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TESIS DOCTORAL

EVALUACIÓN DEL POTENCIAL DE DIVERSAS PLATAFORMAS ANALÍTICAS PARA LA CARACTERIZACIÓN DE COMPUESTOS DE INTERÉS EN ALIMENTOS Y METABOLITOS EN FLUIDOS BIOLÓGICOS

presentada por

Rocío García Villalba

para optar al grado de

Doctor Europeo en Química

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CERTIFICA:

Que el trabajo que se presenta en esta tesis doctoral bajo el título: "**EVALUACIÓN DEL POTENCIAL DE DIVERSAS PLATAFORMAS ANALÍTICAS PARA LA CARACTERIZACIÓN DE COMPUESTOS DE INTERÉS EN ALIMENTOS Y METABOLITOS EN FLUIDOS BIOLÓGICOS",** que ha sido realizado bajo mi dirección y la de los Drs. D. Antonio Segura Carretero y Dña. Alegría Carrasco Pancorbo en los laboratorios que el grupo FQM-297 tiene en el Departamento de Química Analítica y también, parcialmente en el Instituto de Fermentaciones Industriales (IFI, CSIC) en Madrid, en las instalaciones que la compañía Bruker Daltonik GMBH tiene en Bremen (Alemania) y en el Centro Médico de la Universidad de Leiden (LUMC) en Holanda, reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dª. Rocío García Villalba pueda optar al grado de Doctor Europeo en Química.

Y para que conste, expido y firmo el presente certificado en Granada a 22 de Marzo de 2010:

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OBJETO Y JUSTIFICACIÓN

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En los últimos años el desarrollo de potentes plataformas analíticas basadas en el acoplamiento de diferentes técnicas separativas (electroforesis capilar (EC) cromatografía líquida (LC) , nanocromatografía líquida (nanoLC), cromatografía de gases (GC)) con sistemas de detección como la espectrometría de masas, ha permitido que la Química Analítica pueda abordar problemas de gran relevancia en diferentes ámbitos (alimentario, medioambiental, bioquímico…).

En lo que respecta a la industria alimentaria, hoy en día el control de la calidad de los alimentos y su valor nutricional, junto con el estudio del efecto de las condiciones de cultivo, procesamiento, transporte, almacenamiento, la genética y otros factores que afectan a su composición química, han despertado gran interés entre los consumidores. El análisis de los alimentos, es por tanto, un tema importante que requiere el desarrollo de procedimientos analíticos lo más rápidos, potentes, reproducibles, robustos y fiables posibles. Por otro lado, debido a las excelentes propiedades saludables que presentan determinados componentes de los alimentos, como por ejemplo los polifenoles, resulta muy interesante desarrollar estudios metabolómicos de estos compuestos a nivel celular así como en diferentes fluidos biológicos (sangre, orina) para determinar su biodisponibilidad. En este sentido, el uso de potentes plataformas analíticas ofrece la posibilidad de afrontar con éxito el análisis de este tipo de muestras.

La presente memoria tiene como objetivo demostrar el potencial de las plataformas analíticas anteriormente mencionadas para la determinación de componentes alimentarios presentes en matrices de interés (malta, lupulo, soja, aceite de oliva) y para llevar a cabo estudios metabolómicos de los polifenoles del aceite de oliva en células cancerígenas y en fluidos biológicos (orina humana).

En el caso del análisis de malta, lúpulo y soja, el objetivo fundamental fue demostrar el potencial de la electroforesis capilar acoplada a diferentes sistemas de detección (UV y espectrometría de masas) para analizar la influencia de determinados factores: el procesamiento en la malta, las condiciones de almacenamiento en el lúpulo y las manipulaciones genéticas en la soja. La caracterización de estas matrices resulta de gran importancia tanto en el ámbito alimentario, proporcionando mayor información al consumidor, como a nivel industrial, permitiendo el control de los procesos de producción para conseguir un producto de calidad con un mayor valor económico.

Con respecto a la matriz aceite de oliva, dada la importancia de los compuestos fenólicos que constituyen una fracción minoritaria pero compleja relacionada con la estabilidad oxidativa, las propiedades saludables y las características organolépticas, se quiso demostrar el potencial de diferentes plataformas analíticas para llevar a cabo la separación, identificación y cuantificación de este grupo de compuestos tan importantes. Además, teniendo en cuenta los beneficios saludables que presentan los compuestos fenólicos del aceite de oliva, se planteó la utilización de las plataformas analíticas evaluadas para llevar a cabo estudios de absorción y metabolización de estos compuestos en células de cáncer de mama donde habían demostrado propiedades anti-tumorales. Asimismo, para poder extrapolar los resultados obtenidos *in vitro* a lo que ocurre en un organismo in vivo, otro de los objetivos fue demostrar la biodisponibilidad de los polifenoles en muestras de orina humana tras la ingesta de aceite de oliva virgen extra.

RESUMEN

RESUMEN

En esta memoria se reúnen los resultados obtenidos durante la realización de la tesis doctoral titulada "Evaluación del potencial de diversas plataformas analíticas para la caracterización de compuestos de interés en alimentos y metabolitos en fluídos biológicos". Se ha dividido en dos grandes secciones: una introducción con información acerca de las plataformas analíticas utilizadas durante el desarrollo de la tesis, que incluyen las técnicas separativas (CE, HPLC, nanoLC, GC) y los sistemas de detección empleados (UV, FID y MS) y un segunda sección experimental donde se muestra la aplicación de dichas plataformas analíticas. La parte experimental se divide en 3 bloques relacionados con las matrices alimentarias implicadas en los trabajos experimentales (cerveza, soja y aceite de oliva) y 9 capítulos, donde se incluyen los resultados obtenidos durante la realización de la presente tesis doctoral.

En el primer capítulo se emplea un método de CZE-UV para llevar a cabo una comparación del perfil proteico de hordeínas en muestras de cebada tomadas en diferentes etapas del proceso de malteado. El contenido en proteínas de la cebada ha demostrado ser un factor muy importante en maltería y en la industria cervecera, relacionado con parámetros de calidad de la malta, además de ser muy útil para la clasificación de variedades. Sin embargo, hasta el momento en que se desarrolló este trabajo existían pocos estudios donde se evaluara el perfil proteico durante el malteado.

En el segundo capítulo se estudió otra de las materias primas fundamentales en la industria cervecera, el lúpulo, cuyo contenido en ácidos amargos (α y β ácidos) está relacionado con el sabor propio de la cerveza. Se desarrolló un método analítico empleando CE-ESI-IT MS para identificar no sólo los ácidos del lúpulo sino también sus compuestos oxidados bajo tres condiciones distintas de almacenamiento en extractos crudos de diferentes variedades. Los ácidos del lúpulo son muy susceptibles a la oxidación y cuando esto ocurre ya no se pueden convertir en iso-α-ácidos, responsables del sabor de la cerveza. De ahí la importancia de controlar el estado de oxidación del lúpulo y estudiar estos compuestos oxidados.

El tercer capítulo centra su interés en demostrar el potencial de CE-ESI-TOF MS para el estudio de los organismos modificados genéticamente (OMGs), llevando a cabo una comparación a nivel metabolómico entre soja convencional y soja transgénica. Debido a las exigencias de la Unión Europea en cuanto a contenido de los alimentos

transgénicos es necesario desarrollar métodos para evaluar la presencia de los OGMs en diferentes alimentos. Se llevó a cabo la optimización tanto del protocolo de extracción como de las condiciones electroforéticas y del espectrómetro de masas. Se llegaron a identificar una gran cantidad de compuestos pertenecientes a diferentes familias en el perfil metabolómico. El trabajo experimental incluido en este capítulo se desarrolló en el Instituto de Fermentaciones Industriales (IFI) del Centro Superior de Investigaciones Científicas de (CSIC) en Madrid.

El cuarto capítulo, primero de los relativos a la matriz aceite de oliva, describe el estudio bidimensional llevado a cabo mediante HPLC-CE-ESI-TOF MS para analizar en profundidad el perfil polifenólico del aceite de oliva. El procedimiento consiste en aislar diferentes fracciones fenólicas de extractos de aceite de oliva mediante HPLC semipreparativa y, posteriormente analizar estas fracciones aisladas mediante CE-ESI-TOF MS. El empleo de dos técnicas complementarias permitió identificar un gran número de compuestos, algunos de ellos nunca habían sido descritos con anterioridad en bibliografía. Además, estas fracciones resultaron ser muy útiles para cuantificar de forma más veraz algunos de los fenoles y para llevar a cabo estudios *in vitro* de la capacidad antiproliferativa y pro-apoptótica de estos compuestos frente a células de cáncer de mama.

En el quinto capítulo se describe el desarrollo de un método de RRLC-ESI-TOF MS capaz de llevar a cabo la identificación y cuantificación de compuestos fenólicos pertenecientes a diferentes familias en 8 aceite de oliva virgen. La cuantificación se realizó de tres formas diferentes: RRLC-UV, RRLC-MS y una nueva aproximación que tiene en cuenta el contenido total de polifenoles, las áreas relativas de cada compuesto y sus factores de respuesta. En trabajos previos, se había estudiado la actividad anticancerígena de extractos polifenólicos completos de estos 8 aceites de oliva en diferentes líneas celulares de cáncer de mama (para considerar los efectos sinérgicos y antagónicos), observando un descenso de la viabilidad celular diferente en función del extracto ensayado. Con objeto de establecer una relación entre la composición fenólica de estos aceites y su actividad anticancerígena se aplicó un análisis multivariante por componentes principales que logró distinguir con claridad los aceites de oliva con distinta actividad antiproliferativa considerando su contenido polifenólico.

En el capítulo 6 se demuestra el potencial de la nanocromatografia líquida acoplada a espectrometría de masas (nanoLC-ESI-TOF MS) para identificar y

cuantificar los polifenoles del aceite de oliva. Se optimizaron las condiciones cromatográficas y del espectrómetro de masas, se identificaron los compuestos teniendo en cuenta la información proporcionada por el TOF y la información bibliográfica y se establecieron los parámetros analíticos del método. Siempre que una técnica analítica se utiliza por primera vez para afrontar un determinado problema, resulta interesante comparar su potencial y parámetros analíticos con los de otra técnica más ampliamente utilizada. Por ello, los parámetros analíticos y otros aspectos de la técnica de nanoLC se compararon con los obtenidos con HPLC. Parte de este trabajo se desarrolló durante una estancia en la sede de la empresa Bruker Daltonik en Bremen, Alemania.

Siguiendo el enfoque del capítulo 6, en el capítulo 7 se desarrolla una metodología analítica empleando cromatografía de gases con un acoplamiento totalmente novedoso a través de una interfase APCI que transfería los iones a un espectrómetro de masas de última generación (MaXis); una plataforma analítica (GC-APCI-MaXIs MS) nunca antes utilizada en el análisis de polifenoles. El trabajo experimental fue realizado durante una estancia en el Centro Médico de la Universidad de Leiden (LUMC), en Holanda.

En el capítulo 8, aprovechando las ventajas en cuanto a sensibilidad que proporciona la plataforma analítica nanoLC-ESI-TOF MS cuyo potencial para el análisis de polifenoles había sido demostrado en el capítulo 6, el objetivo planteado fue estudiar los procesos de absorción y metabolización de los polifenoles del aceite de oliva en células de cáncer de mama de la línea JIMT-1. Los polifenoles del aceite de oliva virgen han demostrado su actividad anti-tumoral en varios estudios *in vitro* (capítulos 4 y 5), sin embargo, poco se sabe acerca de la eficiencia del proceso de absorción y la transformación metabólica de estos polifenoles a nivel celular. El estudio de estos procesos podría ser útil para comprender los mecanismos involucrados en la actividad anti-tumoral de estos compuestos.

El capítulo 9 centra su interés en el estudio de la biodisponibilidad de los polifenoles del aceite de oliva. El conocimiento de la biodisponibilidad (cómo se absorben, metabolizan y excretan los compuestos en el organismo) es importante para poder extrapolar los resultados obtenidos *in vitro* con modelos celulares a lo que ocurre en un organismo *in vivo*. Se ha utilizado un método de RRLC-ESI-TOF-MS para llevar a cabo un análisis exploratorio de muestras de orina humana tras la ingesta de aceite de oliva virgen extra. A causa de la dificultad a la hora de interpretar los cromatogramas

obtenidos debido a la complejidad de los mismos, se emplearon métodos quimiométricos (PCA, PLS…) que permitieron distinguir las muestras de orina antes y después de la ingesta y establecer los valores m/z responsables de esta discriminación (potenciales biomarcadores). Se llegaron a identificar más de 60 metabolitos de los principales polifenoles del aceite en las muestras de orina. Además se llevaron a cabo estudios cinéticos de los metabolitos identificados como posibles biomarcadores.

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ENGLISH SUMMARY

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This report is a summary of all the results obtained during the doctoral thesis entitled: "Evaluation of the potential of different analytical platforms for the characterization of compounds of interest in food matrices and metabolites in biological fluids". It has been divided into two wide sections: the first one is an introduction with information about the analytical platforms used during the development of the work, included in this thesis, which are composed by separation techniques (CE, HPLC, nanoLC, GC) and detection systems (UV, FID and MS), and the second section shows the application of these analytical platforms. The experimental part has been divided into 3 blocks related to the food matrices involved in the experimental work (beer, soybean and olive oil) and 9 chapters, which include the results obtained during the course of this thesis.

In the first chapter, a CZE-UV method is used to carry out a comparison of the protein profile (hordeins) in barley samples taken at different stages of the malting process. The protein content of barley has proved to be a major factor in malting and brewing industry, related to malt quality parameters, besides being very useful for the classification of varieties. However, there were few studies evaluating the protein profile during malting when this work was carried out.

In the second chapter, we study another key raw material for the brewing industry: hops, whose content in bitter acids (α- and β-acids) is related to the beer flavor. An analytical method using CE-ESI-IT MS was developed to identify not only hop acids but also their oxidized derivatives, obtained under three different storage conditions from crude extracts of different hop varieties. Hop acids are highly susceptible to oxidation and when this happen, they can not be converted into iso- α-acids, which are actually responsible of the beer taste. Hence the importance of controlling the oxidation state of hops and of studying these oxidized compounds. The method was also applied to the determination of iso-α-acids in beer.

The third chapter demonstrates the potential of CE-ESI-TOF MS for the study of genetically modified organism (GMOs), carrying out a comparison between transgenic soybean and its corresponding non-transgenic parental line, both grown under identical conditions. Due to the requirements of the European Union about the content of the transgenic food it is necessary to develop methods to evaluate the presence of GMOs in

different foods. The optimization of the extraction procedure and the electrophoretic and mass spectrometry conditions was carried out and a large number of compounds were tentatively identified by using a metabolic profiling approach. The experimental work included in this chapter was developed in the Institute of Industrial Fermentations (IFI, CSIC, Madrid).

The fourth chapter, the fist one related to olive oil, describes a bidimensional study in which we use HPLC coupled to CE and ESI-TOF MS to separate and characterize more in depth the phenolic fraction of olive oil samples. The method involves the isolation of different phenolic fractions from olive oil extracts by semi-preparative HPLC and then the analysis of these isolated fractions by CE-ESI-TOF MS. The use of two complementary techniques allowed identifying a large number of compounds, some of them described by fist time in literature. Besides, the isolated phenolic fractions proved to be very useful to quantify in a more accurate way some phenols of olive oil samples and to carry out *in vitro* studies about the anti-proliferative and pro-apoptotic capacity of these compounds in different cancer cell lines.

The fifth chapter describes the development of a RRLC-ESI-TOF MS method to identify and quantify the phenolic compounds in eight different virgin olive oils. The compounds were quantified using three different strategies: RRLC-UV, RRLC-MS and a new approach using the total polyphenols content obtained with Folin Ciocalteau, the relative areas and the response factors. In previous studies the anti-carcinogenic activity of phenolic extracts of these eight olive oils was studied in different breast cancer cell lines (to consider the synergistic and antagonistic effects) observing a decrease in cell viability which was different depending on the extract tested. In order to establish a correlation between the phenolic composition of these olive oil extracts and their anti-proliferative abilities, a multivariate analysis by principal component (PCA) was applied.

In chapter 6 the potential of nanoliquid chromatography coupled with mass spectrometry (nanoLC-ESI-TOF MS) to identify and quantify olive oil polyphenols is evaluated. Chromatographic and mass spectrometry conditions were optimized, the compounds were identified based on the information provided by TOF-MS analyzer and previously published reports and the analytical parameters of the method were established. When an analytical technique is applied by first time to face a particular problem, it is quite interesting to compare its potential and performance with those of

other techniques more widely used. Therefore, a comparison between the performance of both nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS methodologies for the separation and quantitation of this type of compounds was made. Part of this work was developed during a stay in the Applications Department of the company Bruker Daltonik in Bremen (Germany).

Following the approach of chapter 6, chapter 7 develops an analytical methodology using gas chromatography (GC) coupled to a new mass spectrometer (MaXis, the last generation of Q-TOF) through a innovative atmospheric pressure chemical ionization (APCI) interface; an analytical platform (GC-APCI-MaXis) never used before for the analysis of polyphenols. The experimental work was done during a stay in the Leiden University Medical Center (LUMC) in Leiden (The Netherlands).

In chapter 8, taking advantage of the sensitivity provided by the analytical platform nanoLC-ESI-TOF MS, whose potential for the analysis of polyphenols was demonstrated in Chapter 6, a nanoLC-ESI-TOF MS method was developed to study the cellular uptake of olive oil phenols in JIMT-1 human breast cancer cells. Polyphenols from virgin olive oil have demonstrated anti-tumoral activity in several *in vitro* studies (chapter 4 and 5). However, little is known about the efficiency of the absorption process and metabolic conversion of extra virgin olive oil-derived polyphenols at the cellular level. The study of these processes could be useful to understand the mechanisms involved in the anti-tumor activity of these compounds.

In chapter 9 the bioavailability of olive oil polyphenols is studied. The knowledge about the bioavailability (how the compounds are absorbed, metabolized and excreted in the body) is very important to be able to extrapolate results obtained *in vitro* with cellular models to what happens in an organism *in vivo*. A RRLC-ESI-TOF MS method has been developed to carry out an exploratory analysis of human urine samples after high intake of virgin olive oil. Due to the difficulty to interpret the results obtained, we used different chemometric methods (PCA, PLS) that allowed us to discriminate between the urine samples before and after the intake and facilitated to find out the m/z values responsible of this discrimination (biomarkers). More than 60 metabolites of the main olive oil phenolic compounds were tentatively identified in the urine samples. Additionally, kinetic studies of the metabolites identified as possible biomarkers were developed, obtaining maximal values after the first two hours for most compounds.

INTRODUCCIÓN:
Plataformas analíticas

A) TÉCNICAS SEPARATIVAS

1. Electroforesis capilar

1.1. Definición e instrumentación

La electroforesis capilar (CE) es una técnica de separación en la que las sustancias a analizar se separan en función de su diferente movilidad, en sentido y velocidad, bajo la acción de un campo eléctrico en el interior de un tubo capilar lleno de una disolución tampón [1 , 2]. El tubo capilar normalmente es de sílice fundida recubierto por una capa polimérica para su protección, y con unas dimensiones que oscilan entre 10-100 cm de longitud y 25-100 µm de diámetro interno.

Las principales características de la CE son:

- Elevada rapidez de análisis.
- Altas eficacias, de 10^5-10^6 platos teóricos por metro de columna.
- Requerimiento de pequeños volúmenes de muestra y reactivos.
- Gran variedad de aplicaciones.
- Facilidad de automatización.

Los componentes básicos de un equipo de CE son: generador de alto voltaje, viales fuente, destino y muestra, capilar (normalmente de sílice fundida recubierto de poliimida), sistema de termostatización, sistema de inyección de la muestra, sistema de detección y sistema de registro (Fig.1).

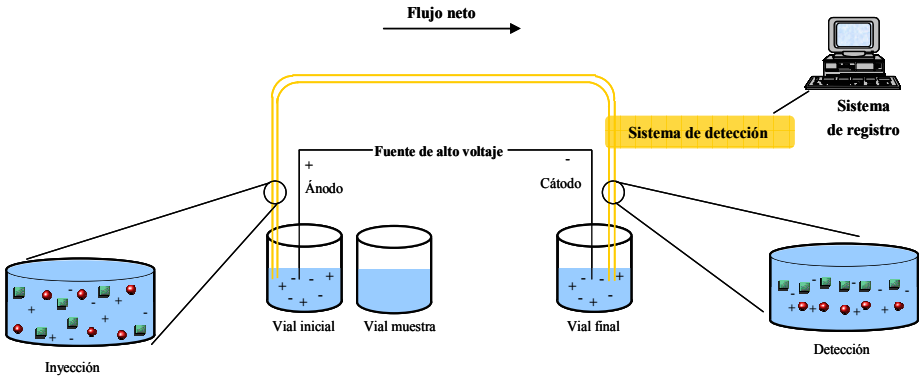

Figura 1. Esquema básico de un equipo de electroforesis capilar (CE)

[1] S. F. Y, Li. "Capillay Electrophoresis: principles, practice and applications". Elsevier, (1992).
[2] P. D. Grossman, J. C. Colburn. "Capillary Electrophoresis. Theory and Practice". Academic Press, San Diego (1992).

1.2. Principios de funcionamiento

Los extremos del capilar se colocan en dos viales rellenos de disolución de separación que contienen cada uno de ellos un electrodo, ambos conectados a una fuente de alto voltaje (hasta 30 kV). La muestra se inyecta dentro del capilar a través de un vial que sustituye temporalmente el vial inicial aplicando un potencial eléctrico (inyección electrocinética) o una presión externa (inyección hidrodinámica) durante unos segundos. Después se vuelve a reemplazar el vial de muestra por el inicial, conteniendo la disolución tampón, y se aplica un potencial eléctrico a lo largo del capilar que produce la separación. Los analitos pueden ser detectados directa o indirectamente con detección óptica (UV-visible, fluorimétrica, fosforimétrica, quimioluminiscente o infrarroja) a través de la ventana del capilar que habitualmente se encuentra cerca del extremo opuesto a donde se hizo la inyección (normalmente cerca del cátodo)- detección on-columna-, o al final del capilar mediante el uso de otros sistemas de detección como la espectrometría de masas o las técnicas eléctricas-detección off- o post-columna-.

De forma básica se puede decir que la migración de las especies químicas dentro del capilar se rige por dos fenómenos, que tienen lugar simultáneamente: electromigración y electroósmosis [3-5].

La **electromigración** consiste en el movimiento neto de las especies cargadas que forman la muestra a través de la disolución tampón dentro del capilar, bajo la influencia del campo eléctrico. Cada analito tiende a migrar hacia su polo correspondiente (cationes al cátodo, aniones al ánodo) a distinta velocidad. A esta velocidad se le denomina velocidad electroforética y se calcula por la siguiente expresión:

$$\nu = \mu_e E$$

donde ν es la velocidad de migración de cada ion (m/s), μ_e es la movilidad electroforética ($m^{2/}V\ s$) y E es el campo eléctrico aplicado (V/m). La intensidad del campo eléctrico es función del voltaje aplicado dividido por la longitud total del capilar.

[3] R. P. Oda, J. P. Landers. "Introduction to Capillary Electrophoresis" en "Handbook of Capillary Electrophoresis". J. P. Landers (Ed.) CRC Press, Florida (1997) 1-49.
[4] M. L. Marina, A. Ríos, M. Valcárcel. "Fundamentals of Capillary Electrophoresis" en "Analysis and detection by Capillary Electrophoresis". M. L. Marina, A. Ríos, M. Valcárcel (Eds) Elsevier, Amsterdam (2005) 1-28.
[5] C. Cruces-Blanco. "Electroforesis capilar". Ed. Universidad de Almería Servicio Publicaciones, Almería (1998).

La movilidad electroforética es un factor que indica cómo de rápido se mueve un ión o soluto a través de la disolución de separación. Es una expresión del balance de fuerzas que actúan sobre cada ión individual; la fuerza del campo eléctrico actúa a favor del movimiento y las fuerzas de fricción y rozamiento, en contra. Puesto que estas fuerzas son constantes durante la electroforesis, la movilidad electroforética es una constante para un ión dado bajo unas condiciones dadas. La ecuación que describe la movilidad electroforética es:

$$\mu_e = \frac{q}{6\pi\eta r}$$

donde q es la carga del ión, η es la viscosidad de la disolución y r es el radio de la partícula o ión en disolución. La carga del ión, q, es estable para los iones totalmente disociados, como son ácidos fuertes o pequeños iones, pero puede estar afectada por cambios de pH en el caso de ácidos o bases débiles. De la ecuación anterior se puede ver que las diferencias en las movilidades electroforéticas serán causadas por las diferencias en la relación entre la carga y el tamaño de ión. Las moléculas pequeñas de gran carga se moverán más rápido a través del capilar que las moléculas con menor carga. Con esta expresión se deduce también que las moléculas neutras, al ser q=0, tendrán una movilidad electroforética igual a cero.

La **electroósmosis** es un fenómeno que se produce siempre que se aplica un campo eléctrico a un sistema líquido (como es el medio electroforético) que esté en contacto directo con una superficie cargada (como es el interior del capilar de sílice fundida). La pared interna del capilar tiene grupos silanoles que, en contacto con el tampón de separación, se ionizan. El grado de ionización se controla principalmente mediante el pH del electrolito de separación (aparecen cargas negativas con disoluciones de pH superior a 2.5-3). De este modo, la pared cargada negativamente del tubo de sílice atrae a los iones con carga positiva del tampón y bajo la acción del campo eléctrico estas cargas positivas se desplazan hacia el cátodo y arrastran con ellas el agua de solvatación asociada. El movimiento global del tampón en el interior del capilar, llamado flujo electrosmótico (EOF), viene determinado por su respectiva velocidad electroosmótica, v_{eo}:

$$v_{eo} = \mu_{e_o} E$$

μ_{e0} es la movilidad electrosmótica definida como:

$$\mu_{eo} = \frac{\varepsilon\zeta}{4\pi\eta}$$

donde ε es la constante dieléctrica del tampón, η su viscosidad y ζ (llamado potencial zeta) el potencial que se genera aproximadamente entre la superficie del capilar y el tampón. El EOF va a afectar a todas las sustancias en el interior del capilar del mismo modo, ya que se trata de una propiedad del sistema capilar-tampón.

Al existir los dos fenómenos de migración (electromigración y electroósmosis), la velocidad de migración de un soluto está relacionada con una combinación de la movilidad electroforética y electroosmótica:

$$v = (\mu_{eo} \pm \mu_e)E$$

El tiempo de migración de la sustancia cargada vendrá dado por la expresión:

$$t_m = \frac{L}{(\mu_{eo} \pm \mu_e)E}$$

donde L es la longitud del capilar hasta el punto de detección.

Un esquema de la existencia de estos dos fenómenos sobre el movimiento neto de las especies contenidas en una muestra se representa en la Figura 2.

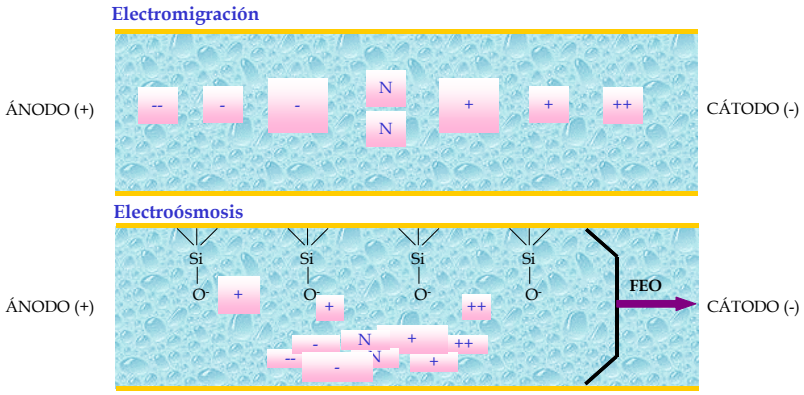

Figura 2. Representación de los fenómenos de electromigración y electroósmosis.

1.3. Modos de separación en electroforesis capilar

Es ampliamente reconocido que la CE es una técnica muy versátil, y esto es causado en parte por los distintos modos de separación disponibles. Además, y a diferencia de la cromatografía líquida donde el cambio de un modo a otro suele requerir el cambio de columna y fase móvil, en CE suele implicar únicamente el cambio de la composición de la disolución reguladora empleada. Los modos de CE más comunes junto con el principio de separación de cada uno de ellos se resumen en la tabla 1 [7].

Modo de separación	*Acrónimo*	*Principio de separación*
Electroforesis capilar en zona	CZE	Carga/tamaño
Cromatografía capilar electrocinética micelar	MEKC	Interacción hidrofóbica/iónica con micelas del surfactante
Electroforesis capilar quiral	CCE	Formación de complejos estereoespecíficos
Electroforesis capilar por afinidad	CAE	Interacciones moleculares entre ligando y analito "objetivo"
Cromatografía capilar electrocinética micelar con microemulsiones	MEEKC	Mecanismos electroforéticos y reparto cromatográfico
Electroforesis capilar en gel	CGE	Tamaño molecular
Isoelectroenfoque capilar	CIEF	Punto isoeléctrico
Isotacoforesis capilar	CITP	Capacidad de migración entre tampones de distinta naturaleza
Electrocromatografía capilar	CEC	Movilidad en una solución libre y retención cromatográfica

Tabla 1. Modos de separación en electroforesis capilar

Los diferentes mecanismos de separación empleados hacen que estos modos sean complementarios entre sí. En algunos casos, una separación puede ser realizada satisfactoriamente por más de un modo electroforético

1.3.1. Electroforesis capilar en zona (CZE)

Es el más versátil y simple de los modos en CE. Fue la primera modalidad que se desarrolló, merced a los trabajos pioneros de Jorgenson y Luckas [8,9] y continúa siendo la más utilizada, con un gran número de aplicaciones en diferentes campos. En el interior del capilar sólo se encuentra el tampón que se emplea para llevar a cabo el análisis, de modo, que una vez inyectada la muestra, la separación de las diferentes sustancias se basa en su distinta relación carga/masa. Tiene la limitación de ser útil sólo para la separación de especies cargadas, no permitiendo separar las neutras.

