth (Comptom, United Kingdom) were the suppliers of the different standards used to build the APCI database [29]. Taxifolin was used as IS to evaluate the reproducibility of the extraction system and the chromatographic runs, and it was purchased from Extrasynthese (Lyon, France).

APCI tune mix and pyridine (99% ultra-pure GC grade) were acquired from Fluka (St. Louis, USA), whereas BSTFA + 1% TMCS was from Pierce (Rockford, IL, USA) and was used as derivatization reagent. It was taken from freshly opened 1 mL bottles.

9.2.2. Sample preparation

Three varieties of avocado fruit ('Hass', 'Sir Prize' and 'Tacambaro') were studied to evaluate the feasibility and potential of the method, and they were provided by the IHSM La Mayora (CSIC), located in Algarrobo-Costa (Málaga, Spain).

The sample extracts were prepared taking into account our previous experience with this matrix [39, 40]. A solid-liquid extraction was carried out by mixing 4 g of freeze-dried sample, 50 ppm of IS and 40 mL of MeOH, shaking in a vortex for 30 min and centrifuging the supernatants at 3000 rpm during 10 min. At the end, the supernatants were evaporated to dryness with a rotary evaporator and reconstituted in 5 mL of MeOH.

Aliquots of 25 µL of this methanolic extract were taken, evaporated and redissolved in the proper volume of derivatization reagent.

A pooled sample of the 3 avocado extracts (e.g. mixing an equivalent volume of each one) was used as analytical QC sample. Data derived from these samples were used to assess system stability.

9.2.3. Derivatization reaction

Avocado extracts were derivatized on-line by adding 50 µL of BSTFA + 1% TMCS to the dried sample and incubating at room temperature for about 60 min, to assure the trimethylsilylation reaction.

9.2.4. GC-APCI-QqTOF MS analysis

The instrument used in this study was an Agilent 7890A GC (Agilent, Palo Alto, USA) equipped with a HP-5-MS column (30 m, 0.25 mm ID and 0.25 µm film thickness) coupled to a MS by using an APCI source, which was equipped with GC transfer line [19]. The MS used was a maXis (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated Qq-TOF MS.

Aliquot of the derivatized samples (1 µL) were used for the injection. Automated injection was done using a programmable MultiPurpose Sampler MPS2 (Gerstel, Mülheim an der Ruhr, Germany). Splitless injection mode was applied by a septumless CIS PTV injector, which operated at a purge time of 60 s, a purge flow of 3 mL/min and its temperature was set at 250°C. Sequential washing steps of the 10 µL syringe were included before and after each injection, as well as a step of sample pumping to remove possible small air bubbles. Helium was used as carrier gas at a constant flow rate of 1 mL/min.

The initial GC column temperature was 140°C and it was kept for 5 min, later on it was increased until 220°C (at a T rate of 3°C/min) keeping that value for 1 min. Then, the temperature changed from 220°C to 241°C at 10°C/min, maintaining that value for 1 min; and finally, increased from 241°C to 310°C at 3°C/min (holding that temperature for 5 min).

All the parameters related to the APCI source and the MS detector were optimized taking into account the area of the MS signal for some of the analytes included in a mix composed by a selection of metabolites belonging to different chemical categories (including, among others, *p*-coumaric acid, ferulic, chlorogenic, quinic, pantothenic and gentisic acids, epicatechin, naringenin, etc.). The ionization conditions chosen were a compromise solution to get the maximum signal for most of the peaks detected in the samples under study. The transfer line to the mass spectrometer was kept at 300ºC. The APCI source and MS were operated in positive mode. The temperature of the APCI vaporizer was 300ºC, the corona discharge needle worked at a voltage of +4000 nA and the pressure of the nebulizer gas (nitrogen) was set at 3.5 Bar. Dry gas (nitrogen) temperature and flow rate were 300ºC and 2 L/min, respectively. Capillary voltage was set at +2000 V and the end-plate offset at −500 V. Data were acquired for mass range from 50 to 1000 *m/z* with 1 Hz acquisition rate. The instrument was calibrated externally using an APCI calibration tune mix. Due to the compensation of temperature drift in the mass spectrometer, this external calibration provided consistent mass values for a complete experimental sequence. Moreover, an additional internal calibration was performed using cyclic-siloxanes –a typical background in GC-MS.

In a first step, MS/MS experiments were carried out in auto-MS/MS mode, where the main detected ions were fragmented under general collision conditions. When necessary, MS/MS experiments were performed selecting the precursor ion and applying different collision energy for each analyte to achieve the best possible fragmentation.

DataAnalysis 4.0 software (Bruker Daltonics, Bremen, Germany) was used for data processing and SmartFormula™ tool was applied for the calculation of elemental composition compounds.

9.2.5. APCI Library

As described by Pacchiarotta et al. [29], the APCI metabolite database used in this study was designed to be freely accessible for the public over the internet. The architecture of the database consists of several tables containing the information about the metabolite, MS spectra and MS^2 spectra. It is accessible through a website facilitating several general search options such as metabolite name, molecular formula or KEGG number. Moreover, a specific MS spectra search is also allowed using the measured *m/z*

value with a set tolerance. Results are returned sorted by *m/z* error. As an extra option, the search can be expanded to seek MS fragments. MS^2 spectra can be searched by entering one or more fragments with their relative intensity. As an option, the search can be expanded to take the precursor ion into account.

Each compound is also correlated with the retention indices calculated for the used column.

9.2.6. GC-EI-Q MS analysis

The instrument and the column used, as well as the chromatographic conditions, were the same as those previously described.

Mass spectrometer was a single Q (5975C inert XL MSD with a triple Axis detector) coupled to GC by EI, which operated at a potential of 70 eV and the ion source temperature was set at 230ºC. The acquisition mode selected was scan and the analyses were performed from *m/z* 40 to 750 mass units. The temperature of the MS Quad was set at 150ºC.

Search was mainly made in the following GC-MS libraries: NIST 2011 EI Mass Spectral Library, Wiley Registry™ 2007 Edition Mass Spectral Library and the Fiehn Lib Metabolite GC/MS Library 2009.

To fully exploit an electronic library identification potential, a deconvolution software is necessary to increase the number of species that can be characterized in a complex sample. Automated Mass Spectral Deconvolution and Identification System (AMDIS) v2.62 from NIST was used.

9.3. Results and discussion

9.3.1. Identification of metabolites in avocado extracts: combining the information of APCI and EI

To demonstrate the applicability of the developed method for the characterization of the avocado fruit metabolome, three different varieties of avocado ('Hass', 'Sir Prize' and 'Tacambaro') have been used.

Accurate *m/z* signals, MS spectra and MS^2 spectra of the compounds detected in avocado fruit extracts were considered to carry out the identification of the metabolites based on the comparison of their signals with those of the substances included in the on-line APCI database and EI mass spectral libraries. ***Table 9.1*** summarizes all the compounds identified using GC-APCI-TOF MS and GC-EI-Q MS. It shows that using on line GC-APCI and EI spectral libraries 85 metabolites can be identified in the avocado extracts.

Figure 9.1(A) and ***9.1(B)*** show representative chromatograms for GC-APCI-MS (BPC of 'Tacambaro' sample and the EICs of all identified compounds) and the BPC obtained when GC-EI-MS was used, respectively.

It is important to emphasize complexity of the samples, which is evident, for example, from a diversity of the identified metabolites. Indeed, the metabolites identified in the avocado extracts belong to several chemical families: amino acids (15), fatty acids and related compounds (16), sugars and derivatives (9), phenolic acids and related compounds (19), flavonoids (5), organic acids (8), vitamins (3), and nucleosides and nucleobases (4). There is as well a small group of compounds that has been named miscellaneous which was composed by 6 compounds (3 sterols, phytohormone, polyol, and organophosphate).

Table 9.1. Metabolite identification by GC-APCI-TOF MS and GC-EI-Q MS considering the information provided by the available databases.

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
1	2.4	234.1335	$C_9H_{24}NO_2Si_2$	L-alanine - 2H + 2 TMS + H	234.1340	0.49	7.0	216.1249/204.1257 /190.1481/ 174.1339/144.0880 /116.0728	–	–	218/190/147/116 /73*	HS, SP, TC
2	2.5	220.1184	$C_8H_{22}NO_2Si_2$	Glycine - 2H + 2 TMS + H	220.1184	- 0.09	59.5	176.1146/149.0486 /102.0906	–	–	204/176/147/102 /73*	HS, SP, TC
3	3.6	267.1232	$C_{13}H_{23}O_2Si_2$	Benzoic acid - 2H + 2 TMS + H	267.1231	0.08	51.4	197.0668/179.0561 /105.0418	–	–	–	HS, SP, TC
4	3.8	309.1729	$C_{12}H_{33}O_3Si_3$	Glycerol - 3H + 3 TMS + H	309.1732	1.13	19.7	219.1220	x	91	218/205/147/73	HS, SP, TC
5	3.9	276.1810	$C_{12}H_{30}NO_2Si_2$	L-leucine - 2H + 2 TMS + H	276.1810	0.00	9.2	186.1324/158.1243	–	–	260/218/158/147 /73*	HS, SP, TC
6	4.2	276.1807		L-isoleucine - 2H + 2TMS + H		0.27	17.4	186.1321/158.1092	x	43	260/218/158/147 /73	HS, SP, TC
7	4.2	196.0778	$C_9H_{14}NO_2Si$	Nicotinic acid - H + 1TMS + H	196.0788	1.08	0.9	–	–	–	195/180/136/106 /78*	HS, SP, TC
8	4.2	248.1489	$C_{10}H_{26}NO_2Si_2$	γ-aminobutyric acid - 2H + 2TMS + H	248.1497	0.72	11.6	158.1003	x	91	232/147/102/73	HS, SP, TC
9	4.3	260.1483	$C_{11}H_{26}NO_2Si_2$	L-proline - 2H + 2TMS + H	260.1497	1.35	22.6	191.0909/170.1071 /142.0999	x	76	216/142/73	HS, SP, TC
10	4.4	263.1132	$C_{10}H_{23}O_4Si_2$	Succinic acid - 2H + 2 TMS + H	263.1129	- 0.29	9.6	173.0668	x	98	247/218/147/73	HS, SP, TC
11	4.8	323.1524	$C_{12}H_{31}O_4Si_3$	Glyceric acid - 3H + 3 TMS + H	323.1525	0.08	11.7	205.1017	x	96	322/292/189/147 /73	HS, SP, TC
12	4.9	257.1129	$C_{10}H_{21}N_2O_2Si_2$	Uracil - 2H + 2TMS + H	257.1136	0.74	10.5	–	x	80	256/241/147/99/ 73	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
13	5.2	231.1775	$C_{12}H_{27}O_2Si$	Nonanoic acid - H + 1 TMS + H	231.1775	- 0.04	26.2	233.1575/215.1475 /141.1326	x	66	215/129/117/73	HS, SP, TC
14	5.3	322.1685	$C_{12}H_{32}NO_3Si_3$	L-serine - 3H + 3 TMS + H	322.1684	- 0.05	7.0	276.1429/232.1192 /204.1245/ 160.0860	x	91	306/278/218/204 /73	HS, SP, TC
15	5.5	274.1649	$C_{12}H_{28}NO_2Si_2$	Pipecolic acid - 2H + 2TMS + H	274.1653	0.45	14.8	184.1132/156.1168	x	96	230/156/133/73	HS, SP, TC
16	5.9	336.1841	$C_{13}H_{34}NO_3Si_3$	L-threonine - 3H + 3 TMS + H	336.1841	0.03	13.7	246.1350	x	93	320/291/218/147 /73	HS, SP, TC
17	6.9	337.1676	$C_{13}H_{33}O_4Si_3$	Dihydroxybutyric acid - 3H + 3 TMS + H	337.1681	0.53	17.3	247.1174	x	91	321/233/189/147 /73	HS, SP, TC
18	8.4	351.1473	$C_{13}H_{31}O_5Si_3$	Malic acid - 3H + 3 TMS + H	351.1474	0.05	18.2	307.1217/233.1013 / 189.1103	x	99	335/233/189/147 /73	HS, SP, TC
19	9.2	411.2227	$C_{16}H_{43}O_4Si_4$	L-threitol - 4H + 4 TMS + H	411.2233	- 0.60	13.3	321.1725/231.1214	x	93	307/217/204/147 /73	HS, SP, TC
20	9.3	274.1287	$C_{11}H_{24}NO_3Si_2$	Pyroglutamic acid - 2H + 2TMS + H	274.1289	0.27	41.4	258.0951/230.1013	x	98	273/258/230/156 /73	HS, SP, TC
21	9.4	350.1634	$C_{13}H_{32}NO_4Si_3$	L-aspartic acid - 3H + 3TMS + H	350.1634	- 0.07	7.8	333.1368/304.1760 /274.1293/ 230.1036/205.1090 /159.0862	x	95	349/232/218/147 /73	HS, SP, TC
22	9.6	225.0933	$C_{11}H_{17}O_3Si$	Vanillin - H + 1 TMS + H	225.0941	0.26	8.4	197.1088	–	–	224/209/194/73*	TC
23	9.8	221.0986	$C_{12}H_{17}O_2Si$	*trans*-cinnamic acid - H + 1 TMS + H	221.0992	0.63	32.0	205.0628/161.0921 /131.0345	–	–	220/205/161/131 /103*	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
24	10.3	425.2022	$C_{16}H_{41}O_5Si_4$	L-threonic acid - 4H + 4 TMS + H	425.2026	0.38	20.3	335.1558/307.1567 /217.056	x	91	292/220/205/147 /73	HS, SP, TC
25	10.8	425.2024				0.12	17.0	335.1520/307.1571 /217.1053	x	94		HS, SP, TC
26	11.0	365.1630	$C_{14}H_{33}O_5Si_3$	Hydroxyglutaric acid - 3H + 3 TMS + H	365.1630	0.06	21.1	275.1123/247.1171 /185.0608	x	90	349/247/147/129 /73	HS, SP, TC
27	11.4	277.1399	$C_{10}H_{25}N_2O_3Si_2$	L-asparagine - 2H + 2 TMS + H	277.1398	- 0.11	15.7	260.1140/259.1286 /221.1891/ 203.1804/187.0916 /159.0981/ 132.0877	–	–	276/159/116/73/ 44*	HS, SP, TC
28	12.3	283.1185	$C_{13}H_{23}O_3Si_2$	4-hydroxybenzoic acid - 2H + 2 TMS + H	283.1180	- 0.19	50.1	267.0897/193.0795	x	96	282/267/223/193 /73	HS, SP, TC
29	12.3	364.1790	$C_{14}H_{34}NO_4Si_3$	L-glutamic acid - 3H + 3 TMS + H	364.1790	- 0.03	6.7	274.1290	x	99	363/348/246/128 /73	HS, SP, TC
30	12.5	310.1654	$C_{15}H_{28}NO_2Si_2$	L-phenylalanine - 2H + 2 TMS + H	310.1653	- 0.12	7.0	220.1177/192.1233	–	–	294/266/218/192 /73*	HS, SP, TC
31	16.1	287.2401	$C_{16}H_{35}O_2Si$	Tridecanoic acid - H + 1 TMS + H	287.2401	- 0.06	31.0	289.2185/271.1737 /215.0840/ 197.1995	x	99	286/271/117/73	HS, SP, TC
32	16.2	371.1475	$C_{16}H_{31}O_4Si_3$	*o*-pyrocatechuic acid - 3H + 3TMS + H	371.1525	4.99	14.7	281.1004/209.0617 /193.0322/ 137.0686	–	–	370/355/193/137 /73*	TC
33	16.5	391.1412	$C_{15}H_{31}O_6Si_3$	Aconitic acid - 3H + 3TMS + H	391.1423	1.12	16.3	301.0917/211.0408	x	99	390/375/229/147 /73	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
34	16.7	313.1284	$C_{14}H_{25}O_4Si_2$	Vanillic acid - 2H + 2 TMS + H	313.1286	0.14	52.3	297.0978/223.0849	x	90	312/297/267/223/73	SP, TC
35	16.9	313.1284	$C_{14}H_{25}O_4Si_2$	Isovanillic acid - 2H + 2 TMS + H		0.22	52.7	297.0982/223.0840	–	–	312/297/267/126/73*	SP, TC
36	17.0	371.1495	$C_{16}H_{31}O_4Si_3$	γ-resorcylic acid - 3H + 3 TMS + H	371.1525	11.76	42.3	281.1070	–	–	355/267/147/73*	SP, TC
37	17.0	327.1432	$C_{15}H_{27}O_4Si_2$	Homovanillic acid - 2H + 2 TMS + H	327.1442	1.07	12.7	209.1004	–	–	326/311/209/179/73*	HS, SP, TC
38	17.3	371.1495	$C_{16}H_{31}O_4Si_3$	Gentisic acid - 3H + 3 TMS + H	371.1525	2.93	13.6	281.1017	–	–	370/355/267/223/73*	SP, TC
39	17.4	–	–	Glycerol phosphate - 4 H + 4 TMS	-	-	-	–	x	94	445/357/299/147/73	HS, SP, TC
40	18.1	309.1332	$C_{15}H_{25}O_3Si_2$	*o*-coumaric acid - 2H + 2 TMS + H	309.1337	0.43	13.9	219.0857	–	–	308/293/161/147/73*	SP, TC
41	18.7	371.1491	$C_{16}H_{31}O_4Si_3$	Protocatechuic acid - 3H + 3 TMS + H	371.1525	2.77	8.0	281.1013	x	60	370/355/193/73	HS, SP, TC
42	18.8	541.2677	$C_{21}H_{53}O_6Si_5$	D-(–)-fructofuranose I - 5H + 5 TMS + H	541.2683	0.62	24.4	451.2177/361.1677/271.1186	x	91	437/217/147/73	HS, SP, TC
43	19.1	481.1924	$C_{18}H_{41}O_7Si_4$	Citric acid - 4H + 4 TMS + H	481.1924	- 0.06	16.7	465.1637/363.1485/273.0988	x	93	465/363/273/147/73	HS, SP, TC
44	19.3	541.2695	$C_{21}H_{53}O_6Si_5$	D-(–)-fructofuranose II - 5H + 5 TMS + H	541.2683	- 1.17	42.0	451.2166/361.1666/271.1208	x	76	437/217/147/73	HS, SP, TC
45	19.9	280.1403	$C_{11}H_{22}N_5Si_2$	Adenine - 2H + 2 TMS + H	280.1408	0.55	22.1	264.1087	x	99	279/264/192/73	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
46	20.0	309.1352	$C_{15}H_{25}O_3Si_2$	*m*-coumaric acid - 2H + 2 TMS + H	309.1337	- 0.34	10.1	219.0907	–	–	308/293/219/203 /73*	HS, SP, TC
47	20.7	553.2675	$C_{22}H_{53}O_6Si_5$	Quinic acid - 5H + 5 TMS + H	553.2683	0.02	11.2	373.1677/259.1187	–	–	–	HS, SP, TC
48	20.8	541.2658	$C_{21}H_{53}O_6Si_5$	Glucose - 5H + 5 TMS + H	541.2683	1.28	15.2	451.2170/361.1669 /271.1178	x	94	217/204/191/147 /73	HS, SP, TC
49	21.1	343.1386	$C_{15}H_{27}O_5Si_2$	Syringic acid - 2H + 2 TMS + H	343.1392	0.55	9.3	327.1076/299.1491 /253.0931/211.086 7	–	–	342/327/312/297 73*	HS, SP, TC
50	21.2	386.1995	$C_{17}H_{36}NO_3Si_3$	Pyridoxine - 3H + 3 TMS + H	386.1998	0.29	22.8	–	–	–	370/295/280/147 /73*	HS, SP, TC
51	22.1	309.1335	$C_{15}H_{25}O_3Si_2$	*p*-coumaric acid - 2H + 2 TMS + H	309.1337	0.18	8.4	219.0909	x	99	308/293/249/219 /73	HS, SP, TC
52	22.3	315.2710	$C_{18}H_{39}O_2Si$	Pentadecanoic acid - H + 1 TMS + H	315.2714	0.18	33.9	317.2494/299.2398 / 243.1238/225.2268	x	49	299/201/117/73	HS, SP, TC
53	22.6	398.1987	$C_{18}H_{36}NO_3Si_3$	L-tyrosine - 3H + 3 TMS + H	398.1998	- 0.09	11.1	326.1552/280.1540 / 218.1035/195.0860 / 179.0905	x	45	280/218/73	HS, SP, TC
54	22.9	615.3254	$C_{24}H_{63}O_6Si_6$	D-mannitol - 6H + 6 TMS + H	615.3235	1.90	23.8	525.2756/345.1727	x	93	319/205/147/103 /73	HS, SP, TC
55	23.3	459.1849	$C_{19}H_{39}O_5Si_4$	Gallic acid - 4H + 4 TMS + H	459.1869	- 0.02	9.1	–	–	–	458/443/281/73*	HS, SP, TC
56	24.3	436.2354	$C_{18}H_{42}NO_5Si_3$	Pantothenic acid - 3H + 3 TMS + H	436.2365	0.01	12.2	–	x	92	420/291/157/103 /73	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
57	24.6	327.2707	$C_{19}H_{39}O_2Si$	Palmitoleic acid - H + 1 TMS + H	327.2714	0.04	3.1	237.2258/219.2171 / 155.0977	x	99	326/311/145/117 /73	HS, SP, TC
58	25.3	329.2868	$C_{19}H_{41}O_2Si$	Palmitic acid - H + 1 TMS + H	329.2870	- 0.01	35.3	331.2666/313.2559 /239.2418	x	99	328/313/145/117 /73	HS, SP, TC
59	26.8	339.1429	$C_{16}H_{27}O_4Si_2$	Ferulic acid - 2H + 2 TMS + H	339.1442	- 0.18	10.8	249.0941	x	48	338/308/249/73	HS, SP, TC
60	28.2	343.3013	$C_{20}H_{43}O_2Si$	Heptadecanoic acid - H + 1 TMS + H	343.3027	1.37	38.4	345.2817/327.2733 /271.1218/ 253.2493/235.1735	x	62	327/129/73	HS, SP, TC
61	28.3	397.1664	$C_{18}H_{33}O_4Si_3$	Caffeic acid - 3H + 3 TMS + H	397.1681	0.04	15.8	307.1172	–	–	396/381//219/73*	HS, SP, TC
62	29.7	409.2177	$C_{21}H_{37}O_4Si_2$	Abscisic acid - 2H + 2 TMS + H	409.2225	4.74	65.4	337.1831/319.1711	–	–	–	HS, SP, TC
63	30.1	353.2862	$C_{21}H_{41}O_2Si$	Linoleic acid - H + 1 TMS + H	353.2870	- 0.03	4.5	263.2369/245.2265 /179.1761	x	99	352/337/129/95/ 73	HS, SP, TC
64	30.2	355.3012	$C_{21}H_{43}O_2Si$	Oleic acid - H + 1 TMS + H	355.3027	- 0.06	9.8	265.2542/247.2413	x	99	354/339/145/117 /73	HS, SP, TC
65	30.3	351.2714	$C_{21}H_{39}O_2Si$	Linolenic acid - H + 1 TMS + H	351.2714	- 0.01	12.8	261.2203/243.2097	–	-	350/335/129/91/ 73*	HS, SP, TC
66	30.5	355.3012	$C_{21}H_{43}O_2Si$	*cis*-vaccenic acid - H + 1 TMS + H	355.3027	0.26	6.8	265.2514/247.2413	x	94	354/339/145/117 /73	HS, SP, TC
67	31.0	357.3164	$C_{21}H_{45}O_2Si$	Stearic acid - H + 1 TMS + H	357.3183	- 0.07	37.9	341.2856/267.2668	x	99	356/341/117/73	HS, SP, TC
68	31.2	369.1547	$C_{17}H_{29}O_5Si_2$	Sinapic acid - 2H + 2 TMS + H	369.1548	0.09	30.1	279.1049	–	-	368/353/338/279 /73*	HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
69	35.1	383.3332	$C_{23}H_{47}O_2Si$	11-eicosenoic acid - H + 1 TMS + H	383.3340	0.73	15.2	293.2847	x	95	382/367/145/117/73	HS, SP, TC
70	36.2	533.2352	$C_{21}H_{45}N_2O_6Si_4$	Uridine - 4H + 4 TMS + H	533.2349	- 0.07	11.1	461.1929/371.1455/349.1704/259.1227/223.0767/185.0910	x	50	517/315/217/147/73	HS, SP, TC
71	37.7	399.3584	$C_{24}H_{51}O_2Si$	Heneicosanoic acid - H + 1 TMS + H	399.3653	6.84	48.0	383.3290/327.3185/309.3079/291.2962	–	–	398/383/132/117/73*	HS, SP, TC
72	39.0	475.3636	$C_{25}H_{55}O_4Si_2$	*α*-glyceryl palmitate - 2H + 2 TMS + H	475.3633	- 0.25	27.7	385.3138	x	90	459/371//239/147/73	HS, SP, TC
73	40.2	556.2630	$C_{22}H_{46}N_5O_4Si_4$	Adenosine - 4H + 4 TMS + H	556.2621	- 0.87	15.6	–	x	99	540/236/217/147/73	HS, SP, TC
74	41.1	919.4389	$C_{36}H_{87}O_{11}Si_8$	Sucrose - 8H + 8 TMS + H	919.4397	0.80	12.3	435.3280/361.1678/315.1021/271.1161/169.0622/149.0379	x	90	437/361/217/147/73	HS, SP, TC
75	42.4	501.3789	$C_{27}H_{57}O_4Si_2$	Monooleoylglycerol - 2H + 2 TMS + H	501.3790	0.12	18.9	411.3291	x	93	485/397/203/129/73	HS, SP, TC
76	44.7	489.1947	$C_{24}H_{37}O_5Si_3$	Naringenin - 3H + 3 TMS + H	489.1943	- 0.02	14.0	–	–	–	488/473/296/179/73*	HS, TC
77	45.0	651.2791	$C_{30}H_{55}O_6Si_5$	Epicatechin - 5H + 5 TMS + H	651.2839	- 0.27	17.0	561.2296/283.1162	x	93	650/368/355/267/73*	HS, SP, TC
78	45.5	651.2836	$C_{30}H_{55}O_6Si_5$	Catechin - 5H + 5 TMS + H		0.31	21.6	561.2369/283.1197	–	–		HS, SP, TC