[7] A. Fernández-Gutiérrez, A. Segura-Carretero, A. Carrasco-Pancorbo. "Fundamentos teóricos y modos de separación" en "Electroforesis capilar: Aproximación según la técnica de detección". A. Fernández-Gutiérrez, A. Segura-Carretero (Eds). Ed. Universidad de Granada. (2005) 11-54.
[8] J. W. Jorgenson, K. D. Luckacs. "Zone electrophoresis in open-tubular glass-capillaries". *Anal. Chem.* 53 (1981) 1298-1302.
[9] J. W. Jorgenson, K. D. Luckacs. "Capillary zone electrophoresis". *Science* 222 (1983) 266-272.

Puesto que las muestras son introducidas normalmente desde el ánodo y el detector se encuentra al lado del cátodo el orden de elución, determinado por la relación carga/tamaño de los analitos, es: cationes, sustancias neutras y aniones (Fig. 3).

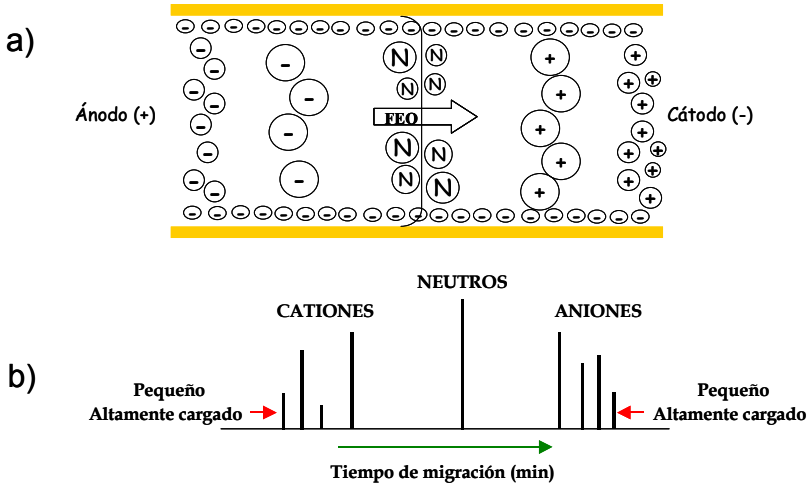

Figura 3. Representación de a) esquema del interior de un capilar en el que se lleva a cabo una separación por CZE y b) electroferograma obtenido por CZE indicando el orden de elución de los distintos analitos.

El hecho de que éste sea el orden de elución se debe a que los cationes se mueven a través del capilar en la misma dirección que el EOF, por lo que sus velocidades de migración serán más rápidas que el propio EOF. Las moléculas neutras, que se mueven a través del capilar empujadas sólo por el EOF, eluyen después de los cationes, pero sin separarse. Los aniones, finalmente, al poseer carga negativa, tenderán a moverse hacia el ánodo en sentido opuesto al EOF, pero generalmente éste es mayor que las velocidades electroforéticas de los aniones, por lo que los aniones se desplazan hacia el cátodo eluyendo en último lugar.

El modo de electroforesis capilar en zona ha sido el que se ha utilizado en todos los métodos electroforéticos puestos a punto en el trabajo que se recoge en la presente memoria.

1.4. Condiciones a optimizar

Las variables más importantes a optimizar en el desarrollo de un método de electroforesis capilar se pueden dividir en dos grupos [10- 12]:

1.4.1. Variables instrumentales

- *Selección del capilar: dimensiones y acondicionamiento*

Habrá que seleccionar la longitud del capilar, relacionada con la resolución entre picos y el tiempo de separación, y el diámetro, que influye en la sensibilidad. Cuanto más largo sea un capilar, mayor es el tiempo de análisis y mayor la separación o resolución entre picos, y cuanto más grande sea el diámetro, mayor será la sensibilidad. Se recomienda utilizar capilares con diámetros entre 50-75 µm ya que diámetros mayores pueden dar lugar a problemas de calentamiento interno. También es importante optimizar el ciclo de lavado del capilar para obtener buena reproducibilidad.

- *Voltaje*

Tanto la velocidad electroforética como la electroosmótica son directamente proporcionales al campo eléctrico. Un voltaje elevado proporciona mayor rapidez y por lo tanto, tiempos de migración más cortos, pero se pueden producir pérdidas en la resolución y eficacia de los picos, ya que la generación de un calor excesivo dentro del capilar a altos voltajes, contribuye significativamente a la dispersión de los analitos y, por tanto, al solapamiento de los picos. El voltaje límite que se puede emplear en la práctica es de 30 kV (en la mayor parte de los instrumentos de CE comerciales) para que no se produzcan efectos de calentamiento del capilar que se conocen con el nombre de efecto térmico o efecto Joule.

[10] L. Arce- Jiménez, A. Segura-Carretero, S. Cortacero-Ramírez. "Aspectos prácticos para el desarrollo de metodologías analíticas mediante electroforesis capilar" en "Electroforesis capilar: aproximación según la técnica de detección". A. Fernández-Gutiérrez, A. Segura-Carretero (Eds). Ed. Universidad de Granada. (2005) 126-155.
[11] G. M. McLaughlin, A. Weston, K. D. Hauffe. "Capillary electrophoresis methods development and sensitivity enhancement strategies for the separation of industrial and enviromental chemicals". *J. Chromatogr. A* 744 (1996) 123-134.
[12] V. Dolník. "Selectivity, differential mobility and resolution as parameters to optimize capillary electrophoretic separation". *J. Chromatogr. A* 744 (1996) 115-121.

- *Temperatura*

El control de la temperatura es siempre importante para evitar el efecto Joule. Además, los cambios en la temperatura afectan a la movilidad de los analitos y al EOF e incluso pueden afectar al pH de la disolución reguladora. El intervalo de temperatura permitido está comprendido normalmente entre 10 y 50ºC. El uso de temperaturas altas en CE disminuye el tiempo de análisis y mejora la forma de los picos, debido a la disminución de la viscosidad, pero también se pierde resolución en especial cuando se trata de analitos muy semejantes o de un número elevado de ellos.

- *Tipo y tiempo de inyección*

La muestra se puede introducir en el capilar usando la modalidad hidrostática o hidrodinámica (gravedad, presión o vacío) y la electrocinética. La inyección hidrodinámica es la modalidad mas comúnmente usada y se puede realizar elevando el vial a una altura determinada por encima del nivel inicial (gravedad), aplicando presión al vial de muestra (presión) o haciendo el vacío en el vial de salida. La inyección electrocinética consiste en colocar el capilar y el ánodo dentro del recipiente con la muestra y aplicar un voltaje pequeño (1-5 kV) durante un corto periodo de tiempo (1-10s) haciendo migrar los componentes de la muestra dentro del capilar.

El tiempo de inyección debe ser la última variable experimental a optimizar en un método. Se usará mayor o menor tiempo en función de la sensibilidad que se quiera conseguir (mayor tiempo de inyección, mayor sensibilidad) o en función del número de analitos que se estén separando. En general, se deben inyectar volúmenes pequeños que puedan ser detectados pero que den lugar a las mejores eficacias.

1.4.2. Variables químicas del medio electroforético

Las características de la disolución de separación (pH, capacidad reguladora, fuerza iónica, presencia de aditivos etc.) juegan un papel más decisivo en la separación electroforética que los parámetros instrumentales.

- *pH*

Es una variable de gran repercusión en la selectividad de los métodos electroforéticos, pues influye en el grado de ionización de especies ácidas o básicas

débiles, y por tanto, en sus movilidades electroforéticas. En general, para la separación de solutos catiónicos se debe trabajar a un pH de una o más unidades por debajo de su pK_b y para solutos aniónicos por encima de su pK_a. Además, teóricamente a medida que aumenta el pH, aumenta la movilidad electroosmótica y por tanto, disminuyen los tiempos de migración. El aumento de la movilidad electroosmótica se debe a que elevados valores de pH producen más disociación de los grupos silanoles Si-OH a $Si\text{-}O^-$ dentro de las paredes del capilar y también porque se produce un aumento del potencial zeta.

- *Capacidad reguladora y composición*

Ésta debe ser alta sin un aumento excesivo de la conductividad en una zona lo más amplia posible de pH, para que se obtenga buena reproducibilidad y una baja fuerza iónica para minimizar el efecto Joule. La elección es generalmente empírica y suele recurrirse a los métodos previamente optimizados.

- *Fuerza iónica*

Tiene una influencia decisiva en las movilidades electroforéticas y electroosmóticas. Normalmente al aumentar la concentración de la disolución de separación (fuerza iónica), se mejora la resolución de los analitos y su forma de pico. Sin embargo, se genera más calor dentro del capilar y es necesario un buen sistema para regular la temperatura (por ejemplo uso de capilares de pequeño diámetro).

- *Presencia de aditivos*

Los aditivos añadidos a las disoluciones tampón son de vital importancia para mejorar la separación electroforética. El empleo de ligandos como ciclodextrinas [13] y poliéteres macrocíclicos permite un notable incremento de la selectividad, mientras que la adición de sales neutras, anfolitos, alquilaminas y polímeros neutros consiguen reducir (o controlar) el EOF y la adsorción de proteínas a la pared del capilar. La adición de disolventes orgánicos (acetonitrilo, alcoholes, etc.) y alcoholes de mayor peso molecular (glicerina, propanol…) puede modificar la polaridad y viscosidad de la disolución de separación, cambiando las movilidades electroforéticas y electroosmóticas de los analitos. Además, pueden usarse para potenciar la solubilidad de analitos hidrofóbicos. También pueden adicionarse

[13] M. R. A. Rodríguez, E. B. Caramao, L. Arce, A. Rios, M. Valcárcel. "Use of cyclodextrins for the separation of monoterpene isomers by micellar electrokinetic capillary chromatography" *J. Microcol. Sep.* 13 (2001) 293-299.

sustancias poliméricas solubles (metilcelulosa e hidroxipropilmetilcelulosa) para reducir el potencial zeta de la pared intena del capilar, y por tanto minimizar el efecto de la electroósmosis y reducir significativamente los fenómenos indeseables de la adsorción.

2. Cromatografía líquida

2.1. Definición e instrumentación

La Cromatografía engloba a un conjunto de técnicas basadas en la distribución de los componentes de una mezcla entre dos fases inmiscibles, una fija o estacionaria y otra móvil. En el caso de la cromatografía líquida, la fase estacionaria es un sólido o un líquido fijado en un sólido y la fase móvil es un líquido. Según como esté dispuesta la fase estacionaria se distingue: *cromatografía plana* donde la fase estacionaria se sitúa sobre un papel o una placa plana (cromatografía en papel o cromatografía en capa fina) y *cromatografía en columna*, donde la fase estacionaria se sitúa dentro de una columna. La cromatografía líquida "clásica" o a baja presión se lleva a cabo en una columna generalmente de vidrio rellena con la fase estacionaria. La muestra se introduce por la parte superior y la fase móvil se hace fluir a través de la columna por gravedad. Las separaciones requieren mucho tiempo y además presentan baja eficacia [14,15]. Con el objeto de aumentar la eficiencia en las separaciones, el tamaño de las partículas de fase estacionaria se fue disminuyendo, lo cual generó la necesidad de utilizar un sistema de bombeo a alta presión de la fase móvil para conseguir un flujo razonable. De esta manera, nació la técnica de cromatografía líquida de alta resolución (HPLC del inglés *High Perfomance Liquid Chromatography*), que es la técnica cromatográfica más empleada en la actualidad. La fase estacionaria es un sólido poroso, generalmente en forma particulada, o bien una fina capa de sustancia líquida ligada a un soporte sólido, contenido en el interior de un tubo habitualmente metálico que da lugar a la columna cromatográfica. La fase móvil es un líquido, ya sea un disolvente o una mezcla de disolventes, a veces con un pH modificado mediante adición de ácidos, bases o sistemas tampón.

[14] R. Cela, R. A. Lorenzo, M. C. Casais. "Cromatografía líquida en columna" en "Técnicas de separación en Química Analítica". Ed. Síntesis S. A. Madrid (2002) 399-498.
[15] M. Valcárcel-Cases, A. Gómez-Hens. "Cromatografía líquida en columna (I). Generalidades" en "Técnicas analíticas de separación". Ed. Reverté S. A. Barcelona (1990) 437-484.

Un sistema de HPLC está formado por [16] (Fig. 4): depósitos para las fases móviles, sistema de bombeo para proporcionar presión a la fase móvil, sistema de inyección de muestras, columna cromatográfica, horno termostatizado, detector y sistema de adquisición de datos. La columna cromatográfica a veces va precedida de una pre-columna para impedir que lleguen a la columna componentes de la muestra que puedan dañar la fase estacionaria.

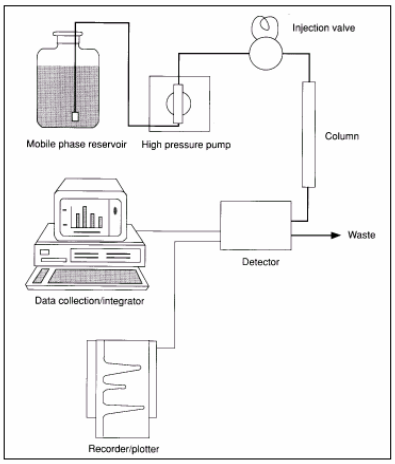

Figura 4. Esquema que incluye los componentes básicos de un sistema HPLC y fotografía de un instrumento real donde se indica la posición de cada uno de esos componentes.

2.2. Principios de funcionamiento

En una separación mediante HPLC la fase móvil impulsada por la bomba transporta una banda de muestra a través de la columna cromatográfica. La inyección se realiza normalmente a través de una válvula de seis vías que permite introducir en el flujo de disolvente, la muestra contenida en un aro o loop de volumen calibrado. Una vez en la columna los analitos interaccionan con la fase estacionaria de tal forma que aquellos que sean más afines con la fase móvil serán menos retenidos por la fase estacionaria y eluirán antes, mientras que aquellos que tengan más afinidad por la fase estacionaria avanzarán más lentamente a través de la columna y eluirán, por tanto, más tarde. La fase móvil puede ser un solvente puro o una mezcla de solventes. Cuando se trata de una mezcla puede programarse la bomba para que tome solventes de diferentes botellas en una proporción determinada y realice la mezcla en una cámara de mezclado. Cuando durante toda la separación se utiliza siempre el mismo solvente, se denomina

[16] J. F. Loro Ferrer. "Cromatografía líquida de alta resolución (HPLC)" en "Manual de Cromatografía". Ed. Dirección General de Universidades e Investigación. Consejería de Educación, Cultura y Deporte. Gobierno de Canarias. (2001) 19-51.

isocrática, sin embargo, es normal realizar un gradiente de composición del solvente a lo largo de la cromatografía para mejorar la eficiencia y acortar la duración del proceso. Una vez eluído cada compuesto debe ser detectado. Para ello, se colocan a la salida de la columna cromatográfica uno o varios detectores que proporcionarán una respuesta al paso de los analitos (absorbancia, emisión fluorescente, conductividad…). El procesado de esta señal produce el cromatograma, en el que se representa la respuesta obtenida por el detector frente al tiempo. La intensidad de cada pico será directamente proporcional al factor de respuesta y a la concentración del analito correspondiente en la muestra.

2.3. Tipos de Cromatografía líquida

Dependiendo de la naturaleza de la fase estacionaria y del tipo de fenómeno físico que provoca la separación, encontramos diferentes tipos de cromatografía líquida [17]. Los más utilizados se resumen en la siguiente tabla.

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

*Muchos autores no la consideran como un tipo de Cromatografía *per se*, sino más bien incluída en la cromatografía de partición o reparto

Tabla 2. Tipos de Cromatografía líquida

El conocimiento de la estructura molecular de los componentes de la muestra puede ser muy útil en la selección de un método de cromatografía líquida. En general, podemos decir que la cromatografía de partición o reparto se aplica a compuestos polares no iónicos; la cromatografía de adsorción separa especies no polares, isómeros e hidrocarburos alifáticos; la cromatografía de intercambio iónico permite analizar compuestos iónicos de peso molecular bajo; los analitos de peso molecular superior a 10.000 se separan mediante cromatografía de filtración sobre gel (de exclusión).

[17] D. A. Skoog, J. J. Leary "Cromatografía de líquidos de alta resolución" en "Análisis instrumental". Ed. McGraw-Hill/Interamericana de España, S. A. Madrid (1996) 730-777.

2.3.1. Cromatografía de partición o de reparto

La cromatografía de partición o de reparto es el tipo de cromatografía de líquidos más ampliamente utilizado y la que hemos empleado cuando HPLC ha sido utilizada para desarrollar el trabajo experimental incluido en algunos capítulos de esta tesis.

El fundamento de este tipo de cromatografía es el reparto o la distribución de los solutos entre una fase móvil líquida y otra estacionaria inmiscible soportada sobre un sólido inerte; es decir, la causa de la discriminación entre los solutos se encuentra, de manera genérica, en las diferencias de solubilidad.

Considerando las polaridades relativas de las fases móviles y estacionarias, se distinguen dos tipos de cromatografía:

- *Cromatografía en fase normal (NP-HPLC)*: la fase estacionaria es polar, mientras que la elución se lleva a cabo con disolventes no polares, como etiléter, cloroformo o *n*-hexano.

- *Cromatografía en fase inversa (RP-HPLC)*: la fase estacionaria es no polar, tratándose normalmente de hidrocarburos tales como C_8 (n-octilo) o C_{18} (n-octadecilo) y la elución se lleva a cabo con una fase móvil de polaridad elevada, como disoluciones acuosas conteniendo metanol, acetonitrilo o tetrahidrofurano.

La cromatografía de líquidos en fase inversa es el modo de HPLC más empleado en la actualidad ya que una gran mayoría de muestras de interés en diversos ámbitos tienen naturaleza hidrofílica. En esta modalidad de fase inversa, el tiempo de retención es mayor para las moléculas de naturaleza apolar, mientras que las moléculas de carácter polar eluyen más rápidamente.

2.4. Condiciones a optimizar

Cuando se pone a punto un método cromatográfico, se persigue obtener buena resolución, precisión, altura de pico y tiempo de análisis lo más corto posible, trabajando a una presión adecuada y consumiendo el menor disolvente posible. La selección de la *fase estacionaria* (columna cromatográfica) y de la *fase móvil*, para la separación de una mezcla determinada, es un problema complicado ya que los componentes de la muestra interaccionan con ambas fases. Normalmente se elige en

primer lugar una fase estacionaria adecuada de una manera general y, seguidamente, se selecciona la fase móvil empíricamente, después de realizar una serie de ensayos. Ésta debe tener una composición tal que sea compatible con la fase fija, que disuelva los componentes a analizar y que permita una buena separación. Luego se optimizan las condiciones de flujo de solvente, cantidad de muestra a inyectar y, en el caso de un detector de absorción se determinará/n la/s longitud/es de onda de detección que resulten más idóneas.

2.4.1. Fase estacionaria

En fase reversa se utilizan fundamentalmente dos tipos de fases estacionarias (C-18 y C-8), estando formadas por grupos no polares unidos a la sílice. Además del tipo de fase estacionaria habrá que seleccionar la longitud de la columna, su diámetro y el tamaño de partícula más adecuado.

- *Diámetro*

El diámetro interno de una columna de HPLC es un aspecto crítico que determina la cantidad de muestra que se puede cargar en en la columna y también influye en su sensibilidad. Las más utilizadas son las de diámetro interno entre 2-5 mm denominadas columnas de rango analítico. Las columnas de diámetro interno más grande (>10 mm) se utilizan normalmente para el aislamiento y purificación de compuestos y se denominan columnas semipreparativas.

- *Tamaño de partícula*

En cuanto al tamaño de partícula puede ser muy variado, siendo las de 5 µm de diámetro las más utilizadas. Partículas más pequeñas ofrecen una mayor superficie y una mejor separación, consiguiendo una mejor eficacia y resolución. Sin embargo, con las columnas convencionales, no se pueden usar partículas de tamaño mucho menor de 5 µm, ya que se produciría un gran aumento de presión, no tolerado por los equipos de HPLC convencionales. Es de hecho, prácticamente imposible trabajar con columnas rellenas con fases estacionarias de menos de 3 µm de diámetro de partícula [18] en instrumentos convencionales. Los aparatos más modernos de HPLC incorporan mejoras para poder trabajar a presiones más altas y, por lo tanto,

[18] S. Eeltink, W. M. C. Decrop, G. P. Rozing, P. J. Schoenmakers, W. T. Kok. "Comparison of the efficiency of microparticulate and monolithic capillary columns". *J. Sep. Sci.* 27 (2004) 1431-1440.

poder utilizar partículas de tamaño más pequeño en las columnas (< 2µm) [19]. Así destacamos los equipos denominados RRLC (Rapid Resolution Liquid Chromatography) [20] y UPLC [21,22] (Ultrahigh-pressure liquid chromatography) que pueden trabajar con valores de hasta 600 y 1000 bares, respectivamente. Esto ha permitido mejorar la eficacia y reducir los tiempos de análisis. (Hay que tener en cuenta que las siglas UPLC son una marca registrada de Waters Corporation y RRLC de Agilent Technologies)

2.4.2. Fase móvil

En cuanto a la fase móvil se pueden aprovechar las modificaciones tanto de su fuerza eluyente (retención), como de su naturaleza (selectividad) de una forma dinámica, modificándolas en el tiempo durante la propia elución.

- *Naturaleza y fuerza eluyente*

Con frecuencia, un solo disolvente no resulta adecuado para una determinada separación, recurriéndose en estos casos a mezclas binarias o ternarias, con objeto de encontrar la más adecuada en lo que a su fuerza eluyente se refiere. Seleccionada la fuerza eluyente, podríamos emplear una elución isocrática, manteniendo constante la composición de la fase móvil, o una elución en gradiente en la cual se cambia la composición de la fase móvil durante el desarrollo del cromatograma. Con ello se consigue aumentar la eficiencia de la separación, con unos efectos similares a los producidos por la programación de temperatura en cromatografía de gases. Un buen lugar para empezar a elegir tipos de gradientes son los catálogos comerciales y los manuales. Tienen una gran cantidad de datos que sugieren buenas condiciones de partida para casi todas las clases de mezclas. Los disolventes que se van a combinar deben ser miscibles a cualquier concentración. Otra modificación de la fase móvil que puede afectar a la retención del compuesto es, por ejemplo, la adición de sales inorgánicas que provoca un aumento lineal en la tensión superficial,

[19] J. M. Cunliffe, S. B. Adams-Hall, T. D. Maloney. "Evaluation and comparison of very high pressure liquid chromatohraphy systems for the separation and validation of pharmaceutical compounds" *J. Sep. Sci.* 30 (2007) 1214-1223.
[20] T. Yoshida, R. E. Majors. "High-speed analyses using rapid resolution liquid chromatography on 1.8-um m porous particles" *J. Sep. Sci.* 29 (2006) 2421-2432.
[21] L. Novakova, D. Solichova, P. Solich. "Advantages of ultra performance liquid chromatography over high-performance liquid chromatography: comparison of different analytical 2 approaches during analysis of diclofenac gel" *J. Sep. Sci.* 29 (2006) 2433-2443.
[22] D. Guillarme, J. Schappler, S. Rudaz, J. L. Veuthey. "Coupling ultra-high-pressure liquid chromatography with mass spectrometry" *Trac-Trends Anal. Chem.* 29 (2010) 15-27.

y como la entropía de la interfase compuesto-disolvente está controlada precisamente por la tensión superficial, se produce un aumento del tiempo de retención.

Otros parámetros a optimizar relacionados con la fase móvil son el pH, el flujo y la temperatura [23].

- *pH*

El pH de la fase móvil afectará la retención si la estructura de las moléculas del soluto resulta afectada. Típicamente, las especies que pueden presentarse en forma protonada y desprotonada en el intervalo de pH de trabajo usual en RP-HPLC se verán afectadas, puesto que ambas formas, manifestarán diferente retención. A efectos prácticos, si se quiere utilizar el pH como un parámetro capaz de alterar sustancialmente la separación, tendrá que ajustarse el pH de la fase móvil de modo que su valor esté comprendido entre el $pK_a \pm 1$ de la especie o especies de interés. Si lo que se quiere es controlar el pH que puede afectar a la retención del compuesto muchos métodos utilizan un tampón como el fosfato de sodio. Estos tampones controlan el pH, pero también neutralizan la carga o cualquier resto de silica de la fase estacionaria que haya quedado expuesta y actúan como contraiones que neutralizan la carga del compuesto. El efecto de los tampones sobre la cromatografía puede variar, pero en general mejoran la separación cromatográfica.

- *Flujo y temperatura*

El flujo y la temperatura afectan a la retención relativa de los diferentes compuestos de una mezcla y, por lo tanto, a su resolución. Aumentando el flujo, se reducen los tiempos de análisis y se mejora la resolución, pero se produce un aumento de presión. Para disminuir la presión se aumenta la temperatura, ya que a temperaturas elevadas la viscosidad del disolvente disminuye, dando lugar a que el eluyente fluya más rápidamente. Hay que modificar simultáneamente ambos parámetros para conseguir separaciones adecuadas. Se deben optimizar cumpliendo con los valores de presión indicados, para tener el máximo tiempo de vida de la columna. No es necesario el control estricto de la temperatura de la columna, pero

[23] K. A. Rubinson, J. F. Rubinson. "Cromatografía líquida" en "Análisis Instrumental". Ed. Pearson Education S. A. Madrid. (2000) 636-676.

las separaciones resultan más reproducibles cuando la temperatura se mantiene constante.

Por otra parte, es necesario tener en cuenta que dado que se utilizan altas presiones, es imprescindible evitar la presencia de partículas que puedan obstruir los conductos y la formación de burbujas que puedan deteriorar el relleno de las columnas y que produzcan inestabilidad en la señal del detector. Para evitar las obstrucciones, los solventes y las muestras a inyectar se filtran con membranas de 0,45 a 0,22 μm. Para evitar la formación de burbujas, los equipos de HPLC cuentan con desgasificadores de solvente por vacío o por burbujeo con He y, en el caso de no contar con los mismos, se deben desgasificar los solventes por medio de ultrasonido o agitación bajo vacío antes de utilizarlos como fase móvil.

2.5. Nanocromatografía líquida

La miniaturización se ha convertido en un importante área de investigación y en las últimas décadas se ha prestado especial atención al estudio y desarrollo de técnicas de separación miniaturizadas. Existen dos tendencias fundamentales en la miniaturización de los sistemas de separación: el diseño y fabricación de sistemas de análisis en microchips y el desarrollo de técnicas de cromatografía líquida o de electromigración en las que la separación se realiza en columnas capilares o capilares de sílice fundida. La técnica de nanoLC fue introducida por primera vez en 1988 por Karlsson y Novotny [24] y desde entonces ha ido emergiendo como una herramienta analítica potente sobre todo en determinados campos como el de la proteómica [25], debido principalmente a la reducción en la cantidad de muestra requerida. Poco a poco se ha ido ampliando el campo de aplicación de esta técnica, y así podemos encontrar en bibliografía trabajos relacionados con la industria farmacéutica, el análisis medio ambiental y de enantiómeros [26] y con la industria alimentaria aunque en mucha menos extensión [27, 28]. En función del diámetro interno de la columna y

[24] K. E. Karlsson, M. Novotny. "Separation efficiency of slurry-packed liquid-chromatography microcolumns with very small inner diameters" *Anal. Chem*. 60 (1988), 1662 -1665.
[25] Y. Ishihama. "Proteomic LC-MS systems using nanoscale liquid chromatography with tandem mass spectrometry" *J. Chromatogr. A* 1067 (2005) 73-83.
[26] J. Hernández-Borges, Z. Aturki, A. Rocco, S. Fanali. "Recent applications in nanoliquid chromatography" *J. Sep. Sci.* 30 (2007) 1589-1610.
[27] S. Fanali, E. Camera, B. Chankvetadze, G. D'Orazio, M. Quaglia. "Separation of tocopherols by nano-liquid chromatography". *J. Pharm. Biomed. Anal.* 35 (2004) 331-337.

de los caudales utilizados, las técnicas de HPLC se clasifican en diversas categorías que se muestran en la Tabla 3. Generalmente se considera que cuando la separación cromatográfica se lleva a cabo en columnas de diámetro interno entre 10 y 100 µm, la técnica se denomina nanocromatografía líquida, cuando se usan columnas de diámetro entre 100 y 500 µm se conoce como cromatografía capilar y para columnas con diámetros alrededor de 800 µm se define como micro- cromatografía.

Parámetro	LC alta eficacia (HPLC)	Micro LC	LC Capilar	Nano LC
Diámetro interno de las columnas	1.5-4.5 mm	0.8 mm	0.18-0.32 mm	0.075-0.1 mm
Longitud del lecho cromatográfico	3-30 cm	5-25 cm	5-25 cm	15-25cm
Diámetro medio de partículas	3-40 µm	3-5 µm	3-5 µm	3-5 µm
Flujo de fase móvil	0.2-2.5 ml/min	10-100 µl/min	1-10 µl/min	0.1-1 µl/min

Tabla 3. Algunos parámetros característicos de las diferentes técnicas de la cromatografía líquida en columna.