Table 9.1. (continued)

		GC-APCI-TOF MS							*GC-EI-Q MS*			
#	**t_r (min)**	***m/z* experimental**	**Predicted molecular formula**	**Compound**	***m/z* theoretical**	**Error (mDa)**	**mSigma value**	**Other signals**	**Presence**	**Qual**	**Fragments**	**Avocado samples containing compound**
79	45.7	455.4285	$C_{28}H_{59}O_2Si$	Pentacosanoic acid - H + 1 TMS + H	455.4279	- 0.57	50.7	–	–	–	454/439/132/<u>117</u>/73*	HS, SP, TC
80	47.8	517.1864	$C_{25}H_{37}O_6Si_3$	Kaempferide - 3H + 3 TMS + H	517.1892	- 0.81	14.7	–	–	–	–	TC
81	50.3	787.3375	$C_{34}H_{67}O_9Si_6$	Chlorogenic acid - 6H + 6 TMS + H	787.3395	0.12	10.9	697.2829/307.1166	–	–	–	HS, SP, TC
82	51.4	663.2495	$C_{30}H_{51}O_7Si_5$	Quercetin -5H + 5 TMS + H	663.2476	- 1.96	153.8	–	–	–	–	HS, SP, TC
83	51.8	473.4153	$C_{31}H_{57}OSi$	Campesterol - H + 1 TMS + H	473.4173	2.05	15.2	<u>383.3673</u>	–	–	–	HS, SP, TC
84	53.2	485.4160	$C_{32}H_{57}OSi$	Stigmasterol - H + 1 TMS + H	485.4173	1.34	28.9	<u>395.3683</u>	–	–	–	HS, SP, TC
85	53.5	487.4313	$C_{32}H_{59}OSi$	β-sitosterol - H + 1 TMS + H	487.4330	1.33	29.1	<u>397.3834</u>	x	99	486/396/357/<u>129</u>/73	HS, SP, TC

Signal underlined means the prevalent ion in the MS spectrum.

* Fragments related to the compound, which is included in the database but not detected in avocado samples.

x: detected

–: no detected

HS: Hass; SP: Sir Prize; TC: Tacambaro

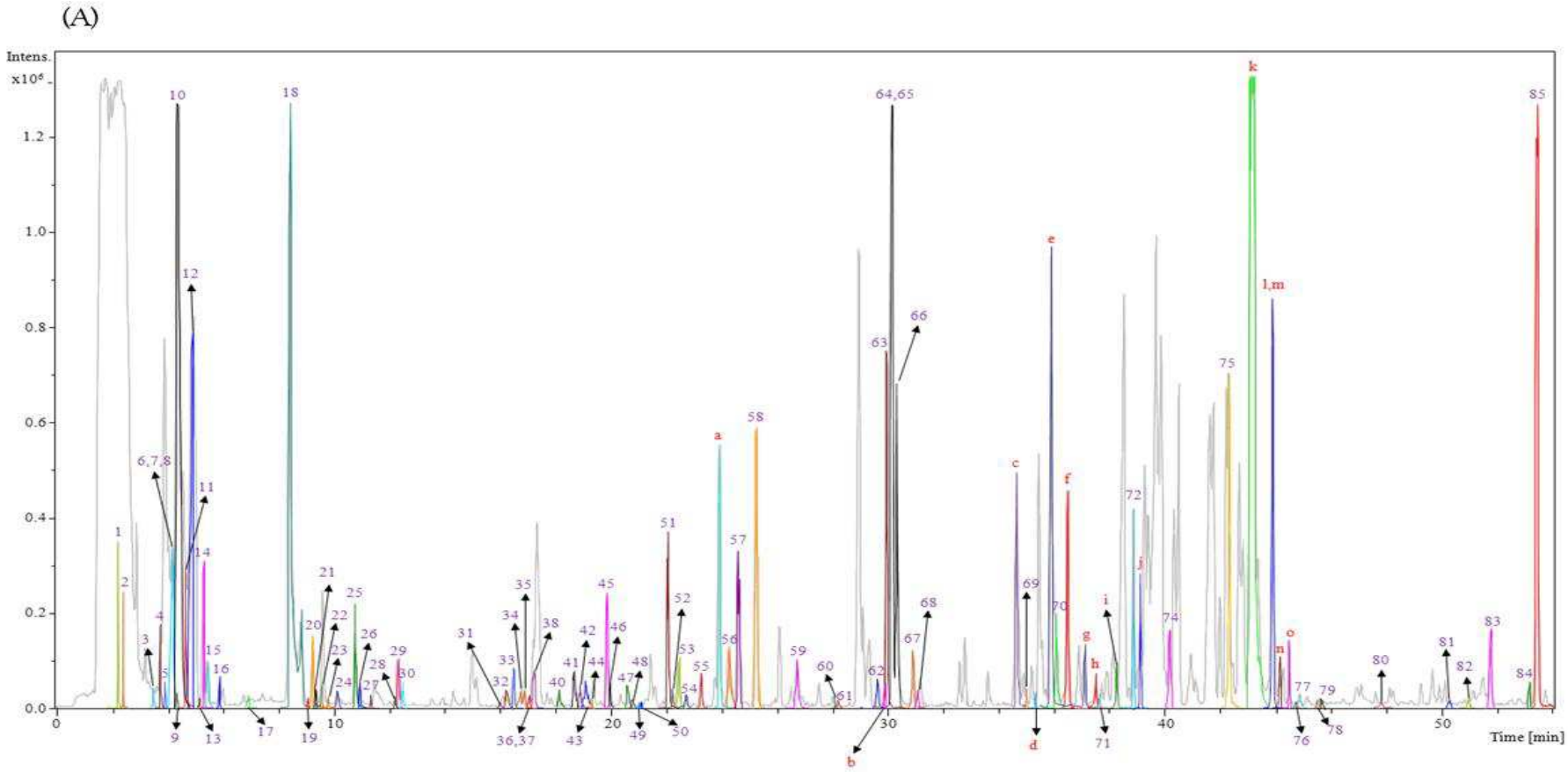

Figure 9.1(A)***.*** BPC of Tacambaro variety and EICs of each compound identified by GC-APCI-TOF MS. EICs are shown in different colours in order to facilitate their identification. The numbers/letters of the compounds identified are enclosed in ***Tables 9.1*** and ***9.3***.

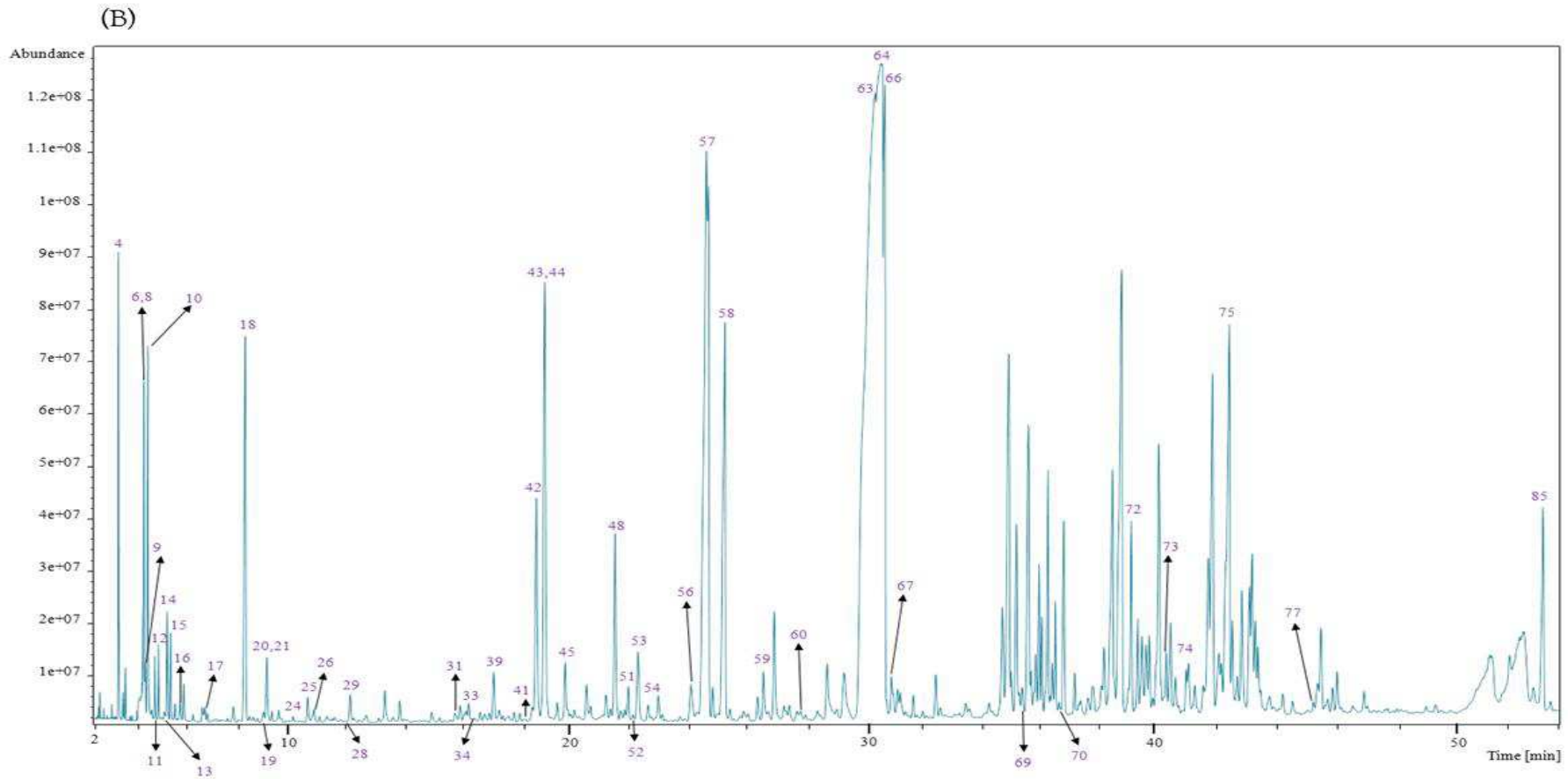

Figure 9.1*(B).* BPC of Tacambaro variety for GC-EI-Q MS. The numbers of the compounds identified are enclosed in ***Table 9.1.***

Figure 9.2 shows the elution windows for the most abundant groups of compounds: organic acids, amino acids, phenolic acids, fatty acids and related compounds, and flavonoids. It is evident that the elution windows are overlapping but nonetheless we can make some general assumptions. For instance, the organic and amino acids elute within the first twenty minutes. Phenolic acids are, more or less, distributed in the middle of the chromatogram, followed by the fatty acids and related compounds area, which is one of the richest fractions of the chromatogram. At the end of the run, flavonoids elute grouped in 6 min approximately.

9.3.2. Comparison of the obtained MS signal in APCI and EI

An overview of the data included in ***Table 9.1*** shows that the identification carried out by both APCI and EI are in good agreement. However, some discrepancies have been experienced too. Since the structural assignment in EI is based on the MS spectrum and in APCI the database search is mainly based on accurate MS and MS^2 data, it seems necessary to compare in depth the MS signals that each platform is using to get the identity of a compound under study. Despite the fact that the analyzers were not the same for both platforms, we assume that the ionization sources have a greater influence in the MS spectra appearance than the analyzers, being the latter more responsible of the accuracy in the *m/z* signals.

The current section is structured in such way that the typical ionization and behavior of the compounds belonging to the different families of analytes found in avocado fruit is discussed. Specific examples of compounds belonging to vitamins, phenolic acids, fatty acids, sterols, organic acids, flavonoids, nucleosides and nucleobases, amino acids and sugars will be included in this section, comparing the EI MS signal and the APCI MS (MS and MS/MS). Moreover, a general statement of the ionization pattern of the different families will be given (see ***Table 9.2***).

When MS/MS experiments were carried out in APCI to build the database, the most influential conditions for the fragmentation were optimized for each single compound included. Although the relative intensity of the fragments was affected by the collision energy, the fragmentation pattern was very reproducible, getting the same *m/z* fragments (oscillating only the relative intensity). The MS/MS analyses were obtained by setting the collision energy in a value high enough for getting a remarkable fragmentation.

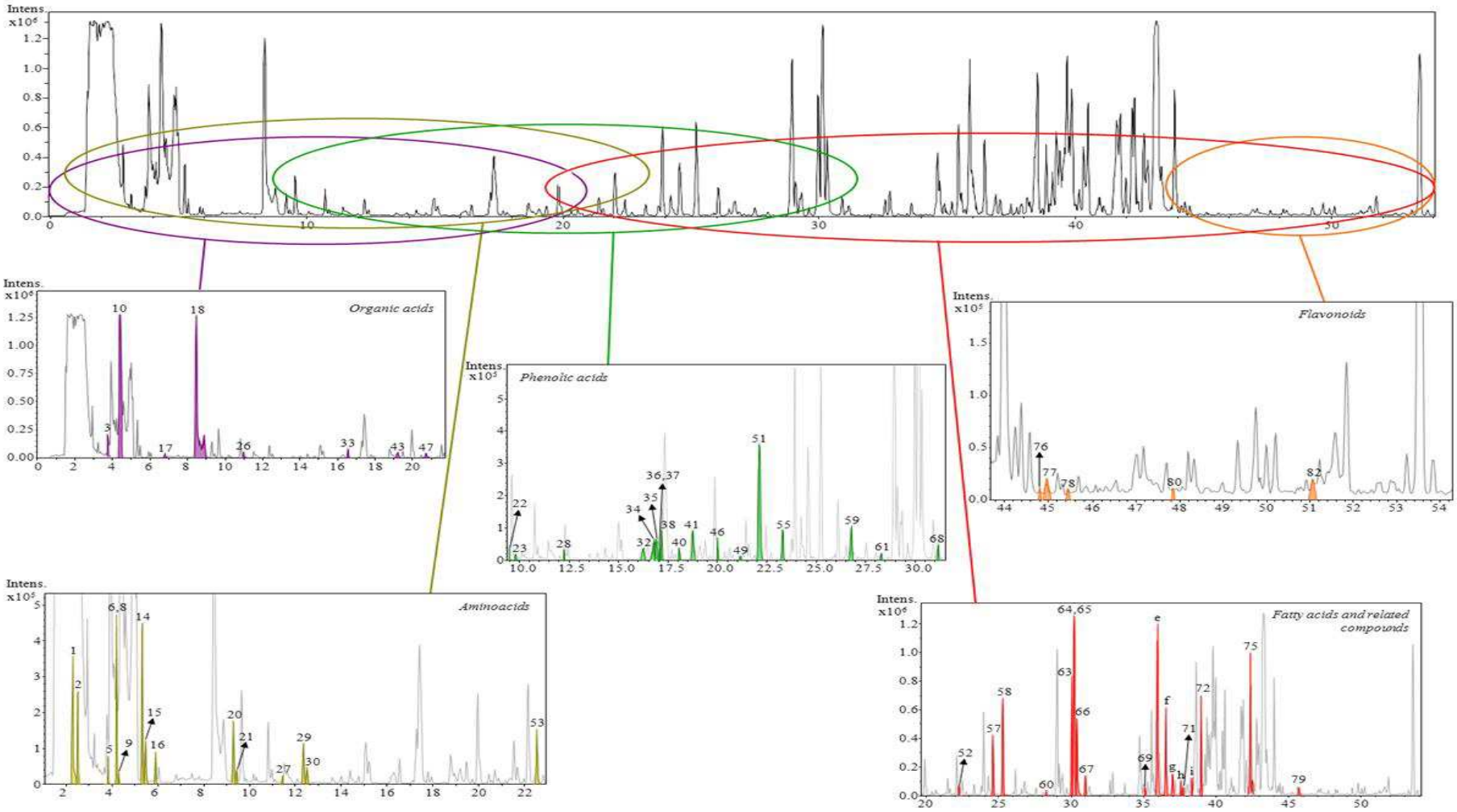

Figure 9.2. Elution window for some of the principal chemical families found in avocado samples.

Table 9.2. APCI (MS and MS/MS) and EI *m/z* signals obtained for compounds belonging to vitamins, phenolic acids, fatty acids, sterols, organic acids, flavonoids, nucleosides and nucleobases, sugars and amino acids.