2.5.1. Instrumentación

El instrumento utilizado en el trabajo experimental que se ha incluido en en dos capítulos de la tesis es un equipo comercial de la empresa Bruker Daltonik (Fig. 5) compuesto por un único módulo que consta de: 4 válvulas (fase A (acuosa) , fase B (orgánica), muestra S y desecho W), 3 sensores de presión, 3 bombas (A, B y muestra S) y 2 sensores de flujo.

[28] S. Fanali, Z. Aturki, G. D'Orazio, M. A. Raggi, M. G. Quaglia, C. Sabbioni, A. Rocco. "Use of nano-liquid chromatography for the analysis of glycyrrhizin and glycyrrhetic acid in licorice roots and candies" *J. Sep. Sci.* 28 (2005) 982-986.

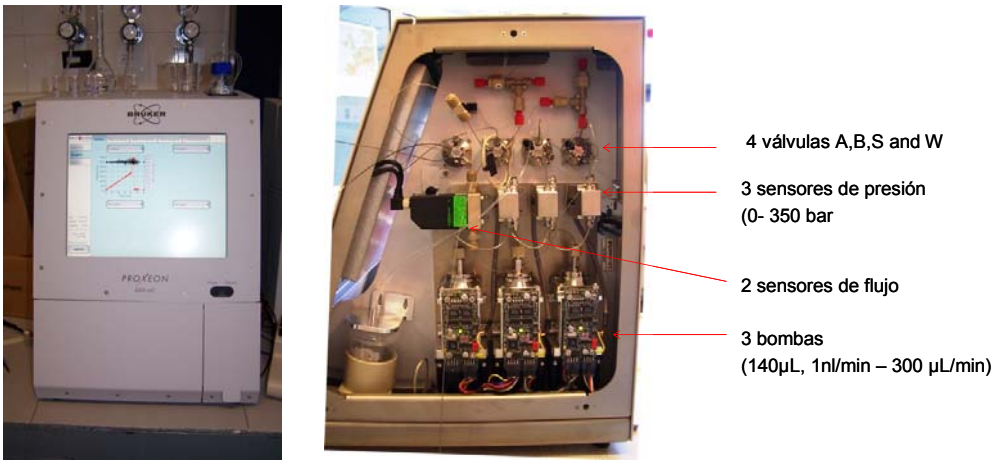

Figura 5. Equipo de nano- LC empleado en el desarrollo experimental.

2.5.2. Principales características

Las principales características que presenta la técnica de nanoLC son:

1) Utiliza *columnas que son capilares*, normalmente de sílice, con un diámetro interno muy pequeño (10-100 µm) y que contienen las fases estacionarias normalmente empleadas en cromatografía líquida (C8, C18). El uso de pequeñas cantidades de fase estacionaria resulta muy interesante porque se pueden emplear materiales de empaquetamiento más caros, como por ejemplo para el análisis de enantiómeros donde se utilizan partículas de sílice modificadas con diferentes selectores quirales. En la mayoría de las aplicaciones se utilizan columnas capilares fabricadas en el propio laboratorio [27]. Se emplean normalmente tamaño de partícula entre 3-5 µm [29], ya que valores más pequeños, aunque aumentan la eficacia y disminuyen los tiempos de análisis, producen un aumento de la presión no tolerado por los equipos de nanoLC.

2) Desde un punto de vista teórico una reducción del diámetro de la columna conlleva un aumento en la *sensibilidad* debido a la disminución de la dilución cromatográfica y al incremento de la eficacia. Sin embargo debido a la baja capacidad de carga, estas columnas de pequeño diámetro presentan volúmenes ideales de inyección del orden de los nanolitros (20-60 nl) por lo que el sistema no puede ofrecer alta sensibilidad. Para conseguir alta sensibilidad se deben utilizar métodos que

[29] H. Liu, J. W. Finch, M. J. Lavallee, R. A. Collamati, C. C. Benavides, J. C. Gebler. "Effects of column length, particle size, gradient length and flow rate on peak capacity of nano-scale liquid chromatography for peptide separation" *J. Chromatogr. A* 1147 (2007) 30-36.

permitan inyectar cantidades de muestra mayores sin producir problemas de solapamiento [30]. Las dos técnicas más utilizadas son la preconcentración on columna y off-columna. En la primera la muestra se disuelve en un disolvente con menor poder de elución que la fase móvil y se inyecta en la columna [31]. La segunda solución consiste en utilizar una precolumna de mayor diámetro que acepta flujos más elevados, donde se preconcentra la muestra antes de transferirla a la columna analítica [32]. Dependiendo de la composición de la *pre-columna* y de la instrumentación disponible se puede utilizar tanta para pre-concetrar la muestra como para un proceso previo de limpieza, como por ejemplo cuando se utilizan reactivos de derivatización [32]. Se recomienda el uso de estas precolumnas ya que la columna capilar se puede bloquear con facilidad cuando se analizan muestras reales.

3) Ofrece la posibilidad de inyectar *pequeñas cantidades de muestra*, lo que resulta muy ventajoso en determinados campos de aplicación donde la disponibilidad de muestra está limitada como en el emergente campo de las Ciencias Ómicas. Sin embargo, como indicábamos antes cuando se inyectan pequeños volúmenes de muestra no se consiguen sensibilidades elevadas, por lo que el analista tiene que llegar a un compromiso entre cantidad de muestra inyectada y sensibilidad.

4) Utiliza *flujos de fase móvil muy bajos* comprendidos entre 100 y 500 nl/min, con los disolventes normalmente empleados en HPLC, mientras que en la cromatografía líquida convencional el flujo está entre 0.2 y 2.5 ml/min. Esto produce una disminución considerable del consumo de reactivos y disolventes, y por ello de los desechos generados y del impacto medioambiental. Además debido a los bajos flujos que se utilizan esta técnica posee una excelente capacidad de acoplamiento directo con técnicas de detección como la espectrometría de masas, que requiere la eliminación previa del disolvente.

[30] H. A. Claessens, M. A. J. Kuyken. "A comparative study of large volume injection techniques for microbore column in HPLC" *Chromatographia*, 23 (1987), 331-336.
[31] M. J. Mills, J. Maltas, W. J. Lough. "Assesment of injection volume limits when using on-column focusing with microbore liquid chromatography" *J. Chromatogr. A* 759 (1997) 284-285.
[32] G. D'Orazio, A. Cifuentes, S. Fanali. "Chiral nano-liquid chromatography-mass spectrometry applied to amino acids analysis for orange juice profiling" *Food Chem*. 108 (2008) 1114-1121.

3. Cromatografía de gases

3.1. Definición e instrumentación

En la Cromatografía de Gases (GC) se utiliza como fase móvil un gas portador inerte, que eluye los componentes de una mezcla a través de una columna que contiene una fase estacionaria inmovilizada. La forma más usual de hacer GC es utilizando como fase estacionaria un líquido que recubre la pared interna de una columna o un soporte sólido, lo que recibe el nombre de *Cromatografía Gas-Líquido (CGL)*. Existe otro tipo de cromatografía de gases menos utilizada, denominada *Cromatografía Gas-Sólido (CGS)* donde el analito se adsorbe directamente sobre las partículas sólidas de la fase estacionaria [33]. El gas portador es un gas inerte generalmente helio, nitrógeno o argón de elevada pureza, con un caudal conocido y controlado. La fase móvil no interacciona con el analito y su única misión es la de transportar la muestra. Las columnas pueden ser con *relleno*, en las que la fase estacionaria líquida está retenida sobre un sólido inerte (soporte) y *capilares,* en las que la fase estacionaria se fija sobre las paredes interiores del capilar. En la inmensa mayoría de los análisis se utilizan columnas capilares, largas y estrechas, hechas de sílice fundida (SiO_2) y recubiertas de poliimida como soporte y como protección de la humedad atmosférica. Los diámetros interiores típicos son de 0.1-0.53 mm y las longitudes típicas de 15 a 100m.

Las partes esenciales de un cromatógrafo de gases son [34] (Fig. 6): fuente de gas portador (botella a presión), sistema de regulación de caudales (válvula reguladora y manómetro), bloque termostatizado de inyección de las muestras, columna termostatada conteniendo la fase estacionaria, detector termostatado con amplificador de señal y registro gráfico, caudalímetro de precisión.

[33] K. A. Rubinson, J. F. Rubinson. "Cromatografía de gases y cromatografía de fluidos supercríticos" en "Análisis Instrumental". Ed. Pearson Education S. A. Madrid. (2000) 680-701.
[34] J. F. Loro Ferrer. "Cromatografía de gases" en "Manual de Cromatografía". Ed. Dirección General de Universidades e Investigación. Consejería de Educación, Cultura y Deporte. Gobierno de Canarias. (2001) 107-156.

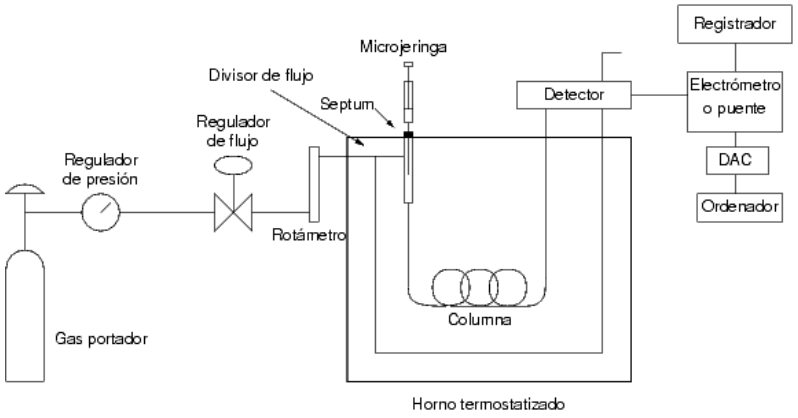

Fig ura 6. Diagrama de un cromatógrafo de gases

3.2. Principios de funcionamiento

La muestra de un líquido volátil (o de un gas) se inyecta, a través de un septum, en la cámara de inyección donde se vaporiza y se arrastra hasta la cabeza de columna. La temperatura inicial de la columna se fija en 40º C por debajo del punto de ebullición del disolvente, que condensa por tanto en la cabeza de la columna. Como los solutos van quedando atrapados lentamente en la porción de disolvente condensado, forman una estrecha banda al principio de la columna. Los analitos, en forma gaseosa, se hacen pasar a través de la columna, arrastrados por el gas portador, y por equilibrios sucesivos entre fase móvil y estacionaria cada componente se desplaza por la columna a velocidades diferentes. La columna debe estar suficientemente caliente a fin de que los analitos alcancen una presión de vapor suficiente para que se eluyan en un tiempo razonable. Finalmente, los analitos después de separados llegan al detector cuya respuesta aparece en la pantalla de un ordenador o un registrador en forma de cromatograma. El detector se mantiene a una temperatura más alta que la columna, de forma que los analitos se encuentran en forma gaseosa[35].

3.3. Preparación de muestra

La clave del éxito para llevar a cabo un buen análisis en cromatografía es preparar la muestra en la forma adecuada para que la misma pueda ser analizada satisfactoriamente. Este proceso puede incluir la extracción de un analito de una matriz compleja, la preconcentración de analitos muy diluidos o la transformación del analito

[35] D. A. Skoog, J. J. Leary "Cromatografía de gases" en "Análisis instrumental". Ed. McGraw-Hill/Interamericana de España, S. A. Madrid (1996) 704-729.

en una forma que pueda ser fácilmente detectable. En concreto en la GC destacamos la extracción de espacio de cabeza (HS: "head-space") y la microextracción en fase sólida, que son métodos para aislar componentes volátiles de matrices complejas y las reacciones de derivatización.

3.3.1. Extracción de espacio de cabeza (HS: "head-space")

El espacio de cabeza, es una técnica analítica ampliamente utilizada para la extracción de analitos a partir de muestras sólidas o líquidas [36,37]. En este método, la muestra se coloca en un vial sellado con un septum y se calienta durante un tiempo determinado a la temperatura fijada. Durante esta operación, la mayor parte de los compuestos volátiles son liberados de la matriz y se transfieren al aire del vial, denominado espacio de cabeza. Se calienta el tiempo suficiente para que se alcance el equilibrio. Seguidamente, con una jeringa se toma una alícuota del aire del vial y se inyecta en el cromatógrafo. La aguja de la jeringa debe calentarse a la misma temperatura que la muestra para evitar condensaciones sobre la misma. Utilizada de esta forma la técnica se denomina "espacio de cabeza estático". Si la mezcla de compuestos volátiles que se originan se arrastran con un gas portador, tenemos una segunda variante llamada "espacio de cabeza dinámico", que implica una mejor volatilización

3.3.2. Microextracción en fase sólida

La microextracción en fase sólida es un método sencillo de extracción de líquidos, aire, o incluso sólidos, sin usar disolventes [38, 39]. El componente clave es una fibra de sílice fundida recubierta de una fina película de 10 a 100 µm, de un líquido no volátil, semejante a las fases estacionarias utilizadas en cromatografía de gases. Se expone la fibra a la disolución de la muestra (o al espacio libre sobre un líquido) durante un tiempo mientras se agita y si conviene también calentando. Sólo se extrae en la fibra una fracción del analito que hay en la muestra. Una vez tomada la muestra, se retira la fibra y se inyecta la jeringa dentro de un cromatógrafo de gases. Se introduce la fibra

[36] Y. Seto. "Determination of volatile substances in biological samples by headspace gas chromatography" *J. Chromatogr. A* 674 (1994) 25-62
[37] M. S. Altaki, F. J. Santos, M. T. Galceran. "Automated headspace solid-phase microextraction versus headspace for the analysis of furan in foods by gas chromatography-mass spectrometry" *Talanta* 78 (2009) 1315-1320.
[38] J. Pawliszyn. "Solid phase microextraction, theory and practice" Wiley-VCH, New York (1997).
[39] C. Dietz, J. Sanz, C. Camara. "Recent developments in solid-phase microextraction coatings and related techniques" *J. Chromatogr. A* 1103 (2006) 183-192.

dentro de la guía caliente del inyector, en cuyo interior el analito se desorbe térmicamente de la fibra, y se introduce sin división durante un tiempo fijo

3.3.3. Reacción de derivatización

Un requisito de los que ha de cumplir un analito para ser susceptible de ser analizado por GC es que sea volátil y térmicamente estable. Sin embargo, la volatilidad se fuerza, en la mayor parte de casos, mediante derivatización química antes del análisis. Los compuestos que contienen grupos funcionales con hidrógenos activos (-COOH, -OH, -NH y -SH) se suelen derivatizar antes de su análisis por GC, ya que estos grupos funcionales tienden a formar enlaces de hidrógeno intermolecular que afectan a la volatilidad, pueden interactuar negativamente con la fase estacionaria de la columna y pueden ser térmicamente inestables. La sililación, acilación y la alquilación son las técnicas de derivatización usadas para alterar estos grupos funcionales mejorando sus características térmicas y cromatográficas [40,41].

- ***Sililación***

Los derivados con silicio son los más ampliamente usados en aplicaciones de cromatografía de gases. En general se forman por substitución de los hidrógenos activos de ácidos, alcoholes, tioles, aminas, amidas y cetonas y aldehídos susceptibles de ser reemplazados por grupos TMS (TriMetilSilil). Existe una amplia gama de reactivos disponibles para la introducción de estos grupos, que difieren en su reactividad, selectividad y reacciones secundarias y en el carácter de los productos de reacción. Existe una amplia literatura disponible que permite seleccionar el reactivo de sililación más adecuado para cada problema analítico particular.

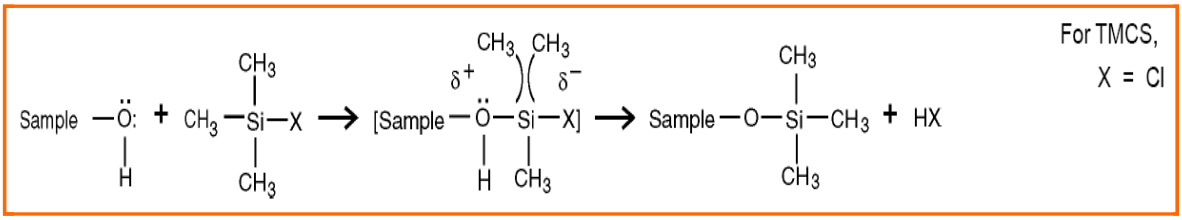

Figura 7 Reacción de derivatización empleando TMS y BSTFA como agente derivatizante

[40] K. Blau, J. Halket. "Handbook of derivatives for chromatography (2nd ed.)" John Wiley & Sons, New York (1993).
[41] D. L. Lin, S. M. Wang, C. H. Wu, B. G. Chen, R. H. Lu. "Chemical derivatization for the analysis of drugs by GC-MS-A conceptual review"*J. Food Drug Anal.* 16 (2008) 1-10.

Los reactivos de sililación y los derivados TMS son inestables frente a reacciones de hidrólisis y deben ser protegidos frente a la humedad. Sin embargo el grado de hidrólisis de cada reactivo y derivado es diferente, y, en algunos casos, es posible preparar derivados en presencia de pequeñas cantidades de humedad, o aislar y purificar derivados por extracción en un solvente orgánico y lavado con soluciones acuosas. Los reactivos que introducen un grupo t-Butilsililo en lugar de un grupo TMS se desarrollaron para presentar una estabilidad hidrolítica mayor. Estos derivados ofrecen una mayor estabilidad frente a la hidrólisis y generan perfiles de fragmentación específicos, lo que los hacen muy útiles en aplicaciones de GC-MS.

La mayoría de los derivados TMS o TBDS tienen una excelente estabilidad térmica y resultan adecuados para una amplia gama de condiciones de inyector y columna. Sin embargo, puesto que los reactivos de sililación modificarán prácticamente todos los hidrógenos activos, es importante que no se inyecten en columnas con fases estacionarias que contengan dichos grupos funcionales.

- ***Acilación***

La acilación es la conversión de compuestos que contengan hidrógenos activos como -H, -SH y -NH en ésteres, tioésteres y amidas mediante la acción de una ácido carboxílico o un derivado de un ácido carboxílico [42]. En aplicaciones cromatográficas, la reacción de acilación se usa principalmente para convertir las clases anteriores de compuestos en derivados que sean más adecuados para cromatografía o que dan una mayor respuesta en la detección cromatográfica que el compuesto original. Un ejemplo importante de esta aplicación es la inserción de grupos perfluoroacetilo en una molécula para incrementar la respuesta en detectores de captura de electrones (ECD). Los acilderivados resultan también útiles en aplicaciones de espectrometría de masas en las que modifican los perfiles de fragmentación de los compuestos a estudiar

- ***Alquilación***

La alquilación es la substitución de un hidrógeno activo por un grupo alifático o alifático-aromático (bencilo). Esta técnica se usa para modificar los compuestos con hidrógenos activos como ácidos carboxílicos y fenoles. La aplicación principal de esta

[42] R. J. Wells. "Recent advances in non-silylation derivatization techniques for gas chromatography" *J. Chromatogr. A* 843 (1999) 1-18.

técnica es la conversión de ácidos orgánicos en ésteres, que permiten mejores cromatogramas que los ácidos libres. Además la reacciones de alquilación pueden usarse para preparar éteres, tioéteres , tioésteres, N-alquilaminas y amidas. A medida que disminuye la acidez de los hidrógenos activos, ha de aumentarse la fuerza del agente alquilante. Al ser las condiciones más extremas y los reactivos más potentes, la selectividad y aplicabilidad de los métodos resulta más limitada.

3.5. Condiciones a optimizar

Por definición estándar, una cromatografía de gases es optimizada cuando se consigue sensibilidad y resolución de una mezcla compleja en el menor tiempo posible. La optimización involucra una serie de variables relacionados con la fase estacionaria (tipo de columna, longitud y diámetro interno), la fase móvil y parámetros operacionales como la temperatura de la columna o los modos de inyección [43].

3.5.1. Fase estacionaria. Selección de la columna

En la inmensa mayoría de los análisis se utilizan columnas tubulares abiertas, también denominadas columnas capilares, largas y estrechas, hechas de sílice fundida (SiO_2) y recubiertas de poliimida como soporte y como protección de la humedad atmosférica (Fig. 8). Las columnas capilares ofrecen mayor resolución, mayor rapidez de análisis y mayor sensibilidad que las columnas empaquetadas, aunque tienen menor capacidad de muestra.

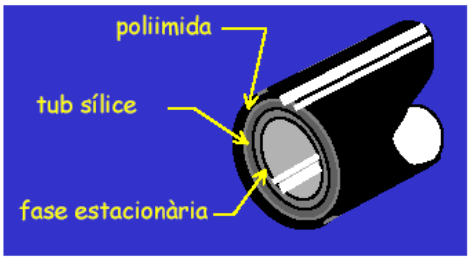

Figura 8. Esquema de una columna tubular abierta

[43] D. C. Harris. "Cromatografía de gases" en "Análisis químico cuantitativo". Ed. Reverté, S. A. Barcelona (2001) 656-686.

La elección básica son fase estacionaria, diámetro y longitud de columna y espesor de la fase estacionaria. Se elegirá un tipo u otro de fase estacionaria líquida en función de la polaridad de los compuestos siguiendo la regla "lo semejante disuelve a lo semejante". Las columnas no polares son las más indicadas para solutos no polares y las muy polares para solutos muy polares. A menudo que la columna envejece, la fase estacionaria se altera, quedan al descubierto grupos silanol (Si-O-H) y aumentan las colas.

En cuanto al diámetro y la longitud, los diámetros interiores típicos son de 0,1-0,53 mm y las longitudes típicas de 15 a 100 m. Fijada la longitud hay que buscar una combinación adecuada de diámetro de columna y espesor de película. Para lograr máxima resolución se precisan *columnas estrechas de espesor muy fino* de fase estacionaria. Permiten análisis rápidos y son especialmente adecuadas para separar mezclas de compuestos de alto punto de ebullición, que se retienen demasiado en columnas de película gruesa. Sin embargo tienen capacidad de muestra muy pequeña, necesitan detectores de gran sensibilidad y no retienen bien a los componentes de punto de ebullición bajo. Las *columnas estrechas de película gruesa* constituyen un buen compromiso entre resolución y capacidad de muestra. Pueden usarse con todos los detectores y con compuestos muy volátiles aunque los tiempos de retención son mayores que los de columnas de película fina. Las *columnas anchas de película gruesa* tienen gran capacidad de muestra, pueden separar compuestos muy volátiles pero dan baja resolución y tienen tiempos de retención grandes. Si una columna cumple la mayoría de los requisitos pero no tiene suficiente resolución, se puede usar una columna más larga del mismo tipo.

3.5.2. Fáse móvil

Ha de ser un gas inerte, que no interaccione con la fase estacionaria ni con el analito, como el helio, el argón, el nitrógeno y el hidrógeno, aunque el que más se usa es el helio. La elección del gas portador se hace, frecuentemente, en función del detector. El nitrógeno, helio y hidrógeno suele utilizarse con los detectores de ionización de llama (FID). El argón con el detector de captura electrónica (ECD). El helio e hidrógeno con el detector de conductividad térmica (TCD), por su elevada conductividad; si bien el hidrógeno es un fuerte reductor, lo que puede limitar su uso. El rango de presiones varía entre 0.7 a 3,5 kg/cm^2 por encima de la presión ambiental, lo que proporciona una

velocidad de flujo desde 25 a 50 ml/min en las columnas de relleno, y de 1 a 25 ml/min en las columnas capilares. Las impurezas que puedan existir en el gas portador degradan la fase estacionaria. Se deben usar gases de una gran calidad, y aun éstos se deben pasar a través de purificadores para eliminar el oxígeno, el agua o trazas de compuestos orgánicos, antes de entrar en la columna.

3.5.3. Temperatura de la columna

Una de las variables más importantes en el desarrollo cromatográfico, es la temperatura de la columna. La temperatura óptima es función de los puntos de ebullición de los componentes de la muestra y del grado de separación deseado. En muestras de parecidos puntos de ebullición la temperatura óptima es ligeramente superior al punto de ebullición medio de los componentes de la muestra. En el caso de muestras complejas, en la que los puntos de ebullición de los distintos componentes son muy diferentes, se recomienda emplear un gradiente de temperatura, aumentando ésta de forma continua o de forma escalonada a medida que avanza la separación. El aumento de la temperatura reduce los tiempos de retención de los compuestos, sin embargo, no se debe elevar mucho la temperatura para evitar la descomposición térmica de los analitos y de la fase estacionaria. En general, para obtener una resolución óptima hay que trabajar a temperatura mínima; sin embargo, el costo de una temperatura baja es un aumento del tiempo de elución y por consiguiente del tiempo requerido para hacer el análisis.

3.5.4. Sistema de inyección

Otra decisión importante es cómo inyectar la muestra. Los requisitos que debe cumplir un sistema de inyección son: producir bandas iniciales lo más estrechas posibles y no alterar la composición de la mezcla que llega a la columna. Existen tres técnicas básicas de inyección de muestras (líquidas o gaseosas) en columnas capilares en función del tipo de muestra: split, split-less y on-column (Fig. 9). Las dos primeras consisten en inyectar y vaporizar la muestra en una cámara de vaporización. La muestra (líquidos) se inyecta mediante una microjeringa calibrada, a través de un disco (septum) de goma, en un inyector caliente que contiene un tubo de vidrio silanizado y que se encuentra en la cabeza de la columna. El bloque de inyección de ordinario está a 50ºC por encima del punto de ebullición del componente menos volátil de la muestra, a fin de

que tenga lugar una rápida vaporización. El volumen de muestra líquida que se inyecta suele ser de 0.1-2 µl. Los productos de descomposición y los componentes no volátiles de la muestra se van acumulando en el tubo de vidrio, que se debe reemplazar periódicamente.

En el sistema *split* una vez vaporizada la muestra, una pequeña fracción del vapor entra en la columna cromatográfica (entre 0.2 y 2% de la muestra), mientras que la mayor parte se desecha fuera del sistema. Es el mejor modo, cuando la concentración del analito es alta o se analizan gases, da una gran resolución y se pueden tratar muestras complejas, aunque para análisis cuantitativo resulta pobre y durante la inyección se pueden perder compuestos volátiles.

El método *split-less* dirige toda la muestra vaporizada a la columna y es el modo más adecuado para el análisis de trazas de analitos o de componentes muy volátiles. Es mejor que el modo con división para compuestos de moderada estabilidad térmica porque la temperatura de inyección es menor pero sigue siendo pobre en análisis cuantitativo porque se pueden perder compuestos menos volátiles durante la inyección.

La inyección *on-column* se lleva a cabo en frío, eliminando la etapa de vaporización que podría producir la descomposición de los compuestos termolábiles. La disolución se inyecta directamente en la columna, sin pasar por un inyector caliente. La cromatografía se inicia calentando la columna. Es el mejor modo de inyección en análisis cuantitativo y en el caso de compuestos sensibles térmicamente pero es una técnica de resolución baja.

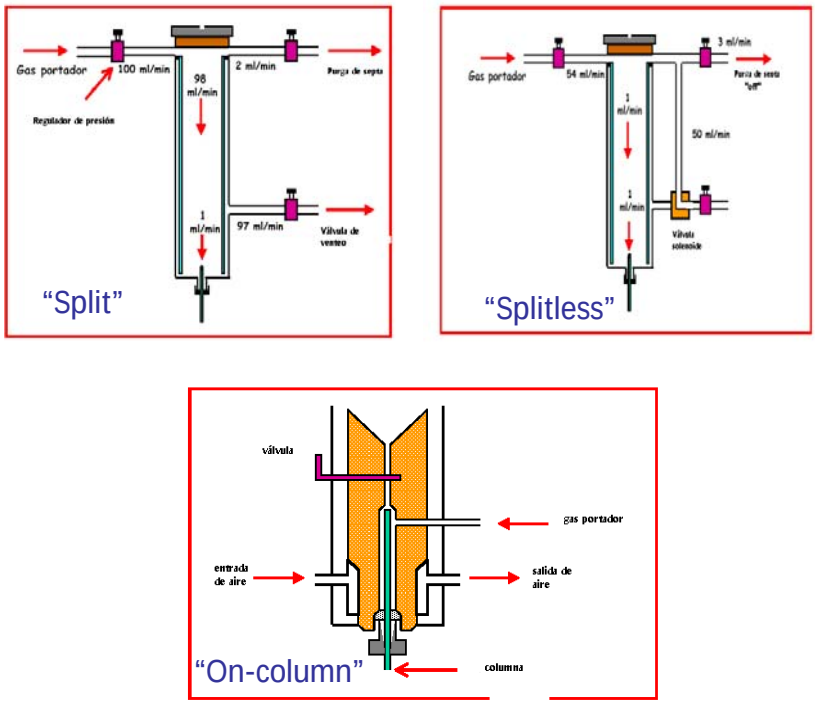

Figura 9. Esquema de diferentes tipos de inyección en GC

B) SISTEMAS DE DETECCIÓN

De forma general, cuando se acopla un detector a un sistema de separación, el analista persigue que el detector cumpla una serie de requisitos que le aseguren que los analitos previamente separados serán detectados adecuadamente:

- Presentar una buena sensibilidad.
- Proporcionar límites de detección bajos (combinación de una alta sensibilidad con una baja fluctuación en la señal de fondo).
- Presentar una determinada selectividad a una serie de analitos o a uno determinado, evitando así posibles interferencias en la señal por parte de otras sustancias presentes en la muestra.
- La respuesta debe ser rápida ante un cambio en la concentración de analito.
- La presencia del detector no debe perjudicar a la eficacia de la separación.
- Proporcionar señales fiables: reproducibles y estables en el tiempo.
- Idealmente, la señal debe ser nula en ausencia de analito.
- Proporcionar cambios en su señal en el margen más amplio posible de concentración o masas del analito, es decir que presente un amplio intervalo lineal.