Compound	*APCI*			*EI*
	MS	*Precursor ion*	MS/MS	MS
Pantothenic acid	**436.2410** $[M-3H+3TMS+H]^+$ (100) 346.1893 [436-90] (10.7)	436.2410 (30 eV)	420 (2.1) 346 [436-90] (50.5) 290 [436-146] (10.1) 217 [436-219] (17.5) **157** (100) 145 [217-71] (60.1) 117 (66.7) 103 (63.1) 73 (62.2)	420 (18.6) 291 (70.8) 261 (9.9) 247 (31.4) 201 (23.5) 157 (36.5) 145 (6.9) 117 (36.3) 103 (60.0) **73** (100)
Ferulic acid	411.1882 $[M-2H+2TMS+73]^+$ (7.9) 339.1429 $[M-2H+2TMS+H]^+$ (40.1) **249.0941** [339-90] (100) 323.1159 [339-16] (19.3)	339.1429 (30 eV)	**249** [339-90] (100) 219 [339-118] (95.2) 191 [219-28] (54.9) 145 [339-193] (81.6) 117 [191-74] (48.7) 89 (9.7) 73 (65.1)	**338** (100) 323 (41.1) 308 (40.3) 293 (14.1) 249 (40.4) 219 (22.3) 191 (11.2) 117 (5.0) 73 (82.1)
Linoleic acid	425.3294 [353+72] (31.3) **353.2895** $[M-H+TMS+H]^+$ (100) 337.2621 [353-16] (6.2) 263.2384 [353-90] (66.7) 245.2279 [263-18] (45.9) 179.1782 (6.8)	353.2895 (20 eV)	337 [353-16] (45.1) **263** [353-90] (100) 245 [263-18] (98.1) 178 (30.4) 129 (26.4) 111 (25.7)	337 (44.8) 262 (15.8) 178 (10.0) 129 (48.2) 117 (37.2) 95 (42.8) **73** (97.8)
Sitosterol	487.4329 $[M-H+TMS+H]^+$ (13.8) **397.3863** [487-90] (100) 243.2108 (10.2) Consecutive losses of CH_4	397.3863 (25 eV)	229 (5.2) 215 (12.5) 161 (67.1) **147** (100) 133 (43.6) 129 (8.7) 109 (27.3)	486 (47.7) 471 (16.1) 396 (88.0) 357 (90.7) 275 (11.8) 255 (15.4) 213 (5.1) 145 (25.7) **129** (100) 73 (64.2) 43 (63.7)
Malic acid	423.1844 [350+73] (5.2) 351.1413 $[M-3H+3TMS+H]^+$ (18.4) 335.1108 [351-16] (6.2) 307.1102 [351-44] (4.8) **233.1028** [351-118] (100) 189.0650 [233-44] (15.1) 160.9989 [233-73] (4.2) 116.9347 (2.2)	351.1413 (25 eV)	189 (18.4) 147 (15.7) 91 (20.1) **73** (100)	335 (13.8) 245 (27.6) 233 (50.7) 189 (20.4) 147 (86.3) 133 (23.9) **73** (100)
		233 (25 eV)	189 (10.2) 147 (81.4) **73** (100)	

Table 9.2. (continued)

Compound	*APCI*			
	MS	*Precursor ion*	MS/MS	MS
Epicatechin	651.2839 [M-5H+5TMS+H]$^+$ (72.8) 561.2298 [651-90] (27.5) 383.3059 (4.1) 369.1701 (3.9) 353.1450 (5.1) **283.1163** (100) 267.1213 [283-16] (6.2) 223.0637 (4.1)	651.2839 (15 or 35 eV)	561 (4.5) 369 (12.2) 355 (38.9) **283** (100) 267 (28.8) 179 (12.3)	650 (13.0) 383 (10.2) **368** (100) 355 (35.3) 297 (7.4) 267 (12.0) 179 (8.5) 73 (72.2)
		283.1163 (25 eV)	**267** [283-16] (100) 255 [283-28] (34.8) 209 [255-46] (16.9) 167 [209-42] (10.1) 149 [167-18] (9.0) 91 (6.7) 73 (23.6)	
Adenine	361.1623 (3.5) **280.1417** [M-2H+2TMS+H]$^+$ (100) 264.1127 [280-16] (5.5) 217.0763 (1.9) 205.0684 (2.5) 169.0097 (3.3) 148.9704 (4.9)	280.1417 (25 eV)	264 (10.1) 192 [264-72] (43.2) 91 (23.2) **73** (100)	279 (24.7) **264** (100) 192 (11.6) 73 (39.7) 45 (10.3)
Serine	**322.1685** [M-3H+3TMS+H]$^+$ (100) 276.1429 [322-46] (19.8) 232.1192 [322-90] (22.2) 204.1245 [322-118] (8.3) 160.0806 [322-118-44] (5.5)	322.1685 (25 eV)	204 [322-188] (27.5) 188 [204-16] (7.7) 167 (7.3) 147 (14.8) 114 [204-90] (22.4) 91 (16.2) **73** (100)	306 (3.3) 278 (6.3) 218 (56.2) **204** (100) 188 (10.0) 147 (25.3) 100 (21.8) 73 (93.8)
Fructofuranose	541.2665 [M-5H+5TMS+H]$^+$ (13.8) **451.2165** [541-90] (100) 373.1678 (42.2) 361.1630 [451-90] (94.8) 271.1015 [361-90] [451-180 (fructose)] (11.7) 226.0554 (13.4) 169.0080 (14.1) 148.9746 (13.8)	451.2165 (25 eV)	271 [451-180] (11.9) 243 [451-208] (24.3) 217 [451-180-54] (29.2) 169 [242-73] (61.9) 155 [169-14] (63.4) 129 (26.7) 117 (19.3) 91 (28.2) **73** [117-44] (100)	437 (37.8) 319 (13.3) 306 (21.8) 217 (98.3) 204 (65.4) 191 (9.3) 147 (50.8) 129 (14.4) 117 (9.8) 103 (20.4) **73** (100)
		361.1630 (25 eV)	243 [361-118] (8.6) 155 (25.1) 129 (14.3) 91 (25.1) **73** (100)	

- In bold letter, the most intense *m/z* signal.
- The relative intensity of the *m/z* signals present in the MS and MS/MS spectra of the compounds under study is shown between brackets.
- Collision energy used in MS/MS mode was chosen in order to achieve a proper fragmentation of the analytes (not vey drastic) and clean MS/MS spectra.
- Reproducibility of MS/MS was tested by using other collision energies.
- No decimal figures in MS/MS data to contain the size of the table.

Vitamins

When pantothenic acid is studied in APCI, the native structure with 3 silylated groups is observed, being the predominant signal in the MS spectrum the pseudo molecular ion $[M\text{-}3H+3TMS+H]^+$ (436.2354). It is possible to observe the loss of TMSOH (mass 90.0501 Da) too. In EI, as expected, the fragmentation is much more abundant. Although the relative intensity of each fragment was not the same, it was found that the MS spectrum in EI and the MS/MS spectrum in APCI were quite similar.

Other vitamins also showed the complete silylation of their active hydrogens and a very high resistance to the in-source fragmentation process. The loss of TMSOH was very common within this family.

Phenolic acids

As an example of phenolic acids, ferulic acid can be chosen. The MS pattern for this compound was characterized by a very intense (but not the predominant one) MS signal corresponding to the complete silylated form (339.1429 $[M\text{-}2H+2TMS+H]^+$), showing the loss of CH_4 (16.0308 Da) and the loss of TMSOH (90.0501 Da). Adducts formation in source, as described by Wachsmuth et al. [26], was also observed, getting in the MS spectrum the *m/z* signal 411.1882 ($[M\text{-}2H+2TMS+73]^+$).

The spectrum achieved in EI for ferulic acid had the following *m/z* signals: 338, 323 (338-15), 308 (323-15), 293 (308-15), 249 (338-90), 219, 191, 117, and 73, being very similar to those observed for the MS/MS fragmentation in APCI of the precursor ion 339.3187.

In general, it can be said that the MS pattern for hydroxycinnamic acids is characterized by the signal corresponding to the complete silylated form and the losses of CH_4 and TMSOH. Hydroxybenzoic acids showed a very similar ionization pattern, being possible to observe the loss of 43.9898 Da (CO_2) as well.

Fatty acids

The pseudo molecular ion corresponding to the derivatized form of the carboxylic group was the main feature of the MS spectrum of linoleic acid (353.2862 $[M\text{-}H+TMS+H]^+$). The MS spectrum also showed the loss of 90.0501 Da (TMSOH), giving an

intense signal with *m/z* 263.2384. Other *m/z* signals found in the spectrum were: 245.2279, 179.1782, 337.2621 and 425.3294 (353+72 (adduct-in source)). The EI spectrum showed *m/z* signals which were in good agreement with those detected when MS/MS mode was used in APCI.

Other example of this family could be oleic acid. As observed for linoleic acid, the APCI MS spectrum showed losses of 90, 108 (90+18), 16 and the pseudo molecular ion+72 (adduct-in source). When the MS spectrum produces by oleic acid in EI is studied, the following *m/z* features are detected: 339, 117, 73, 130, 145, 222, 264, and 55; whilst in APCI MS/MS it is possible to see (with 25 eV): 339, 265, 247, 121, 131, and 73.

For fatty acids, in the MS/MS spectra, apart from the classical losses of TMSOH and the cluster series with consecutive losses of CH_2, it was possible to observe two additional signals corresponding to the loss of CH_4 from the protonated molecular ion and its substitution with a H_2O molecule.

Sterols

Although the molecular ion of the silylated form of β-sitosterol is observed in the MS spectrum, the predominant signal is the loss of 90 Da (giving *m/z* 397.3863). *m/z* 397.3863 is the pseudomolecular ion–H_2O, behavior which has been previously described for sterols when they have been studied by LC-APCI-MS [41]. Moreover, the *m/z* signal 243.2108 (397-154) is also observed. Some other fragments produced by consecutive losses of CH_2 (14.0156 Da) can be detected in the MS spectrum.

When the signals produced by this compound in EI are studied, the following *m/z* features are detected: 129 (486-357), 357 (486-129), 396, 486, 73, 145, 255, and 470. In MS/MS mode in APCI, by using collision energies within the range between 15 and 35 eV, the spectra are quite similar. The highest *m/z* signal is 147, observing as well 161, 133, 109, 129, 229, etc.; the major part of the fragments are related by CH_2-differences.

Similar behavior was found for some other sterols, such as stigmasterol and campesterol, whose prevalent signal in MS was the loss of 90 Da (giving *m/z* 395.3669 and 383.3673 respectively). Its MS/MS spectrum was characterized by subsequent losses of CH_2.

Organic acids

Malic acid can be chosen as example of the organic acids group. The pseudo molecular ion of the silylated form (with 3 TMS groups) is detected with remarkable intensity, although the predominant signal is 233.1028 (351-118), being 118 the loss of TMSOH+CO. 351 losses firstly 44 (CO_2) and then 74 (TMS); the loss of 16 can be observed too. In the same way, *m/z* 233 shows the losses of 44 (*m/z* 189.0650) and 72 (*m/z* 161.0680). Adducts formation occurs in source leading to detect 423.1891 (351+72).

As general statement for the organic acids group, it can be said that the loss of a TMS group and the loss of a CO_2 group are constant features in the spectra of the compounds belonging to this family.

Flavonoids

It can be claimed that flavonoids are characterized by the loss of 16.0313 (CH_4) and an additional fragment related to a TMS group (this is observed for apigenin, luteolin, chrysin, naringenin…). The MS spectra of epicatechin and catechin show some peculiarities, since the loss of 90 (C_3H_9SiOH) is more likely to be detected than for other compounds belonging to this family. In APCI, the MS spectrum of epicatechin shows as features: 651.2839 (with 5 TMS groups), 283.1163 (predominant *m/z* signal in the spectrum), 561.2298, 383.3059, 267.1213, 353.1450, and 369.1701.

Naringenin can be considered as one of the typical examples of flavonoids, since it produces as most intense signal in the MS spectrum the pseudo molecular ion-16. Its spectrum in EI is very similar to the MS/MS APCI spectrum.

Nucleosides and nucleobases

The highest signal in the MS spectrum of adenine (nucleobase-a purine derivative) corresponds to $[M-2H+2TMS+H]^+$, which is 280.1417; 280 is losing 16 to produce 264.1127.

In EI 264 is the prevalent signal in a very clean spectrum, together with 73, 279, 192, and 217. When *m/z* 280 is considered as precursor ion and MS/MS experiments are carried out in APCI, the *m/z* fragments obtained are (25 eV): 73, 91, 192, 217, and 264.

Uracil (*m/z* 257.1129 $[M-2H+2TMS+H]^+$), for instance, shows in its spectrum not only the loss of 16, but also the loss of 12.

Some compounds that have been included within the same group as nucleobases are the nucleosides (such as adenosine and uridine), and their behavior therefore is in between the nucleobases and sugars; losses of 16 are very common, together with consecutive losses of 90.

Sugars

The pseudo molecular ion completely silylated+H of fructofuranose is found in the MS spectrum; however, the prevalent signal is 541-90 (451.2165), which subsequently undergoes another loss of 90 (361.1630). The signal with *m/z* 271.1015 (361-90) is detected as well. 373.1745 is present in the spectrum with considerable intensity, being 169.0080, 148.9746 and 226.0554 other detected features in APCI MS spectrum.

When EI is used as source, the main signals in the spectrum can be easily compared with the MS/MS APCI signals: when 451 is the precursor ion, 73, 155, 169, 217, and 91 are detected as *m/z* fragments; for 361 as precursor ion, 73, 91, and 169 are the main *m/z* signals.

Although the classical derivatization strategy for sugars consists of two steps (oximation and silylation), methoxyamination was not used in this case to simplify the procedure and check the usefulness of our database after a simple silylation (as for the other families of compounds). All the sugars are represented as pseudo molecular ions. As previously observed by Pacchiarotta et al. [29], the fragmentation resulted in consecutive losses of TMSOH, giving at least one of the following signals: 307, 217 and 205.

Amino acids

The prevailing signal of serine in APCI MS is 322.1685 $[M-3H+3TMS+H]^+$. Apart from this signal, it is possible to observe some other *m/z* signals as indicated in ***Table 8.2.***

In EI, the most intense *m/z* signal is 204; when MS/MS is carried out in APCI (322 as precursor ion), the fragments present in the MS/MS spectra are: 204 (322-188), 147, 114 (204-90), 91, 73, 188 (204-16), and 167.

As Pacchiarotta et al. [29] highlighted, the presence of two functional groups with different reactivity towards the silylation reagent is a characteristic feature of amino acids, being the carboxylic group more reactive than the amino group. Since reactivity is very different, the silylation of both kinds of functional groups is not always complete, being very typical that amino acids give more than one chromatographic peak very often. In general, amino acids spectra show the following losses: TMSOH (90), TMSOH+CO (118) and TMS (73).

9.3.3. Tentative identification of other metabolites in avocado fruit extracts

In an attempt to take advantage of the capabilities of the Qq-TOF mass spectrometer used with GC-APCI (which gives the analyst an accurate *m/z* signal (both in MS and MS/MS mode) together with the isotopic distribution), a tentative identification of some compounds previously described in the matrix under study [39, 42, 43] –but not available as commercial pure standards– was performed. Taking into account the structure of these compounds and the number of active groups, it was possible to calculate, theoretically, the molecular weight of the complete silylated form and search them in the chromatogram. ***Table 9.3*** shows the predicted molecular formula (together with the retention time, *m/z* theoretical and experimental, error, mSigma value, other signals in MS and possible name of the compound) of 15 metabolites tentatively identified by GC-APCI-MS. These metabolites are mainly alkanols, furans or fatty acids derivatives and all of them were found in the three avocado samples under study. Some of these compounds do not show the derivatized form, since they do not have any active hydrogen which could be replaced by a trimethylsilyl group.

9.3.4. Distribution of the compounds identified in avocado

Table 9.1 showed that both APCI and EI provide a fairly comparable number of the identifications, although some differences between two platforms are evident as well. Figures 3 and 4 illustrate those differences. Our purpose was not to accomplish a comprehensive comparison of the analytical performance of both platforms (if so the comparison would not be fair as designed, since the analyzers are different), we intended to check the usefulness of a very recently developed GC-APCI database to identify as many compounds as possible in a complex matrix, evaluating if their results were in good

agreement with those obtained using one of the most widely used GC-MS platforms (GC-EI-Q MS).

An Euler diagram (***Figure 9.3(A)***) shows the total number of the identified analytes and the fractions scored by each platform (GC-APCI and GC-EI). The diagram clearly illustrates an overlap between the techniques (51 compounds) and, at the same time, it emphasizes significantly higher number of unique ID's contributed by GC-APCI. A possible explanation of this difference is the wider range of the polarities and molecular weights amendable to a GC-APCI analysis, without forgetting the important role of the high resolution MS analyzer used for GC-APCI.

The last point is illustrated by ***Figure 9.3(B)***, where the compounds identified by APCI and EI respectively are grouped according to the chemical families they belong to. The most remarkable differences between both platforms are related to phenolic acids and flavonoids, although they can be observed for fatty acids and amino acids as well. With regards to the phenolic acids and flavonoids the differences can be accounted to the poor ionization of those compounds in EI mode. The difficulty for EI in terms of fatty acids in this case is that the mass spectral library for EI does not include some of the compounds that have been found in *Persea americana*. Although miscellaneous group shows the same height in the graphic for both GC-MS methodologies, it is necessary to stand out that the compounds detected are not the same, and neither APCI nor EI identified the totality of the compounds of this group (6).

Table 9.3. Tentative identification of metabolites found in avocado fruit by GC-APCI-TOF MS.

#	t_r (min)	*m/z* experimental	Predicted molecular formula	Compound	*m/z* theoretical	Error (mDa)	mSigma value	Other signals
a	24.0	<u>279.2682</u>	$C_{19}H_{35}O$	2-pentadecylfuran + H	279.2682	0.04	2.5	–
b	29.9	307.2978	$C_{21}H_{39}O$	2-heptadecylfuran + H	307.2995	1.72	5.5	–
c	34.7	503.3764	$C_{26}H_{59}O_3Si_3$	Avocadene – 3H + 3 TMS + H	503.3767	0.23	24.8	413.3268/<u>323.2761</u>/233.2238/205.1039/137.1243
d	35.4	<u>249.2185</u>	$C_{17}H_{28}O$	Avocadenofuran + H	249.2213	2.83	10.2	–
e	36.0	<u>293.2464</u>	$C_{19}H_{33}O_2$	Methyl linolenate I + H	293.2475	1.15	31.5	–
f	36.6	<u>291.2318</u>	$C_{19}H_{31}O_2$	Benzyl laurate I + H	291.2319	0.04	18.1	–
g	37.0	<u>293.2456</u>	$C_{19}H_{33}O_2$	Methyl linolenate II + H	293.2475	1.89	16.9	–
h	37.6	<u>291.2324</u>	$C_{19}H_{31}O_2$	Benzyl laurate II + H	291.2319	-0.58	4.2	–
i	38.3	<u>299.2378</u>	$C_{17}H_{35}O_2Si$	Myristoleic acid – H + 1 TMS + H	299.2401	2.29	15.6	–
j	39.2	349.2920	$C_{22}H_{41}OSi$	2-(1-Pentadecenyl)furan – H + 1 TMS + H	349.2921	0.15	20.0	<u>259.2432</u>/ 205.1096
k	43.2	<u>393.3195</u>	$C_{24}H_{45}O_2Si$	1-hydroxy-2,12,15-heneicosatrien-4-one – H + 1 TMS + H	393.3183	-0.14	13.7	303.2676
l	43.9	<u>391.3030</u>	$C_{24}H_{43}O_2Si$	1-hydroxy-2,5,12,15-heneicosatetraen-4-one – H + 1 TMS + H	391.3027	-0.32	3.9	301.2518
m	44.0	451.3239	$C_{26}H_{47}O_4Si$	Persenone A – H + 1 TMS + H	451.3238	-0.13	21.3	–
n	44.2	453.3395	$C_{26}H_{49}O_4Si$	Persin – H + 1 TMS + H	453.3395	-0.04	17.6	–
o	44.6	<u>455.3542</u>	$C_{26}H_{51}O_4Si$	Persenone B – H + 1 TMS + H	455.3551	0.91	32.8	305.2819

The *m/z* of the prevalent ion of the MS spectra is underlined.

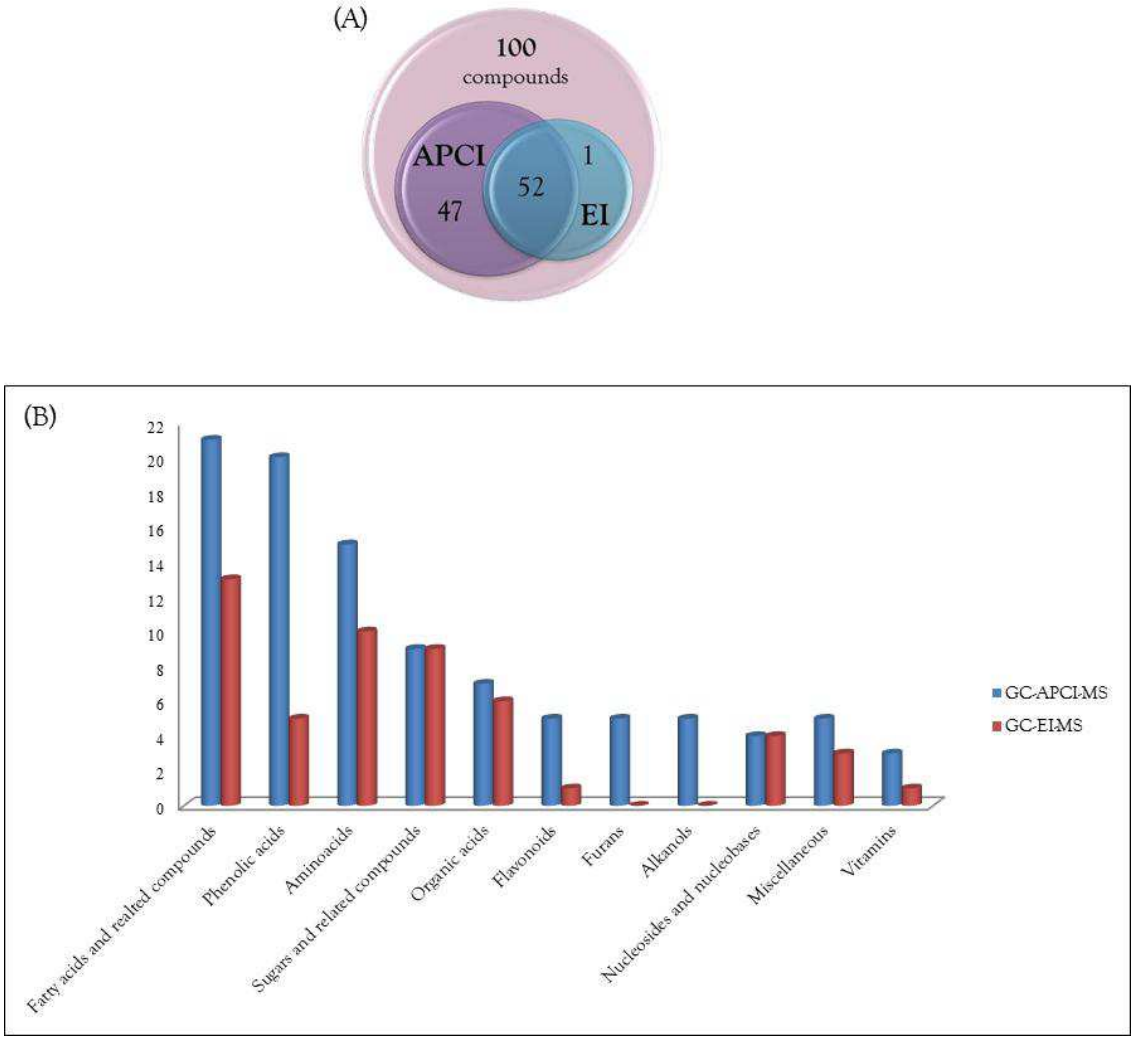

Figure 9.3. Comparison of the number of compounds identified by using the two platforms. (A) Euler diagram for a holistic view of the results and (B) column graph for comparison of the number of identified compounds by families. * The compounds considered were all the identified in the study (see ***Tables 9.1*** and 9***.3***).

Considering the total area of the chromatograms and the result of the addition of the analytes areas belonging to the same chemical category, the percentages corresponding to each chemical group vary (see ***Figure 9.4***). We did not try to make a very accurate quantitative estimation, since we are aware that the relative responses of the analytes under study vary a lot depending on the ionization source. In this figure, from our point of view, it is possible to identify the chemical categories of compounds which represent a higher percentage of the global profile in the chromatograms (in APCI and EI); higher percentages are logically related to a proper efficiency of the ionization and considerable abundance of the compounds belonging to a particular family.

Figure 9.4(A) represents a distribution where the percentage of each chemical family (in terms of sum of areas and total area of the chromatogram) is estimated for GC-APCI-MS and ***Figure 9.4(B)*** shows the distribution for GC-EI-MS. At a first glance, the sectors diagram for GC-APCI-MS is more balanced and almost all the families of compounds are properly detected. However, the diagram for GC-EI-MS shows the opposite, because the biggest part of the graph (≈81%) is occupied by a group of unknown and the group of fatty acids and related compounds. Fatty acids group represents a high percentage of the total area in APCI (22% approx) as well, and the group of unknown compounds covers also a considerable percentage (≈23%), but to a lesser extent if compared with EI.

It is remarkable that the percentage that the miscellaneous group represents is higher for EI (8.4%) than for APCI (3.1%); a possible explanation for this can be found in the fact that the only compound identified by EI, that is not present in GC-APCI, is enclosed in this group.

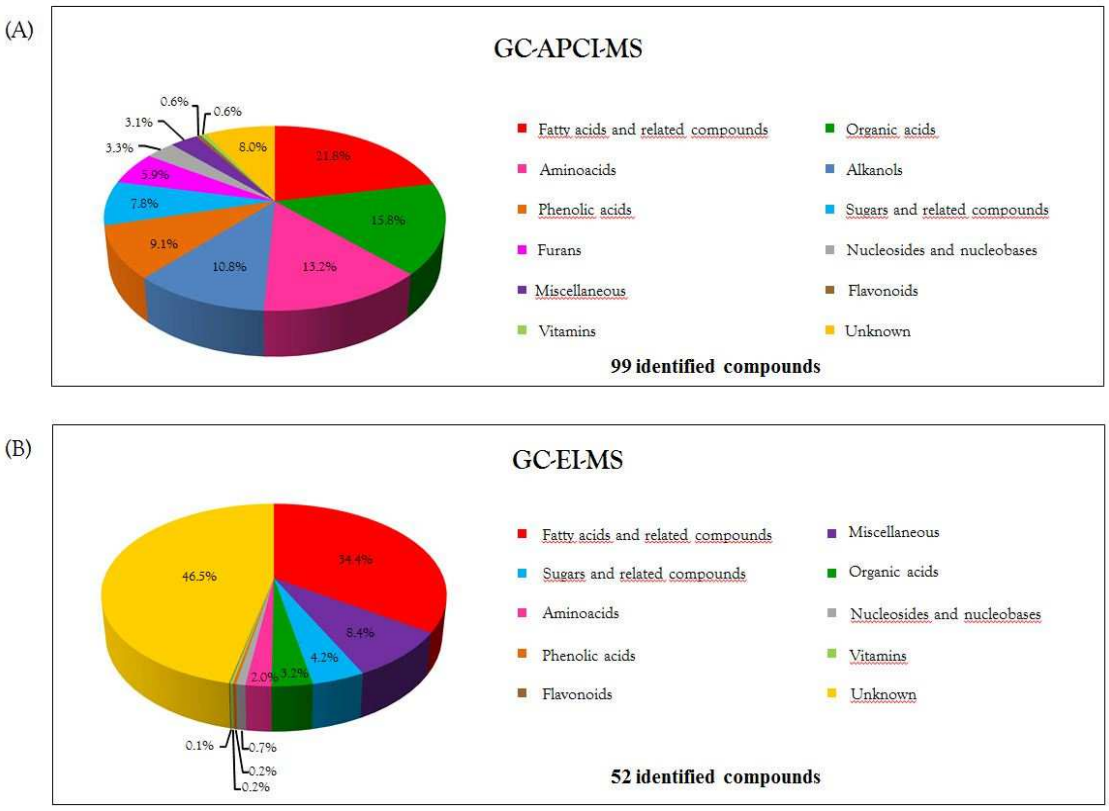

Figure 9.4. Sector diagrams for the distribution of each chemical group in avocado fruit depending on the technique applied. (A) Distribution in APCI, (B) Distribution in EI. * The compounds considered were all the identified in the study (see ***Tables 9.1*** and ***9.3***).

9.3.5. Limitations of the GC libraries

Considering the results included in this paper, it is possible to claim that both APCI and EI databases are very useful to achieve the identification of compounds present in a sample; however it is very clear that both have some drawbacks as well. Pros and cons of the two databases used will be discussed within this section (see ***Table 9.4***).

Table 9.4. Advantages and drawbacks of GC-EI-MS and GC-APCI-MS databases used in the current study.

	GC-EI-MS database	**GC-APCI-MS database**
Advantages	▪ Automated ▪ Quite quick search of matches with database ▪ No optimization of fragmentation parameters ▪ About 250,000 compounds	▪ Free and publicly available ▪ Suitable for more polar and biggest molecules ▪ MS/MS spectra* ▪ All possible silylated forms for a compound
Disadvantages	▪ IUPAC names ▪ No specific fragments ▪ Small range of polarities and molecular weights ▪ Only native or some of the possible silylated forms for some compounds	▪ Non automated ▪ Limited number of compounds (being updated) ▪ MS conditions-dependent ▪ Optimization of fragmentation parameters needed ▪ Considerable cost*

* Facts related to the kind of analyzer used.

EI database is the most common and widely used in several fields and it contains a huge number of compounds, including drugs, metabolites, poisons, pesticides, fungicides and common sample contaminants, among others. On the contrary, an on-line APCI library has appeared only recently and includes rather limited number of compounds (although it is constantly being updated). However, APCI database includes some analytes which cannot be studied by EI, since their molecular weight or polarity is outside of the range of application of this traditional source. For instance, APCI allows the detection of flavonoids or phenolic acids.

Automation and the promptness of the search of matches against the database are one of the main benefits of EI library and, at the same time, the principal problem of APCI database, where the search is manual so far. The names provided by the APCI database are the common ones (although includes IUPAC names as well), whilst EI

usually works with IUPAC nomenclature, which could complicate the search. When EI databases are used together with deconvolution softwares the number of species that can be characterized in a complex sample is considerably increased.

EI libraries sometimes do not consider all the silylated forms of a compound when a search is carried out, finding only the free or native forms and, eventually, some of the silylated ones; nonetheless, APCI database takes into account all the possible silylated forms of a compound. Anyway, this is a limitation of the EI libraries that can be easily addressed with the manual addition of the derivatized spectra.

As the search in APCI can be made considering several search parameters, such as the accurate *m/z* signal, any *m/z* signal of a MS/MS fragment (for fragments related to the in-source or in-funnel fragmentation), and the name of the compound, the tool is very flexible. The database even includes retention indexes. It can happen that a compound which is included in the database does not appear as "match" when the search is carry out by name, being found when the molecular formula is used to search the possible matches.

In general, it is possible to say that in EI, the fragmentation parameters do not need to be optimized, as a potential of 70 eV has been considered as a universal value, appropriate for almost every compound under study. However, the ionization in APCI is more conditions-dependent and, for this reason, some parameters like collision energy have to be optimized for getting a proper fragmentation for each particular analyte.

Using 70 eV as optimum potential and the fact that there is no need of optimizing it, is not always an advantage, since the excessive fragmentation produced by EI does not generate specific fragments for each compound, finding that the predominant *m/z* signals in almost any MS spectrum are 73, 116, 147, 192 or 255. Several research groups have tried to address this issue, Fialkov et al. [44, 45], for instance, demonstrated that electron ionization of vibrationally cold molecules in the supersonic molecular beams (cold EI) provided mass spectra with enhanced molecular ion, yet with good library search results and superior identification probabilities. The issue is not observed for APCI, because softer collision energies preserve the pseudo molecular ion or, when the fragmentation is more drastic, the most intense signal of the spectra is the result of losing a TMS (73) or TMSOH (90) group.

The approach used in the current work was mainly qualitative; quantification of the identified compounds was not intended, however, it seems necessary to stand out that EI, being a physical form of ionization, is only slightly affected by the presence of the matrix. APCI could be more vulnerable in this regard, being potentially subjected to matrix effects that could be an obstacle in the quantitative performance of the technique. Further studies evaluating these possible matrix effects seem to be necessary.

Traditionally the robustness of the MS data in CI under vacuum conditions (PCI and NCI) has not been considered as one of its strongest capabilities, which is precisely one of the strengths of EI (the good reproducibility and comparability among instruments). However, this problem has been faced in the new generation APCI interfaces, where the dependency on the instrument employed has been reduced. To illustrate this fact, ***Figure 9.1. Supporting information*** is included, where the robustness of APCI is quite evident when the MS and MS/MS spectra of several pure standards and the same compounds in avocado samples are compared. L-tyrosine, quinic and palmitic acid were considered as examples.

Other positive characteristic of APCI is that even if a compound under study is not included in the database, the analyst always gets a tentative molecular formula and can still work on its identification (although this is obviously related to the kind of analyzer used, and it would be applicable to a GC-EI system coupled to a high resolution MS analyzer (may be without preserving the pseudo molecular ion, in this case, due to the harsh fragmentation)).

Although the APCI database includes information about retention index, the search in these two libraries is mainly based on mass spectra comparison. This fact could be an inconvenient when analytes with the same molecular weight (isomers), that have almost identical fragmentation, have to be identified. In these occasions, if a commercial standard is available, retention times are required to distinguish between the isomers. In the current work was necessary to use standards of different isomers, because in the avocado chromatograms different peaks with the same mass and the same MS spectra were found. These compounds were *p*-, *o*- and *m*-coumaric acids; vanillic and isovanillic acids; α-, β-, γ-resorcylic, *o*-pyrocatechuic, protocatechuic and gentisic acids; epicatechin and catechin; l-leucine and l-isoleucine; and oleic and *cis*-vaccenic acids. All of them were

included in the APCI library. ***Figure 9.2. Supporting information*** shows some examples of mass spectra of isomers found in avocado extracts. As we can see in ***Figure 9.2(A) Supporting information***, *p*-, *o*- and *m*-coumaric acids present the same fragmentation patterns, although the relative intensities of each one are not exactly the same. Something similar it is observed in ***Figure 9.2(B)*** and ***2(C) Supporting information***, where the mass spectra of oleic and cis-vaccenic acids, and l-leucine and l-isoleucine are shown, respectively. The predominant signal in MS is the molecular ion, except in the case of coumaric acids, where the loss of TMSOH (90.0501) produces the most intense *m/z* signal. In view of this figure, it is more than evident that the discrimination between isomers is not possible just based on MS; in these cases, the use of a standard is essential to achieve a reliable identification.

9.4. Conclusions

The first application of a very recently developed GC-APCI-TOF MS database to characterize the metabolome of a tropical fruit with a very complex composition such as *Persea americana* has been carried out. The results obtained by GC-APCI-MS have been compared with those achieved using a more conventional platform, such as GC-EI-MS. Moreover, a comparison of the MS signals observed in both ionization sources has been made considering different analytes belonging to several chemical families, observing that the MS spectra in EI are quite similar to the MS/MS spectra in APCI.

Considering the wide range of application, its robustness, and the reliability of the database used, GC-APCI-MS has to be definitely considered as a very promising routine (bio) analytical technique.

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Supporting information

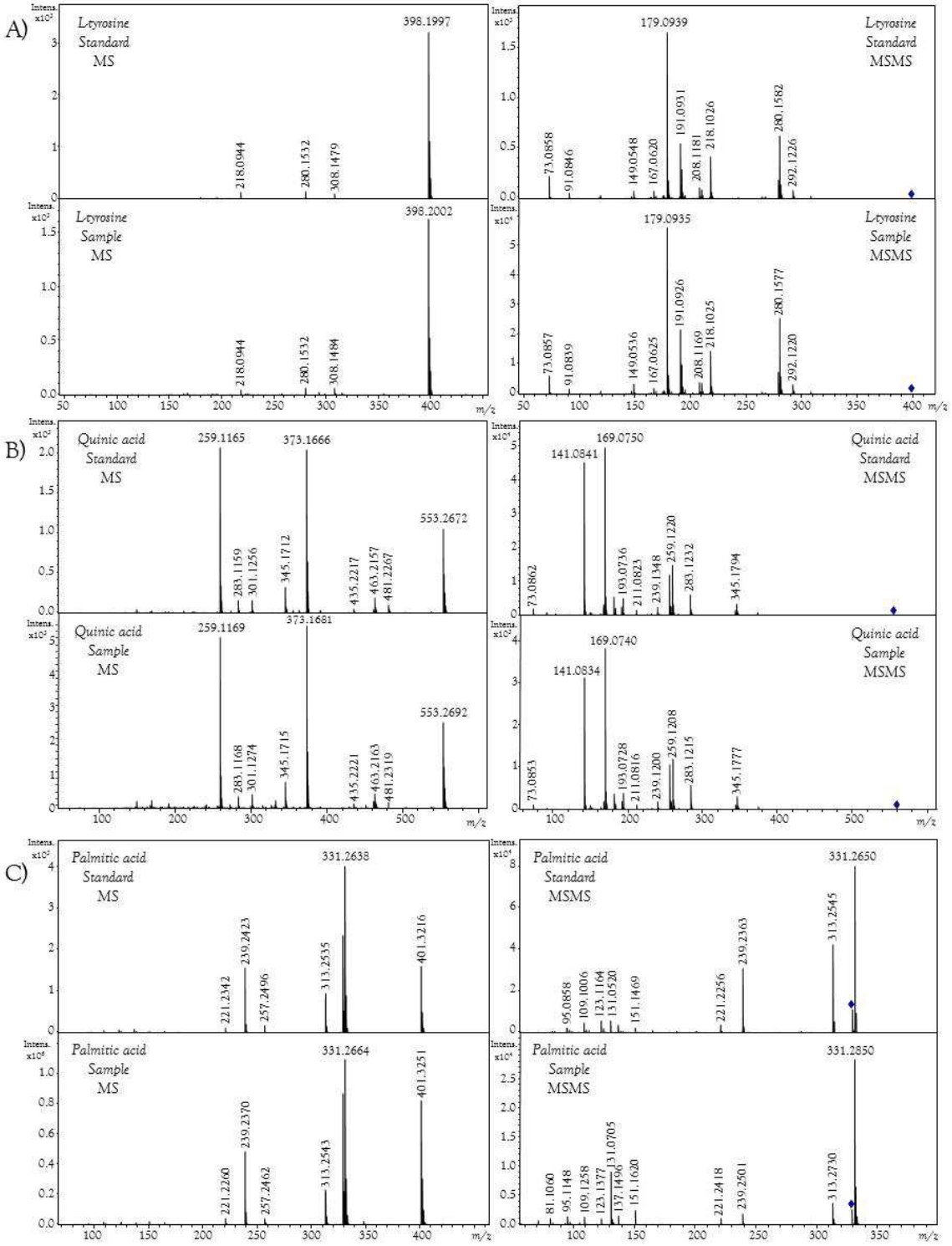

Figure 9.1. Supporting information. Reproducibility of APCI-MS fragmentation. MS and MS^2 of L-tyrosine (A), quinic acid (B) and palmitic acid (C) are shown, both for the standard and the same compound in avocado samples.

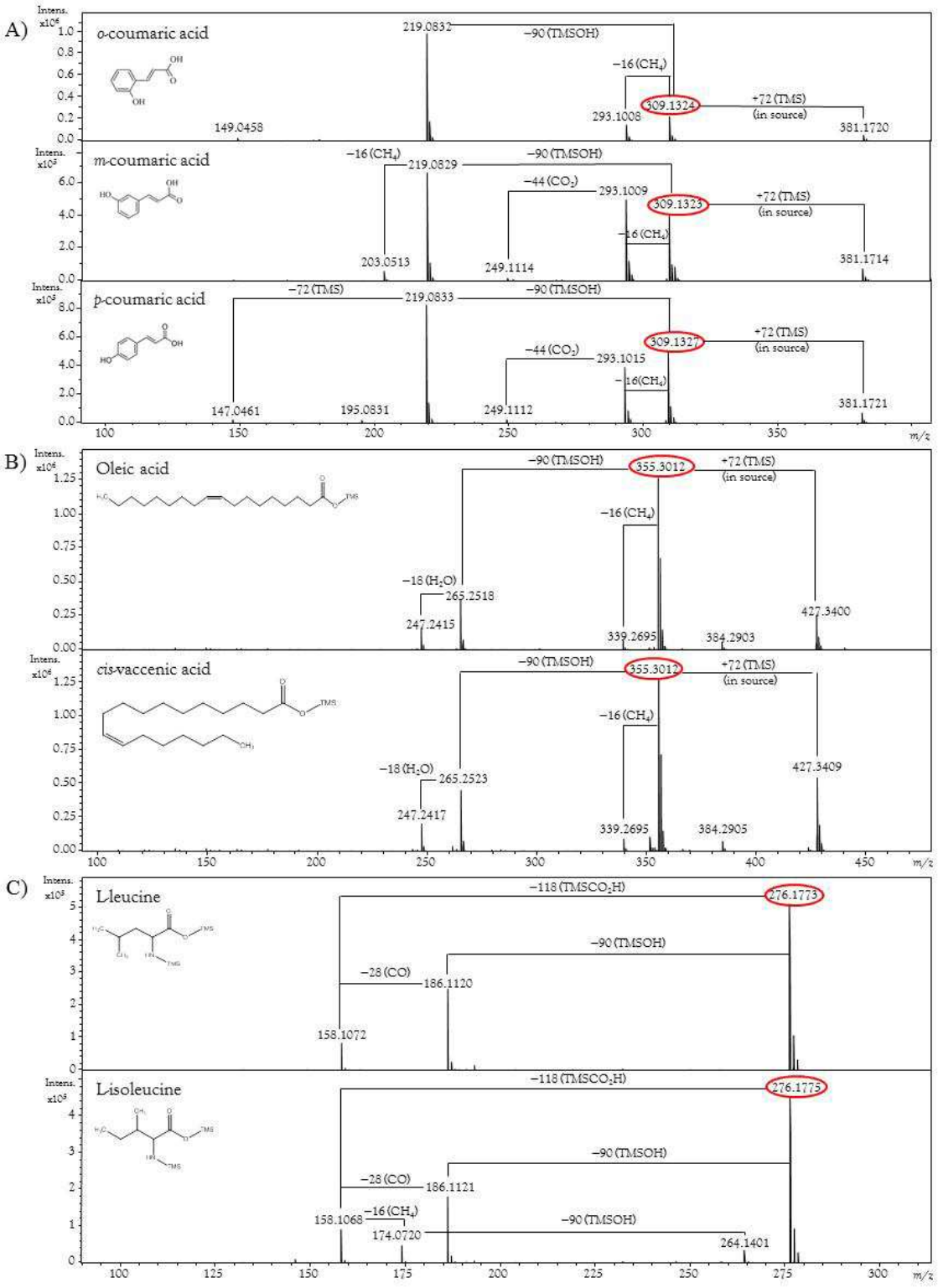

Figure 9.2. Supporting information. MS spectra of some isomers of phenolic acids (A), fatty acids (B) and amino acids (C) found in avocado fruit. The principal losses of each one are shown. The signal of the complete silylated form is highlighted by a red circle.

Chapter 10

Gas chromatography-flame ionization detector/atmospheric pressure chemical ionization-time of flight mass spectrometry data together with statistics for the exploratory analysis of avocado extracts from different varieties at two ripening degrees

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To be submitted

Abstract

In order to investigate avocado fruit ripening, untargeted GC-APCI-TOF MS metabolic profiling analyses were carried out. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were used to explore the pseudo-polar and polar metabolites profiles from fruit samples at 2 different ripening degrees.

These results not only confirmed published metabolic data but also reveal new insights into avocado fruit composition and metabolite changes, demonstrating therefore the value of metabolomics as a functional genomics tool.

Keywords: Gas chromatography / Food metabolomics / Atmospheric pressure chemical ionization / *Persea americana* / Flame ionization detector / Principal component analysis

10.1. Introduction

The avocado fruit is a complex matrix with an increasing interest due to the beneficial effects that have been related to its consumption [1-5], including cancer-preventive properties, antioxidant capacities, cardiovascular health improvement, weight management and healthy aging, among others. This is in part due to its content of monounsaturated, polyunsaturated and saturated fatty acids; however these effects cannot be just assigned to one particular group of compounds but to a variety of nutrients and phytochemicals. Avocado´s mesocarp is an excellent source of different kind of metabolites that show high variations in terms of concentration; sugars, amino acids, carbohydrates, carotenoids, tocopherols, proteins, phenols, vitamins, sterols, tannins, fatty acids, coumarins, etc. [6-8]. The nutritional quality of the avocado fruit is a function of the fruit´s metabolite content, which is affected by the genotype, agricultural practice, environment, but also by physiological changes that take place during fruit growth and ripening.

Avocado fruits can be kept on the tree for long periods once physiological maturity (defined as the capability of ripening naturally after harvest) is reached. The identification of the physiological maturity for avocado fruits is not a simple task, as this maturation is not accompanied by changes in external appearance [6]. The large increase in avocado production during the last decades and the continuous growth of the industry have stimulated search for means and methods to determine fruit maturity and the best time

of harvesting. One of the earliest maturity indexes was oil concentration in the fruit, which is difficult to measure accurately but it is closely related to percentage of dry matter. Although additional maturity indexes such as fruit growth rate, softening enzymes or fruit firmness have also been tested, % of dry matter is the most commonly used index to estimate harvesting maturity [9]. Commercially, the quality of avocado fruit is rated according to size, shape, estimated oil content (dry matter), absence of defects, and firmness [10]. This has been extensively discussed in literature [7, 11-16]. Some authors have also checked the variation in the composition of avocado fruit in terms of particular chemical families of compounds during ripening or storage, considering fatty acids, sugars, carbohydrates, or some other analytes [6, 17-21]. There is not optimum strategy described so far; what is clear is that in order to establish practices in harvesting or to devise methods of testing the maturity of the avocado fruit, it is necessary to know the composition of this fruit throughout its life cycle and the changes taking place during ripening.