Son varios los detectores que se pueden acoplar a las técnicas de separación descritas y, se podrían clasificar en tres categorías: detectores ópticos, electroquímicos y "otros", que incluiría los detectores radioquímicos y la espectrometría de masas.

En el trabajo experimental que se recoge en la presente memoria, se ha empleado la detección espectrofotométrica Ultravioleta- Visible (UV-Vis) acoplada a CE y HPLC, el detector de ionización de llama (FID) con GC y la espectrometría de masas (MS) con todas las técnicas de separación descritas (CE, HPLC, nanoLC y GC). Se procederá a la descripción más detallada de los mencionados sistemas de detección en los próximos apartados.

1. Absorción UV-Visible

Los métodos basados en la absorbancia de la luz UV-Vis son los más utilizados en equipos comerciales de CE y HPLC debido a su posibilidad para determinar un gran número de compuestos y grupos funcionales y su facilidad de manejo. Este detector

tiene su fundamento en la interacción entre la radiación UV-Vis y la materia, dando origen al fenómeno conocido como absorción de la radiación. Son muchas las moléculas que pueden absorber radiación UV-Vis y ello hace que este modo de detección pueda considerarse muy cercano al detector universal. Este comportamiento presenta la ventaja de que al ser sensible a la gran mayoría de las especies químicas puede utilizarse para resolver muchos problemas analíticos pero el inconveniente de su baja selectividad en caso de que se requiera. Además, como los espectros moleculares de absorción son de bandas anchas, cualquier compuesto que coeluya con el analito probablemente interferirá en la cuantificación porque será muy difícil seleccionar una longitud de onda de medida de absorción del analito a la que el coeluyente sea totalmente transparente aunque tengan espectros bien diferenciados. Consecuentemente, la utilización del detector UV-Visible obliga a establecer métodos con un adecuado poder de resolución del analito respecto de todos los compuestos absorbentes contenidos en la muestra. Presenta además buena sensibilidad, gran rango dinámico lineal y buena estabilidad a los cambios de flujo y temperatura.

Estos detectores pueden ser de tres tipos: longitud de onda fija, longitud de onda variable y diodo array. Los detectores de *longitud de onda fija* son los más simples y utilizan la intensa raya de emisión a 254 nm de una lámpara de mercurio. Los instrumentos más versátiles, de *longitud de onda variable*, tienen lámparas de deuterio, xenón, o volframio y un monocromador, con el que se puede elegir la longitud de onda óptima, de ultravioleta o visible, para detectar los analitos estudiados. El *diodo array* es el más moderno y más potente de los tres, conduce la luz mediante un sistema de diodos alineados, evita la dispersión y registra el espectro completo de cada soluto que pasa por el detector.

Cuando este detector se utiliza acoplado a CE, una pequeña sección de la capa de imida, que envuelve al capilar para darle resistencia, se tiene que eliminar mediante calentamiento con una pequeña llama y la detección se lleva a cabo a través de esta ventana. Debido a la utilización de capilares con diámetros internos muy pequeños la sensibilidad del detector UV-Vis en el acoplamiento con CE es mucho menor que la de otros sistemas de detección. Además, tanto la disolución portadora en CE como los

disolventes utilizados en LC tienen que ser cuidadosamente seleccionados de manera que no absorban a la longitud de onda de trabajo [44,45].

Para llevar a cabo la identificación y cuantificación de los compuestos utilizando la absorción UV-Vis como sistema de detección, habrá que disponer de patrones comerciales o patrones aislados mediante HPLC preparativa. Aun así, con este sistema, una identificación inequívoca es difícil de conseguir.

2. Detector de ionización de llama

El detector de ionización de llama (FID) es uno de los detectores más generalizados en cromatografía de gases junto con los de conductividad térmica (TCD), captura electrónica (ECD) y MS. Básicamente es un quemador de hidrógeno/oxígeno, donde se mezcla el efluente de la columna (gas portador y analito) con hidrógeno (ver Fig. 8). La temperatura del detector ha de ser mayor o igual que la de la columna para evitar la condensación de los compuestos. Inmediatamente este gas mezclado se enciende mediante una chispa eléctrica, produciéndose una llama de alta temperatura. La mayoría de los compuestos orgánicos al someterse a altas temperaturas pirolizan y producen productos intermedios iónicos y electrones que al ser conductores eléctricos facilitan un mecanismo de paso de corriente eléctrica a través de la llama. Este hecho se aprovecha estableciendo una diferencia de potencial de unos centenares de voltios entre la parte inferior del quemador y un electrodo colector situado por encima de la llama. La corriente generada se utiliza como señal de detección, pero al ser demasiado baja se debe amplificar mediante un amplificador de alta impedancia antes de ser registrada.

El detector de ionización de llama es quizás el detector más utilizado en GC, debido a las ventajas que presenta en cuanto a su buena sensibilidad incluso con columnas de paso estrecho, amplio intervalo de respuesta lineal, volumen muerto casi inexistente, poco ruido, robustez y sencillez. Es el detector que normalmente se usa con columnas capilares. Una desventaja del detector de ionización de llama es que destruye la muestra.

[44] J. J. Berzas-Nevado, G. Castañeda Peñalvo. "La detección espectrofotométrica UV-Visible en electroforesis capilar" en "Electroforesis capilar: aproximación según la técnica de detección". A. Fernández-Gutiérrez, A. Segura- Carretero (Eds). Ed. Univ. Granada. (2005) 157-187.
[45] A. L. Crego, M. L. Marina. "UV-Vis absorbance detection in capillary electrophoresis" en "Analysis and detection by capillary electrophoresis". M. L. Marina, A. Ríos, M. Valcárcel (Eds). Elsevier, Amsterdam (2005) 225-296.

Se aplica a compuestos orgánicos, es selectivo para sustancias que contienen uniones C-H en su estructura, pero es poco sensible a grupos como carbonilo, aminas, alcoholes y halógenos y totalmente insensible a gases no combustibles como H_2O, CO_2, SO_2 y óxidos de nitrógeno. Este hecho, más que limitar el ámbito de aplicación de este detector, lo hace más selectivo permitiendo el análisis de muestras contaminadas con alguno de los compuestos mencionados. El proceso de ionización que se da en la llama es complejo, pero se cree que la respuesta de este detector, está directamente relacionada con el número de enlaces carbono e hidrógeno del compuesto a analizar. Esto produce que sea un detector sensible a la masa (al número de átomos de carbono que salen de la columna) más que a la concentración, por lo tanto no le afectan demasiado los cambios en el flujo de salida.

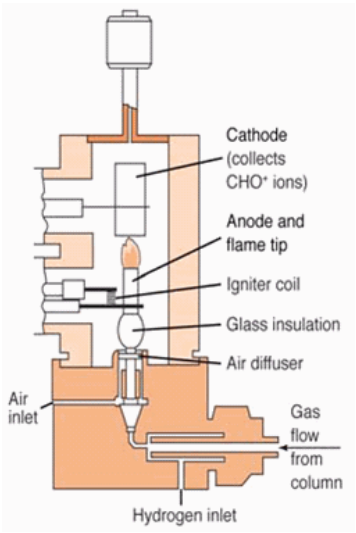

Figura 10. Detector de ionización de llama

3. Espectrometría de masas

La espectrometría de masas (MS) es una técnica que se basa en ionizar moléculas gaseosas acelerarlas en un campo eléctrico, y luego separarlas de acuerdo con su relación masa-carga [46]. El proceso de ionización a veces suministra suficiente energía para que las moléculas se rompan en diversos fragmentos. La corriente total de todos los iones generados se registra en un intervalo amplio seleccionado de masas y el cromatograma que representa la corriente iónica total frente al tiempo de elución se

[46] A. Ei-Aneed, A. Cohen, J. Banoub. "Mass spectrometry, review of the basics; electrospray, MALDI, and commonly used mass analyzers" *Appl. Spectrosc. Rev.* 2009 (44) 210-230.

denomina TIC (Total Ion Current o Total Ion Chromatogram). También se puede hacer un seguimiento de un ión seleccionado, si se busca un compuesto o una clase de compuestos determinados (EIC o EIE, Extract Ion Chromatogram o Extract Ion Electropherogram). El eje de abscisas de un espectro de masas es m/z, la masa del ion (m, en unidades de masa atómica) dividida por el número de cargas que lleva (z). La mayoría de los iones tienen una sola carga, de modo que m/z es equivalente a la masa del ión en unidades de masa atómica.

La MS es probablemente, de entre todas las herramientas analíticas al alcance del científico, la de aplicación más general. Recientemente, se ha utilizado no sólo en investigación, sino también en análisis de rutina de los procesos industriales, en control de calidad, etc.

Sus principales cualidades son:

- Capacidad de identificación ya que proporciona un espectro característico de cada molécula.
- Cuantitativa: permite medir la concentración de las sustancias.
- Gran sensibilidad: habitualmente se detectan concentraciones del orden de ppm o ppb
- Universal y específica.
- Proporciona información estructural sobre la molécula analizada.
- Suministra información isotópica.
- Es una técnica rápida: se puede realizar un espectro en décimas de segundo, por lo que puede monitorizarse para obtener información en tiempo real sobre la composición de una mezcla de gases

Se puede utilizar directamente sin necesidad de poner delante una técnica separativa (experimentos de infusión directa) [47], sin embargo la combinación de una técnica de separación de alta eficacia (GC, LC y CE) con la espectrometría de masas como sistema de detección, da lugar a una herramienta muy útil en el análisis de muestras complejas [48, 49].

[47] M. J. Lerma-García, G. Ramis-Ramos, J. M. Herrero-Martínez, E. F. Simo-Alfonso. "Classification of vegetables oils according to their botanical origin using profiles established by direct infusion mass spectrometry" *Rapid Commun. Mass Spectrom*. 22 (2008) 973-978.
[48] E. Ibañez, A. Cifuentes. "New analytical techniques in food science". *Crit. Rev. Food Sci. Nutr*. 41 (2001) 413-450.
[49] K. B. Tomer. "Separation combined with mass spectrometry". *Chem. Rev.* 101 (2001) 297-328.

Un esquema de lo que sería el acoplamiento de cualquier técnica separativa con la espectrometría de masas se muestra en la Figura 2, en la que indicamos los acoplamientos utilizados en la parte aplicada de esta memoria de tesis

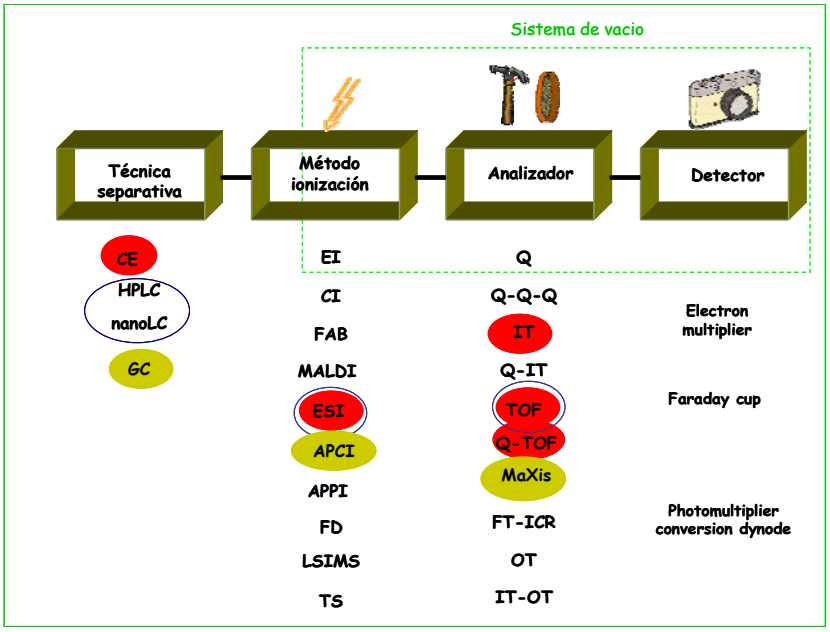

Figura 11. Esquema de un acoplamiento entre una técnica separativa y MS indicando con el mismo color (en el sombreado o trazado) los acoplamientos utilizados en la presente memoria.

Los analitos salen de la técnica separativa correspondiente y se introducen en la fuente de ionización donde se evaporan, se ionizan y se aceleran. Los iones acelerados pasan a uno de los numerosos tipos posibles que hay de analizadores de masas donde se separan, de modo que diferentes tipos de iones llegan al detector a diferentes tiempos. Esta pequeña corriente de iones se amplifica y da la señal de salida que se registra y procesa a través de un sistema informático. La versatilidad de la espectrometría de masas se debe en parte al amplio abanico de posibilidades de cada una de las tres secciones de un espectrómetro de masas: fuente de ionización, analizador y detector. En concreto el resultado que se obtenga depende notablemente de cuáles sean la interfase (o fuente de ionización) y el analizador utilizados.

El mayor problema que surge a la hora de acoplar las diferentes técnicas separativas con la espectrometría de masas está relacionado con la forma de introducir la mezcla en la fuente de ionización. Para ello se han descrito diversos tipos de

interfases donde se produce la evaporación y/o ionización de las moléculas [50,51]. Como característica general a todas ellas se puede señalar que una ionización exitosa requiere que una cierta energía sea transferida al analito. Se suelen clasificar atendiendo al grado de fragmentación que provoquen en la estructura del compuesto a estudiar. Técnicas de ionización fuertes, como impacto electrónico (EI) y otras más suaves entre las que se pueden encontrar electrospray (ESI), ionización química a presión atmosférica (APCI), fotoionización a presión atmosférica (APPI), ionización química (CI), bombardeo atómico (FAB), desorción/ionización láser asistida por matriz (MALDI), etc. En el siguiente esquema se clasifican algunas de las interfases de uso más extendido considerando el rango de analitos y peso molecular de los mismos que pueden analizar.

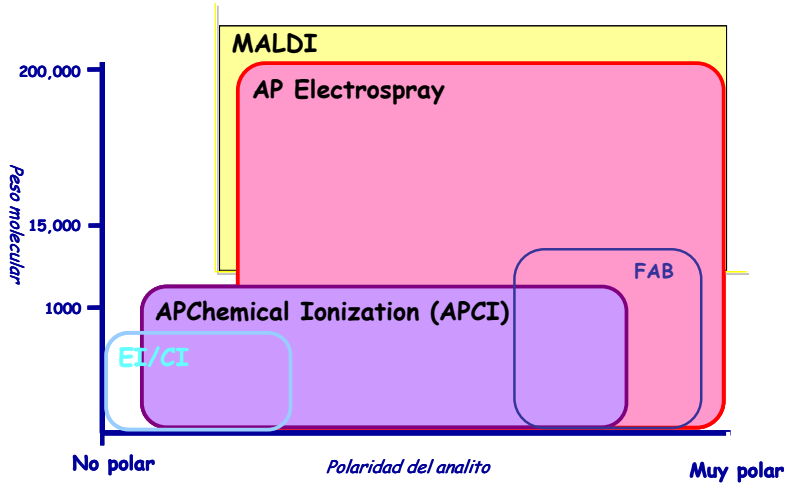

Figura 12. Rango al que trabajan los sistemas de ionización más comunes.

AP: Presión atmosférica

En lo que concierne al analizador de masas, el analista puede encontrar disponibles numerosas opciones, entre las que podemos señalar como más comunes: cuadrupolos (Q), trampas de iones (IT), triples cuadrupolos (QQQ), tiempos de vuelo (TOF), transformada de fourier-resonancia de ión ciclotrónica (FT-ICR), orbitrap (OT), acoplamientos entre varios de los mencionados, etc. Todos ellos se distinguen en la exactitud que ofrecen al determinar la masa molecular de los analitos (error entre la masa exacta determinada y el valor teórico), la capacidad o incapacidad para determinar

[50] Gelpi, E. "Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments" *J. Mass Spectrom.* 37 (2002) 241-253.
[51] E. Edwards, J. Thomas-Oates. "Hyphenating liquid phase separation techniques with mass spectrometry: on-line or off-line" *Analyst* 130 (2008) 13-17.

distribuciones isotópicas (True isotopic pattern), el poder hacer MS^n (de gran utilidad para determinar estructuras químicas), la velocidad de barrido, el rango de masas en el que pueden medir, y la resolución (el valor de la masas dividido entre la diferencia de masa Δm entre dos iones muy próximos) [52].

En el trabajo desarrollado en esta tesis se ha utilizado la espectrometría de masas en, prácticamente todas las aplicaciones, acoplada a los diferentas técnicas separativas empleadas (CE, HPLC, nanoLC y GC).

3.1. Interfases utilizadas para el acoplamiento

Para poder acoplar cada una de estas técnicas separativas con la espectrometría de masas hay que solventar varias dificultades.

Algunas de las técnicas de separación empleadas, CE, HPLC y nanoLC, trabajan en fase líquida mientras que en un espectrómetro de masas las sustancias para ser analizadas deben entrar en fase gaseosa. Esto obliga a la utilización de una interfase adecuada que transfiera los iones desde una fase líquida a una fase gaseosa. A pesar de la variedad de interfases desarrolladas para estos acoplamientos, la más utilizada es la interfase ESI. Esta interfase además de permitir la transferencia directa de los compuestos desde el capilar de separación hasta el espectrómetro de masas, permite un análisis eficaz de compuestos polares, lábiles, y/o de compuestos con un alto peso molecular (normalmente hasta 100000 Da). Por otro lado, es fácil de implementar, es sensible y puede usarse en un amplio intervalo de aplicaciones.

En el caso de la GC, al ser una técnica que trabaja en fase gaseosa al igual que la espectrometría de masas, el acoplamiento es más compatible. Cuando se utiliza la cromatografía de gases capilar, que es lo más habitual, el acoplamiento directo resulta más fácil ya que emplea caudales de fase gaseosa muy bajos. En este caso, el obstáculo que se presenta a la hora de realizar el acoplamiento es que el efluente que emerge de la columna cromatográfica sale a presión atmosférica y debe introducirse en el interior del espectrómetro de masas que trabaja a alto vacío, sin que este se pierda o disminuya de forma significativa. Las fuentes de ionización usadas en el 90% de los casos son el

[52] A. G. Marshall, C. L. Hendrickson. "High-resolution mass spectrometers". *Annu. Rev. Anal. Chem.* 1 (2008) 579-599.

impacto electrónico (EI) y la ionización química (CI) [53], que requieren de condiciones especiales de vacío. Con la idea de superar las limitaciones que presentan estas interfases, en el trabajo desarrollado en la presente tesis se ha utilizado un acoplamiento novedoso empleando ionización química a presión atmosférica (APCI).

3.1.1. Ionización por electrospray (ESI)

Esta técnica ha revolucionado el campo de los acoplamientos entre técnicas analíticas de separación que trabajan en fase líquida y la espectrometría de masas. En ocasiones no se considera el ESI como un procedimiento de ionización en sí, ya que no produce iones, sino la transferencia de estos desde una fase líquida a una fase gaseosa; sin embargo, de lo que no hay duda es de que tiene el potencial suficiente para, a través de colisiones ión-molécula, producir cambios en la naturaleza y cargas de los iones en fase gas.

En el proceso de formación del electrospray, el cual se lleva a cabo a presión atmosférica, intervienen diversos mecanismos al mismo tiempo. La muestra con la ayuda de un gas nebulizador, se carga y dispersa simultáneamente. El disolvente se va evaporando (desolvatación) de las microgotas formadas y éstas van aumentando su densidad de carga eléctrica. Como consecuencia, las gotas se encogen y los iones que se encuentran en la superficie se ven forzados a aproximarse entre sí debido al campo electrostático que se aplica entre la salida del capilar y la entrada al equipo MS (±2-5 kV). En cierto momento, la repulsión de los iones se hace mayor que la tensión de la superficie que mantiene unidas las gotas en forma esférica, y las pequeñas gotas se rompen (el punto donde se pronostica que las gotas se romperán se llama límite de Rayleigh, pero la ruptura se suele producir un poco antes de que se alcance dicho punto). Debido a fuerzas de repulsión coulómbica aumenta la tensión superficial de las microgotas y éstas acaban “explotando” (“explosiones de Coulomb”), formándose así una serie de pequeñas gotas cargadas que seguirán sufriendo procesos de evaporación y explosión sucesivos hasta que finalmente se forman iones cargados desnudos que pasan a fase gaseosa con una o más cargas y son atraídos hacia la entrada del espectrómetro de masas como consecuencia del voltaje aplicado [54,55].

[53] F. G. Kitson, B. S. Larsen, C. N. McEwen. “Gas chromatography and mass spectrometry: a practical guide” Academic Press, New York. (1996).
[54] J. C. Severs, R. D. Smith. “Electrospray ionization mass spectrometry”. R. B. Cole (Ed.). John Wiley & Sons, New York (1997).

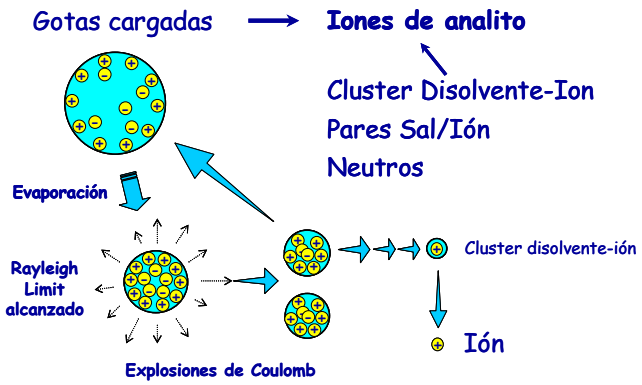

Figura 13. Esquema donde se explica el proceso de formación del electrospray.

La ionización se puede llevar a cabo en el modo positivo o negativo. En el modo positivo, se podrán formar iones múltiplemente protonados $[M+nH]^{n+}$ donde n es el número de protones cargados positivamente en la molécula. Del mismo modo, es posible también la formación de aductos con iones sodio, litio, potasio, amonio, etc. En el modo negativo, se observa normalmente la desprotonación de las moléculas, pudiéndose formar también iones múltiplemente desprotonados $[M-nH]^{n-}$.

Interfases ESI utilizadas con diferentes técnicas separativas

El acoplamiento de técnicas separativas que trabajan en fase líquida con el espectrómetro de masas a través de la interfase ESI presenta diferentes dificultades en función de la técnica separativa empleada que se solventan utilizando diferentes diseños de este tipo de interfase ESI.

➢ *Acoplamiento CE-ESI-MS*

En el acoplamiento CE-ESI-MS el primer problema que se plantea es la incompatibilidad del flujo procedente del capilar de separación (de hasta 100 nl/min), con el flujo necesario para la formación de un electrospray estable (1-200 µl/min). Otro problema es mantener el circuito eléctrico que se requiere para llevar a cabo la separación en CE y que se forma entre los extremos del capilar al aplicar la diferencia de potencial. Además hay que tener en cuenta la compatibilidad de las disoluciones tampón empleadas con el espectrómetro de masas, por lo que se deben utilizar como

[55] R. Martin-Smith. "Instrumentation" en "Understanding Mass Spectra. A basic approach". K. L. Busch (Ed). John Wiley & Sons, New York (1999) 1-40.

disoluciones tampón compuestos volátiles que se evaporen con facilidad evitando el incremento del ruido de fondo y la obstrucción y contaminación del sistema de detección. Con objeto de solventar estos problemas, se han desarrollado diferentes tipos de interfases para CE-ESI-MS [56-] teniendo como objetivo la formación de un electrospray estable y el mantenimiento de la corriente eléctrica en el interior del capilar. En la siguiente tabla se muestran las principales características de los tres tipos de interfases ESI: sin flujo adicional, con flujo adicional y con unión líquida.

Interfase	*Contacto eléctrico*	*Spray*	*Ventajas*	*Inconvenientes*
Sin flujo adicional	-Recubrimiento conductor -Electrodo en el interior capilar	Del capilar de separación (0-100 nl/min, nano-ESI)	Alta sensibilidad	-No muy estable (recubrimiento inestable, obstrucción…) -Dependiente del FEO
Con flujo adicional	Líquido adicional	Líquido adicional (1-6 µl/min)	-Disponibilidad comercial -Independiente del FEO -Spray estable	Baja sensibilidad
Unión líquida	Depósito con electrolito de separación entre capilar de separación y el de transferencia	Del capilar de transferencia (nano-ESI)	-Alta sensibilidad -Independiente del FEO -Más estable que ESI sin flujo adicional	-Ensanchamiento de los picos -Menos estable que ESI con flujo adicional (obstrucción del capilar de transferencia…)

Tabla 4. Principales características de los tres tipos de interfase ESI más utilizados.

La interfase ESI con flujo adicional, ha sido la empleada en el acoplamiento CE-MS en esta memoria y es la que procedemos a explicar.

Interfase ESI con flujo adicional

Esta interfase esta formada por tres tubos concéntricos, el primero de ellos es el propio capilar de separación que se encuentra rodeado de un tubo de acero inoxidable por el que se hace fluir el líquido adicional, y por un tercer tubo por el cual se introduce un gas nebulizador que favorece la formación del electrospray [60].

[56] B. F. Chao, C. J. Chen, F. A. Li, G. R. Her. "Sheathless capillary electrophoresis-mass spectrometry using a pulsed electrospray ionization source" *Electrophoresis* 27 (2006) 2083-2090.
[57] A. D. Zamfir, N. Dinca, E. Sisu, L. Peter-Katalinic. "Copper-coated microsprayer interface for on-line sheathless capillary electrophoresis electrospray mass spectrometry of carbohydrates". *J. Sep. Sci*. 29 (2006) 414-422.
[58] J. M. Ding, P. Vouros. "Advances in CE/MS. Recent developments in interfaces and applications". *Anal. Chem. News Featur.* 71 (1999) 378A- 385A.
[59] J. Cai, J. Henion. "Capillary electrophoresis-mass spectrometry". *J. Chromatogr. A* 703 (1995) 667-692.
[60] C. Simó, A. Cifuentes. "Electroforesis Capilar: Detección mediante espectrometría de masas" en "Electroforesis capilar: aproximación según la técnica de detección". A. fernández-Gutiérrez, A. Segura-Carretero (Eds) Ed. Univ. Granada. (2005) 409-438.

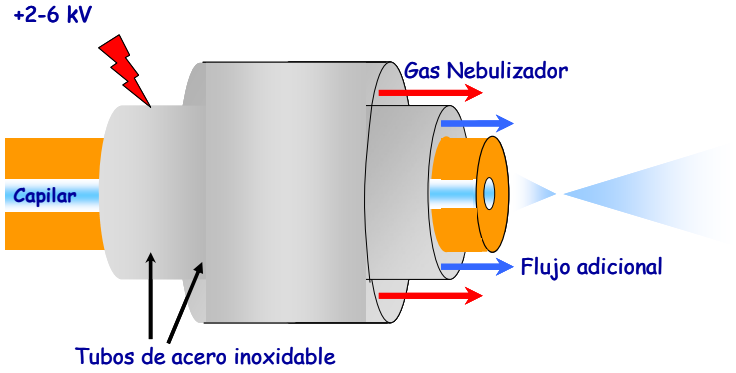

Figura 14. Esquema de una interfase ESI con flujo adicional.

Como se ha comentado anteriormente, para que una interfase ESI trabaje de modo estable requiere flujos del orden de µl/min, de modo que el flujo con el que típicamente se trabaja en CE (del orden de los nl/min) se aumenta mediante el empleo del líquido adicional, facilitando el acoplamiento CE-MS y dando lugar a la formación de un electrospray estable. Además, el flujo adicional facilita el cierre del circuito eléctrico con el interior del capilar, ya que el líquido añadido permite el contacto entre el electrolito de separación que fluye del interior del capilar y el tubo metálico (conectado a tierra) que rodea el capilar de separación (ver la anterior figura).

Se trata de una interfase robusta y de fácil manejo, aunque presenta algunas limitaciones, como puede ser la disminución de la sensibilidad resultante de la dilución que el líquido adicional produce sobre las bandas de los analitos que salen del capilar. Por otro lado, es necesario considerar otros parámetros como son la composición y el flujo de la disolución adicional, presión del gas nebulizador, situación del capilar con respecto al tubo concéntrico que lo rodea, naturaleza del electrolito de separación, etc., ya que van a influir tanto sobre la intensidad de la señal MS como sobre la resolución de la separación [61,62].

En líneas generales, se pueden dar las siguientes recomendaciones experimentales para llevar a cabo con éxito el acoplamiento CE-MS con interfase ESI de flujo adicional:

[61] J. Samskog , M. Wetterhall, S. Jacobsson, K. Markides. "Optimization of capillary electrophoresis conditions for coupling to a mass spectrometer via a sheathless interface". *J. Mass Spectrom.* 35 (2000) 919-924.
[62] K. Huikko, T. Kotiaho, R. Kostiainen. "Effects of nebulizing and drying gas flow on capillary electrophoresis/mass spectrometry". *Rapid Commun. Mass Spectrom.* 16 (2002) 1562-1568.

1) El capilar debe sobresalir con respecto al tubo concéntrico que lo rodea un tercio aproximadamente de su diámetro externo (p.ej., un típico capilar de 360 µm de diámetro externo deberá sobresalir aprox. 120 µm).

2) El flujo adicional debe ser suficientemente conductor como para cerrar el circuito eléctrico con el interior del capilar de separación, pero con una fuerza iónica no demasiado alta para evitar descargas en la fuente de ionización.

3) Normalmente el flujo adicional debe contener un porcentaje alto de disolvente orgánico, y una pequeña cantidad de ácido o base para mejorar la ionización de las sustancias en estudio según se trabaje con ESI en modo positivo o negativo.

4) Los flujos de líquido adicional y de gas nebulizador deben mantenerse en valores suficientemente bajos como para permitir un electrospray estable, evitando la generación del efecto de succión o excesiva dilución de los analitos.

A pesar de las limitaciones e inconvenientes de esta interfase, actualmente es la más utilizada en el acoplamiento CE-MS, siendo hasta la fecha la única disponible comercialmente.

➢ *Acoplamiento HPLC-ESI-MS*

En el acoplamiento HPLC-ESI-MS, al contrario de lo que ocurre en CE, el principal obstáculo es la enorme cantidad de disolvente que acompaña al analito, con flujos en muchos casos comprendido entre 2-3 ml/min. Los espectrómetros de masas son aparatos de alto vacío que no pueden recibir fácilmente estos caudales de disolvente, especialmente aquellos que operan con masas exactas como los analizadores de tiempo de vuelo, que se ven muy afectados por la humedad que entra al sistema dando lugar a grandes desviaciones en la exactitud de masas. Las interfases ESI normalmente empleadas para HPLC aceptan flujos comprendidos entre 0.1-1 ml/min, aunque realmente el flujo recomendado a la entrada del espectrómetro de masas está entre 0.2-0.5 ml/min, dependiendo del analizador. Para solucionar el problema de los flujos elevados se recomienda utilizar divisores de flujo entre la salida de la columna cromatográfica y la entrada de la interfase, aunque esto supondrá una disminución de la sensibilidad producida por la eliminación de parte de la muestra. Otra posible solución es utilizar columnas cromatográfica más estrechas, de 2,1 mm de diámetro con caudales

de 0.2 ml/min, que si se pueden introducir directamente en el espectrómetro de masas. Además, cuando se usa espectrometría de masas, se deben evitar los aditivos no volátiles en la fase móvil (como el tampón fosfato). En fase inversa, se deben utilizar combinaciones agua y metanol o acetonitrilo con modificadores orgánicos volátiles.

Figura 15. Interfase ESI utilizada en el acoplamiento HPLC-ESI-MS

Se pueden dar las siguientes recomendaciones experimentales para llevar a cabo con éxito el acoplamiento HPLC-MS con interfase ESI:

1) Utilizar tubos de conexión adecuados entre el cromatógrafo, el divisor y la interfase para minimizar el ensanchamiento de la banda fuera de la columna.
2) Se recomienda lavar con isopropanol-agua el nebulizador, los tubos de conexión y la válvula después de una serie de análisis. Una aguja limpia y no dañada es esencial para alcanzar buenas condiciones de electrospray.
3) Siempre que sea posible adicionar una pequeña cantidad de ácido o base a la fase móvil para mejorar la ionización.
4) Seleccionar condiciones adecuadas del gas nebulizador (presión, flujo y temperatura) para obtener un spray estable. Estas condiciones dependen del flujo de fase móvil que llegue a la interfase, cuanto mayor sea el flujo, mayor serán los valores de estos parámetros.

- *Acoplamiento nanoLC-ESI-MS*

En el acoplamiento nanoLC-ESI-MS al igual que ocurre con CE el flujo que proviene de la columna capilar es demasiado pequeño (del orden de los nanolitros) para

conseguir un electrospray estable [63]. Para solucionar este problema se han utilizado interfases nanoESI con flujo adicional similares a las empleadas en el acoplamiento CE-ESI-MS [64] aunque existen pocas referencias bibliográficas ya que pronto se desarrollaron las interfases nanoESI sin flujo adicional que permiten trabajar con flujos muy pequeños (50- 500 nl/min) obteniendo alta sensibilidad [65]. Son fuentes con un diseño diferente al utilizado en otro tipo de acoplamientos (HPLC-ESI-MS o CE-ESI-MS), ya que están especialmente diseñadas para flujos bajos. En vez de un nebulizador utilizan una aguja o capilar interno ("emitters") para transportar la muestra desde la columna capilar hasta la cámara de nebulización. La diferencia de potencial que se genera entre la punta del capilar y la entrada del espectrómetro de masas produce la ionización y en muchos casos no se requiere de un gas nebulizador adicional debido a los pequeños volúmenes de disolvente utilizados. Los capilares internos pueden ser de sílice [32] con algún tipo de unión conductora que permita establecer el potencial necesario para la formación del electrospray o pueden estar fabricados de acero inoxidable [66]. Muchas aplicaciones encontradas en bibliografía utilizan los capilares de sílice que además presentan la ventaja de que la columna cromatográfica se puede empaquetar directamente en ellos y de esta forma eliminar los volúmenes muertos post-columna [67,68]. Los capilares de sílice tienen un diámetro en la punta de 1-2 µm y producen sprays estables en el rango de 20-300 nl/min. En algunos trabajos se utilizan fuentes nanoESI disponibles comercialmente pero insertando capilares preparados en el propio laboratorio, para solucionar problemas concretos [[69].

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[63] M. Karas, U. Bahr, T. Dulcks. "Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine" *Anal. Chem.* 366 (2000) 669-676.

[64] C. E. Parker, J. R. Perkins, K. B. Tomer, Y. Shida, K. O'Hara, M. Kono. "Application of nanoscale packed capillary liquid chromatography (75µm id) and capillary zone electrophoresis/electrospray ionization mass spectrometry to the analysis of macrolide antibiotics" *J. Am. Soc. Mass. Spectrom.* 3 (1992) 563-574.

[65] A. Amirkhani , M. Wetterhall, S. Nilsson, R. Danielsson, J. J. Bergquist. "Comparison between different sheathless electrospray emitter configurations regarding the performance of nanoscale liquid chromatography-time-of-flight mass spectrometry analysis" *J. Chromatogr. A* 1033 (2004) 257-266.

[66] J. N. Alexander, G. A. Schultz, J. B. Poli. "Development of a nanoelectrospray mass spectrometry source for nanoscale liquid chromatography and sheatless capillary electrophoresis" *Rapid Commun. Mass Spectrom.* 12 (1998) 1187-1191.

[67] Y. Song, Z. Quan, Y. M. Liu. "Assay of histamine by nano-liquid chromatography/tandem mass spectrometry with a packed nanoelectrospay emmiter" *Rapid Commun. Mass Spectrom.* 18 (2004) 2818-2822.

[68] D. Y. Bang, D. Kang, M. H. Moon. "Nanoflow liquid chromatography-tandem mass spectrometry for the characterization of intact phosphatidylcholines from soybean, bovine brain, and liver" *J. Chrom. A* 1104 (2006) 222-229.

[69] S. Fanali, Z. Aturki, G. D'Orazio, A. Rocco. "Separation of basic compounds of pharmaceutical interest by using nano-liquid chromatography coupled with mass spectrometry" *J. Chromatogr. A* 1150 (2007) 252-258.

En los últimos años la empresa Bruker Daltonik ha desarrollado un nuevo nebulizador (nanoFlowESI sprayer) optimizado para flujos pequeños que se emplea con la fuente de ionización estándar utilizada en los acoplamientos con HPLC y CE (Figura 7). No se requieren modificaciones de la fuente manteniendo los beneficios de la nebulización ortogonal y el gas de secado. Esta interfase es la que hemos utilizado en la parte experimental de esta memoria para el acoplamiento nanoLC-ESI-TOF MS. El nebulizador contiene un capilar muy estrecho de sílice y una entrada para el gas de nebulización (nitrógeno). Se lleva a cabo la introducción directa del efluente de la columna cromatográfica al espectrómetro de masas ya que la columna va conectada directamente a la interfase.

En general en las fuentes nanoESI la eficacia de la ionización es mayor ya que los iones se producen con más eficacia a medida que el tamaño medio de las microgotas del spray disminuye. Además el pequeño tamaño del spray formado y la proximidad de la punta del capilar a la entrada del espectrómetro de masas permiten la introducción de una fracción mayor de los iones hacia el interior.

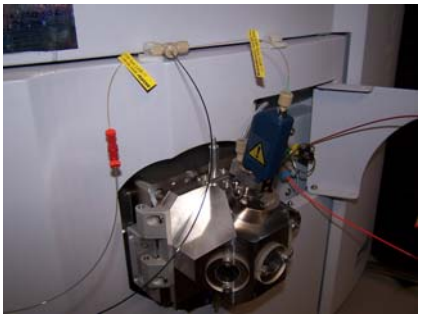


Figura 16. Interfase nano-ESI utilizada en el acoplamiento nano-ESI-MS

Se pueden dar las siguientes recomendaciones experimentales para llevar a cabo con éxito el acoplamiento nanoLC-MS la interfase ESI utilizada en esta memoria:

1) El capilar de sílice del interior de la interfase debe sobresalir ligeramente con respecto a al tubo que lo rodea. Para evitar que se rompa la punta de sílice se recomienda colocar la interfase con la punta en el interior y una vez colocada sacarla poco a poco hasta obtener una señal de fondo adecuada y estable.

2) Se recomienda utilizar conexiones adecuadas entre la columna y la entrada del nebulizador para evitar volúmenes muertos y posibles fugas difíciles de detectar

debido al flujo tan pequeño. Estas conexiones hay que cambiarlas periódicamente.

3) Seleccionar condiciones adecuadas del gas de nebulización (presión, flujo y temperatura). Se recomienda utilizar temperaturas bajas puesto que el flujo es muy pequeño.

3.1.2. Interfases GC-MS. Nuevo acoplamiento APCI.

Tal y como se comentaba anteriormente, la utilización de GC acoplada a un espectrómetro de masas requiere sistemas especiales de conexión ya que el efluente que emerge de la columna cromatográfica sale a presión atmosférica y debe introducirse en el interior del espectrómetro de masas que trabaja a alto vacío. Hasta el momento, los acoplamientos más habituales se han llevado a cabo con fuentes de ionización de impacto electrónico (EI) e ionización química (CI).

- *Impacto electrónico (EI)*

Las moléculas gaseosas entran en la fuente de ionización y son bombardeadas por electrones emitidos por un filamento calentado (generalmente tungsteno) con una energía cinética de 70 electronvoltios. Esta energía es suficiente para ionizar las moléculas ($M + e^- \longrightarrow M^+ + 2\ e^-$) y para romperlas en fragmentos más pequeños. El ión M^+ tiene una relación m/z correspondiente a la masa molecular del analito y se llama ión molecular. Dependiendo de su estructura y de la cantidad de energía absorbida, este ión puede atravesar el analizador intacto o fragmentarse generando iones más pequeños. Este proceso de fragmentación tiene lugar en la mayoría de los casos, donde el ion molecular M^+ puede ser poco abundante e incluso no estar presente. Una fragmentación muy intensa de moléculas grandes crea dificultades para interpretar el espectro de masas. También se pueden generar iones negativos.

- *Ionización química (CI)*

La fuente de ionización química (CI) es una forma indirecta de generar iones, en la que utiliza un reactivo en forma gaseosa. Estos reactivos, generalmente metano, isobutano o amonio, son introducidos en un canal de reacción, e ionizados por impacto electrónico. Posteriormente, estos iones son mezclados nuevamente con una corriente

gaseosa del reactivo, para generar otros iones por transferencia de protones o iones. Finalmente, esta corriente de iones es mezclada con una corriente gaseosa de moléculas de analito que son igualmente ionizadas. También se pueden generar iones negativos con reacciones similares. Es una técnica más suave que la EI y produce menos fragmentación.

La formación de los diferentes fragmentos iónicos puede modificarse mediante la elección del gas de reacción. En muchas ocasiones se utiliza metano en la fuente de ionización. Electrones enérgicos convierten CH_4 en diversos productos reactivos:

$$CH_4 + e^- \longrightarrow CH_4^+ + 2\,e^-$$
$$CH_4^+ + CH_4 \longrightarrow CH_5^+ + CH_3$$

CH_5^+ es un gran dador de protones, que reacciona con el analito para dar MH^+, que de ordinario es el ion más abundante en los espectros de masas con ionización química.

$$CH_5^+ + M \longrightarrow CH_4 + MH^+$$

El uso tan extendido de ambas interfases es debido a la multitud de bases de datos comerciales que existen y que facilitan notablemente la identificación de muchos de los compuestos presentes en muestras complejas. Sin embargo, debido a que la fragmentación de los compuestos con ambas interfases suele ser bastante grande, a veces podemos encontrar problemas para identificar el ión molecular (precursor) y por consiguiente, identificar un determinado compuesto.

En el trabajo desarrollado en uno de los capítulos de esta memoria se va a utilizar un método de ionización alternativo e innovador para lograr con éxito el acoplamiento GC-MS resolviendo los problemas clásicos a los que tenía que enfrentarse el analista. Se ha utilizado como fuente de ionización la ionización química a presión atmosférica (APCI). Este acoplamiento no ha sido accesible comercialmente hasta hace unos meses y hay un número muy reducido de aplicaciones en las que se use [70].

[70] A. Carrasco-Pancorbo, E. Nevedomskaya, T. Arthen-Engelans, T. Zey, G. Zurek, C. Baessmann, A. M. Deelder, O. A. Mayboroda. "Gas chromatography/atmospheric pressure chemical ionization- time of flight mass spectrometry: analytical validation and applicability to metabolic profiling" *Anal. Chem.* 81 (2009) 10071-10079.

- *APCI*

El gas que proviene del cromatógrafo se desplaza por un capilar de transferencia ("transfer line") situado entre la columna capilar y la entrada del espectrómetro de masas. Este capilar debe estar a una temperatura elevada para evitar la condensación de los compuestos. Los compuestos que salen de este capilar pasan a la zona de ionización donde una descarga producida por una corona produce los electrones necesarios para conseguir la ionización química a presión atmosférica. La ionización del sustrato es un proceso muy eficaz, y los iones generados entran después en el analizador de masas que se encuentra a alto vacío a través de un skimmer. La sensibilidad es mayor que en la ionización química convencional debido a la mayor eficacia de ionización cuando ésta transcurre a presión atmosférica. APCI es aplicable a compuestos de baja a moderadamente alta polaridad, proporciona masas moleculares y es aplicable hasta masas de 2000 Da. Se considera complementaria a ESI. Además con el uso de esta fuente revolucionaria, GC se puede acoplar a espectrómetros de masas de alta resolución como TOF, Q-TOF, MaXis ya que con las fuentes de ionización clásicas estos acoplamientos resultaban más difíciles dando lugar a pobres resoluciones y exactitud de masa baja [71,72]. El uso de esta fuente permite acoplar ambas técnicas separativas (LC y GC) al mismo instrumento.

En la Figura 15 se muestra la interfase APCI utilizada en el acoplamiento, indicando específicamente el capilar de transferencia ("transfer line"), uno de los puntos críticos de este sistema. Uno de los inconvenientes de este diseño es la dificultad de posicionar de una manera reproducible el capilar de transferencia con respecto a la entrada al espectrómetro de masas y la pérdida de eficacia que se produce durante la migración de los analitos a lo largo del capilar de transferencia.

[71] T. Arthen-Engeland, R. Dunsbach. "New generation of GC/High resolution TOF-MS:APCI/APLI source for increased flexibility" Technical Note Bruker Daltonik GmbH, Bremen, Germany.
[72] C. McEwen, R. McKay. "A combination atmospheric pressure LC/MS:GC/MS ion source: advantages of dual AP-LC/MS:GC/MS instrumentation" *J. Am. Soc. Mass Spectrom*. 16 (2005) 1730-1738.

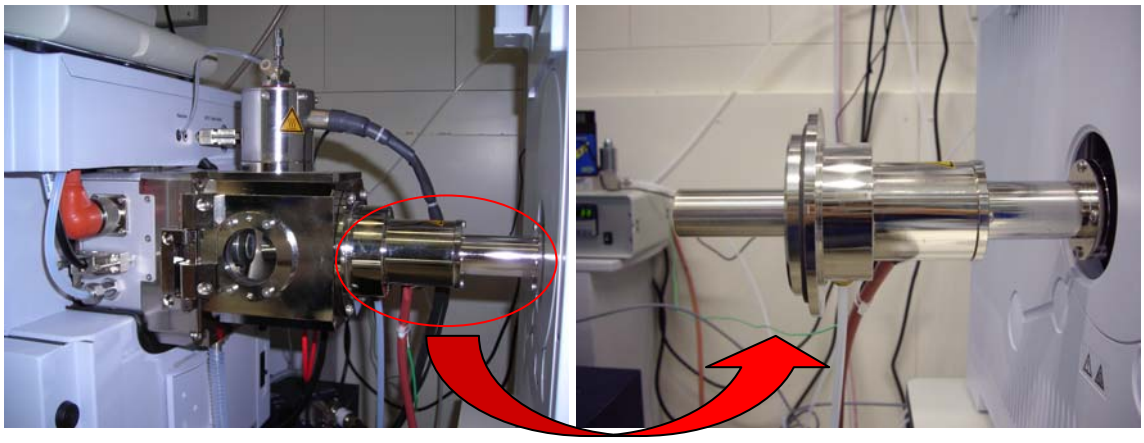


Figura 17. Interfase APCI para acoplamiento entre GC y MS

3.2. Analizadores de masas

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

Como ya se ha indicado con anterioridad existen diferentes tipos de analizadores pero los utilizados en el trabajo experimental de esta memoria fueron la trampa de iones (IT), el tiempo de vuelo (TOF), el cuadrupolo acoplado al tiempo de vuelo (Q-TOF) y una nueva generación de TOF denominado MaXis. Estos analizadores, tal y como mencionábamos antes, difieren en cómo los fragmentos de un compuesto se separan de acuerdo a su relación masa/carga y también en la velocidad al adquirir datos, el límite de detección, la resolución y la calidad del espectro de masas que se obtiene.

3.2.1. Trampa de iones

El analizador de trampa de iones (IT) consiste fundamentalmente en un electrodo anular y dos electrodos laterales de geometría hiperbólica, que poseen una perforación que permite la entrada y la salida de los iones. Los iones formados en la fuente entran en el analizador donde se aplican diferentes voltajes generando un campo eléctrico tridimensional en la cavidad de la trampa. Este campo atrapa y concentra los iones dada su trayectoria de oscilación estable. La naturaleza de la trayectoria depende del potencial y de la relación masa/carga (m/z) de los iones. Una vez que los iones se

encuentran atrapados dentro del analizador se puede llevar a cabo, en función del objetivo del análisis, tanto el análisis de las masas como el aislamiento de un ión precursor y la posterior fragmentación de este ión. Para llevar a cabo la determinación de todas las especies que entran o se forman en la trampa, los potenciales de los electrodos se alteran sometiendo a los iones confinados a una rampa lineal de radiofrecuencia (RF) de modo que son progresivamente expulsados como resultado de desestabilizaciones de la órbita que mantienen dentro de la trampa. Una vez que estos iones llegan al detector, la señal se procesa y da lugar al espectro de masas.

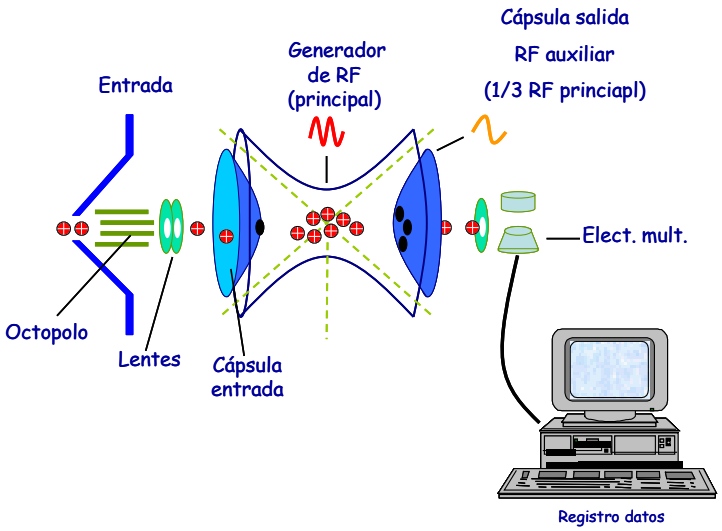


Figura 18. Esquema de un analizador de trampa de iones (y del optopolo y lentes previas al analizador).

El siguiente esquema nos muestra las partes que posee la IT (con una fuente de ionización ESI) empleada para llevar a cabo parte del desarrollo experimental de la presente memoria.

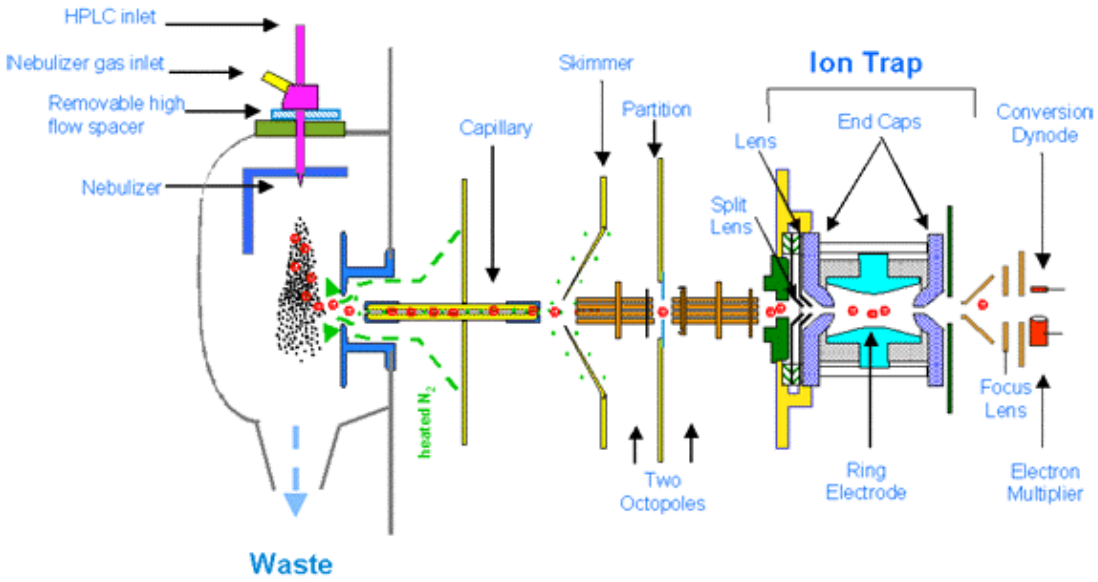


Figura 19. Esquema de un espectrómetro de masas ESI-IT.

Está formado por cuatro partes: la interfase (ESI en este caso); la zona de "transporte y convergencia" de iones formada por skimmers, optopolos y lentes; el analizador (IT); y el detector.

La primera zona es la cámara de formación del spray (interfase) donde, como ya comentamos en la correspondiente sección, se nebuliza la solución de la muestra y se ioniza a través de un proceso de desolvatación. La zona de "transporte y focalización" de iones posee cuatro zonas que están a alto vacío originado por una serie de bombas que hay en el sistema. Los iones pasan a través de un capilar de vidrio hasta un skimmer que elimina el volumen del gas de secado; a continuación pasan por los octopolos que los transporta y guían desde justo detrás del skimmer hasta el detector atravesando una serie de lentes. Por último los iones entran en el analizador de trampa de iones donde se separan en función de su relación m/z.

Una vez que los iones se encuentran atrapados dentro de este analizador se puede llevar a cabo tanto el análisis de sus masas (obteniéndose el espectro de MS) como el aislamiento de uno o varios iones precursores y su posterior fragmentación (dando lugar a lo que se denomina espectros de MS-MS o espectros MS^2).

El analizador IT es uno de los más usados debido a su coste, a la velocidad de barrido y a la posibilidad de realizar fragmentaciones sucesivas de los iones seleccionados, aunque la sensibilidad y la resolución no es la mejor.

Algunas de las especificaciones más destacables de la trampa de iones son:

- El rango de masas que puede analizar es 50-2200 m/z.
- Resolución: podemos tener una resolución normal o máxima según la velocidad de escaneo de 1.000 a 15.000
- Posibilidad de llevar acabo análisis MS/MS. Permite llevar a cabo fragmentaciones sucesivas de los iones seleccionados dando lugar a espectros MS^n que aportan información sobre la estructura de los analitos.
- Equipo relativamente barato y presenta menos dificultades técnicas.

3.2.2. Tiempo de vuelo

El analizador de tiempo de vuelo (TOF) discrimina según la distinta velocidad que adquieren los iones en el interior del analizador en función de su relación m/z [73]. Está basado en el principio de que todos los iones generados en la fuente de ionización tienen la misma energía cinética, por lo que su velocidad es inversamente proporcional al cuadrado de su masa. Se aplica un voltaje determinado para acelerar los iones, lanzándolos a un tubo de alto vacío con una energía cinética constante. Los iones que tienen la misma energía cinética pero diferentes valores de m/z adquieren distinta velocidad de forma que no todos llegarán al extremo contrario a la vez. Los iones de mayor m/z "volarán" a menor velocidad que los de menor m/z. La resolución entre los iones de diferente m/z será mejor cuanto mayor sea longitud del tubo (habrá una mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energías de los iones formados en la fuente [55].

El siguiente esquema nos muestra las partes que posee el MicroTOF empleado para llevar a cabo parte del trabajo experimental de la presente memoria.

[73] K. A. Rubinson, J. F. Rubinson. "Espectrometría de masas" en "Análisis Instrumental". Ed. Pearson Education S. A. Madrid. (2000) 522-577.

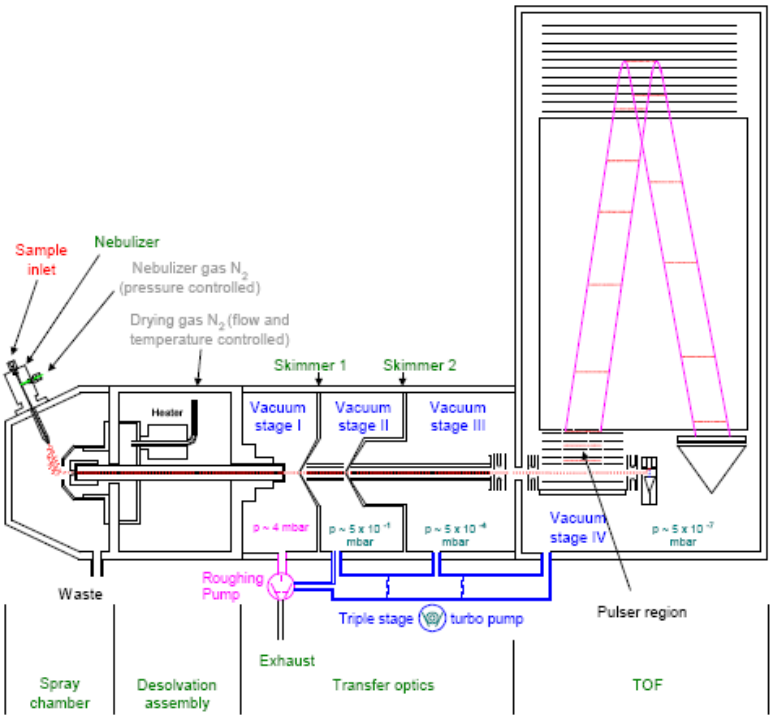


Figura 20. Esquema detallado de las partes de las que consta un ESI-MicroTOF.

Por orden, vamos encontrando la cámara de formación del spray (spray chamber), la unidad de desolvatación (desolvation unit), la zona de "guía y convergencia" de iones (ion guide and beam focusing), la zona de aceleración ortogonal (orthogonal acceleration), el detector de referencia, el camino o tubo de vuelo (flight path), el reflector (reflector) y el detector (detector). Cuatro de los compartimentos han de estar sometidos a alto vacío.

La muestra entra en la cámara de nebulización, donde tiene lugar la formación del electrospray. Los iones formados atraviesan la unidad de desolvatación, que separa las zonas a presión atmosférica de la primera zona a alto vacío, y que consta de un calentador del gas de secado y un capilar de cristal. Se llega a través de ella a la zona de transmisión o transferencia óptica que consta de tres módulos que están a alto vacío, separados entre sí por varios skimmers. El primero de ellos (stage I) es el único que está conectado a una bomba externa rotatoria; mientras que los tres módulos (stages I, II y III) están conectados a una bomba turbo molecular que posee distintos niveles de vacío. Están separados entre sí por varios skimmers. Los dos hexapolos son los que transfieren los iones hasta la zona de alto vacío, mientras que las lentes (1-5) enfocan o dirigen dichos iones.

La zona de aceleración ortogonal contiene dos de las últimas lentes mencionadas anteriormente (lentes 4 y 5) y acelera los iones hacia el tubo de vuelo aplicando un campo eléctrico intermitente.

En función de su masa, los iones se introducen en mayor o menor medida en el reflector. Detrás del mismo hay zonas de tensión que repelen los iones que le llegan; lógicamente, los iones pequeños serán repelidos con más facilidad.

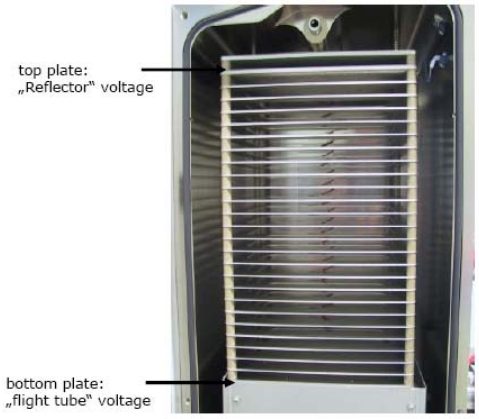


Figura 21. Imagen de de la parte final del tubo de vuelo donde se sitúa el reflector.

El detector es un detector de impacto electrónico que consiste en una serie de placas a alto voltaje que convierten el impacto de los iones en señales eléctricas. En el detector hay millones de poros muy pequeños que están internamente recubiertos con una capa semiconductora; cada uno de ellos trabaja como un multiplicador de electrones independiente. También hay un detector de referencia.