The comprehensive qualitative and quantitative analysis of all the metabolites within a matrix is a very ambitious goal still far from complete achievement for any system; however there is no question about the fact that an unbiased, global and exploratory type of analysis seems to be the most appropriate approach for studying avocado metabolites and their dynamic changes, getting a global picture about its composition and changes during the ripening process. To the best of our knowledge, this kind of approach has been just applied in very few occasions for the analysis of this fruit [8, 21]. Indeed, most of the studies published so far focused on the determination of a particular group of compounds [17, 19, 22-26]. As a result, despite the fact that the fruits of *Persea americana* are increasingly consumed worldwide, its composition is far from being well-described.

Advances in MS (coupled to different separation techniques (GC, LC and CE, or even by means of direct injection experiments)) have enabled the analysis of metabolites in a previously unimaginable scale [27-30]. The continually growing interest in plant metabolomics give us the chance of applying our knowledge of plant metabolism and biochemical composition in a wide range of fields, such as plant-based nutrition [31, 32]. It is obvious that all "omics" approaches will rely heavily upon bioinformatics for the storage, retrieval, and analysis of large datasets; and metabolomics is no exception. The

complex, essentially multivariate nature of metabolic measurements requires dedicated efforts to extract biologically relevant information from them. The most commonly used strategies of pre-processing, explorative data analysis using unsupervised and supervised methods, prediction, visualization and validation tools in order to achieve a proper biological interpretation have been described in very interesting reviews [27, 33-36]. The most popular approaches include unsupervised methods such as PCA, or HCA. If the objective of data analysis is classification or regression, predictive models have to be created; in this category one can find linear discriminant analysis (LDA), PLS-DA and OPLS-DA. Support vector machines and artificial neural networks work with derived input variables can be used as well in these kinds of studies.

The purpose of the present study was to investigate the changes in metabolic composition from unripe to ripe avocado fruits that can be observed when the fruit is harvested. The biological changes were determined by GC-APCI-TOF MS using a non-targeted profiling approach useful to determine a wide range of metabolites. This platform has been applied just in very few metabolomics studies [37-41]; its potential for carrying out this short of exploratory analysis has not been previously evaluated. All the data obtained by the untargeted approach used underwent a variety of chemometric analyses, including PCA and PLS-DA, to identify typical changes in some of the chemical constituents of the edible part of avocado fruit among the samples belonging to different varieties and during ripening. An increasing understanding of the metabolic changes underlying fruit ripening and of the pathways responsible for the biosynthesis of nutritionally relevant metabolites may help finding a proper indicator of avocado fruit maturity. Moreover, if further studies keep facilitating a deep knowledge about the composition and biochemical basis of this tropical fruit, clinical studies on the health benefits of avocado consumption could be conducted with guaranty of success.

10.2. Materials and methods

10.2.1. Chemicals and standards

BSTFA + 1% TMCS from Pierce (Rockford, IL, USA) was used as derivatization reagent in freshly opened 1 ml vials. The APCI tune mix and pyridine (99% ultra-pure GC grade) were supplied by Fluka (St. Louis, USA). Methanol was chosen as solvent to prepare sample extracts and it was purchased from Panreac (Barcelona, Spain). Taxifolin

was used as IS to evaluate the reproducibility of the extraction system and the chromatographic runs, and it was purchased from Extrasynthese (Lyon, France). Comercially available pure standards were used with purposes of identity confirmation. Some organic acids (citric and malic acids), pantothenic acid (vitamin B_5), β-sitosterol, D-mannoheptulose, D-mannitol, L-aspartic acid and several fatty acids (stearic, *cis*-vaccenic, linoleic and myristoleic acids) were supply by Sigma-Aldrich (St. Louis, USA).

10.2.2. Samples, extraction and derivatization reaction

The samples selected in this study were part of the germplasm collection from the IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). Fruits were harvested from plants of 13 varieties grown under identical environmental conditions (soil, rain, light, etc.) and analyzed at two ripening degrees (fruits just harvested and fruits ready for consumption)). The varieties analyzed were 'ColinV 33', 'Gem', 'Harvest', 'Hass', 'Hass Motril', 'Jiménez 1', 'Jiménez 2', 'Lamb Hass', 'Marvel', 'Nobel', 'Pinkerton', 'Sir Prize' and 'Tacambaro'. It is important to highlight the fact that 4 of these varieties are ('Hass Motril', 'Jiménez 1', 'Jiménez 2' and 'Tacambaro') are putative mutants of 'Hass' and so far they are undistinguishable with genetic markers, although they present some differences at the phenotypic level.

Pulp of 2-3 pieces of fruit was homogenized, frozen and freeze-dried to prepare sample extracts using the protocol previously described by Hurtado-Fernández et al. [8, 25]. Briefly, 4 g of the freeze-dried (and homogenized) sample were mixed with 40 mL of pure MeOH and 50 ppm of IS. A vortex was used for shaking the tubes during 30 min. The supernatants were taken and centrifuged at 3000 rpm for 10 min, and after that, were evaporated to dryness and redissolved in 5 mL of MeOH (50/50, v/v). Aliquots of 25 μL of this methanolic extract were taken, evaporated and redissolved in the proper volume of derivatization reagent.

A programmable MultiPurpose Sampler was used to carry out the on-line derivatization reactions of the standard solutions and avocado extracts by adding 50 μL of BSTFA + 1% TMCS to a dried aliquot of the sample and incubating at room temperature for 60 min.

A QC sample was used to assure the stability of the system and it was made by mixing equivalent volumes of the extracts of each variety of avocado fruits and was injected every 5 samples throughout the batch.

10.2.3. Analytical equipments

The chromatographic separations were carried out in an Agilent 7890A GC system (Agilent, Palo Alto, USA). The system was equipped with a HP-5-MS column (30 m, 0.25 mm ID and 0.25 µm film thickness) and coupled to two detectors running in parallel (FID and MS), being required a column flow splitter with deactivated capillaries (0.6 m, 0.25 mm ID, 0.25 µm film thickness) to allow the simultaneous acquisition with both detectors. Automated injection of aliquots of the derivatized samples (1 µL) was done using a programmable MultiPurpose Sampler MPS2 (Gerstel, Mülheim an der Ruhr, Germany). A septumless CIS PTV injector was used to carry out the injections in splitless mode. The purge time of the PTV injector was 60 s, using a purge flow of 3 mL/min and setting the temperature at 250°C. Sequential washing steps of the 10 µL syringe were included before and after each injection, as well as a step of sample pumping to remove possible small air bubbles. Helium was used as carrier gas at a constant flow rate of 1 mL/min.

The temperature of the column varied according to the gradient described as follows: the initial column temperature was kept at 140°C for 5 min, then increase at a temperature rate of 3°C/min until 220°C, keeping that value for 1 min. Later on, the temperature raised from 220°C to 241°C at 10°C/min and it was held for 1 min; and finally, the temperature was increased from 241°C to 310°C at 3°C/min, maintaining that temperature for 5 min.

As it was mentioned above, two different detectors were used; FID and an ultra high resolution TOF MS MaXis (Bruker Daltonics, Bremen, Germany). FID operated by applying the following conditions: 300°C of front detector temperature, hydrogen flow of 40 mL/min and 370 mL/min for air flow. The MaXis was coupled to the GC system by using a multipurpose source as APCI interface (operating in positive mode), which was equipped with GC transfer line [42] kept at 300 °C. The APCI vaporizer temperature was 300°C, the corona discharge needle worked at a voltage of +4000 nA and the nebulizer gas (nitrogen) pressure was set at 3.5 Bar. Dry gas (nitrogen) temperature and flow rate

were 300ºC and 2 L/min, respectively. Capillary voltage was set at +2000 V and the end-plate offset at −500 V. Both an external and internal calibrations were performed injecting an APCI tune mix solution and using cyclic-siloxanes, respectively [37]. Spectra were acquired within the mass range between 50 and 1000 *m/z* with 1 Hz frequency.

Rational chemical formulas were generated based on internally calibrated monoisotopic masses within 10 mDa mass error, using the SmartFormula tool within the DataAnalysis™ software package (Bruker Daltonik). The identification of the metabolites under study was achieved taking into account the information provided by a recently developed APCI library [43]. To study the FID data, ChemStation B.03.02 (Agilent Technologies, Palo Alto, USA) software was used.

10.2.4. Statistical analysis

The GC-MS data files were exported as mzXML files and aligned by using in-house developed alignment algorithm msalign2 tool (http://www.ms-utils.org/msalign2/); peak picking was performed using XCMS package (The Scripps Research Institute, La Jolla,USA) using the default settings except for bandwidth (bw parameter) for grouping of features set to 10.

The generated data matrix was imported to SIMCA-P 12.0 software package (Umetrics, Umeå, Sweden). The data were mean centred and unit variance-scaled prior to statistical analysis. The validity and the degree of overfitting of the PLS-DA models were checked using a 200 permutations test.

10.3. Results and discussion

10.3.1. Fruit metabolite screening by GC-APCI-TOF MS/GC-FID

The applicability of our method for analyzing real avocado extracts of 13 varieties at two ripening degrees had to be checked. Very complex GC profiles were obtained by using each detector. Although the main goal of the study was carrying out an exploratory analysis of the profiles, the identification of an important number of metabolites could be achieved bearing in mind previously published results [38, 43, 44].

Figure 10.1 shows an example of an avocado chromatogram achieved both by GC-FID and GC-APCI-TOF MS, where it is possible to appreciate the complexity of the profiles.

Avocado fruits are harvested from the trees when they reach physiological maturity but they are still unripen and, consequently, they can have different phytochemical composition at different moments during the ripening process. Changes in metabolite levels may be dramatic or subtle. The dramatic changes will be easily recognized; however, subtle changes will require fine statistical processing to determine whether or not the observed changes are significant. A single GC-MS metabolite profile can yield more than 300 distinct components. This provides a wealth of information to be interpreted and leads to significant challenges in processing the data. To simplify the task, using techniques to reduce the dimensionality of the data set and to visualize the data is very common approach.

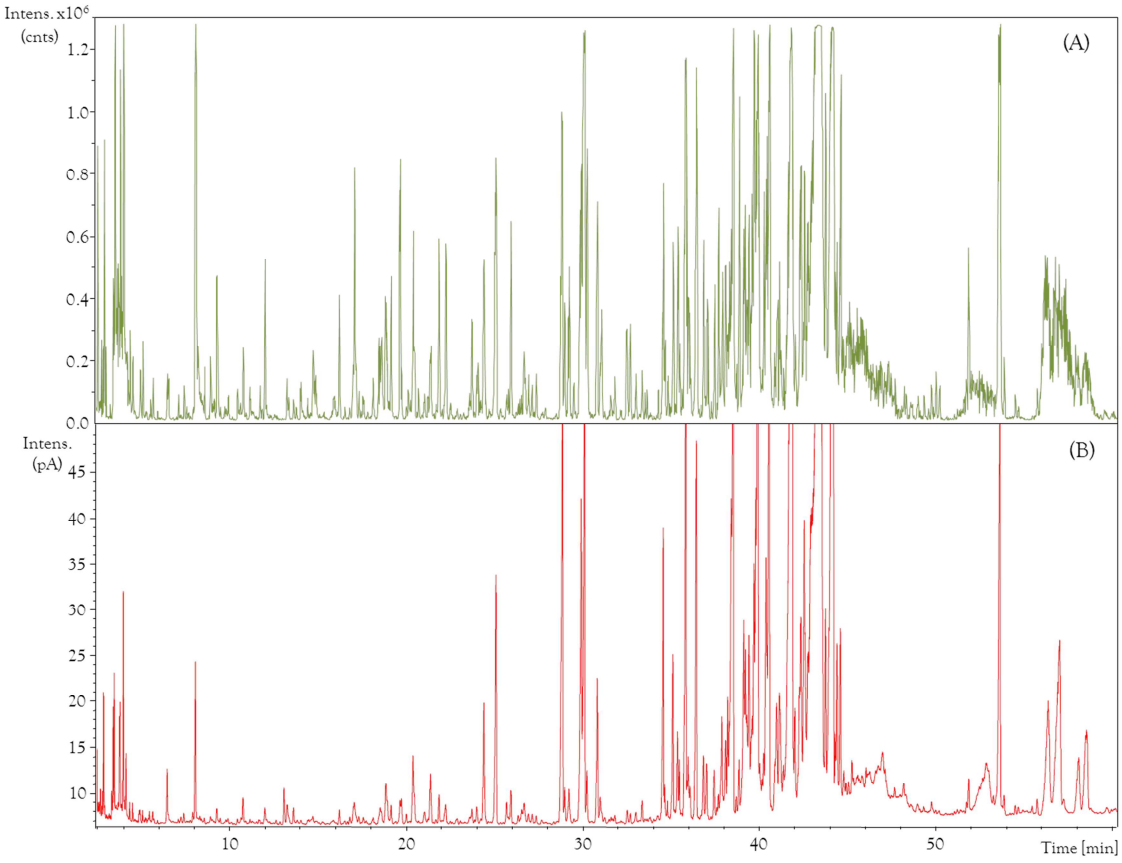

Figure 10.1. BPCs of avocado *cv.* Lamb Hass at the second ripening degree obtained by (A) GC-APCI-TOF MS and (B) GC-FID.

The subsequent sections are dedicated to the treatment of the data obtained by GC-APCI-TOF MS. The statistical analysis of the data from GC-FID is actually ongoing in our lab, and for this reason the results are not included in this chapter.

10.3.2. Exploratory analysis of avocado metabolites by GC-APCI-TOF MS

In previous sections of this paper, we have tried to address that the targeted approaches have been used much more extensively than untargeted ones [19, 22-24], even if they could make more difficult to get a holistic insight into the ripening process. As stated before, in metabolomics, multivariate analysis with an appropriate algorithm should be performed depending on data structure and preliminary mining intention. Exploratory analyses tend to be used most often in this discipline. The mission of the analysis is mainly for the characterization of data structure and preliminary mining of significant tendencies included in the data. It is evident that exploratory data analysis should be performed before conducting further analysis, such as multiple regression or classification.

Evaluating the structure of the data for unripe and ripe samples

One of the main objectives of this work is to evaluate how the ripening can affect the metabolic composition of different avocado varieties. To illustrate it, the following figure (***Figure 10.2***) shows the plots where the scores and loadings of the PCA both for unripe and ripe samples are included. Observations or scores (avocado varieties) are represented by the brown hexagons and for them only their position on the chart is important. The loadings are the hexagons with different colors and they represent the metabolic profile of the samples under study. Usually, loading values (p) correspond to the covariances between the variables and the score vectors; here, however, the loadings are rescaled as correlations (with values between −1 and +1).

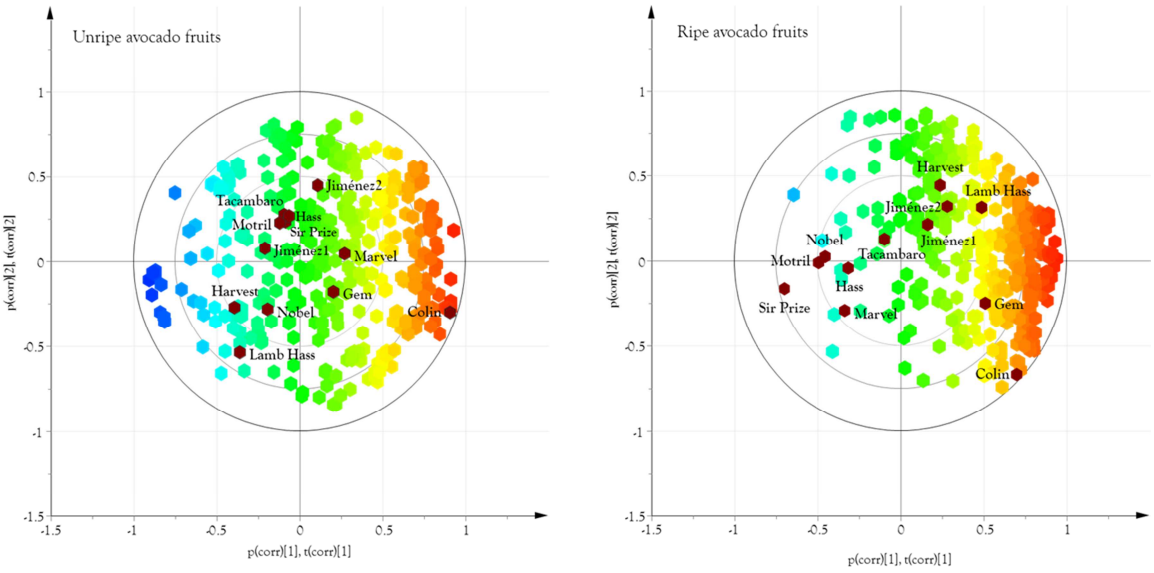

Figure 10.2. Biplot that shows the effect caused by the ripening process on the metabolic evolution of different avocado varieties.

Paying attention to the loadings, just the visual comparison of these two plots can give us an overall impression about the significant metabolic evolution that avocado fruits experiment during the ripening process, being more than evident the existing differences between both matrices. Another, piece of the evidence supporting the idea of the "deep" metabolic transformation is the calculation of the RV coefficient for samples at the first ripening degree and the second one, which has been 0.224. This low RV value indicates a low correlation between the matrices and a consistently high degree of the dissimilarity the unripe and ripe stages.

However, since any research project has only limited resources it is important to make use of PLS-DA on the entire dataset to reveal and identify the most important components in the metabolic shift during the ripening process.

Discriminant metabolites to separate unripe and ripe avocado samples

First, we applied an unsupervised multivariate method (PCA) to identify the most important sources of the variability in the data. ***Figure 10.3*** shows the score plot of PCA model considering all the samples under study. It is evident that the difference in the ripening degree is the most important source of the variability in the data. The second component reflects the changes among avocado fruits varieties.

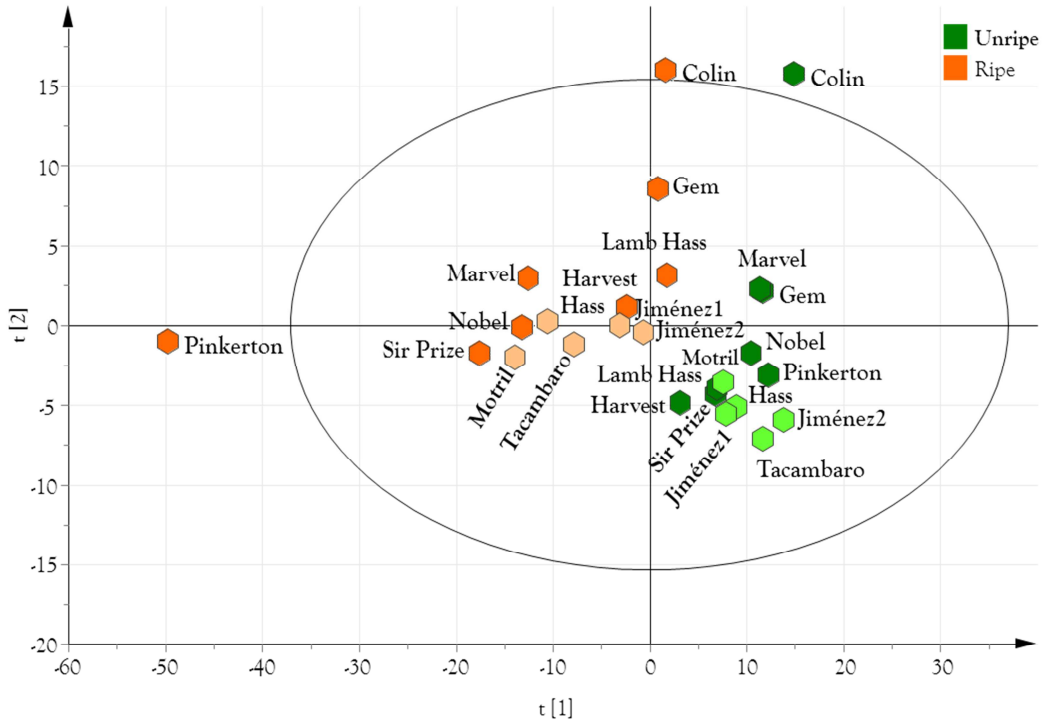

Figure 10.3. Score plot of PCA model considering both unripe and ripe samples. R2X = 0.647, Q2 = 0.465

Three ecological races or botanical varieties of avocado adapted to different climate conditions have traditionally been recognized: Mexican, Guatemalan and West Indian. These races are distinguishable on the basis of morphological, physiological and horticultural traits [45-47]. Most commercial avocado cultivars are interracial hybrids developed from chance seedlings. Thus, the most important cultivars in subtropical climates, such as 'Hass', 'Bacon' and 'Fuerte' are Guatemalan x Mexican hybrids with different degrees of hybridation [48]. One of the interesting points of the current study is that, as has been previously mentioned, some putative 'Hass' mutants varieties ('Jiménez 1', 'Jiménez 2', 'Tacambaro' and 'Hass Motril') are included in this study [45]. It is remarkable the fact that these mutant varieties are clustered within the same area of the plot and also very close to 'Hass'. As expected, this small distant among the mutants of 'Hass' and Hass *cv.* (they present a different color from the rest of the samples to easily identify them) is observed both in unripe and ripe avocado samples. 'Colin V33' can be considered as an outlier when compared with the rest of the samples and, in fact, studies with molecular markers also show a high genetic distance between 'Colin V33' and 'Hass' [45]. Something similar was observed for 'Pinkerton' and 'Gem' at ripe states. Thus, 'Pinkerton', 'Gem' and 'Colin V 33' are the varieties which show characteristic metabolic

profiles, fact explained by the noticeable degree of separation from the others when the two first principle components are considered.

It is quite interesting to observe the grouping of the different ripening degrees. In general, samples belonging to the first ripening state are laying closer than ripe samples, meaning that the metabolome of unripe fruits is more similar than in the case of ripe ones.

To corroborate further on the metabolic changes associated with ripening, we have built a two class PLS-DA model using the degree of ripening as class characteristic. ***Figure 10.4*** represents a cross-validated score plot of PLS-DA model (R2X=0658, R2Y=0.991, Q2=0.78). In this figure, 'Colin V 33' and 'Pinkerton', in ripe and unripe state, respectively, were the varieties that showed fewer similarities with the rest of the members of their respective categories.

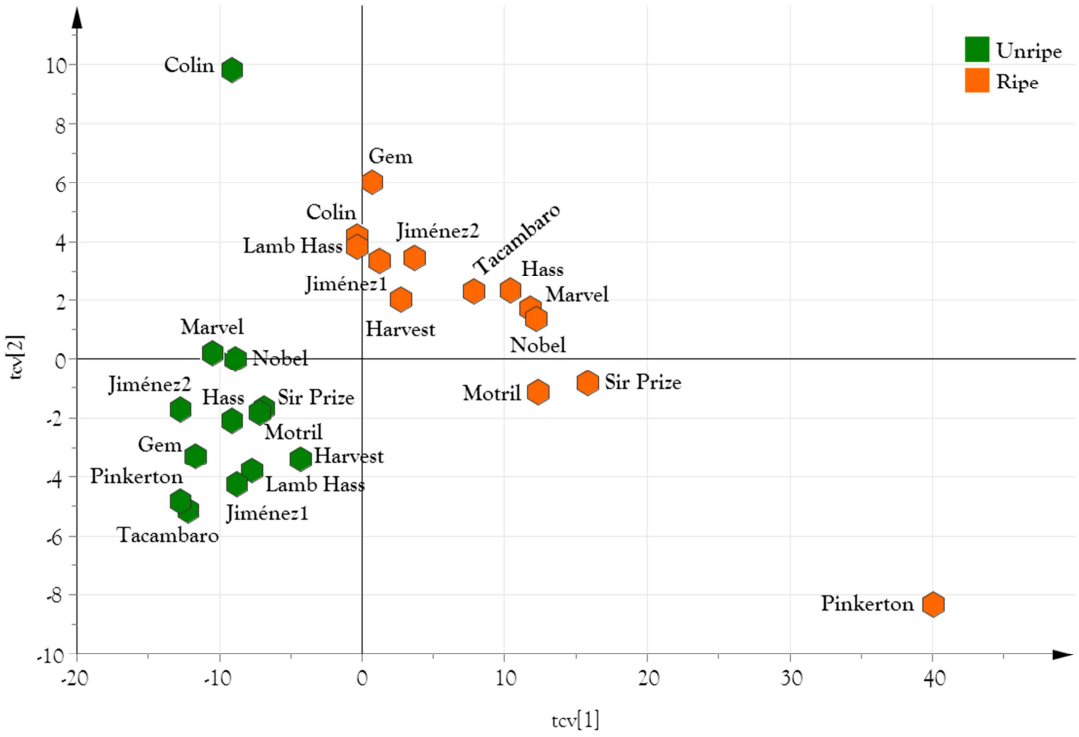

Figure 10.4. Cross-validated score plot of PLS-DA model.
R2X = 0.658, R2Y = 0.991, Q2 = 0.78

The features, most influential for the model, can be selected on the basis of variable importance in the projection (VIP) scores. ***Table 10.1*** summarizes all discriminative features with VIP values >1.4. Taking into account the capabilities of the UHR-TOF MS used and our previously published results [38, 43], we tried to provide a tentative

assignment for the features shown in the table. Compounds included in the table are sorted based on the VIP value, from the highest to the lowest.

Table 10.1. *m/z* signal of the complete silylated form, retention times and other fragments of the possible classifiers of avocado variety and ripening degree obtained by statistical analysis PLS-DA.

t_r	***m/z* signal complete silylated form**	**Other fragments**	**Tentative assignment**
27.1	643.3159	539.2510/463.2192/373.1693/ 345.1732/283.1192/191.0948	D-mannoheptulose – 6H + 6 TMS
24.0	279.2682	–	2-pentadecylfuran + H
9.4	350.1634	333.1368/304.1760/274.1293/ 230.1036/205.1090/159.0862	L-aspartic acid – 3H + 3 TMS
43.3	393.3195	303.2676	1-Hydroxy-2,12,15-heneicosatrien-4-one –H + 1 TMS
8.7	351.1473	307.1217233.1013/ 189.1103	Malic acid – 3H + 3 TMS
31.0	357.3164	341.2856/267.2668	Stearic acid – H + 1 TMS
19.2	481.1924	465.1637/363.1485/273.0988	Citric acid – 4H + 4 TMS
30.4	355.3012	265.2514/247.2413	*cis*-vaccenic acid – H + 1 TMS
53.6	487.4313	397.3834	β-sitosterol – H + 1 TMS
36.0	293.2464	–	Methyl linolenate + H
24.3	436.2354	–	Pantothenic acid – 3H + 3 TMS
23.0	615.3254	525.2756/345.1727	D-mannitol – 6H + 6 TMS
30.1	353.2862	263.2369/245.2265/179.1761	Linoleic acid – H + 1 TMS
44.1	451.3239	–	Persenone A – H + 1 TMS
38.3	299.2378	–	Myristoleic acid – H + 1 TMS

As is appreciated in ***Table 10.1***, a tentative identity for 15 metabolites has been achieved, some of which has been extensively described in literature. The identification of some of these compounds have been corroborated by using pure standards and also making use of the available GC-APCI-TOF MS database [43].

D-mannoheptulose seems to be the most influential compound in the sample classification. It is one of the most abundant carbohydrates in avocado fruit and it is considered as a primary photosynthetic product, acting as a source of energy [49]. It has

been demonstrated that the concentration of this C7 sugar decrease as fruit ripens, fact that has driven to postulate that it can act as inhibitor of the ripening process [50]. The next one is 2-pentadecylfuran, an alkylfuran also called avocadofuran, which has been studied because of its antibacterial, antifungal and insecticidal activities [5, 51]. It is observed that several fatty acids also play an important role on the sample classification, and this can be due to the fact that the fat content is frequently correlated with the maturity of the fruit and with the quality or the condition of the fruit after harvest or storage [15]. Other interesting compounds are persenone A –compound quite abundant in avocado with chemopreventive and anti-inflamatory activities– and β-sitosterol [5, 52, 53].

It is obvious that further experiments are required in order to get a more information about the identity of these important compounds. The required experimental is already ongoing in our lab.

Similarities among genetically related samples and their metabolic differences from the rest

As a result of the increasing importance of avocado cultivation in Spain, several cultivars and rootstocks have been studied over the last years in order to optimize avocado cultivation. These cultivars and rootstocks include both genotypes obtained in different countries as well as local selections obtained after prospection of elite trees which could be well adapted to local environmental conditions. The optimization of genetic resource management requires a precise identification of the genotypes as well as a correct evaluation of the amount and distribution of genetic diversity within a species. Genetic markers have been used in avocado to identify accessions and clarify genetic relationships between different species and genotypes [45]. To the best of our knowledge very few studies considered as aim the metabolic comparison of putative mutants of 'Hass' [8, 24]. Herewith, we intended to do so considering the global metabolic profile achieved by GC-APCI-TOF MS. To achieve this purpose, we represent the data by using a Cooman´s plot, where 'Hass' related varieties were used for PCA class modeling with ripening degree as class id, being the rest of the sample used as prediction set.

Figure 10.7. Cooman´s plot obtained for the 13 avocado varieties under study.

The plot contains four regions separated by 95% confidence limits in a 2D graph in which samples below the horizontal line belong to the ripe 'Hass'-related cultivars (95%), while samples in the left region of the vertical line belong to ripe 'Hass'-related cultivars (95%). In the current cases the rest of the sample as outliers, they are outside the critical distance to the models; in other words, they do not belong to any of these "control" groups. Therefore, this model should be able to predict whether any avocado fruit is mutant of 'Hass' paying attention to its metabolic composition. Furthermore, these results can also bring about information related to possible metabolic differences among these mutants, fact that could be extremely interesting since, currently, it is not possible to distinguish these and other mutants of 'Hass' at a molecular level, detecting only in some cases differences in flowering date and production.

Nowadays, there is an increasing interest on genetic engineering, since genetic transformations are an environmentally sustainable alternative way for crop improvements [54]. Genetic engineering tend to apply strategies that are usually targeted to specific characteristics of the plant and, for this reason, a single horticultural trait can be altered without significantly affecting the phenotype of the cultivar. In the particular case of avocado cultivars, certain breeding objectives are difficult to achieve conveniently due to several obstacles, such as the few avocado flowers that yield mature fruits (around a

0.1% of the total of the flowers produced) or the slow maturation of avocado tree [55, 56]. Avocado genetic improvements are mainly limited by the availability of genes that are associated with specific horticultural traits. The most important traits, from a commercial point of view, are the flavor, fruit quality and precocity, tree growth rate, etc. Thus, the use of genetic tools are primarily focused on the acceleration of the breeding cycle, the improvement of the breeding efficiency, the fruit ripening control and the increase of disease resistance. Avocado has been genetically transformed by using certain genes that are involved in the resistance against specific diseases, because of the high economic impact that the diseases can cause, not only due to the loss of fruits (decrease of production), but also due to the expensive cost of fungicide treatments [54, 57, 58]. Furthermore, in the last decades, it has been possible to isolate several genes from avocado which are associated with the fruit ripening. These genes have been used to improve avocado cultivars, considering the fact that a greater length of time from harvest to flesh softening can extend the shelf life of avocado fruits and favor its long distance transport [54, 56]. However, it is important to take into account that the gene expression is closely related to the metabolic composition of a fruit, and also that the ripening process use to be associated with the activation of different pathways that can influence the content of several compounds usually related with specific organoleptic properties. Hence, the genetic transformation to control the avocado ripening should be carefully made, since the modification of the metabolites could lead to alterations in the overall nutritional quality [59].

10.4. Conclusions

The use of GC-APCI-TOF MS together with different statistic tools has demonstrated to be a very powerful and valuable platform to achieve a better understanding about the effect that the ripening process have on the metabolic profile of different avocado varieties. This is the first time in which an untargeted approach is used to study this influence on avocado fruits and it has been possible to identify tentatively 15 of the metabolites most influential on the sample classification. These results can help to identify possible ripening markers that facilitate the knowledge about the optimum moment of the harvest season to collect the fruits, being an alternative to the current parameter used (estimated oil content).

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Chapter 11

Potential of gas chromatography-electron ionization-mass spectrometry together with pattern recognition methods to identify ripening markers of avocado fruit

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To be submitted

Abstract

Herewith the usefulness of a GC-EI-Q MS methodology for the identification of potential ripening or variety markers of avocado fruit has been evaluated. A selection of 13 avocado varieties (grown under identical pedoclimatic conditions) at two diverse ripening degrees was made and their derivatized methanolic extracts were analyzed by a GC-MS. Even though the method was able to identify about 80 metabolites within a single run, the approach used was more exploratory and data pre-processing and data analysis steps were carried out, implying filtering (based on frequency of occurrence, analysis of the variance, and abundance in the respective classes), normalization, and PCA and PLS-DA analysis. 17 compounds were selected as the most influential to be considered in PCA and clustering analysis by k-means. Some of these compounds were mannoheptulose, *p*-coumaric, eicosenoic, aspartic and abscisic acids. *p*-coumaric increased its concentration over the ripening, whilst mannoheptulose, eicosenoic and aspartic acids showed an opposite trend. To corroborate further on the metabolic changes associated with ripening, a two class PLS-DA model was built, identifying the features more influential for the model.

Keywords: Food metabolomics / Gas chromatography / Electron ionization / *Persea americana* / Principal component analysis / Partial Least Squares-Discriminant Analysis

11.1. Introduction

Avocado, the fruit of the subtropical tree *Persea americana*, native to Central America and Mexico, is nowadays grown and consumed in many other parts of the world [1]. Due to the high economic importance of avocado, the fruit industry is showing a remarkable interest in processing and enhancing the value of this crop. The fruit is widely consumed as a food, the plant is also used for medicinal purposes and the oil is commonly utilized for manufacturing foodstuff, cosmetics, and health care products [2, 3]. Apart from its particular sensory properties, the consumption of avocado-derived products is getting considerable attention due to its high nutritional value and reported health benefits [2, 4-7]. These positive health-related effects are probably linked to its content of essential nutrients and various bioactive compounds; however, the global composition of this fruit has not been deeply studied yet. Avocado can be considered as a proper source of sugars,

amino acids, carbohydrates, carotenoids, tocopherols, proteins, phenols, vitamins, sterols, fatty acids, etc. [3, 8-13]; however, some work is still needed to achieve the comprehensive metabolic characterization of avocado and to facilitate the understanding of its biochemistry and the biochemical basis of its healthy properties.

This knowledge could be very useful as well to identify the horticultural maturity of avocado. One of the unique aspects of the biology of avocado is the way it matures on the tree. The avocado fruit does not ripen until harvested [14] and may be retained on the tree for several months after reaching physiological maturity (i.e. ability to ripen) [15]. Hodgkin demonstrated that eating quality of avocados improved over the harvest period [16, 17], fact which could be related to an increasing oil content. Measuring oil content of avocados is time consuming and difficult; therefore, other strategies are nowadays used to rate the avocado fruit quality, such as size, estimated oil content (dry matter), absence of defects, and firmness [18]. This topic has been extensively discussed, and the evolution of fatty acids, sugars, carbohydrates, or the content of some other analytes in avocado has been checked during ripening or storage by several research groups [8, 19-23], however, the optimum strategy to identity maturity has not been described so far.

Within this context, it is important to stand out that the use of metabolomics technologies is opening up great expectations for a wide range of biological targets, and food applications related to quality and nutrition are rapidly emerging [24]. The availability of cutting-edge technologies makes that researches rather focus on the determination of the entire metabolome of a biological system than use selective targeted approaches. This is a fundamental shift from the characterization of specific classes of plant primary or secondary metabolites [25-28]. Significant advances have been accomplished in agriculture and food research to distinguish and characterize related materials through the combined use of advanced analytical chemistry techniques to acquire metabolic profiles with different kinds of statistical tools to extract relevant information from the dataset [29-33]. Practical steps in chemometrics analysis include the experiment design, data pre-processing and data analysis [34-37], and they all are very crucial to assure the achievement of a proper biological interpretation of the system under evaluation.

GC was probably the first analytical technique used in the field of metabolomics [38], even before that this concept started to be used. Since then, GC still remains as one of the most important tools in this area, being its chromatographic resolution and the possibility of being easily coupled to MS some of its most remarkable advantages. Most of the commercial GC-MS systems use EI and CI as ionization sources, although APCI has been used in some applications over the last years [39-41]. Some of these applications have tried to demonstrate that GC-APCI-TOF MS can be a real alternative to conventional GC-MS; however, the existence of just one spectral database for APCI [42], in contrast with the comprehensive databases (with automatic search and containing a huge number of compounds) available for EI sources represents an important drawback.

The aim of this work was to evaluate biological changes in the metabolic composition of avocado fruits (coming from 13 varieties at 2 diverse ripening degrees) by using a GC-EI-Q MS methodology and a non-targeted profiling approach. The data were studied using chemometrics tools, which included pre-processing, dimension reduction and statistical analysis. To the best of our knowledge this is the first time in which a very unique avocado sample selection –as the one used here– undergone these kinds of analyses.

11.2. Materials and methods

11.2.1. Chemicals and standards

BSTFA + 1% TMCS from Pierce (Rockford, IL, USA) was used as derivatization reagent in freshly opened 1 ml vials. MeOH was chosen as solvent to prepare sample extracts and it was purchased from Panreac (Barcelona, Spain). Taxifolin was used as IS to evaluate the reproducibility of the extraction system and the chromatographic runs, and it was purchased from Extrasynthese (Lyon, France).

11.2.2. Samples, extraction and derivatization reaction

The samples selected in this study were part of the germplasm collection from the IHSM La Mayora (CSIC, Algarrobo-Costa, Málaga, Spain). Fruits from 13 different varieties ('ColinV 33', 'Gem', 'Harvest', 'Hass', 'Hass Motril', 'Jiménez 1', 'Jiménez 2', 'Lamb Hass', 'Marvel', 'Nobel', 'Pinkerton', 'Sir Prize' and 'Tacambaro') were grown

under identical environmental conditions, and harvested at two ripening degrees (fruits just harvested and fruits ready for consumption).

The pulp of 2-3 pieces of fruit was homogenized, frozen and freeze-dried to prepare sample extracts using the protocol previously described by Hurtado-Fernández et al. [10, 43]. Briefly, 4 g of the freeze-dried (and homogenized) sample were mixed with 40 mL of pure methanol and 50 ppm of IS, using a vortex for 30 min. The supernatants were taken and centrifuged (at 3000 rpm for 10 min), afterwards, they were evaporated to dryness and redissolved in 5 mL of MeOH. Aliquots of 25 µL of this methanolic extract were taken, evaporated and redissolved in the proper volume of derivatization reagent.

The derivatization reaction of the avocado extracts was carried out by adding 50 µL of BSTFA + 1% TMCS to a dried aliquot of the sample and incubating at room temperature for 60 min.

A QC sample was used to assure the stability of the system; it was made by mixing equivalent volumes of the extracts of each variety of avocado fruits and it was injected every 5 samples throughout the batch.

11.2.3. Analytical instrument

The chromatographic separations were carried out in an Agilent 7890A GC system (Agilent, Palo Alto, USA). The system was equipped with a HP-5-MS column (30 m, 0.25 mm ID and 0.25 µm film thickness) and coupled to a MS detector. Mass spectrometer was a single Q (5975C inert XL MSD with a triple Axis detector) coupled to GC by EI, which operated at a potential of 70 eV and the ion source temperature was set at 230ºC. The acquisition mode selected was scan and the analyses were performed from m/z 40 to 750 mass units. The temperature of the MS Quad was set at 150ºC.

Automated injection of aliquots of the derivatized samples (1 µL) was done using a programmable MultiPurpose Sampler MPS2 (Gerstel, Mülheim an der Ruhr, Germany). A septumless CIS PTV injector was used to carry out the injections in splitless mode. The purge time of the PTV injector was 60 s, using a purge flow of 3 mL/min and setting the temperature at 250ºC. Sequential washing steps of the 10 µL syringe were included before and after each injection, as well as a step of sample pumping to remove possible small air bubbles. Helium was used as carrier gas at a constant flow rate of 1 mL/min.

The temperature of the column varied according to the following gradient: the initial column temperature was kept at 140ºC for 5 min and then it increased at a temperature rate of 3ºC/min until 220ºC, keeping that value for 1 min. Later on, the temperature raised from 220ºC to 241ºC at 10ºC/min and it was held for 1 min. Finally, the temperature was increased from 241ºC to 310ºC at 3ºC/min, maintaining that temperature for 5 min.

11.2.4. Data pre-processing and statistical analysis

Deconvolution and Identification Software (AMDIS) (included with NIST mass spectral database) was used for data pre-processing. The mass and compounds filters were adjusted so that the software could identify as many metabolites possible with a run. Mass Profiler Professional (version 2.1.5) was used for statistical analysis. The data pre-processing and data analysis steps were as follows: setting the importation filters and alignment parameters, selecting normalization criteria, defining the sample groups, setting data filters, and evaluating data clustering with PCA. Once these steps were completed, the data were evaluated through statistical tools such as fold-change and significance analysis. The final analysis steps were to construct and test a classification model by using PLS-DA. Further data processing included identification of the compounds used in the model by the mass spectral database search and assignment of a tentative identity. Search was mainly made in the following GC-MS libraries: NIST 2011 EI Mass Spectral Library, Wiley Registry™ 2007 Edition Mass Spectral Library and the Fiehn Lib Metabolite GC/MS Library 2009.

11.3. Results and discussion

11.3.1. Fruit metabolite screening by GC-EI-Q MS

At this point, we proceeded to analyze the avocado extracts which were prepared from the samples coming from 13 different varieties at 2 diverse ripening stages. ***Figure 11.1*** shows the complexity of one of the GC-EI-Q MS profiles obtained.

As stated before, we were using a non-targeted exploratory analysis, fact which means that in principle, to find the identity of the metabolites detected in the profiles was not a priority. However, the advantage of using an instrument such as the one selected in this chapter is that EI spectra allow library searching and provide fragmentation data; this

together with the previously published results [44] gave us the chance to identify about 80 metabolites (see ***Table 11.1***).

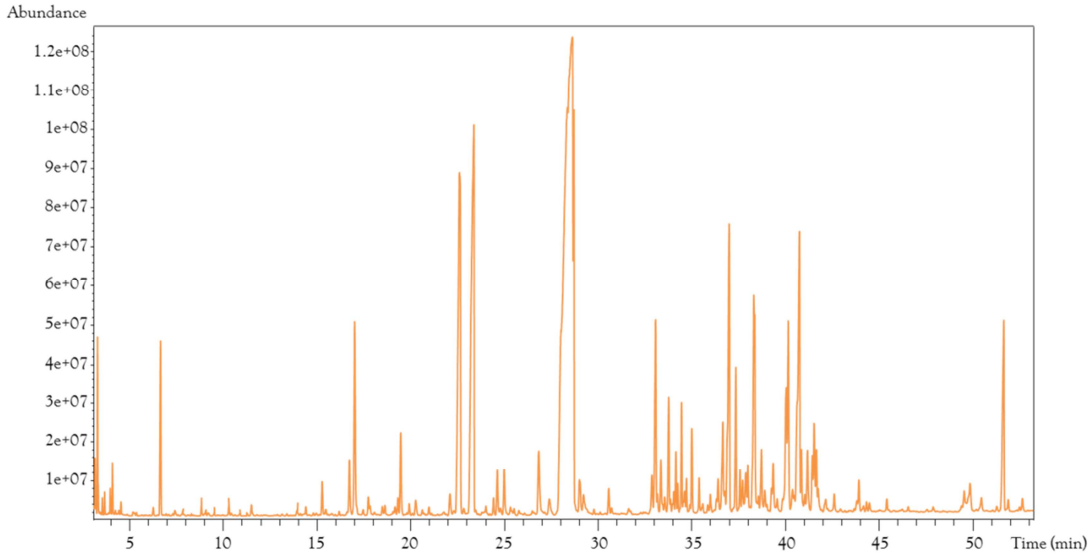

Figure 11.1. Metabolic profile of avocado Hass *cv.* obtained by GC-EI-Q MS.