Este analizador de tiempo de vuelo (TOF) es rápido y sensible, permite la determinación de masas exactas empleando TIP (True Isotopic Pattern) para el análisis en dos dimensiones, y da óptimos resultados en un rango muy amplio sin requerir tediosos procesos rutinarios de re-calibración. Es decir, aporta una mayor fiabilidad de los resultados aplicando un método analítico casi bidimensional: combinando la determinación de masas exactas con el análisis de la distribución isotópica. Una de sus principales ventajas es que estos proporcionan una resolución elevada, lo que permite obtener valores de masa molecular muy exactos; además permiten obtener espectros de masas con una transmisión iónica eficaz y proporcionan ciclos muy rápidos

Algunas de sus especificaciones más notables son:

- El rango de masas que puede analizar en modo estándar es 50-3000 m/z.
- Resolución: 10000 (en modo normal (MicrOTOF)) y 15000 en modo MicrOTOF focus.
- Exactitud: 3 ppm con calibración interna y 5 pp con calibración externa.

3.2.3. Q-TOF

La espectrometría de masas es una técnica que permite acoplar varios analizadores en serie de manera que se puede obtener además de la masa molecular del analito, información estructural acerca del ión molecular seleccionado. Cuando un primer espectrómetro de masas es acoplado a otro en serie, la técnica se conoce como espectrometría de masas en tandem (MS/MS). En el caso de que se tenga interés en un determinado ion (fragmento o ion molecular), éste se selecciona, se separa y se introduce en la cámara de colisión. Por choque con el gas de colisión que se encuentra en la cámara, la energía cinética de estos iones seleccionados se transforma en energía vibracional de forma que los iones fragmenten y se introduzcan en el segundo analizador de masas, donde se separan y analizan. Las técnicas de ionización blandas como es el caso de la ESI inducen escasa fragmentación en la molécula aportando poca información estructural de la misma, por lo que el empleo de esta espectrometría de masas en tandem resulta muy interesante. Probablemente, el instrumento más utilizado para llevar a cabo este tipo de análisis sea un espectrómetro de triple cuadrupolo, aunque existen otros muchos entre los que se encuentran el espectrómetro de masas híbrido cuadrupolo –tiempo de vuelo (Q-TOF) empleado en uno de los trabajos desarrollados en esta memoria. En la siguiente figura se muestran las partes que posee el Q-TOF empleado para llevar a cabo parte el desarrollo experimental de la presente memoria.

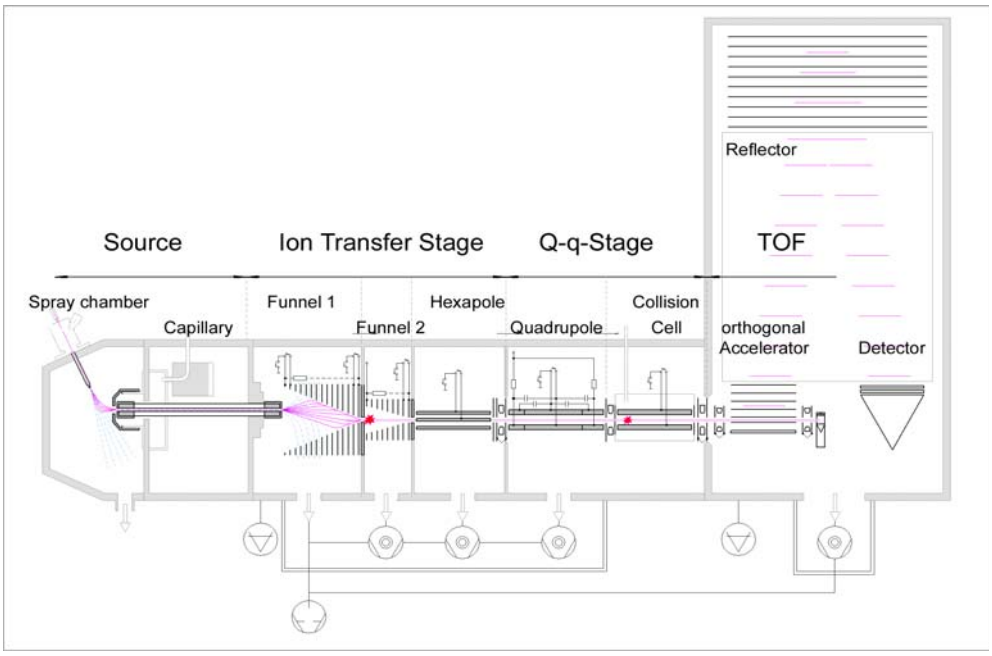


Figura 22. Esquema detallado de las partes de las que consta un Q-TOF

Está formado por las mismas partes que un TOF con la diferencia de que se introduce un cuadrupolo para aislar los analitos que se quieren fragmentar seguido de una celda de colisión donde se produce la fragmentación. Los iones fragmentados se aceleran en el acelerador ortogonal y se introducen en el tiempo de vuelo donde se separan en función de su relación m/z de la misma forma que veíamos en la descripción del TOF. Además se sustituyen los skimmer del TOF por un nuevo diseño tipo funnel que evita que se pierdan iones aumentando la sensibilidad.

Algunas de sus especificaciones más notables son:

- El rango de masas que puede analizar en modo estándar es 50-20000 m/z.
- Alta resolución 17500-20000 FWHM
- Combina exactitud de masas (1-2 ppm con calibración interna y 5 ppm con calibración externa) y distribución isotópica tanto de precursores como de fragmentos.
- Excelente sensibilidad con la tecnología funnel.
- Buena reproducibilidad en todo el rango de masas.

3.2.4. MaXis

Los analizadores de tiempo de vuelo han experimentado un gran desarrollo en los últimos años. Así una nueva generación de TOF denominada MaXis ha sido introducida no hace mucho tiempo por la empresa Bruker Daltonik. La instrumentación es básicamente la misma que un TOF con la diferencia de que el tubo de vuelo es más largo lo que hace que se consigan resoluciones más altas (de 40,000 - 60,000 FWH) en un amplio rango de masas. Esta alta resolución puede resultar muy útil para el análisis de isómeros y muestras complejas en proteómica y metabolómica. Además permite realizar MS/MS y posee excelente exactitud de masas, alta sensibilidad y alta velocidad de adquisición de datos ideal para el acoplamiento con técnicas separativas con tiempos de análisis cortos como UPLC.

Algunas de sus especificaciones más notables son:

- El rango de masas que puede analizar es muy amplio
- Resolución muy alta 40000-60000 FWHM tanto en MS como MS/MS
- Excelente exactitud de masas (orden sub-ppm)
- Excelente sensibilidad en el MS/MS en un rango de masas amplio (novel IonCooler™ technology)
- Buena reproducibilidad en todo el rango de masas.
- 20 Hz full spectra acquisition at ultra-high mass resolution for high-speed liquid chromatography

PARTE EXPERIMENTAL: Matrices estudiadas y metodologías propuestas

Esta segunda sección denominada "PARTE EXPERIMENTAL: MATRICES ESTUDIADAS Y METODOLOGÍAS PROPUESTAS" se divide en tres bloques que corresponden con cada una de las matrices que se consideraron objeto de estudio durante el desarrollo experimental de la presente memoria: cerveza, soja y aceite de oliva. En cada bloque, se incluye una introducción con información acerca de las matrices estudiadas, seguida por los capítulos donde se recogen los resultados obtenidos en los estudios experimentales relativos a esa matriz. Cada uno de los capítulos consta de una pequeña introducción en la que se explican las motivaciones que nos llevaron a la realización de este trabajo experimental y los objetivos que se persiguieron. Tras eso, se adjunta el artículo ya publicado o en proceso de revisión, que se obtuvo como consecuencia de la experimentación realizada, y en algunos casos, al trabajo, le sigue una información adicional en forma de anexo.

Así en los tres primeros capítulos se pretendía demostrar el potencial de la CE para afrontar con éxito diferentes situaciones que pueden darse en la industria alimentaria. Los dos primeros trabajos están relacionadas con la industria cervecera: en el capitulo 1 se utiliza CZE para comparar el perfil proteico de muestras de cebada tomadas en diferentes etapas del proceso de malteado, y en el capítulo 2, se demuestra el potencial de CE-ESI-IT MS para analizar los ácidos del lúpulo y sus compuestos de oxidación en diferentes condiciones de almacenamiento. En el tercer capítulo relativo a la soja se utilizó CE-ESI-TOF MS para comparar a nivel metabolómico soja convencional y soja modificada genéticamente.

Los siguientes capítulos englobados en el bloque 3 están relacionados con la matriz aceite de oliva, y más concretamente con sus compuestos fenólicos. Se comenzó en el capítulo 4 con un estudio profundo del perfil polifenólico del aceite de oliva mediante un análisis bidimensional empleando HPLC-CE-ESI-TOF MS. Las fracciones fenólicas aisladas mediante HPLC semipreparativa se utilizaron para cuantificar algunos de los polifenoles del perfil y para estudiar su actividad anticancerígena en diferentes modelos celulares *in vitro*. En el capítulo 5, se puso a punto un método fácil y rápido utilizando RRLC-ESI-TOF MS para identificar y cuantificar extractos polifenólicos de diferentes aceites de oliva que habían dado muy buenos resultados en ensayos *in vitro* con células cancerígenas, intentando establecer una relación entre su contenido polifenólico y su actividad anticancerígena. En los capítulos 6 y 7 se demostró el potencial de dos nuevas plataformas analíticas nanoLC-ESI-TOF MS y GC-APCI-

MaXis MS para el análisis de polifenoles del aceite de oliva, desarrollando dos métodos analíticos que podrían resultar muy útiles en estudios futuros donde se quisiera determinar polifenoles en otro tipo de muestras (muestras biológicas, por ejemplo). En el capítulo 8 se estudió la absorción y metabolismo de los polifenoles del aceite de oliva en células cancerígenas, empleando nanoLC-ESI-TOF MS. Y puesto que para extrapolar los buenos resultados obtenidos *in vitro* es necesario conocer la biodisponibilidad de estos compuestos *in vivo* en el capitulo 9 se empleó un método RRLC-ESI-TOF MS seguido de un estudio estadístico multivariante para llevar a cabo un análisis exploratorio de muestras de orina humana después de una ingesta forzada de aceite de oliva virgen.

BLOQUE I:
CERVEZA

BLOQUE I. CERVEZA

La cerveza es una bebida alcohólica, no destilada, de sabor amargo desarrollada por los pueblos de los imperios mesopotámicos y egipcios, que se fabrica con granos de cebada u otros cereales cuyo almidón, una vez modificado, es fermentado en agua y frecuentemente aromatizado con lúpulo. De ella se conocen múltiples variantes con una amplia gama de matices debido a las diferentes formas de elaboración y a los ingredientes utilizados [74]. Aunque la elaboración de la cerveza se puede hacer con cualquier cereal (trigo, mijo, arroz), la más habitual es la obtenida a partir de cebada, sometida previamente a un proceso de malteado o germinación que da lugar a la malta. Durante este proceso se liberan diversas enzimas y se realizan los cambios necesarios en la estructura molecular de los diferentes componentes de la semilla (especialmente la degradación del endospermo) para obtener de ella la mayor cantidad de moléculas de azúcares fermentables y nutrientes básicos para la levadura.

1. Ingredientes básicos y proceso de elaboración

Los cuatro ingredientes básicos que, por regla general, intervienen en la elaboración de la cerveza son: malta, agua, lúpulo y levadura y el proceso de elaboración consta de las siguientes etapas [75,76]:

- ***Molienda***

Se muelen los granos de malta para obtener gránulos muy pequeños, sin llegar a convertirlos en harina, conservando la cáscara lo más intacta posible para utilizarla posteriormente como elemento filtrante.

- ***Maceración***

Se mezcla la malta molida con agua en la cuba de maceración, con el objetivo de liberar las enzimas que se han producido durante el procedimiento de malteado que reducen las cadenas largas de azúcares en otras más simples y fermentables. Las

[74] V. Berhoef. "La Enciclopedia de la Cerveza" Ed. Libsa, Madrid (2001).
[75] I. S. Hornsey. "Elaboración de cerveza: microbiología, bioquímica y tecnología" Ed. Acribia, S. A., Zaragoza (2002).
[76] I. S. Hornsey "A history of beer and brewing" RSC, Cambridge (2003)

enzimas se activan al contactar con el agua y como cada tipo de enzima tiene su temperatura ideal para activarse, la mezcla se hace pasar por diferentes etapas cada una de ellas con condiciones óptimas para cada tipo de enzima. Al final de la etapa de maceración se aumenta la temperatura hasta los 82ºC, destruyendo todas las enzimas.

- ***Filtración***

Se separa el extracto acuoso, denominado mosto, de los sólidos agotados (bagazos). La mezcla líquida contiene todo lo que se ha extraído del grano y que es soluble en agua.

- ***Cocción***

Después de la filtración, el mosto se lleva a la cuba de cocción, donde se cuece durante 60-90 minutos. Esta cocción sirve principalmente para esterilizar el mosto, destruyendo todos los microorganismos que hayan podido introducirse, concentrarlo, evaporando el agua y coagular algunas proteínas. Además, durante esta etapa se introducen los diferentes lúpulos, que imparten al mosto sus características aromáticas propias. Los que aportan principalmente amargor se añaden al principio de la cocción mientras que los aromáticos, al ser más volátiles entran al final de la etapa. Una vez finalizada la cocción se filtra el mosto para retirar los restos de lúpulo y las partes espesas que se han formado durante el proceso.

Figura 1. Tanques de cobre originales de cocción del mosto.

- ***Refrigeración y oxigenación***

Al término de la cocción el mosto se refrigera y oxigena para que se convierta en un medio ideal para el crecimiento de las levaduras y para la fermentación.

- ***Fermentación***

El mosto se lleva al tanque de fermentación, donde se añade la levadura. La levadura primero se reproduce muy activamente consumiendo el oxígeno contenido en el mosto y cuando se acaba el oxígeno empieza a consumir el azúcar transformándolo en alcohol y anhídrido carbónico. Las cervezas de baja fermentación tardan en fermentar de 8 a 10 días, y las de alta fermentación, de 4 a 6 días. Las cervezas más artesanas son envasadas con adiciones de azúcar (o de mosto) y de levadura fresca, lo que provoca una segunda fermentación en la botella, responsable de la efervescencia de la cerveza.

- ***Maduración***

Al acabarse la fermentación se lleva la cerveza a los tanques de maduración, donde va madurando a una temperatura que ronda los 0º C. En las cervezas de baja fermentación el proceso de maduración (*lagering*) es muy importante, porque este tiempo les proporciona un carácter más profundo. Por esta razón a las cervezas de baja fermentación se les suele llamar también "lager". Las cervezas de alta fermentación, especialmente las que sufren una segunda fermentación en la botella, tienen un tiempo más corto de lagering, ya que su cuerpo se consigue durante la segunda fermentación dentro de la botella.

- ***Envasado***

Hay que distinguir los dos tipos de envasado que pueden presentarse:
a) *Para las cervezas de baja fermentación y las de alta fermentación sin segunda fermentación en botella o barril*

Antes de llevar la cerveza a la máquina de llenado se inyecta CO_2 en los tanques hasta conseguir la saturación deseada, para que la cerveza salga de su recipiente con una buena capa de espuma.

b) *Para las cervezas con segunda fermentación en botella o barril*.

Antes de embotellar se añade una pequeña cantidad de azúcar, una dosis de levadura y se asegura una buena mezcla de los nuevos ingredientes con la cerveza. La segunda fermentación en las cámaras calientes dura hasta dos semanas y es el mejor seguro de calidad que existe para la cerveza. Como el poco aire que puede quedar en la botella y que podría dar lugar a una oxidación en el futuro se ha absorbido durante la segunda fermentación, no hace falta pasteurización, y la cerveza sigue evolucionando y madurando dentro de la botella.

Figura 2 Diagrama del proceso de elaboración de la cerveza

En el trabajo desarrollado en los dos primeros capítulos de esta tesis se estudiaron dos de las materias primas utilizadas en el proceso de elaboración de la cerveza: cebada y lúpulo.

2. Cebada

La cebada es un importante cereal de cultivo usado para alimentación humana y animal, además de ser el cereal preferido para la producción de malta en la industria cervecera. Los azúcares que contiene el grano de cebada no son inmediatamente accesibles y, en una fase previa, es preciso activar unas enzimas presentes en el propio grano que reducirán las largas cadenas de almidón para liberar azúcares. Esta operación

conocida como malteado consiste simplemente en una germinación controlada de los granos de cereal durante la cual se forman las enzimas y se modifican suficientemente las reservas alimenticias de manera que puedan ser hidrolizadas adicionalmente durante la maceración. El término malta se refiere a los granos a los que se les aplica este proceso. Puede provenir de diferentes cereales, siendo la cebada el cereal malteado más común por ser muy rico en almidón, sustancia que da origen al extracto fermentable, y pobre en proteínas, aunque en cantidades más que suficientes para proporcionar los aminoácidos necesarios para el crecimiento de la levadura y las sustancias nitrogenadas que desarrollan un papel importante en la formación de espumas.

2.1. Proceso de malteado

El proceso de malteado consta de las siguientes etapas [77,78]:

- ***Almacenamiento y limpieza***

La cebada cervecera se cosecha y se almacena durante un corto periodo de tiempo de 6 a 8 semanas para que todas las semillas sean capaces de germinar, sacándolas de un estado parecido al de la hibernación, oxigenando los silos o almacenes debidamente. Esta oxigenación no debe ser muy elevada para mantener las constantes vitales de la cebada en el punto justo donde el balance entre el consumo de la propia glucosa -debido a la respiración de las semillas- y la producción de sustancias no deseadas por la respiración intramolecular que se produce en ausencia de oxígeno, las mantenga en el mínimo vital. A continuación ha de limpiarse la cebada, eliminando granos y sustancias extrañas que contenga.

- ***Remojo***

Consiste en sumergir las semillas en un tanque parcialmente lleno de agua a unos 15ºC, para que sea absorbida y aumente rápidamente el contenido en agua de los granos. El contenido del tanque se airea intensamente, insuflando aire a través del agua de remojo mediante el uso de tuberías perforadas o por succión. El remojo se interrumpe, por drenaje, a las 12-24 horas y cada grano de cebada permanece recubierto de una película de agua, a través de la cual puede disolverse el oxígeno del aire del

[77] J. Hough. "Biotecnología de la cerveza y de la malta" Ed. Acribia S.A., Zaragoza (1990).
[78] W. Kunze "Technology brewing and malting" VLB, Berlin (1999).

entorno. Tras unas pocas horas de "descanso al aire", la cebada se sumerge de nuevo en agua limpia; la alternancia de remojo y "descanso al aire" continúa hasta que la cebada ha alcanzado una humedad de aproximadamente el 42%. Para entonces, es probable que el grano haya comenzado a germinar. La respiración aumenta significativamente cuando el embrión se activa, lo que crea una demanda masiva de oxígeno en el agua de remojo (de aquí la necesidad de hacer borbotear aire y de los descansos al aire durante el remojo).

- ***Germinación***

Cuando los granos dan muestras de que han comenzado a germinar, se transfieren al equipo de germinación. El contenido de humedad, que normalmente se haya en torno al 42%, permanece constante durante la etapa de germinación. El tipo de germinador más frecuente es una caja de base rectangular provista de un falso fondo perforado sobre el que se deposita un lecho de malta. A través del lecho, y habitualmente de abajo a arriba, se hace pasar una corriente de aire saturado de agua, a unos 15ºC, con lo que se asegura la disponibilidad de oxígeno, la eliminación de dióxido de carbono por la respiración de los embriones y el mantenimiento de una temperatura constante en todo el lecho.

Desde el punto de vista fisiológico, existe una continuidad entre el remojo y la germinación. El crecimiento embrionario se inicia durante el remojo, pero como las reservas de nutrientes inmediatamente disponibles son limitadas, resulta necesario movilizar las del endospermo, constituido por células de gran tamaño provistas de granos de almidón grandes y pequeños, recubiertos de proteína. Para poder acceder a estos nutrientes se produce la secreción por parte del embrión de enzimas que degradan las proteínas, el almidón y las paredes celulares del endospermo. En algunos casos se rocía la cebada remojado con ácido giberélico que estimula la producción de estas enzimas hidrolíticas. El proceso de germinación tarda entre 4 y 6 días para la cebada.

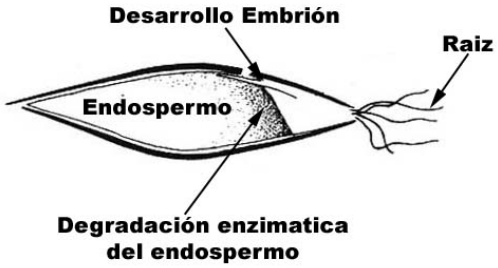


Figura 3. Grano de cebada durante la germinación

- ***Secado y tostado***

Cuando se estima que la activación enzimática de la germinación se encuentra en su punto óptimo, se para el proceso reduciendo la humedad del grano hasta su mínimo (4%) mediante un secado y tostado por aire caliente.

2.2. Tipos de maltas

Según la temperatura del aire que usemos y el tiempo que apliquemos a las semillas durante el secado y tostado, se obtienen diferentes tipos de maltas [79]:

- *Malta básica*: maltas claras, poco horneadas con gran poder enzimático, que suelen formar la parte más grande o la totalidad de la mezcla. En concreto estas maltas son llamadas *lager*, *pale* o *pils*, según el fabricante.
- *Maltas aditivas*: Son maltas de color que va de ámbar a negro, muy horneado y con poco o nada de poder enzimático. El grado de tostado de la malta determina el color de la cerveza .Se puede llegar al punto de quemarla, produciendo *malta negra*. Suelen ser usados en pequeñas cantidades para incidir sobre el color o el gusto de la cerveza o por algún motivo técnico propio de la elaboración.
- *Maltas mixtas*: Estas maltas están más tostadas que las maltas base pero conservan propiedades enzimáticas suficientes, al menos, para sus propios azúcares, de manera que pueden ser usados como base o como aditivos. En esta categoría encontramos las maltas de color caramelo y ámbar, conocidas en Inglaterra como maltas *cristal* (y derivados) y en Alemania como maltas *caramelo*.

[79] D.E. Briggs,"*Malts and Malting*" Kluwer Academic / Plenum Publishers (1998).

Figura 4. Diferentes tipos de maltas en función de las condiciones de secado y tostado

2.3. Calidad de la cebada y malta

Como en otros cereales, la cebada utilizada en maltería y elaboración de cerveza necesita cumplir unos requerimientos de calidad específicos relacionados con el poder germinativo, el tamaño de los granos y el contenido de proteínas, entre otros [80]. El interés fundamental del malteador es obtener una cebada que germine fácil y uniformemente. En cuanto a las proteínas, se requiere un contenido relativamente bajo, entre el 9 y el 11.5 %. Porcentajes más elevados son indeseables no sólo porque baja la cantidad de glúcidos, sino porque la fracción insoluble (hordeína y glutelina) puede producir turbidez en la cerveza. No obstante, una cantidad mínima de proteína es necesaria para asegurar la producción de espuma, una buena actividad enzimática y una cantidad de aminoácidos suficientes para el desarrollo de las levaduras en la elaboración de cerveza. Existen bastantes trabajos donde se relaciona un contenido bajo en proteínas totales con una buena calidad de la malta obtenida.

2.4. Clasificación de las proteínas de la cebada

Una vía tradicional, pero útil, de clasificar las proteínas de los cereales en general, es la basada en su solubilidad en diferentes disolventes (Tabla 1).

Proteína	Soluble en	Representada en el grano de cebada como
Albúminas	Agua	Enzimas
Globulinas	Soluciones salinas	Enzimas
Glutelinas	Soluciones ácidas o alcalinas	Proteína de reserva
Prolaminas	Soluciones alcohólicas	Proteína estructural

Tabla 1. Clasificación, basada en la solubilidad, de las proteínas de los cereales.

[80] Y. Li, P. B. Schwarz, J. M. Barr, R. D. Horsley. "Factors predicting malt extract within a single barley cultivar" *J. Cereal Sci.* 48 (2008) 531-538.

Las prolaminas son las proteínas dominantes de la matriz proteica que rodea los gránulos de almidón dentro de las células del endospermo y en la cebada, esta fracción recibe el nombre de hordeínas [81] y se clasifican en 4 familias de polipéptidos:

- Ricas en sulfuros: fracciones B (28-45 kDa) y alfa (20 kDa)
- Pobres en sulfuros: fracción C (49-72 kDa)
- Alto peso molecular: fracción D (100 kDa)

2.5. Análisis de proteínas de cebada y malta: técnicas analíticas más utilizadas

Muchas de las características de calidad mencionadas dependen del cultivo o variedad utilizados, siendo por esto importante contar con métodos que permitan una segura diferenciación o discriminación de cultivares. Originariamente la identificación de cultivares estaba basada en características morfológicas, sin embargo, el creciente número de variedades con semillas de apariencia similar y su alteración durante el proceso de malteado hizo necesaria el uso de otras técnicas para determinar la pureza varietal de lotes de malta y cebada. Típicamente, estos métodos se han basado en la medida de las proteinas del endosperma de la semilla (hordeinas) a las que pueden aplicarse distintas técnicas analíticas.

Los métodos más utilizados para la identificación de hordeínas en cebada han sido la electroforesis en gel de poliacrilamida (PAGE), la cromatografía líquida de alta resolución (HPLC) y también encontramos algunas aplicaciones con MALDI-TOF para la clasificación de variedades [82]. En los últimos años la electroforesis capilar ha demostrado ser muy útil para el análisis de proteínas de almacenamiento en diferentes cereales de cultivo como trigo, avena, arroz y maíz [83], y también encontramos separaciones rápidas y con alta resolución de hordeínas de cebada [84,85]. Por medio

[81] L. Vapa, D. Radovic. "Genetics and molecular biology of barley hordeins" *Cereal Res. Commun.* 26 (1998) 31-38.
[82] J. Salplachta, J. Bobalova. "MALDI-TOF mass spectrometry of hordeins: rapid approach for identification of malting barley varieties" *J. Mass. Spectrom*. 44 (2009) 1287-1292.
[83] S. R. Bean, G. L. Lookhart. "Ultrafast CE analysis of cereal storage proteins and its application to protein characterization and cultivar differentiation" *J. Agric. Food Chem*. 48 (2000) 344-353.
[84] G. L. Lookhart, S. R. Bean, B. L. Jones. "Separation and characterization of barley (Hordeum vulgare L.) hordeins by free zone capillary electrophoresis". *Electrophoresis* 20 (1999) 1605-1612.
[85] Y. Yan, Y. Jiang, J. Yu, M. Cai, Y. Hu, D. Perovic. "Characterization of seed hordeins and varietal identification in three barley species by high-performance capillary electrophoresis" *Cereal Res. Commun.* 31 (2003) 323-330.

de esta metodología se pudieron obtener electroferogramas característicos para cada una de las variedades de cebada, observando diferencias sustanciales en la distribución de proteínas entre las distintas fracciones de hordeínas (B, C y D). Además de utilizar estas técnicas para diferenciar variedades basándose en el perfil proteico, se pueden encontrar en bibliografía algunos trabajos donde se relaciona el contenido en determinadas hordeínas de la cebada (especialmente las D y B- hordeinas) con los principales parámetros que determinan la calidad de la malta [86,87].

3. Lúpulo

El lúpulo (Humulus lupus) es una de las materias primas esenciales de la cerveza y desde la antigüedad se le han atribuido múltiples beneficios para la salud [88]. Es una de las tres especies de plantas del género *humulus*, de la familia de las cannabináceas, de hoja perenne y dióica, lo cual significa que las flores masculinas y femeninas crecen sobre plantas diferentes. Para la elaboración de cerveza se utilizan sólo las flores de las plantas femeninas (también llamadas conos o piñas) antes de que sean fecundadas, aunque en países como Inglaterra y Bélgica también utilizan, a veces, lúpulos femeninos fecundados que son más fuertes y amargos.

Figura 5. Flores femeninas del lúpulo (conos)

Originariamente, el lúpulo silvestre crecía en los valles húmedos de Oriente Próximo y los cerveceros babilonios y egipcios ya lo usaban. Pero su generalización

[86] J. L. Molina- Cano, J. P. Polo, A. Sopena, J. Voltas, A. M. Pérez-Vendrell, I. Romagosa. "Mechanism of malt extract development in barleys from different european regions: II. Effect of barley hordein fractions on malt extract yield" *J Inst Brew* 106 (2000) 117-123.
[87] G. Simic, R. Sudar, A. Lalic, Z. Jurkovic, D. Horvat, D. Babic. "Relationship between hordein proteins and malt quality in barley cultivars grown in Croatia". *Cereal Research Commun*. 35 (2007) 1487-1496.
[88] J. F. Stevens, J. E. Page. "Xanthohumol and related prenylflavonoids from hops and beer: to your good health!" *Phytochemistry* 65 (2004) 1317-1330.

llegó en el siglo XVI, al ser impuesto como uno de los tres únicos ingredientes de la cerveza bávara por la "Ley de la pureza" de 1516 que limitaba los ingredientes permitidos en su elaboración a tres: cebada (o trigo), lúpulos y agua (en 1516 se desconocía la acción de la levadura). Actualmente se cultiva en más de 50 países.