In ***Table 11.1*** it is possible to see the metabolites identified within this matrix, it includes the retention time, compound name, *m/z* signal of the completely silylated form and fragments detected for the identified compounds. Having a look at the identity of the analytes included in ***Table 11.1***, one can understand that the described method was useful to get information about amino acids, fatty acids and related compounds, sugars and derivatives, phenolic acids and related compounds, flavonoids, organic acids, sterols, vitamins, and nucleosides and nucleobases, etc.

Table 11.1. Compounds identified in avocado samples by GC-EI-Q MS.

t_r (min)	Compound	m/z completely silylated form	Qual	Fragments
3.3	Glycerol – 3H + 3 TMS + H	308	91	218/205/147/73
3.5	L-isoleucine – 2H + 2TMS + H	275	43	260/218/158/147/73
3.6	-aminobutyric acid – 2H + 2TMS + H	247	91	232/147/102/73
3.7	L-proline – 2H + 2TMS + H	259	76	216/142/73
3.8	Succinic acid – 2H + 2 TMS + H	262	98	247/218/147/73
4.1	Glyceric acid – 3H + 3 TMS + H	322	96	322/292/189/147/73
4.2	Uracil – 2H + 2TMS + H	256	80	256/241/147/99/73

Table 11.1. (continued)

t_r (min)	Compound	m/z completely silylated form	Qual	Fragments
4.4	Nonanoic acid – H + 1 TMS + H	230	66	215/129/117/73
4.5	L-serine – 3H + 3 TMS + H	321	91	306/278/218/204/73
4.7	Pipecolic acid – 2H + 2TMS + H	273	96	230/156/133/73
5.1	L-threonine – 3H + 3 TMS + H	335	93	320/291/218/147/73
6.0	Dihydroxybutyric acid – 3H + 3 TMS + H	336	91	321/233/189/147/73
7.4	Malic acid – 3H + 3 TMS + H	350	99	335/233/189/147/73
8.2	L-threitol – 4H + 4 TMS + H	410	93	307/217/204/147/73
8.3	Pyroglutamic acid – 2H + 2TMS + H	273	98	273/258/230/156/73
8.3	L-aspartic acid – 3H + 3TMS + H	349	95	349/232/218/147/73
9.3 9.7	L-threonic acid – 4H + 4 TMS + H	424	91 94	292/220/205/147/73
10.0	Hydroxyglutaric acid – 3H + 3 TMS + H	364	90	349/247/147/129/73
11.2	4-hydroxybenzoic acid – 2H + 2 TMS + H	282	96	282/267/223/193/73
11.3	L-glutamic acid – 3H + 3 TMS + H	363	99	363/348/246/128/73
15.1	Tridecanoic acid – H + 1 TMS + H	286	99	286/271/117/73
15.4	Aconitic acid – 3H + 3TMS + H	390	99	390/375/229/147/73
15.6	Vanillic acid – 2H + 2 TMS + H	312	90	312/297/267/223/73
16.2	Glycerol phosphate – 4 H + 4 TMS	460	94	445/357/299/147/73
17.5	Protocatechuic acid – 3H + 3 TMS + H	370	60	370/355/193/73
17.8	D-(–)-fructofuranose I – 5H + 5 TMS + H	540	91	437/217/147/73
18.0	Citric acid – 4H + 4 TMS + H	480	93	465/363/273/147/73
18.3	D-(–)-fructofuranose II – 5H + 5 TMS + H	540	76	437/217/147/73
18.8	Adenine – 2H + 2 TMS + H	279	99	279/264/192/73
20.5	Glucose – 5H + 5 TMS + H	540	94	217/204/191/147/73
21.0	*p*-coumaric acid – 2H + 2 TMS + H	308	99	308/293/249/219/73
21.2	Pentadecanoic acid – H + 1 TMS + H	314	49	299/201/117/73

Table 11.1. (continued)

t_r (min)	Compound	m/z completely silylated form	Qual	Fragments
21.4	L-tyrosine – 3H + 3 TMS + H	397	45	280/218/73
22.0	D-mannitol – 6H + 6 TMS + H	614	93	319/205/147/103/73
23.2	Pantothenic acid – 3H + 3 TMS + H	435	92	420/291/157/103/73
23.6	Palmitoleic acid – H + 1 TMS + H	326	99	326/311/145/117/73
24.3	Palmitic acid – H + 1 TMS + H	328	99	328/313/145/117/73
25.8	Ferulic acid – 2H + 2 TMS + H	338	48	338/308/249/73
27.1	Heptadecanoic acid – H + 1 TMS + H	342	62	327/129/73
29.1	Linoleic acid – H + 1 TMS + H	352	99	352/337/129/95/73
29.3	Oleic acid – H + 1 TMS + H	354	99	354/339/145/117/73
29.4	*cis*-vaccenic acid – H + 1 TMS + H	354	94	354/339/145/117/73
30.0	Stearic acid – H + 1 TMS + H	356	99	356/341/117/73
34.5	11-eicosenoic acid – H + 1 TMS + H	382	95	382/367/145/117/73
35.6	Uridine – 4H + 4 TMS + H	532	50	517/315/217/147/73
38.2	-glyceryl palmitate – 2H + 2 TMS + H	474	90	459/371//239/147/73
39.4	Adenosine – 4H + 4 TMS + H	555	99	540/236/217/147/73
40.3	Sucrose – 8H + 8 TMS + H	918	90	437/361/217/147/73
41.6	Monooleoylglycerol – 2H + 2 TMS + H	500	93	485/397/203/129/73
44.0	Epicatechin – 5H + 5 TMS + H	650	93	650/368/355/267/73
52.7	-sitosterol – H + 1 TMS + H	486	99	486/396/357/129/73

* Signal underlined means the prevalent ion in the MS spectrum.

Although it is possible to visually distinguish the chromatograms obtained for samples from different variety or ripening stage and compare them, the process is tedious, subjective and non-quantitative. In addition, minor differences between very closely related compounds might be missed if the analyst proceeds in this way. These drawbacks are taken into account by multivariate analysis. The chemical pattern recognition methods, such as PCA and SIMCA, are now greatly appreciated for providing reasonable characterization of plant or food extracts.

11.3.2. Exploratory analysis of avocado metabolites by GC-EI-Q MS

Despite all the efforts to get rid of the unwanted analytical variability in the final data, this can be only minimized. Pre-treatment and pre-processing usually include normalization, scaling, baseline correction, peak alignment, and other methods used on the dataset to make samples analyzable and comparable. A common lost in repeatability is the drift in peak position in the chromatograms, which can be caused by the sample matrix, column ageing, or some other factors. In chromatography-MS, a combination of alignment and peak picking is most regularly used. Normalization is a step that removes the variation between data from different samples related, for instance, to the sampling procedure; in other words, removes the variability which is not coming from the samples themselves [35].

In the current case, the alignment was based on spectral pattern and retention time. The extracted spectra needed to have a cross correlation factor of 0.6 and a retention time match of 0.05 min to be considered the same component. The huge number of features detected in the evaluated samples was reduced based on frequency of occurrence in the samples, analysis of the variance, and abundance in the respective classes of the samples at different stages of the data treatment. The frequency filter reduced the number of tentative markers to 158; these unique entities were selected to carry out the statistical analysis. Numerous features occur only once or twice and were removed during the data filtering step.

Afterwards, the amount of fold-change (increase) in the concentration of any given compound was determined. This analysis identifies entities with large abundance differences between the data classes, that is, those that differed in concentration by two fold, three fold, four fold, and so forth between green and mature samples. The fold change was set at ≥ 4 [log 2(FC) ≥ 2]. Next, ANOVA was used to determine if the differences between those compounds that met the fold-change criteria were statistically significant. Using a probability p value of ≤ 0.05, the 158 entities from the frequency filter were reduced to 17 significant compounds. The compounds with the lowest p values and highest fold changes were selected for further stages of the analysis; the 17 selected metabolites were used to perform a PCA and clustering analysis by k-means method.

Figure 11.2 shows the PCA-3D plot, where it is possible to observe the general structure of the dataset and the clustering of the samples. This analysis was made considering four principal components, which explained 76.35 % of the total variance. Unripe samples were separated from ripe ones mainly on the basis of X axis. However, the metabolic variability among the different varieties at the same ripening stage was explained by Y and Z-axis. The first component (X axis) explained 53.14% of the variance; while the first two principal components (X and Y-axis) explained 62.69%.

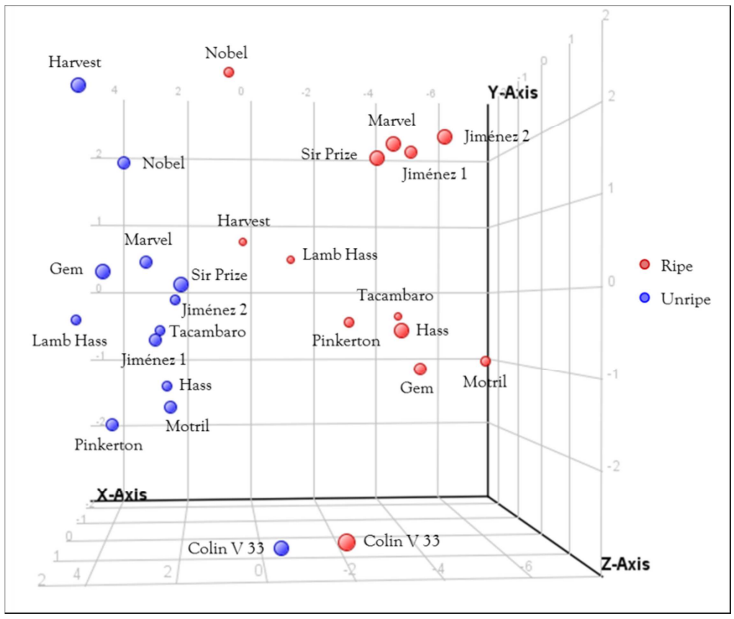

Figure 11.2. PCA-3D plot for the 13 avocado varieties at 2 ripening degrees.

Table 11.2 includes a list where the influence of each variable considered to carry out the PCA is showed.

Tabla 11.2. List of the compounds responsible of the natural grouping of the samples under study after apply PCA and its influence in each principal component.

t_r	Compound	PC1 (53.14%)	PC2 (9.65%)	PC3 (7.64%)	PC4 (5.97%)
4.7	Compound 1	- 0.160	0.212	0.318	0.374
8.3	Compound 2	0.157	- 0.010	0.528	- 0.187
9.7	Compound 3	0.199	0.087	0.407	0.168
12.3	Compound 4	- 0.109	0.504	- 0.008	0.092
17.0	Compound 5	0.170	- 0.045	0.452	- 0.267
21.0	Compound 6	- 0.219	0.088	- 0.025	0.268

Tabla 11.2. (continued)

t_r	Compound	PC1 (53.14%)	PC2 (9.65%)	PC3 (7.64%)	PC4 (5.97%)
23.1	Compound 7	0.269	0.217	- 0.064	0.035
25.1	Compound 8	0.259	- 0.120	- 0.053	0.292
26.1	Compound 9	0.278	0.176	0.002	0.014
26.4	Compound 10	0.276	0.091	- 0.022	- 0.024
26.7	Compound 11	0.236	0.020	0.068	0.045
27.5	Compound 12	0.256	- 0.073	- 0.061	0.235
28.5	Compound 13	0.192	- 0.135	- 0.359	0.053
29.2	Compound 14	0.200	0.275	- 0.065	- 0.216
30.8	Compound 15	0.249	0.304	- 0.114	- 0.081
31.8	Compound 16	- 0.157	0.034	0.221	0.400
34.5	Compound 17	0.215	- 0.188	- 0.051	0.400

Values highlighted in red are the most influential metabolites in each component.

As stated before, it was clear that the force driving the separation among the samples under study in the 3D-PCA plot was the ripening state. To study further the evolution of the levels of the 17 variables selected for the classification, we calculated the clustering achieved by k-means method, resulting in three clustering models: the metabolites whose concentration remarkably increases during ripening; those that decrease their levels as the fruit ripens; and the compounds with more stable concentration.

Table 11.3 summarizes the information of the clustering models, including the retention time and the compound belonging to each cluster. The information provided by this type of analysis is quite interesting since it allows us to uncover the most prominent patterns in the data, letting the analyst test for entities displaying either similar or mirror-image abundance profiles. Indeed, interesting relationships may be revealed, because entities that exhibit similar behavior across a set of experimental conditions may share similar reaction pathways.

Table 11.3. Clustering groups achieved after applying a k-means method.

Cluster	t_r	Compound
Cluster 0: Compounds with decreasing concentration over ripening	8.3	Compound 2
	17.0	Compound 5
	25.1	Compound 8
	26.1	Compound 9
	27.5	Compound 12
	34.5	Compound 17
Cluster 1: Compounds with increasing concentration over ripening	21.0	Compound 6
	31.8	Compound 16
Cluster 2: Compounds with more stable concentration	4.7	Compound 1
	9.7	Compound 3
	12.3	Compound 4
	23.1	Compound 7
	26.4	Compound 10
	26.7	Compound 11
	28.5	Compound 13
	29.2	Compound 14
	30.8	Compound 15

The next step was to try to accomplish the identification of the 17 metabolites used in the PCA and clustering analysis. While it is not necessary to know their identity, identification could lead to an improvement understanding the ripening mechanism of this tropical fruit.

Table 11.4 encloses the information about the identification of the 17 compounds, including the retention time, the automatically generated prediction obtained by using the NIST 2011 Database (searching as well in Wiley and Fiehn libraries), the m/z signal of the completely silylated form, fragmentation patterns, and Qual (confidence in the identification). There is a column close to the one which includes the NIST prediction,

where we indicate the identification that we consider as correct. In some cases, it is the same as predicted by the databases (may be just changing the name in order to give a simpler and more intuitive one), but in others, we suggest a different metabolite. This alternative identity is given when the predicted identity does not seem plausible (with very low Qual or chemical inconsistencies (metabolites not derivatized, unlikely present in the chromatogram with the retention time that they have, not likely present in avocado fruit, etc.)). In these particularly challenging cases, we carried out a more careful search, extracting the background, studying the fragmentation patterns more in depth, taking into account the Kovats indices, etc.. It has been possible to identify 10 of the 17 selected compounds.

Tabla 11.4. Identification of some of the 17 most influential metabolites of avocado fruit in the classification of the samples under study.

t_r	#	Identification	*m/z* complete silylated form	Fragments	Qual
4.7	Compound 1	Pipecolic acid – 2H + 2TMS	273	273/230/156/147/73	98
8.3	Compound 2	Aspartic acid – 3H + 3TMS	349	349/232/218/147/73	97
9.7	Compound 3	Threonic acid – 3H + 3TMS	424	292/220/205/147/73	95
12.3	Compound 4	Compound 4	–	–	–
17.0	Compound 5	Compound 5	–	–	–
21.0	Compound 6	*p*-coumaric acid – 2H + 2TMS	308	308/293/249/219/179/147/73	99
23.1	Compound 7	Compound 7	–	–	–
25.1	Compound 8	Compound 8	–	–	–
26.1	Compound 9	Mannoheptulose – 6H + 6TMS	642	539/373/217/204/191/147/73	–
26.4	Compound 10	Heptadecenoic – H + 1TMS	340	340/325/145/129/117/73	96
26.7	Compound 11	Compound 11	–	–	–
27.5	Compound 12	Compound 12	–	–	–
28.5	Compound 13	Abscisic acid – 2H + 2TMS	409	409/319/259/217/205/174/73	–
29.2	Compound 14	Linoleic acid – H + 1TMS	352	352/337/262/129/95/73	99
30.8	Compound 15	D-sorbitol – 6H + 6TMS	614	319/307/217/205/147/103/73	90
31.8	Compound 16	–	–	–	–
34.5	Compound 17	Eicosenoic acid – H + 1TMS	382	382/367/292/145/129/117/73	97

Two of the most influential metabolites in the first principal component were the compounds 6 and 9, which have been identified as *p*-coumaric acid and mannoheptulose, respectively. The mentioned hidroxycinnamic acid increased its concentration during the ripening (cluster 1), whilst the carbohydrate showed the opposite behavior (its concentration tended to decrease with the ripening process (Cluster 0)). Some authors have previously found the same trend for both metabolites. In the case of *p*-coumaric acid, it has been identified as a potential ripening marker (not just in the case of avocado fruit, but also in other fruits) [13, 23, 46, 47]. The decrease of mannoheptulose –together with other C7 sugars– during the ripening, as well as during the storage of avocado fruit, has been also reported by several researchers [48-50], who hypothesized that it may play an important role on the metabolism and fruit-ripening process. It has been study the possible role of the C7 sugars as inhibitors of the ripening, considering the fact that avocado does not ripen till the fruit is detached from the tree [51]. Heptadecenoic acid was one of the other metabolites with high factor loadings regarding PC1, compound which, in general terms, showed a quite stable concentration over the ripening process.

In relation to the second principal component, the metabolites pointed out as the most relevant were heptadecadiene and eicosenoic acid (compounds 4 and 17, respectively). Eicosenoic acid was clustered in the group of compounds with decreasing concentration as the avocado fruit ripens. What we have found for eicosenoic acid is that it has been previously described for other fruits such as ackee fruit [52], where the total fatty acids showed a decline with ripening in different fruit tissues.

The analytes which seemed to have more influence in the third and fourth principal components were the compounds 2 and 13 –identified as aspartic and abscisic acids–, and, compound 5, compound 16 and eicosenoic acid (compound 17), respectively. Aspartic acid was clustered in the group of compounds that decrease with the ripening process (cluster 0). This trend observed for aspartic acid is, in principle, opposite to the one found for tomato during on-vine ripening, where an increase of concentration was observed for the major hexoses, cell wall components, and some amino acids (such as aspartic acid) [53]. Abscisic acid was one of the compounds included in cluster 2, meaning that its concentration did not fluctuate remarkably during the ripening. However, it has been observed that, in general, the levels of this phytohormone tend to

be higher in developing avocado fruits and decrease over the course of fruit growth, probably because of the oxidative catabolism [54].

11.3.3. Classification model

Classification aims to produce general hypotheses that are described by several variables and identified by known labels corresponding to the class information. Samples are assigned to groups considering a prediction model that determines the classification taking into account certain entities that have already been identified. There are different tools to perform a class prediction model, being PLS-DA one of the most used.

The unsupervised nature of the PCA algorithm provides a means to achieve unbiased dimensionality reduction, thus it offers an informative first look at the dataset structure and relationships between groups. However, supervised pattern recognition methods, such as PLS-DA, rely on the class membership of each observation and they are used to discriminate between two classes and assess possible predictors belonging to each class. PLS-DA is commonly applied in metabolomics studies and, if it is guided by well-separated PCA scores, it has greater probabilities of producing relevant results [45].

In this work, we have built a two class PLS-DA model using the degree of ripening as class characteristic. PLS-DA is used to sharpen the partition between groups of observations, obtaining the maximum possible separation between the classes. The first step was to train the model with the data, and afterwards the model had to be tested and validated. The accuracy of the model was 92.3%. These results demonstrate the feasibility of developing a model to accurately predict whether an avocado sample belongs to ripe or unripe class. However, this separation is quite obvious, not being necessary to have a prediction model to assure the correct classification, what we wanted to address was the identification of the possible classifiers (most influential variables for the model).

11.4. Conclusions

Continuous efforts are being made to get a global overview of the composition of what we eat. The aim of enlarging this knowledge is double: to understand their bioactivities and possible healthy effects and to enhance product quality control. It is in the latter context where the application that we present here fits. Avocado fruit is quite peculiar because of the way in which it matures on the tree and also because it is highly

perishable compared to other products. A reliable determination of its maturity is very difficult; any of the strategies used so far is optimal. In an attempt to improve the knowledge about the biochemical metabolic changes that avocado fruit undergoes during ripening, we present the application of a GC-EI-Q MS method. This platform has been considered sometimes as obsolete when compared with those with other powerful analyzers; however, we demonstrate here that GC-EI-Q MS together with the use of statistics (data pre-processing and PCA, clustering analysis and PLS-DA) has given very valuable information, being possible to obtain the identification of metabolites very influential in the classification of the samples under study. Some of these compounds were *p*-coumaric, eicosenoic, aspartic and abscisic acids, and mannoheptulose. *p*-coumaric was the only of the mentioned analytes increasing its concentration over the ripening, whilst mannoheptulose, eicosenoic and aspartic acids showed an opposite trend. Despite the good results achieved, there is still some work to do to get an overall overview and understanding about fundamental biochemistry of avocado and the biosynthesis of its relevant metabolites.

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Chapter 12

Existing complementarity between different analytical techniques used to perform metabolomics studies

Nowadays the use of a single analytical platform does not allow the analysis of each and every one of the metabolites present in a complex matrix, due to the numerous compounds that it could contain, which besides present diverse physical and chemical properties. For this reason, the application of different analytical techniques is recommendable to achieve a deep and global knowledge about the whole metabolome of a fruit or vegetable, taking advantage of the strengths of each technique [103]. Vibrational spectroscopies, such as IR, provide a detailed and reproducible spectrum that will reflect the molecular composition of the analyzed sample, but assignment of IR bands to individual compounds is not normally possible. UV and PDA also provide reproducible and characteristic spectrum but only chromophore bearing compounds are detectable. The UV spectrum gives useful structural information, but often indicates the metabolite class rather than its exact identity. The requirement of unambiguous annotation essentially limits the metabolic profiling to NMR spectroscopy and MS-based approaches. HR-NMR spectroscopy allows the detection of a wide range of structurally diverse metabolites simultaneously. It is a nondestructive and inherently quantitative technique that requires relatively simple sample preparation and fast data acquisition per analysis. Other advantages of NMR include robustness and high reproducibility. Its major drawback is its poor sensitivity compared to MS. MS-based techniques have a wide dynamic range and high selectivity and sensitivity but require molecules to be ionized (charged). The largest disadvantage of MS in comparison with NMR is its lower reproducibility. MS-based approaches usually require extensive sample preparation, which can cause metabolite losses, and depending on the ionization technique, metabolites may be differentially ionized. Combination with a separation technique reduces the complexity of the mass spectra and provides additional information on the physico-chemical properties due to the time dimension added [104, 105]. Despite good separation, highly complex extracts, can still be prone to significant ion suppression/matrix effects. This, together with variable ionization frequencies, makes MS-based quantification more difficult and totally dependent on available reference standards.

In this context, GC-MS can provide a comprehensive analysis of a large variety of metabolites, but requires them to be volatile. This requirement is readily accomplished by chemical derivatization, but at the cost of additional time, processing, and variance.

Compared to GC, LC does not require sample volatility, simplifying sample preparation, and is able to detect simultaneously thousands of metabolites. Whilst metabolite separation in GC and LC is based on their interaction with the stationary phases, in CE it is based on their mass to charge ratio providing different information on the chemical properties. CE provides sensitivity, separation efficiency and smaller sample and solvent volumes. The main disadvantages of CE are the larger variation in migration time and peak shapes and its technical limitations for the analysis of non-polar metabolites.

To illustrate the complementary of hyphenated platforms, such as reversed-phase LC, GC and CE coupled to UV and MS, ***Figure x.1*** shows the metabolic profiles obtained for different avocado samples and the chemical classes identified with each one.

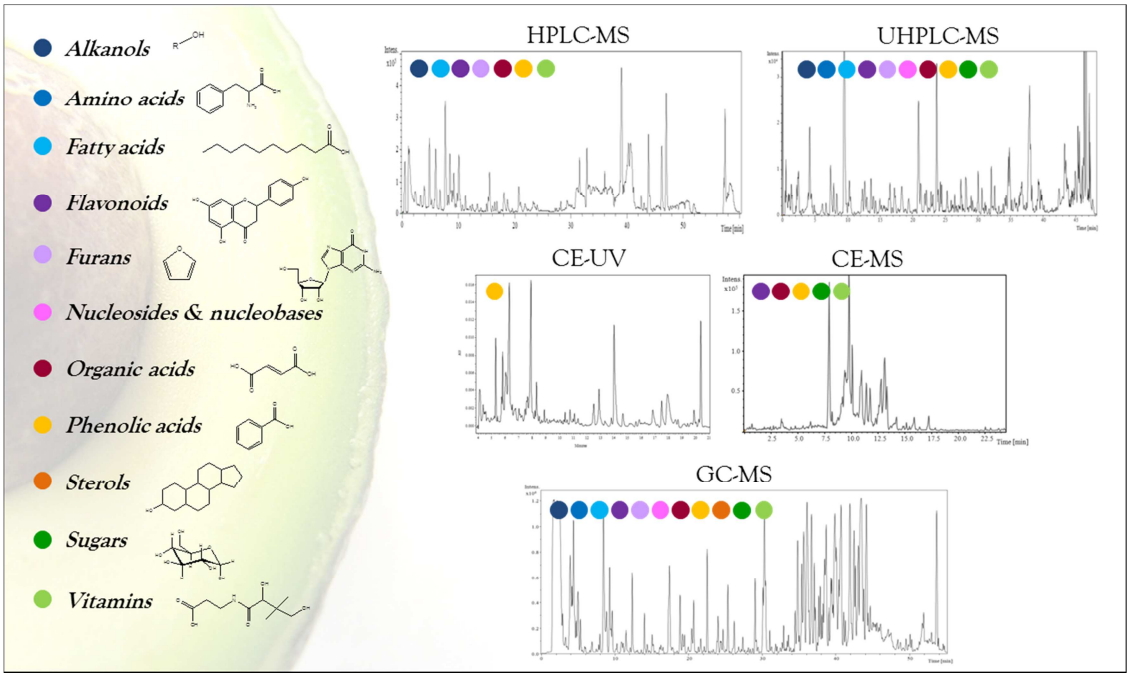

Figure 12.1. Example of the metabolites identified in avocado samples by using different analytical techniques.

Combining the information obtained by HPLC-MS, UHPLC-MS, CE-MS, CE-UV, and GC-MS methods developed (using targeted and/or non-targeted approaches), we obtained multiple levels of information useful for structural elucidation, which enabled the identification and quantification of a comprehensive set of metabolites commonly present in the avocado pulp, at the same time that allowed us to assess the relative strengths and weaknesses of the hyphenated platforms concerning the analysis of the avocado extracts.

CE-UV/MS were the least informative platforms in terms of the number of metabolites tentatively identified: all of the compounds assigned in the CE profiles could also be identified using LC or GC. However, polar metabolites are not well retained and separated on reversed-phase columns, making CE a powerful and complementary technique for the analysis of such metabolites in short analysis times. Concerning reproducibility, migration times shifted more in CE-MS than the retention times in UHPLC-MS, with RSD values smaller than 11.45% and 7.01%, respectively. The UHPLC-MS showed smaller LODs in the negative mode for the same phenolic compounds quantified by CE-MS. The smaller sensitivity of CE-MS might be explained considering the dilution of the sample by using the coaxial sheath flow liquid and the smaller injection volume.

As can be seen in ***Figure 12.1***, peaks in the UHPLC profile are sharper and better resolved in a shorter analysis time than in the HPLC one; ion suppression was greatly minimized in this way, which improved the ability to identify and quantify a wider number of compounds/classes of compounds. Thus, it appears that UHPLC may be the preferred choice among the liquid separation techniques for metabolic profiling. GC-MS and UHPLC-MS provided the detection of the largest sets of compounds belonging to a bigger number of metabolite classes. The GC-MS method provided the detection of low-molecular-weight metabolites (including amino and organic acids, phenolic compounds, sterols or fatty acids, among others) with a boiling point low enough after chemical derivatization to allow their elution from the GC column. The UHPLC-MS reversed-phase method provided the complementary detection of higher-molecular-weight compounds of medium-to-high lipophilicity from the same classes mentioned above, with the exception of sterols; on the contrary, glycosides of phenolic compound could not be detected by GC.

It should be noted that, even though platforms such as GC or UHPLC coupled to MS are able to detect thousands of compounds, they cannot provide a comprehensive overview of the whole metabolome when used independently, and hence, it is becoming common practice the use of several analytical platforms to combine the information obtained and meet the demanding requirements of metabolomics.

CONCLUSIONS/ CONCLUSIONES

CONCLUSIONS

1. Several analytical methodologies have been successfully optimized, validated and applied to perform different metabolomics studies –mainly using metabolic profiling approaches– of avocado fruits.

2. More than 200 metabolites have been identified in a total of 17 different avocado varieties. The determined compounds belong to several chemical families such as fatty acids, aminoacids, sugars, organic acids, vitamins, phytohormones, phenolic acids, flavonoids, alkanols, furans, nucleosides and nucleobases, sterols, etc.

3. The applicability of a CE-UV method for the identification and quantification of the phenolic acids present in 13 different avocado samples at two ripening degrees has been demonstrated. The influence of the ripening process on the concentration of these metabolites has been evaluated, showing that HCA derivatives –which increase as fruit ripens– are the main responsible of the differences between ripening stages. However, it has been observed that HBA derivatives play a crucial role when a classification among avocado varieties is intended.

4. The metabolic evolution of 10 specific polar compounds has been studied in 18 samples from Reed avocado *cv.* which were picked at different moments of the harvest season (over 3 months). The analytes under study were carefully selected taking into account their presence (not only in the chosen variety) and their abundance. CZE coupled to MS has been used; MS operated both in *Full Scan* and *MRM*. The majority of the metabolites (8) have shown a decrease of its concentration as the picking date is closer to the end of season. When the analysis of the same samples was carried out by LC-ESI-TOF MS, it seemed particularly interesting the remarkable variation of the concentration of some unknown metabolites; two of them have been tentatively identified as two phenolic acids-related sugars.

5. A powerful HPLC-DAD-ESI-TOF MS method has been developed to go deep into the knowledge about avocado composition, in terms of phenolic compounds (phenolic acids and flavonoids), of 3 avocado varieties. The potential of this methodology has been proved, identifying unequivocally a total of 17 metabolites and other 25 compounds, tentatively, in a single run.

6. A quite ambitious metabolic profiling approach has been used for the identification of as many compounds as possible with the avocado fruit by UHPLC-UV/ESI-TOF MS. After the complete optimization and validation of this sensitive method, it was successfully applied for the identification of around 200 metabolites (being certain about the presence of 60 and quantifying 20 of them). The use of multivariate statistics analysis has been very useful to pinpoint a set of compounds differentially regulated during the process of ripeness of 13 varieties of avocado fruit.

7. The identification of horticultural maturity for avocado and the best harvesting time is not a simple task, as maturation is not accompanied by changes in external appearance. Usually, oil content and dry matter in the flesh are controlled to determine when the adequate picking time is. Trying to offer some other alternatives, an interesting study has been carried out to evaluate those changes produced in avocado composition depending on fruit harvesting date. Four varieties were selected and, from them, a proper number of fruits were collected at different time points over their season. The extracts coming from these samples have been studied by applying a HPLC-UV/FL/ESI-IT MS method that allows a comprehensive metabolic profiling of this tropical fruit.

8. A new coupling of GC and MS through an APCI source -typical interface for LC- has been tested and applied, together with a GC-FID method, to determine quantitative changes in the metabolic profile of *Persea americana*. Both detectors have been compared considering both their analytical parameters and their quantitative results. GC-FID has shown better results in terms of precision but lower sensitivity and lower number of quantified metabolites. It can be considered as general statement –paying attention to the evolution of the studied metabolite concentrations– the fact that the concentration of organic acids, flavonoids and vitamins tend to decrease with the ripening process, whereas phenolic acids (HCA derivatives) usually increase their

concentration as the fruit ripens. Despite results for GC-FID are quite good, it is evident that it can not compete with GC-APCI-TOF MS, which, in contrast, allows an easier identification and an accurate quantification of a higher number of metabolites.

9. Taking into account the fact that the analytical platform GC-APCI-TOF MS had been scarcely used and the GC identification problems associated to the sample derivatization, it seemed very necessary building a metabolite database. A spectral library has been built, including information about retention indices, and MS and MS/MS spectra of a wide variety of compounds belonging to several chemical families, which can be frequently found both in food matrices and biofluids. When created, it contained approximately 150 metabolites, although it is continuously updated.

10. The tremendous potential of GC-APCI-TOF MS for metabolomics studies and the applicability of the recently developed database have been evidenced. The very first application of this analytical coupling for the qualitative analyisis of different avocado extracts has shown very promising results, achieving the identification of about 100 metabolites that belong to different chemical classes. These results have been compared with those achieved by using a consolidated analytical tool in all the routine laboratories, GC-EI-Q MS, showing that GC-APCI-TOF MS should be definitively considered as part of the "metabolomics toolbox".

11. Finally, it is important to point out that the avocado metabolome complexity makes extremely difficult to carry out the comprehensive characterization of this fruit by using a single omics technology. The different studies gathered in this Doctoral Thesis have highlighted that we can consider as mandatory to combine the use of several analytical methodologies to reach a deep knowledge about the composition of the matrix under study, due to the complementary information that they can provide.

CONCLUSIONES

1. Diversas metodologías analíticas han sido desarrolladas y aplicadas con éxito para llevar a cabo distintos estudios metabolómicos —principalmente determinación de perfiles metabólicos— en frutos de aguacate.

2. Se han identificado alrededor de 200 metabolitos en un total de 17 variedades de aguacate, matriz cuya composición era bastante desconocida hasta el momento. Los compuestos determinados pertenecen a diversas familias químicas como ácidos grasos, aminoácidos, azúcares, ácidos orgánicos, vitaminas, fitohormonas, ácidos fenólicos, flavonoides, alcanoles, furanos, nucleósidos y nucleobases, esteroles, etc.

3. Se ha demostrado la aplicabilidad de un método de CE-UV para la identificación y cuantificación de ácidos fenólicos presentes en 13 muestras de aguacate en dos estados de maduración distintos. La influencia de la maduración en la concentración de estos metabolitos ha sido evaluada, mostrando que los derivados del HCA —que aumentan conforme la fruta madura— son los principales responsables de las diferencias metabólicas observadas entre los estados de madurez. Sin embargo, se ha visto que los derivados del HBA juegan un papel más importante cuando lo que se persigue es lograr una discriminación entre diferentes variedades de este cultivo.

4. La variación del perfil metabólico de 18 muestras de aguacate de la variedad Reed (recogidas en momentos distintos de la campaña) ha sido evaluada llevando a cabo la determinación de 10 compuestos polares. Dichos analitos fueron seleccionados tras verificar su presencia en diversas variedades de aguacate y comprobar que se encontraban a niveles de concentración razonablemente altos. Para su determinación se recurrió a CZE acoplada a MS, detector cuyas prestaciones analíticas fueron evaluadas tanto en *Full Scan* como en *MRM* (aproximaciones no específica y específica respectivamente). La concentración de la mayoría de los metabolitos estudiados —8 de ellos— experimentó un descenso a medida que la fecha de recolección era más próxima

al final de la campaña. Las mismas muestras han sido analizadas mediante LC-ESI-TOF MS, encontrando como particularmente interesante la variación significativa de la concentración de 2 metabolitos a lo largo del proceso de maduración. Dichos compuestos han sido identificados tentativamente como dos ácidos fenólicos glicosilados.

5. Se ha desarrollado un potente método HPLC-DAD-ESI-TOF MS para conocer más en profundidad la composición de la fracción fenólica (considerando ácidos fenólicos y flavonoides) de 3 variedades de aguacate. El potencial de esta metodología se ha puesto de manifiesto al identificar, en un solo análisis, un total de 17 metabolitos inequívocamente y otros 25 compuestos de manera tentativa.

6. El potencial de una nueva metodología UHPLC-UV/ESI-TOF MS para llevar a cabo la caracterización más global y profunda posible del perfil metabólico del aguacate ha sido evaluado. Tras la exhaustiva optimización y validación de todos los parámetros analíticos de interés, el método ha sido exitosamente utilizado para la identificación de aproximadamente 200 metabolitos, estando seguros de la identidad de 60 compuestos, de los cuales 20 se han cuantificado. El uso de análisis estadísticos multivariante ha sido de gran utilidad para poner de manifiesto el comportamiento diferenciado de un grupo de compuestos durante el proceso de maduración de frutos de aguacate provenientes de 13 variedades distintas.

7. El momento preciso para recoger los frutos de aguacate es un parámetro que no es fácil de establecer y, en el que, como es evidente, agricultores y productores a gran escala están tremendamente interesados. Normalmente, se controlan tanto el contenido en aceite como la materia seca de la pulpa para intentar determinar cuándo es más adecuado llevar a cabo la recogida de los frutos. Tratando de ofrecer nuevas alternativas, se ha realizado un estudio muy interesante en el que se evalúan los cambios producidos en la composición del aguacate en relación al momento en que ha sido recogido. Se seleccionaron 4 variedades de aguacate y, de las mismas, se obtuvieron un buen número de muestras recogidas en distintos momentos de la campaña. Los extractos de dichas muestras se han estudiado aplicando un método HPLC-UV/FL/ESI-IT MS que permite un análisis completo del perfil metabólico de esta fruta tropical.

8. Un nuevo acoplamiento de GC y MS mediante una interfase APCI –ampliamente empleada en LC– ha sido utilizado en la determinación cuantitativa de los cambios producidos en el perfil metabólico de *Persea americana*. Además de APCI-TOF MS, la evolución del perfil matabólico se ha evaluado empleando GC-FID. Ambos detectores se han comparado teniendo en cuenta sus parámetros analíticos y los resultados cuantitativos obtenidos. GC-FID ha proporcionado mejores resultados en términos de precisión, pero peores en cuanto a sensibilidad y número de compuestos cuantificados. De forma general, prestando atención a la evolución de la concentración de los metabolitos estudiados, los ácidos orgánicos, flavonoides y vitaminas tienden a disminuir con el proceso de maduración; mientras que los ácidos fenólicos (derivados del HCA) suelen aumentar su concentración a medida que la fruta madura. A pesar de los buenos resultados obtenidos para GC-FID, es evidente que no puede competir con GC-APCI-TOF MS, plataforma que facilita considerablemente la identificación y permite la precisa cuantificación de un mayor número de metabolitos.

9. Teniendo presente que el acoplamiento GC-APCI-TOF MS había sido escasamente empleado hasta el momento y los problemas que conlleva la identificación en GC (asociados a la derivatización de las muestras) cuando no se dispone de bases de datos espectrales, parecía necesario el crear una biblioteca espectral con la señal de metabolitos en GC-APCI-MS. Se ha creado una base de datos que incluye información acerca de los índices de retención y los espectros de MS y MS/MS de una gran variedad de compuestos pertenecientes a diversas familias químicas, los cuales son frecuentemente encontrados en matrices alimentarias y fluidos biológicos. En el momento de su creación, la base de datos contenía información de unos 150 metabolitos; está siendo continuamente actualizada.

10. El gran potencial de GC-APCI-TOF MS para su uso en estudios metabolómicos y la aplicabilidad de la recién desarrollada base de datos ha sido demostrado. La primera aplicación de esta herramienta analítica para el análisis cualitativo de diferentes extractos de aguacate ha derivado en resultados muy prometedores, logrando la identificación de 100 metabolitos con muy diversa estructura química. Estos resultados se compararon con aquellos obtenidos empleando un acoplamiento muy consolidado y asentado en los laboratorios de rutina, GC-EI-Q MS, evidenciando que GC-APCI-

TOF MS debe ser definitivamente incluida dentro de la "caja de herramientas metabolómicas".

11. Finalmente, es importante señalar que la complejidad que presenta el metaboloma del aguacate dificulta enormemente el poder conocer su composición de un punto de vista global utilizando únicamente una de las muchas tecnologías ómicas disponibles hoy en día. Los diferentes estudios recogidos en la presente Tesis Doctoral han puesto de manifiesto la gran importancia del uso de varias metodologías analíticas (complementarias entre ellas) para llegar a conocer de forma minuciosa la matriz bajo estudio.

ABBREVIATIONS

[Buffer]	Buffer concentration
3D-FL	Three dimensional fluorescence
Ac Pin	(+)-1-acetoxypinoresinol
ACN	Acetonitrile
AcOH	Acetone
AD	Amperometric detection
AHBA	4-amino-2-hydroxy-benzoic acid
ANOVA,	Analysis of variance
AOAC	Association of Official Analytical Chemists
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photo-ionization
ASE	Accelerated solvent extraction
BHT	Butilated hydroxytoluene
BPC	Base peak chromatogram
BPE	Base peak electropherogram
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CCE	Chiral capillary electrophoresis
CD	Cyclodextrin
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CGE	Capillary gel electrophoresis
CI	Chemical ionization
CIEF	Capillary isoelectric focusing
CITP	Capillary isotachophoresis
CL	Chemical luminescence
CZE	Capillary zone electrophoresis;

DA	Discriminant analysis
DAD	Diode array detector
DHPE	2,3-dihydroxyphenylethanol
DOA	Decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA)
EA	Elenolic acid
ED	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
EIC	Extracted ion chromatogram
EIE	Extracted ion electropherogram
ELSD	Evaporative light scattering detector
EOF	Electroosmotic flow
ESI	Electrospray ionization
Et2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
EVOO	Extra virgin olive oil
FAOSTAT	Food and Agriculture Organization of the United Nations
FID	Flame ionization detector
FL	Fluorescence detector
FT-ICR	Fourier transform ion cyclotron resonance
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
HA-LAESI	Heat-assisted laser ablation electrospray ionization
HCA	Hierarchical cluster analysis
HPLC	High performance liquid chromatography
HR-EI	High resolution electron impact
HR-FAB	High resolution fast atom bombardment
HR-NMR	High resolution nuclear magnetic resonance
HYTY	Hydroxytyrosol
i.d.	Internal diameter of capillary
ICP	Inductively coupled plasma

IHSM	Instituto de Hortofruticultura Subtropical y Mediterránea
IMS	Ion mobility spectrometry
IR	Infrared spectroscopy
IS	Internal standard
IT-OT	Ion trap-orbitrap
K-NN	K-nearest neighbor classification
LC	Liquid chromatography
L_{ef}	Effective length of capillary
LIF	Laser induced fluoresncence
Lig Agl	Ligstroside aglycone
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Light scattering detector
m/z	Mass/charge ratio
MAE	Microwave assisted extraction
MALDI	Matrix assisted laser desorption ionization
MEEKC	Microemulsion electrokinetic capillary chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSTFA	N-methyl-N-trimethylsilyltrifluoroacetamide
NACE	Non-aqueous capillary electrophoresis
NaOH	Sodium hydroxide
NIR	Near infrared spectroscopy
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
OES	Optical emission spectrometry
Ol Agl	Oleuropein algycone
OPLS	Orthogonal partial least squares
PCA	Principal component analysis

PDA	Photodiode array detector
PLE	Pressurized liquid extraction
PLS	Partial least squares
PTV	Programmed temperature vaporizing
Q	Quadrupole
QC	Quality control
Q-IT	Quadrupole ion trap
Qq	Double-quadrupole
QqQ	Triple-quadrupole
RF	Response factor
RI	Refractive index
RSD	Relative standard deviation
SC	Sodium cholate
SDS	Sodium dodecyl sulphate
SFE	Supercritical fluid extraction
SIMCA	Soft independent modeling class analogy
SLE	Solid-liquid extraction
SPE	Solid phase extraction
SPME	Solid phase microextraction
SPR	Surface plasmon resonance
SWE	Subcritical water extraction
T	Temperature
TBAOH	Tetrabutylammonium hydroxide
$\mathbf{t_{inj}}$	Injection time
$\mathbf{t_r}$	Retention time
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TOF	Time of flight
Tween 20	Polyoxyethylene sorbitan monoalurate
TY	Tyrosol
UHPLC	Ultra high performance liquid chromatography
UHR-TOF	Ultra high resolution time of flight

USDA	United States Department of Agriculture
UV	Ultraviolet detection
UV/Vis	Ultraviolet/visible detection
V	Voltage
VIP	Variable importance in the projection
VOO	Virgin olive oil