3.1. Composición del lúpulo

El lúpulo es el responsable fundamental del amargor de la cerveza, utilizado para compensar el excesivo dulzor proporcionado por la malta. Antes de su aceptación y generalización, algunos cerveceros venían utilizando diversas hierbas y especias con este fin. Además, el lúpulo posee excelentes cualidades aromáticas. Las flores de la planta del lúpulo contienen en su interior unas glándulas de color amarillo llenas de una resina llamada lupulina, que es el ingrediente que aportará a la cerveza su sabor amargo y los aromas propios. Del amargor son responsables los ácidos amargos y los aromas proceden de aceites elementales constituidos en especial por compuestos bastante volátiles y delicados a base de ésteres y de resinas. Además de los principios amargos y los aceites esenciales podemos encontrar en el lúpulo otros compuestos como azúcares, polifenoles, taninos, pectinas, proteínas etc, que se muestran en la Tabla 1 [89].

Compuestos mayoritarios	Concentración (% w/w)
α-ácidos	2-17
β-ácidos	2-10
Aceites esenciales	0.5-3.0
Polifenoles y taninos	3-6
Monosacáridos	2
Aminoácidos	0.1
Proteínas	15
Lípidos y ácidos grasos	1-5
Pectinas	2
Cenizas-sales	10
Celulosa-ligninas	40-50
Agua	8-12

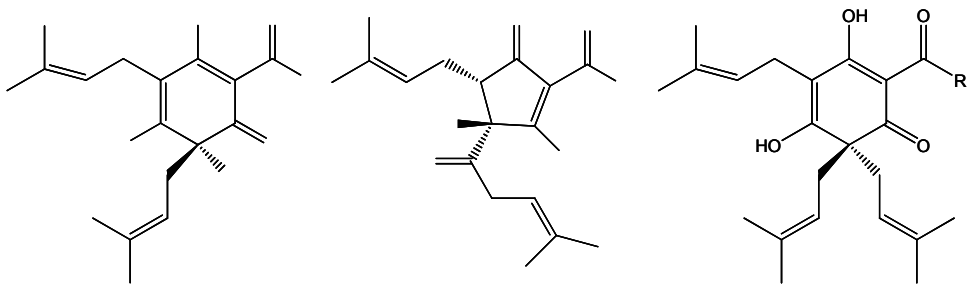
Tabla 2. Principales compuestos del lúpulo

[89] J. L. Benítez, A. Foster, D. De Keukeleire, M. Moir, F. R. Sharpe, L. C. Verhagen, K. T. Wetwood. "Hops and hop products" en "Manual of Good Practice" European Brewery Convention (1997)

3.2. Componentes amargos del lúpulo

Son aportados principalmente por los llamados α- ácidos y β-ácidos. Los más importantes, los α-ácidos o humulonas, representan una familia de compuestos que incluyen: humulona, cohumulona y adhumulona, y en menor proporción prehumulona y posthumulona, ilustrados en la Figura 6. Se diferencian en la cadena lateral unida al carbono 2 del anillo hexacarbonado. Durante la cocción del lúpulo en el mosto, los α-ácidos se reorganizan o se isomerizan del modo que se indica en la figura dando lugar a los iso α-ácidos o isohumulonas, mucho más amargos y mucho más solubles en el mosto que los α-ácidos [90]. Además, estos compuestos tienen propiedades tensioactivas, lo que permite la estabilización de la espuma, e inhiben el crecimiento de bacterias en la cerveza.

Además de los α-ácidos, el lúpulo también contiene β- ácidos o lupulonas (Fig 2): lupulona, colupulona, adlupulona, prelupulona, postlupulona. Estos compuestos tienden a oxidarse durante la ebullición dando una serie de derivados amargos, aunque su contribución al amargor final de la cerveza es menor que el de los α-ácidos isomerizados.



α- ácidos Iso-α- ácidos β- ácidos

Definición de R	α-ácido	Iso-α- ácido	β-ácido
CH_2-CH- $(CH_3)_2$	Humulona	Isohumulona	Lupulona
CH- $(CH_3)_2$	Cohumulona	Isocohumulona	Colupulona
CH-$(CH_3)_2$-CH_2-CH_3	Adhumulona	Isoadhumulona	Adhulupona
CH_2-CH_3	Posthumulona	Isoposthumulona	Postlupulona
CH_2-CH_2-CH-$(CH_3)_2$	Prehumulona	isoprehumulona	Prelupulona

Figura 6. Estructuras de los ácidos amargos

Las proporciones relativas de α- y β- ácidos dependen de la variedad de lúpulo y, para una determinada variedad, de las condiciones de cultivo.

[90] D. De Keukeleire. "Fundamentals of beer and hop chemistry". Quim. Nova 23 (2000) 108-112.

3.3. Variedades de lúpulo

Existen numerosas variedades botánicas de lúpulo aunque tradicionalmente se ha hablado de tres categorías en función del nivel de α-ácidos y de aceites esenciales:

- *Lúpulos amargos*: son los que aportan más ácidos amargos que aromas
- *Lúpulos aromáticos*: son los que aportan más elementos aromáticos que amargos
- *Lúpulos mixtos*: son los que aportan ambas características juntas aunque menos acentuadas.

Sin embargo, las clasificaciones actuales tienden a no diferenciar las variedades en grupos, sino a evaluarlas de manera individualizada en función de sus características propias, muy influenciadas por el clima y las condiciones de cultivo.

3.4. Oxidación del lúpulo

Las sustancias amargas del lúpulo, especialmente los α-ácidos, son muy susceptibles a la oxidación y degradación durante el almacenamiento, generando productos de oxidación muy diversos [91]. Los cambios que tienden a producirse son: oxidación de cadenas laterales, pérdida de las mismas y transformación de los anillos hexagonales en pentagonales. Cuando los α-ácidos se oxidan ya no pueden ser isomerizados en iso-α-ácidos, lo cual merma significativamente su capacidad de amargor, afectando al sabor de la cerveza. Estos fenómenos son acelerados por el oxígeno, temperatura y humedad, lo que hace que el almacenamiento y conservación del lúpulo sean muy delicados. Los cerveceros deben tener esto muy en cuenta y, por ello, tratan de conseguir lúpulos lo más frescos posibles y de guardarlos en frío (cámaras frigoríficas) y en condiciones anaeróbicas (libres de oxígeno). Además, con el fin de conservar sus propiedades, el lúpulo está presente en el mercado en diferentes formas que van desde el lúpulo deshidratado hasta extractos de lúpulo. Lógicamente, en cada manipulación se van perdiendo características y no es lo mismo utilizar un lúpulo fresco o congelado que un aceite de concentrado de lúpulo. La variedad y el frescor del lúpulo influyen muy sensiblemente en la calidad final de la cerveza.

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3.5. Análisis de los ácidos de lúpulo: técnicas analíticas más utilizadas

Teniendo en cuenta que el contenido en ácidos del lúpulo y su estado de oxidación afectan a la calidad final de la cerveza, resulta interesante desarrollar métodos para el análisis de este tipo de compuestos. Los métodos clásicos de análisis aprobados por la ASBC (American Society of Brewing) y EBC (European Brewery Convention) se basaban en medidas espectrofotométricas y conductimétricas aunque más tarde se desarrollaron métodos más específicos que utilizaban cromatografía líquida de alta resolución (HPLC) [89,92]. Además, en bibliografía encontramos otros métodos de HPLC-UV y HPLC-MS [93] para la identificación y cuantificación de los ácidos del lúpulo así como diferentes modalidades de electroforesis capilar acoplada a detección UV-Vis [94]. Sin embargo, estudios donde se evalúen los efectos del almacenamiento en la composición del lúpulo son muy escasos. En un trabajo llevado a cabo por Canbas y col. [95] se estudiaron los efectos de la temperatura de almacenamiento en el contenido de α y β- ácidos empleando métodos espectrofotométricos y en otro trabajo más reciente se utiliza conductimetría y HPLC para evaluar las pérdidas de estos ácidos durante el almacenamiento [96].

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CAPÍTULO 1:
Análisis del perfil proteico (hordeínas) de muestras de cebada en diferentes etapas del proceso de malteado mediante CZE

El contenido en proteínas de la cebada, es un parámetro muy importante relacionado con la calidad de la malta obtenida. Por este motivo, se han desarrollado diferentes metodologías analíticas para el análisis del perfil proteico de cebada, que además ha resultado muy útil para la diferenciación de variedades. Durante el proceso de malteado o germinación se producen muchas modificaciones en el grano de cebada que también influirán en la calidad de la malta final. El objetivo de este primer capítulo de la tesis fue desarrollar una metodología analítica por electroforesis capilar en zona con detección ultravioleta-visible para llevar a cabo una comparación del perfil proteico de hordeínas en muestras de cebada tomadas en diferentes etapas del proceso de malteado.

Antes de la germinación las proteínas de la cebada ya constituyen una mezcla muy compleja. A esto hay que sumarle que durante la germinación se producen procesos de degradación que generan nuevos compuestos más simples y en algunos casos parte de los compuestos nitrogenados se utilizan en la síntesis de nuevas proteínas. Todo esto hace que las proteínas de la cebada en germinación no sean una mezcla simple, de fácil caracterización y que en algunos casos se obtengan electroferogramas confusos.

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Free-Zone Capillary Electrophoresis Analysis of Hordein Patterns at Different Stages of Barley Malting

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We have carried out a comparison of hordein patterns at different stages of the malting process using free-zone capillary electrophoresis (FZCE). FZCE has proved to be a suitable technique for the separation and characterization of hordeins in barley seeds. Assays of protein extraction and electrophoretic procedures led us to conclude that hordeins were best extracted with 40% ethanol and analyzed using 50 mM phosphate−glycine, pH 2.5, containing 20% ACN and 0.05% HPMC, at 12.5 kV and 45 °C, with 10 s hydrodynamic injection at 0.5 psi and 50 μm i.d. × 31 cm uncoated fused-silica capillary. Our results afford useful information about changes in the composition of these proteins in barley during malting.

KEYWORDS: Proteins; hordeins; barley; malt; free-zone capillary electrophoresis

INTRODUCTION

Barley is an important cereal crop, used as both animal and human food, as well as the preferred grain for malt production in the brewing industry. Barley grain is converted into malt by soaking in water until it sprouts under controlled conditions to form enzymes and modify its nutrient reserves (*1*). Malt quality is greatly influenced by the barley protein content.

Grain protein comprises a complex mixture of polypeptides that are classified by their extractability and solubility characteristics: albumins (soluble in water), globulins (insoluble in water but soluble in dilute salt solutions), glutelins (insoluble in the above solutions but soluble in weak acid or basic solutions), and prolamins (insoluble in the above solutions but soluble in alcohol/water mixtures) (*2*−*4*).

The alcohol-extractable fraction of barley grain contains the main endosperm storage proteins, the hordeins. Hordeins accumulate in protein bodies of the starchy endosperm during seed development and comprise 30−50% of the total grain protein. They are extremely heterogeneous in composition in different barley cultivars. In accordance with electrophoretic mobility, hordeins are classified into four groups of polypeptides, known as B, C, D, and γ. The B hordein (sulfur-rich) and C hordein (sulfur-poor) are the two main fractions, while D hordein (high molecular weight) and γ hordein (sulfur-rich) are minor components. B and C hordeins account for 70−80% and 10−20% of the hordein fraction, respectively, while γ and D hordeins make up less than 5% of the total hordein fraction. The proportions of the different hordein fractions present in mature grain depend on cultivar and growth conditions (*5*).

Hordeins are a dominant component of the protein matrix that surrounds the starch granules within the cells of the endosperm. One of the most important physical−chemical changes that occurs during malting is the degradation of the hordeins in this matrix and their conversion into soluble peptides and amino acids to provide substrates for the synthesis of proteins in the growing embryo. The degradation of hordeins during malting is also necessary to allow enzymes access to the starch, thus facilitating its complete hydrolysis (*5*, *6*).

Albumin and globulin proteins are relatively resistant to proteolysis, whereas our results show that hordeins undergo a dramatic breakdown during malting, with D hordein being degraded more rapidly than B hordein and C hordein being the most resistant (*7*−*9*). Protein content is a key characteristic used for accepting or rejecting barley for malting, and therefore it is important to have methods that can separate, characterize, and identify barley proteins. The two most commonly used identification techniques, polyacrylamide gel electrophoresis (PAGE) and high-performance liquid chromatography (HPLC), have recently been reviewed (*10*−*13*). Matrix-assisted laser desorption ionization (MALDI)−time of flight mass spectrometry (TOF-MS) also seems to be a useful alternative technique for the identification of these barley prolamins, with a detection sensitivity of about 50−100 ng total protein loaded (*14*). Another approach to identify barley varieties is isoelectric focusing. Isoelectric focusing performed with immobilized pH gradients was found to be superior to other commonly used electrophoretic methods for the identification of barley cultivars, but evaluation was more complicated because of the larger number of protein bands detected (*15*).

In recent years a new, more powerful technique for the analysis of storage proteins in cereals, capillary electrophoresis (CE), has been developed. Using only a small sample, it is

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capable of rapid, high-resolution, quantitative digital analysis of storage proteins in such cereal crops as wheat, oats, rice, and maize (*16*−*23*). In particular, free-zone capillary electrophoresis (FZCE) produces little hazardous waste and is easily automated, which makes it an attractive method for separating hordeins (*24*, *25*). Lookhart et al. and Yan et al. have described the optimum conditions for the extraction, separation, and characterization of hordein subclasses and applied their method to differentiate barley cultivars.

We describe here a simpler and faster FZCE method to characterize hordein patterns during different stages of malting.

EXPERIMENTAL PROCEDURES

Chemicals and Samples. Hordeins were extracted either with 1-propanol or with ethanol (Panreac, Barcelona, Spain) using DL-dithiothreitol from Sigma Chemical Co. (St. Louis, MO) as reducing agent.

Sodium dihydrogen phosphate 1-hydrate, glicine, and acetonitrile, used for the CE running buffers, were from Panreac (Barcelona, Spain), hydroxypropylmethyl-cellulose (hypromellose, HPMC) was from Sigma Chemical Co. (St. Louis, MO), and phosphoric acid was from Merck (Darmstadt, Germany). The buffers were prepared by weighing the concentrations indicated in doubly distilled water and adjusting the pH to 2.5. All solutions were filtered through a 0.5 μm Millipore (Bedford, MA) membrane filter before being injected into the capillary. Distilled water was deionized using a Milli-Q system (Millipore, Bedford, MA).

Ten barley samples of the variety Scarlett taken at different stages of the malting process were provided by the company Intermalta S. A. (Spain): sample M1, initial barley of variety Scarlett; sample M2, green malt at the end of steeping; samples M3, M4, M5, M6, M7, and M8, green malt obtained from six different substages of germination. Each sample is soaked and kept in forced ventilation for 17 h at a humidity of around 40% and temperature between 17 and 22 °C. When the barley passes from one stage to another it is watered abundantly, except for samples M7 and M8. Samples M9 and M10 were malt obtained from the two substages of the roasting. The first substage (sample 9) is carried out with a hot air flow of 130.000 m^3/h per 50 tons for 17 h at a maximum temperature of 65 °C. The malt is dried until reaching 14−16% humidity. In the second substage, the air flow is less (80.000 m^3/h per 50 tons), and the process is carried out for 12 h with an increase in temperature from 60 to 80 °C.

Equipment. CE experiments were made with a Beckman P/ACE System MDQ capillary electrophoresis instrument equipped with a built-in 0−30 kV high-voltage power supply and a diode array detector and GOLD software for system control and data handling. All capillaries (fused silica) had an internal diameter of 50 μm and were 31.2 cm in total length (Beckman Instrument Inc., Fullerton, CA). Temperature was controlled using a fluorocarbon-based cooling fluid.

Electrophoretic Procedure. CE separation was carried out on a fused silica capillary (50 μm i.d., 375 μm o.d., total length 31.2 cm; a detection window was created at 21 cm from the capillary inlet). New capillaries were preconditioned by rinsing with 1 M phosphoric acid for 10 min, followed by a 5 min rinse with Milli-Q water and 15 min with buffer (50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC). To ensure good repeatability, after each separation the capillary was rinsed with 1 M phosphoric acid for 5 min, followed by a 5 min flush with Milli-Q water. The capillary was equilibrated with the running buffer for 15 min before each sample injection.

Samples were injected hydrodynamically under low-pressure (0.5 psi) for 10 s into the anodic end. Electrophoretic separation was performed at 12.5 kV, and the temperature was kept at 45 °C. All solutions and buffers were filtered through a 0.20 μm syringe filter. UV detection was performed at 200 and 280 nm simultaneously.

Hordein Extraction Procedure. The barley grains taken at different stages of the malting process were dried in an oven for 2.5 h at 50 °C to remove humidity. The dried grains were milled to powder in a commercial grinder, and 300 mg of the milled samples was extracted with 1 mL of 40% ethanol for 5 min using a vortex. The extracts were then centrifuged at 4800 rpm for 10 min. The supernatants containing the hordeins were filtered through 0.20 μm filters and transferred to a microvial before being analyzed by capillary electrophoresis.

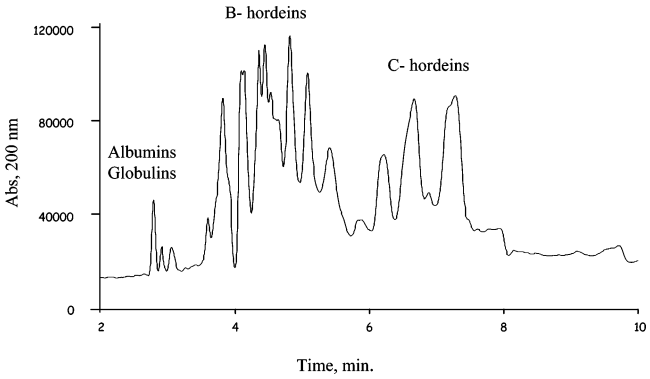

Figure 1. Identification of the different groups of peaks in the optimum electropherogram obtained with a malt sample provided by the company Grupo Cervezas Alhambra. Extraction conditions: ethanol 40%. Experimental conditions: buffer 50 mM phosphate–glycine containing 20% ACN and 0.05% HPMC; 50 μm i.d. fused silica capillary, 31.2 cm detector and total length, 12.5 kV, 10 s hydrodynamic injection at 0.5 psi.

RESULTS AND DISCUSSION

Optimization of the CE Method. We studied the effects of different separation parameters to obtain the best conditions of selectivity, sensitivity, and resolution.

The CE method was optimized using the extract obtained with 40% ethanol from a malt sample provided by Grupo Cervezas Alhambra S.L. The main obstacle to the separation of proteins was the tenacious affinity of ionized silanols toward a proteinaceous surface due to the multiple ionizations occurring on the silica surface at any pH above 3 and to the multiple charges typically present on a protein surface. One simple strategy consists of the use of electrolyte solutions at acidic pH values for suppressing silanol dissociation (*26*). A final pH of 2.5 was chosen for the running buffer because of the basic properties of the hordeins and their positive electrophoretic mobility at acidic pH.

First, we studied different running buffers at this pH, such as 100 mM phosphoric acid, 50 mM phosphate containing 20% ACN, 50 mM phosphate containing 20%ACN and 0.05% HPMC, and 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC. The best resolution and fastest separation was achieved using 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC as running buffer. These results are in accordance with the bibliography because in general organic solvents in low-pH phosphate buffers, particularly ACN, tend to improve the resolution of CE separations of storage proteins in wheat (*17*, *21*), barley (*24*), maize, and sorghum (*18*).

To obtain better resolution between the hordein peaks, we also tested the effect of the ionic strengths of the running buffer: 25 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, 75 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, and 100 mM phosphate−glycine containing 20% ACN and 0.05% HPMC. The resolution did not improve when the buffer concentration was increased, and for lower concentrations, the ionic strength was not enough to obtain separation.

We also tested the effects of different voltages, temperatures, and injection times. The applied voltage was varied between 10 and 30 kV, and a voltage of 12.5 kV was found to produce

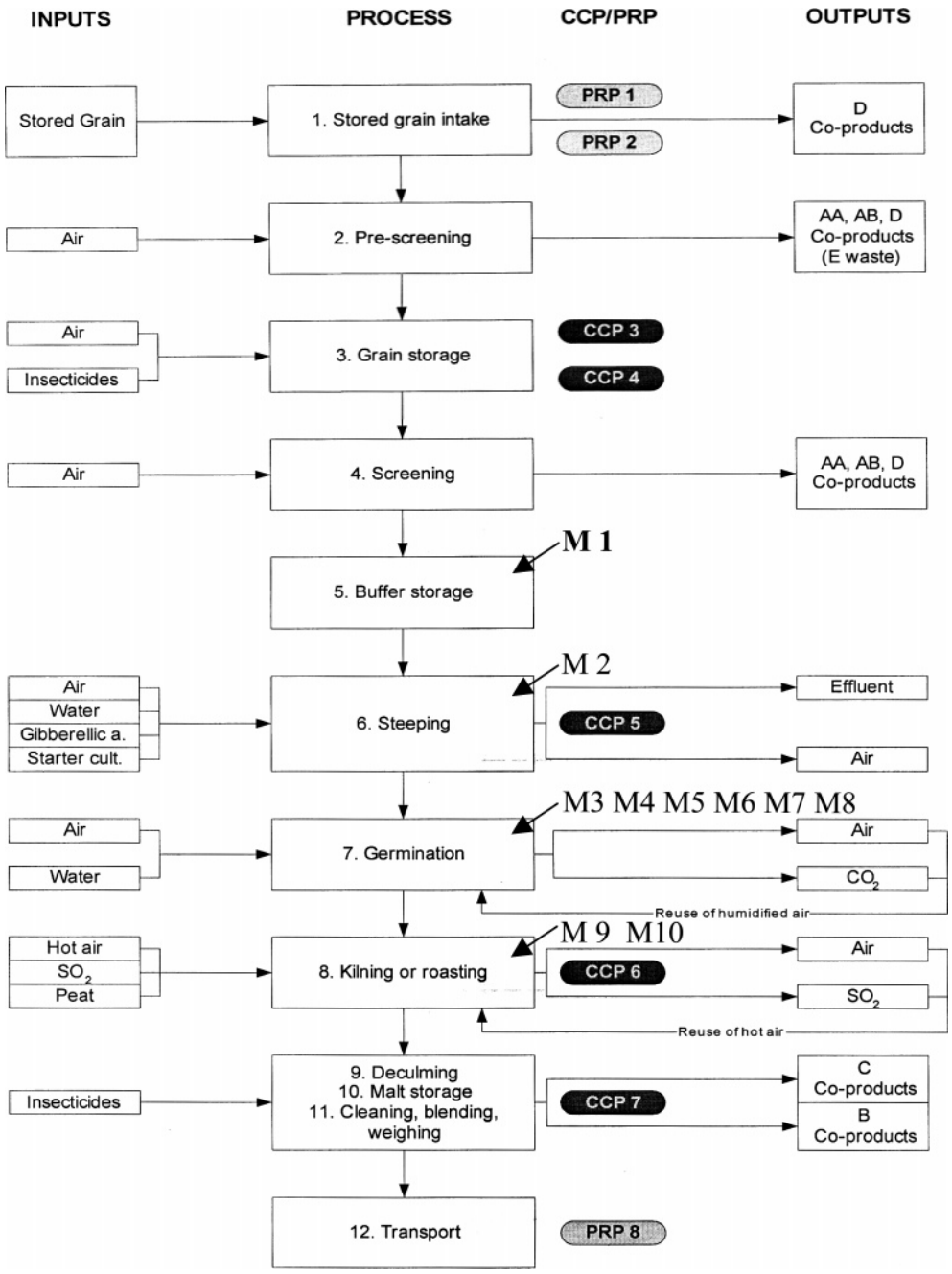

Figure 2. Diagram of the different stages of the malting process indicating where the ten samples have been taken: sample M1, initial barley of variety Scarlett; sample M2, green malt at the end of steeping; samples M3, M4, M5, M6, M7, and M8, green malt obtained during the six successive substages of germination; samples M9 and M10, malt obtained during the two substages of roasting.

fast separation and good resolution. Two temperatures (30 and 45 °C) were also tested, and the best resolution was obtained at 45 °C. Assays of different injection systems, hydrodynamic injection (between 5 and 10 s) and electrocinetic injection (8 s, 10 kV) proved that hydrodynamic injection for 10 s produced the best results.

Consequently, we chose the following CE conditions: a running buffer of 50 mM phosphate−glycine containing 20% ACN and 0.05% HPMC, pH 2.5, voltage 12.5 kV, 45 °C, 10 s hydrodynamic injection at 0.5 psi, and 50 μm i.d. × 31 cm uncoated fused-silica capillary. The main differences between our method and those of Lookhart et al. and Yan et al. are the running buffer, the temperature, and the dimension of the capillary, which have allowed us to obtain shorter analysis times (<10 min).

Repeatability was tested by analyzing seven consecutive injections under the optimum conditions described above, and the retention time was 1.5%.

Comparative Studies of Extraction Procedure. To extract the hordeins in the samples, we studied three types of possible mixtures: 1-propanol 50% with 1% DTT, ethanol 40%, and ethanol 40% with 1% DTT. All the solvents were compared under the same optimum CE conditions described above.

The time needed to extract the hordeins was investigated previously by extracting samples for 5 to 60 min with a wide variety of solvents and temperatures. These assays proved that 5 min was enough to extract hordeins (*13, 24*). Therefore we extracted for 5 min 300 mg of milled samples of grain dried in an oven for 2.5 h at 50 °C with 1 mL of the different solvents studied using a vortex. The extracts were then centrifuged at

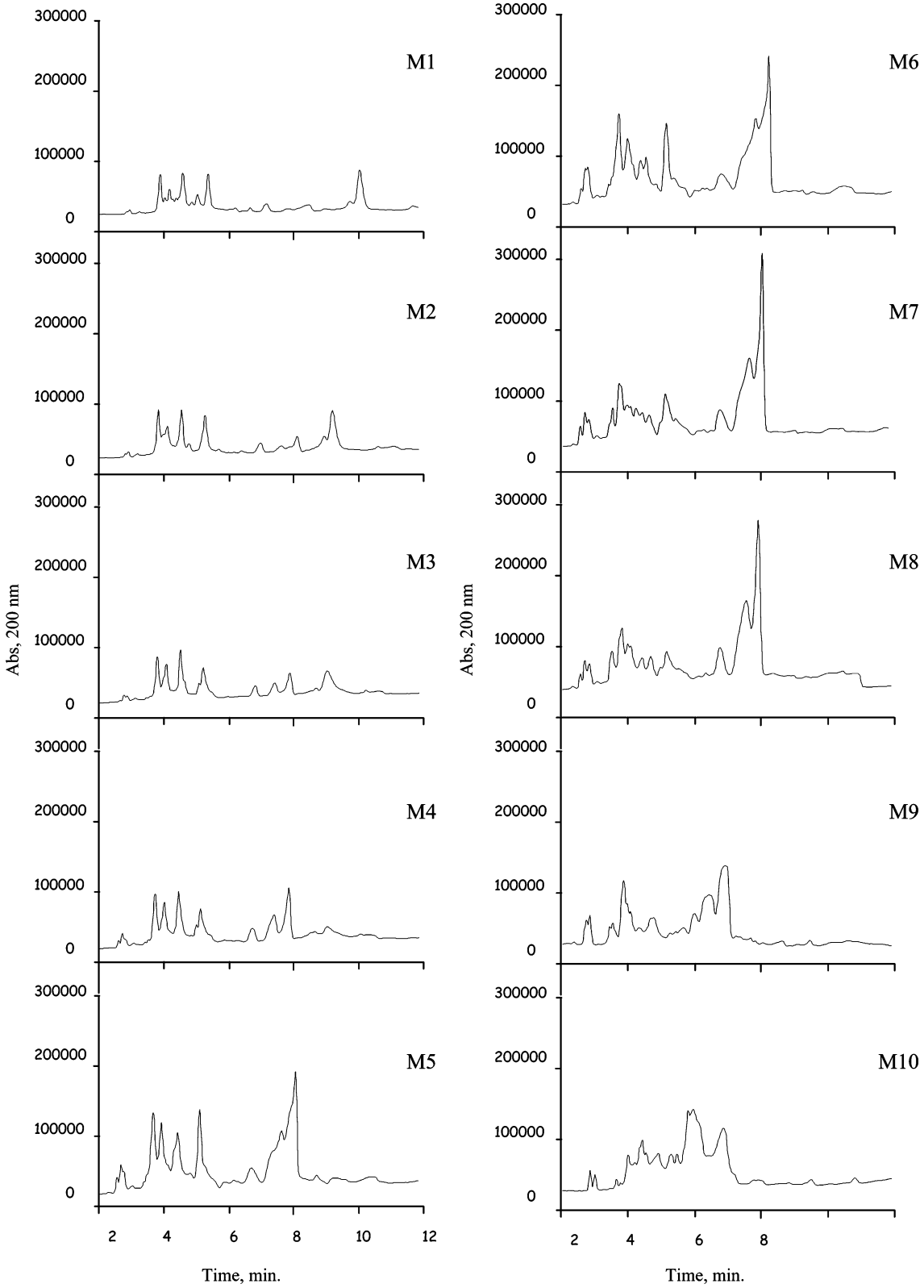

Figure 3. Comparison of hordein patterns of ten samples taken at different stages of the malting process. The separation conditions were as described in Figure 1.

4800 rpm for 10 min. The supernatants containing the hordeins were filtered through 0.20 μm filters and transferred to a microvial before being analyzed by capillary electrophoresis.

The results obtained with 40% ethanol extracts showed better resolution than those with 50% 1-propanol and 1% DTT. Apart from this, the addition of a reducing agent did nothing to improve the resolution of the hordein peaks. Therefore, the samples were successfully separated by FZCE after extraction with 40% ethanol under nonreducing conditions, and the resolution was better than that with the reduced samples.

Identification of Hordeins. The optimum electropherogram of the protein pattern can be seen in **Figure 1**. The proteins were resolved into three groups of peaks. The first group was identified as albumins and globulins since previous work on

the separation of wheat proteins by FZCE showed that the albumin and globulin proteins had the highest mobilities of the proteins tested (*27*). The other two well-separated groups apparently represented the B and C hordeins. The hordein CE patterns were similar to those reported by Lookhart et al. (*24*) and by Yan et al. (*25*).

Application. The aim of this study was to follow the behavior of hordein samples taken at different stages of the malting process with FZCE.

The different stages of the malting process can be seen in **Figure 2**. The barley used for the malting process is stored under suitable conditions with enough oxygen to keep the embryo alive after it is screened to remove other grains or foreign objects. The clean grains are then steeped in oxygenated water until reaching a moisture content of 45%. Then the grains are allowed to germinate, and at the correct moment, germination is interrupted to roast the grains with a hot air current. The malted grain is stored and should remain stable for months or even years. The barley proteins before and after germination comprise a complex mixture difficult to characterize. This is because during the germination carbohydrates are consumed in a respiratory process, so the protein content seems to increase and nitrogenous substances are used to synthesize proteins of the embryo (*1*).

A set of ten samples comprising different phases of the malting process (M1−M10) were analyzed to study the capacity of FZCE to differentiate the protein profiles of malting and to test any changes that might take place during this process.

The comparison of hordein patterns at different stages is shown in **Figure 3**. This comparison shows important changes among the ten samples during the main phases of the process. During the first stages of the malting process, reception and steeping (samples M1 and M2), the patterns of B and C hordeins were approximately the same, indicating that significant changes do not occur in these proteins during steeping. The samples taken at these stages also exhibited fewer peaks in the C hordein range. During the germination stages more differences could be seen. Although the B hordeins did not show any significant changes during the first two stages, a slight increase in the height of the peaks could be observed in the last sample. But the main qualitative and quantitative differences among samples were found in the area of the C hordeins. C hordeins increased in quantity throughout the six stages of the germination process, exhibiting the highest peaks in the samples taken during the last two stages (M7, M8). In the roasting process, the extreme conditions of temperature and humidity brought about an important change in the protein patterns of both B and C hordeins.

Capillary electrophoresis allows a fast determination of the hordein fractions in barley, and our results provide useful information to help evaluate the changes in proteins during malting to differentiate and monitor the stages of this process. Since C hordeins show the most significant changes throughout the stages, they could be used as reliable indicators to follow the malting process.

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Apéndice

Creemos interesante añadir este apéndice para intentar aclarar y ampliar los resultados obtenidos en este trabajo, incluyendo además información obtenida de estudios llevados a cabo en los últimos años.

Las proteínas fueron resueltas en tres grupos de picos identificados basándonos en estudios previos: albúminas y globulinas, B-hordeínas y C-hordeínas. Al tratarse de una muestra de malta en cada uno de los grupos encontramos tanto las proteínas originales como derivados producidos durante el proceso de malteado.

Como se puede observar en los electroferogramas obtenidos se produjeron cambios en las tres zonas siendo la zona de las C-hordeínas la más afectada:

- *Zona de las albúminas y globulinas*. Aumenta bastante en las primeras etapas de la germinación. Según algunos autores esto podría ser debido a la hidrólisis de proteínas más hidrofóbicas y de mayor peso molecular [97] y también por la síntesis de nuevas proteínas durante el proceso de germinación [98].

- *Zona de B-hordeinas*. Presenta el mismo perfil electroforético durante las primeras etapas de la germinación. Transcurridas 51 horas (en mitad de la germinación) se observa un ligero aumento del contenido de B-hordeinas que rápidamente desciende en las últimas etapas. Este aumento y posterior reducción podría ser debido a que durante la germinación se reducen las uniones disulfuro, liberando las B-hordeinas y, posteriormente, estas hordeínas se degradan enzimáticamente [99]. En las últimas etapas de la germinación aparecen desdoblamiento de picos debido a la disociación de las proteínas en subunidades.

- *Zona de C-hodeinas*. Es la que presenta mayores cambios. En las primeras etapas de la germinación se observa la desaparición de un pico alrededor de 10 minutos que podría corresponder a una C-hordeína de alto peso molecular, y la

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[99] I. Celus, B. Kristof, J. A. Delcour. "The effects of malting and mashing on barley protein extractability" *J. Cereal Sci.* 44 (2006) 203-211.

aparición de un pico de menor peso molecular que va aumentando a lo largo del proceso de germinación hasta que desaparece totalmente en la etapa de secado. Podría tratarse de un polipéptido que finalmente sufre una nueva degradación. Al final del proceso de germinación se observan una serie de picos que corresponderán parte a las C-hordeínas originales y otros a productos de degradación.

Los resultados obtenidos proporcionan información sobre los cambios en la composición proteica de la cebada que pueden resultar útiles para la evaluación de su calidad durante el proceso de malteado.

CAPÍTULO 2:
Análisis de los ácidos del lúpulo y sus derivados oxidados y de los iso-α-ácidos de la cerveza mediante CE-ESI-IT-MS

Varios métodos analíticos se han utilizado para la identificación y cuantificación de los ácidos del lúpulo. Sin embargo, a pesar de que el estado de oxidación de estos compuestos es un factor crítico que afecta a la calidad de la cerveza obtenida, son pocos los estudios que evalúen estos procesos de oxidación y los derivados originados.

El objetivo de este segundo capítulo de la tesis fue poner a punto un método de electroforesis capilar en zona acoplado a espectrometría de masas para identificar, no sólo los ácidos del lúpulo, sino también sus compuestos oxidados bajo tres condiciones distintas de almacenamiento: almacenamiento normal del lúpulo, después de someterlo a una oxidación forzada durante 2h a 80ºC, y tras mantenerlo durante 2 años a temperatura ambiente en presencia de luz. Lo que se pretendía era observar los compuestos de oxidación formados y las condiciones en las que aparecían. Además, este estudio se llevó a cabo utilizando 4 variedades diferentes de lúpulo: Nugget, Saaz, Mágnum y Columbus cuyas características generales se muestran en la Tabla 3.

Variedad	**Contenido en α-ácidos (w/w %)**	**Contenido en aceites (ml/100g)**	**Estabilidad en el almacenamiento**
Columbus	12.0-15.0	1.0-1.5	Baja
Magnum	14.0-16.0	1.6-2.1	Buena
Saaz	3.0-4.0	0.4-0.6	Pobre
Nugget	11.0 -13.5	1.7-2.3	Muy buena

Tabla 3. Principales características de las variedades de lúpulo utilizadas en el estudio

Se quería demostrar que la electroforesis capilar acoplada a espectrometría de masas podía ser una herramienta analítica adecuada para el análisis de este tipo de compuestos y para el control de su estado de oxidación con objeto de obtener una cerveza de calidad.

Asimismo, la metodología desarrollada por CZE se pretendía aplicar por primera vez para el análisis cualitativo y cuantitativo de los principales iso-α-ácidos de la cerveza. Estos compuestos han sido ampliamente estudiados en bibliografía, pero empleando principalmente cromatografía líquida con diferentes detectores (UV y MS) [100-102].

[100] A. Khatib, H. K. Kim, E. G. Wilson, R. Verpoorte. "High performance liquid chromatographic method for iso-alpa-acids" *J. Liq. Chromatogr. Relat. Technol* 29 (2006) 293-302.

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Analysis of Hop Acids and Their Oxidized Derivatives and Iso-α-acids in Beer by Capillary Electrophoresis–Electrospray Ionization Mass Spectrometry

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This study investigates the applicability of on-line coupling of capillary electrophoresis with electrospray ionization tandem mass spectrometry (CZE-ESI-MS) for the separation and characterization of α- and β-acids and oxidized hop acids from crude extracts of different hop varieties. CZE-ESI-MS with negative-ion electrospray ionization proved to be a suitable technique for the determination of these types of natural compounds and their oxidized derivatives. The CZE parameters (pH, concentration, and buffer type) and ESI-MS parameters (nature and flow rate of the sheath liquid, nebulizer pressure, drying gas flow rate, temperature, and compound stability) were optimized. The optimized method provides the potential for a fast qualitative determination of hop acids and their oxidation compounds. The method was also applied to the determination of iso-α-acids in beer.

KEYWORDS: Acetone hop extract; hop acids; iso-α-acids; capillary zone electrophoresis; mass spectrometry

INTRODUCTION

Extracts of hop cones, the female flowers of *Humulus lupulus* L., are used for adding aroma and flavor in the beer-brewing process. Hops contain hundreds of components but of particular interest are the so-called resins, containing mainly hop acids, hop oil, and polyphenols. These three classes of resin are important as biochemical markers to differentiate hop varieties. The hop acids, part of the soft resin fraction, consist of two related series, the α-acids (humulone, cohumulone, and adhumulone) and the β-acids (lupulone, colupulone, and adlupulone) (*1*). Besides the two series of normal-, co-, and ad-homologues there are also some minor hop acids in the plant, including posthumulone/postlupulone, prehumulone/prelupulone and adprehumulone (*2*). These are present as a complex mixture of varying composition and concentrations. The relative proportions of α-acids and β-acids as well as the content of co-homologues depend on the hop variety and, for any given variety, on the growing conditions.

Many different varieties of the *H. lupulus* L. species exist, each one with its own different composition and agronomic characteristics. Traditionally, hop varieties have been classified into two groups, namely, "aroma" or "bitter" types, depending on their α-acid content and flavor characteristics.

Hops are prone to oxidation and chemical deterioration. Both the α- and β-acids are very susceptible to oxidation and degradation during storage. Their oxidation products affect beer flavor significantly because once α-acids have been oxidized, they can no longer be isomerized into iso-α-acids; thus, the hops' bittering potential decreases, and the aroma becomes unpleasant and "cheesy". It is also important to package the hops properly, which involves keeping them in refrigerated storage at temperatures of between 0 and 5 °C, removing as much oxygen as possible, and storing them in an oxygen barrier material (*3*, *4*). Apart from the storage conditions, each variety has a particular tendency to be oxidized, and so the oxidation state of hops is an important quality factor that needs to be looked at closely for quality control in the brewing industry.

During the brewing process the virtually insoluble α-acids of the hop extract are converted into the more soluble iso-α-acids, which give the typical bitter taste to the beer. In addition to imparting bitter taste, iso-α-acids exhibit other interesting features: they have tensioactive properties, thereby stabilizing the beer foam, and they inhibit the growth of Gram-positive bacteria.

An analysis of the hop acids in hops is important for quality control, and many methods have been developed to provide a

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quantitative analysis of the α-acids in hops and hop products. A widely used empirical method extracts the bitter components into solvents and measures them quantitatively by spectrophotometry. HPLC with UV detection is also routinely used to analyze bitter acids (*5*−*12*). Nevertheless, UV is neither sensitive nor selective enough for the direct identification of minor hop acids in complex mixtures. The instability and structural similarity of the bitter hop acids cause difficulty in routine analysis. Recently, the detection of these compounds by HPLC coupled to mass spectrometry was investigated. The six major bitter hop acids have been analyzed by HPLC coupled with atmospheric pressure ionization tandem mass spectrometry (APCI-MS-MS) (*2*) and with negative electrospray ionization mass spectrometry (*13*).

Other techniques such as capillary electrophoresis (CE) in its different modes have been applied to the analysis of hop acids, that is, capillary zone electrophoresis (CZE) (*14*), micellar electrokinetic chromatography (MEKC) (*15*, *16*), and microemulsion electrokinetic chromatography (MEEKC) (*17*−*19*), using a UV detector. The iso-α-acids have also been determined using MEKC-UV (*20*−*23*).

The aim of this work has been to develop the first fast and simple capillary electrophoresis−electrospray ionization mass spectrometry (CE-ESI-MS) method for the identification of hop acids and their oxidation compounds in four varieties of hops: Saaz, Nugget, Magnum, and Columbus. We have also determined iso-α-acids in beer to demonstrate the applicability of this method.

MATERIALS AND METHODS

Chemicals. The hop acid standard, an international calibration extract ICE 2, composed of a mixture of α-acids (34.94% humulone + adhumulone and 14.45% cohumulone) and *β*-acids (12.02% lupulone + adlupulone and 12.92% colupulone), and the iso-α-acid standard ICS-I2, with a mixture of 64.3% *trans*-iso-α-acids (iso-humulone, iso-adhumulone, and iso-cohumulone) were from Labor Veritas, Zürich, Switzerland.

Ammonium acetate, ammonium carbonate, acetic acid, and diethylamine were from Panreac (Barcelona, Spain), ethanolamine and diethanolamine were from Aldrich (Steinheim, Germany), and ammonia was from Merck (Darmstadt, Germany), all of which were used for the CE running buffers at different concentrations and pH values. Buffers were prepared by weighing the quantity indicated in doubly distilled water and adding 2 M ammonium hydroxide to adjust the pH. Triethylamine (TEA) was from Aldrich, and HPLC grade 2-propanol used in the sheath flow, acetone, and sodium hydroxide were from Panreac. All solutions were filtered through 0.45 *μ*m Millipore (Bedford, MA) membrane filters before being injected into the capillary. Distilled water was deionized using a Milli-Q system (Millipore). DSC-Diol and DSC-C18 solid-phase separation (SPE) cartridges were from Supelco (Bellefonte, PA).

Instrumentation. For CE separations we used a P/ACE System MDQ (Beckman Instruments, Fullerton, CA) equipped with a UV−visible detector and a 0−30 kV high-voltage built-in power supply. A bare fused-silica capillary with 50 *μ*m i.d. was from Composite Metal Services (Worcester, U.K.). The detection length to the UV detector was 7 cm, and the total length was 100 cm (corresponding to the MS detection length). The instrument was controlled by a PC running System 32 Karat software from Beckman.

CE was coupled using an electrospray interface (ESI) (model G1607A from Agilent Technologies, Palo Alto, CA) to the MS detector (Bruker Daltonics, Squire 2000). A commercial coaxial sheath-flow interface was used (vide infra). The coaxial sheath liquid and the electrical contact at the electrospray needle tip were delivered by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL). An ESI-MS interface provided both a coaxial sheath liquid makeup flow and a nebulization gas to assist droplet formation. Both the drying gas and the nebulization gas were nitrogen. The mass spectrometer was used in the negative-ion mode, and the capillary voltage was set at 4000 V. The ion trap scanned within the *m*/*z* 300−700 range at 13000 u/s during separation and detection in the scan mode. The maximum accumulation time for the ion trap was set at 5.00 ms, the target count was set at 20000, and the trap drive level was set at 100%. 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 made electrical contact between the capillary effluent and water for the electrospray needle. The instrument was controlled by a PC running Esquire NT software from Bruker Daltonics.

Before the first use, the uncoated capillaries were conditioned using a rinse with 0.1 M NaOH for 10 min followed by a rinse with water for 5 min and finally a rinse with running buffer for 30 min. Capillary conditioning between runs was carried out by flushing the column for 3 min with water and finally for 5 min with the separation buffer. At the end of the day the capillary was rinsed with water for 30 min and dried for 10 min.

Hop Samples and Beer. Hop pellets of the varieties Saaz and Nugget and bottles of "extra" beer were obtained from the company Grupo Cervezas Alhambra S.L. (Granada, Spain), whereas the varieties Columbus and Magnum were provided by S.A. Española de Fomento del Lúpulo (León, Spain).

It is known that drying temperatures >65 °C cause variable losses of hop acids (*3*), so we induced the oxidation of the α- and *β*-acid standards and four hop varieties: Saaz, Nugget, Columbus, and Magnum. The hop pellets were received in intact, lightproof packaging. Ten grams was reduced to powder with a mortar and heated for 2 h at 80 °C in an oven. We also studied the natural oxidation of Saaz and Nugget by keeping pellets of the harvest of the year 2000 in a plastic vessel at room temperature and in the presence of light for 2 years.

Extraction of Hop Acids and Oxidized Derivatives of the Hop Pellets. To recover the hop acids present in the pellets and their oxidized derivatives, we studied different organic solvents (methanol, ethanol, and acetone) with water (0:100, 25:75, 50:50, 75:25, and 100:0). The extraction protocol was as follows: 2.5 g of hop pellets, previously reduced to powder with a mortar, was extracted three times with 50 mL of acetone/water (75:25 v/v) for 10 min each time. The extracts were combined and brought to dryness in a rotary evaporator under reduced pressure at 60 °C to get rid of any residual solvent. During the last step, 2 mL of acetone/water (50:50 v/v) was added to dissolve the extract, which was then passed through a 0.20 *μ*m membrane filter and analyzed by CE-ESI-MS.

The extraction protocol of the naturally oxidized derivatives and the compounds obtained by induced oxidation was the same for both methods and for all four varieties of hops studied.

Extraction of Isomerized Hop Acids in "Extra" Beer. We assayed two different cartridges, DSC-Diol and DSC-C18, and obtained the best results with DSC-C18. Thus, the subsequent extraction protocol of isomerized hop acids was as follows: a DSC-C18 cartridge placed in a vacuum elution apparatus was conditioned by passing 20 mL of acetone/water (75:25 v/v) and then 10 mL of water through it. Subsequently, 100 mL of degassed "extra" beer was passed through the column. The isomerized hop acids were recovered by passing four portions of 5 mL of acetone/water (75:25 v/v). The final volume was dried in a rotary evaporator under reduced pressure at 60 °C. The residue was reconstituted in 2 mL of acetone/water (50:50 v/v) and passed through a 0.20 *μ*m filter before CE-ESI-MS analysis.

General Procedure. The optimum conditions used for the CE-ESI-MS separation method were as follows: running buffer, 160 mM ammonium carbonate/ammonium hydroxide; pH 9; voltage, 20 kV; 7 s injection time; sheath liquid, 2-propanol/water, 50:50 v/v, with 0.1% TEA delivered at a flow rate of 3 *μ*L/min; drying gas flow rate, 4 L/min at 150 °C; nebulizing gas pressure, 6 psi; and MS analyses carried out using a compound stability of 25%.

RESULTS AND DISCUSSION

Development of the CE-ESI-MS Method. The effects of different separation parameters were studied to obtain the best selectivity, sensitivity, and resolution conditions.

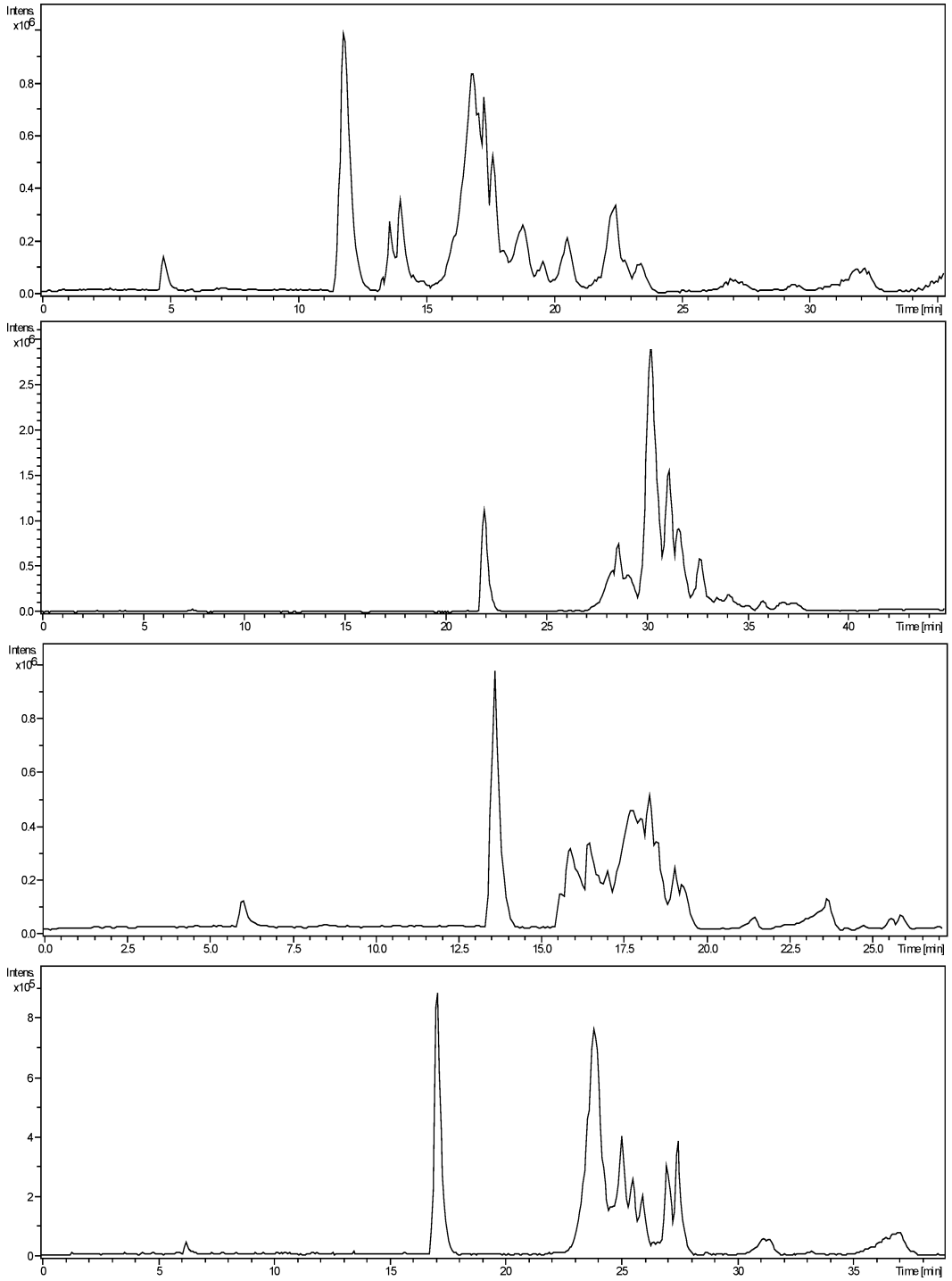

Figure 1. Comparison between different running buffers: (**a**) ammonium carbonate/ammonium hydroxide, 160 mM at pH 9; (**b**) diethylamine/ammonium hydroxide, 500 mM at pH 10.5; (**c**) ammonium hydroxide/acetic acid, 500 mM at pH 9.5; (**d**) ammonium acetate/ammonium hydroxide, 160 mM at pH 10.5. Experimental conditions: 50 μm i.d. fused-silica capillary, 100 cm detector and total length, 20 kV, 7 s of hydrodynamic injection at 0.5 psi; sheath liquid, 2-propanol/water, 50:50 v/v, containing 0.1% TEA; flow rate, 3 μL/min; dry gas, 4 L/min, 150 °C; nebulizing gas pressure, 6 psi. MS analyses were carried out using negative polarity. Compound stability was 25% MS scan *m*/*z* 300–700 (target mass *m*/*z* 550). Sample was oxidized hop pellets of the variety Saaz.

The CE-ESI-MS method was optimized using the extract obtained with 75:25 v/v acetone/water from oxidized Saaz hops because this extract was the most complex and its electropherogram presented the greatest number of peaks. First, the optimum concentration and pH of four volatile running buffers, ammonium carbonate/ammonium hydroxide, diethylamine/ammonium hydroxide, ammonium hydroxide/acetic acid, and ammonium acetate/ammonium hydroxide, were established. The pH of ammonium carbonate/ammonium hydroxide was assayed between 8.5 and 10 at a concentration of 100 mM, and pH 9 showed the best resolution. The concentration was then studied between 100 and 190 mM at pH 9, the best resolution being found with 160 mM.

After studying the influence of pH between 9.5 and 10.5 at a concentration of 100 mM, we chose pH 10.5 as optimum. Diethylamine/ammonium hydroxide was assayed in the range between 100 and 500 mM, and the best resolution was found to occur at a concentration of 500 mM.

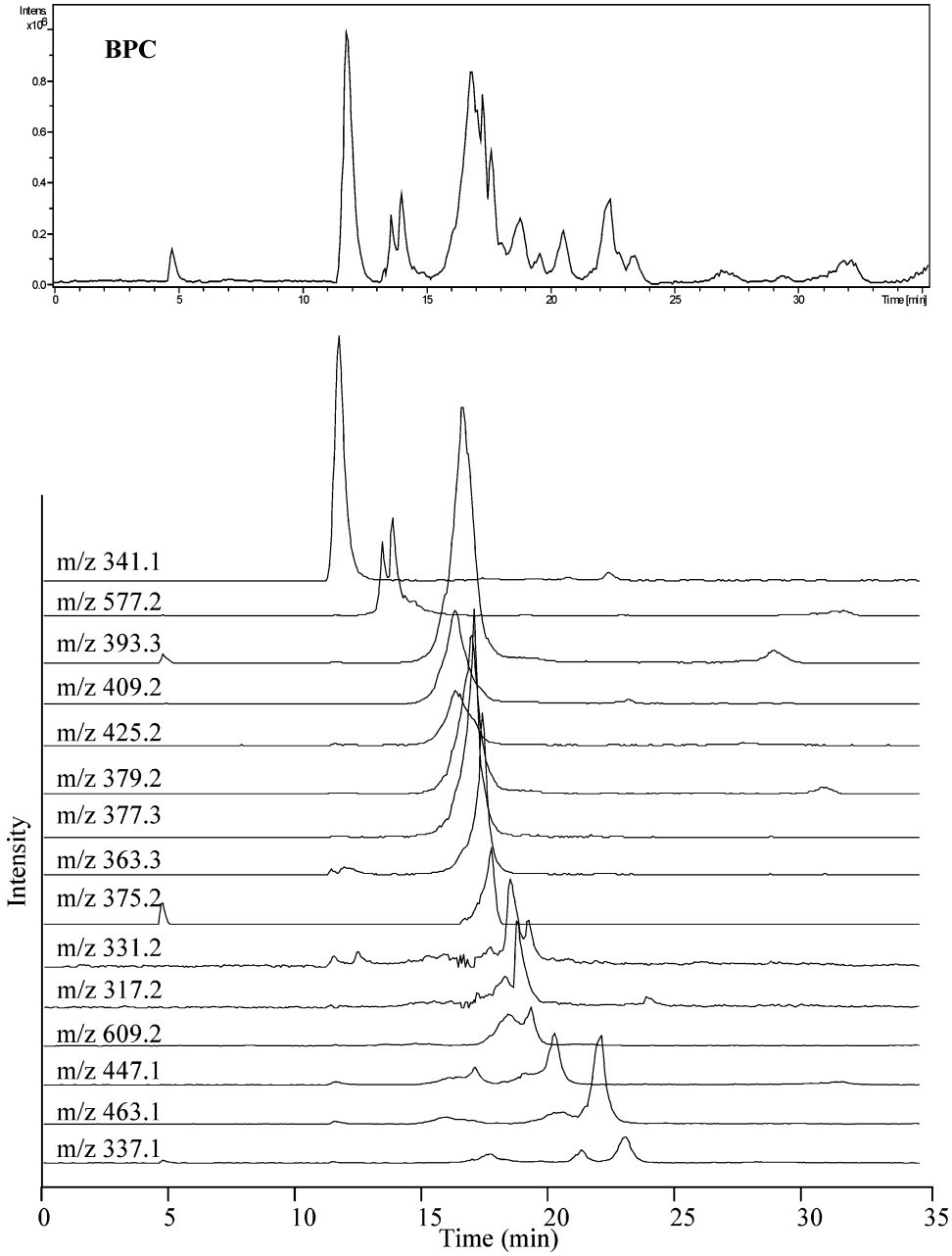

Figure 2. Base peak electropherogram and extracted ion electropherogram. Conditions: buffer ammonium carbonate/ammonium hydroxide, 160 mM at pH 9; 50 μm i.d. fused-silica capillary, 100 cm detector and total length, 20 kV, 7 s of hydrodynamic injection at 0.5 psi; sheath liquid, 2-propanol/water, 50:50 v/v, containing 0.1% TEA; flow rate, 3 μL/min; dry gas, 4 L/min, 150 °C; nebulizing gas pressure, 6 psi. MS analyses were carried out using negative polarity. Compound stability was 25%.

The pH and concentration using ammonium hydroxide/acetic acid as running buffer were also examined. The effect of pH was studied by using a concentration of 500 mM of ammonium hydroxide and adjusting the pH with acetic acid to between 9 and 10. We chose pH 9.5 as the optimum value, and the concentration was then assayed between 100 and 500 mM at this pH. Higher concentrations were tried, but these produced noises in the baseline.

The pH of ammonium acetate/ammonium hydroxide was assayed by varying it between 8 and 11 when using a concentration of 160 mM. The greatest number of peaks appeared at pH 10.5. We then studied the concentration effect between 80 and 180 mM and found the best result with a concentration of 160 mM. **Figure 1** shows the optimum electropherograms found in the different studies carried out with the four running buffers under optimum conditions. Of all of the conditions studied, ammonium carbonate/ammonium hydroxide at pH 9 at a concentration of 160 mM offered the most information about the compounds of interest.

To obtain better resolution between peaks, different percentages (5, 10, and 15%) of organic solvents such as 2-propanol and sodium dodecyl sulfate (SDS) at 5 and 10 mM were added to the buffer without success.

The voltage was varied between 10 and 30 kV, and finally a voltage of 20 kV was selected to obtain the best resolution. The injections were made at the anodic end using N_2 pressure of 0.5 psi for 7 s (1 psi = 6894.76 Pa). These conditions were chosen for the optimization of the ESI parameters.

It is well-known that the choice of sheath liquid has significant effects on sensitivity and on the electrical contact between CE and ESI (*24*, *25*). Generally, a small amount of volatile TEA is used for ESI-negative detection (*26*). Next, the sheath liquid

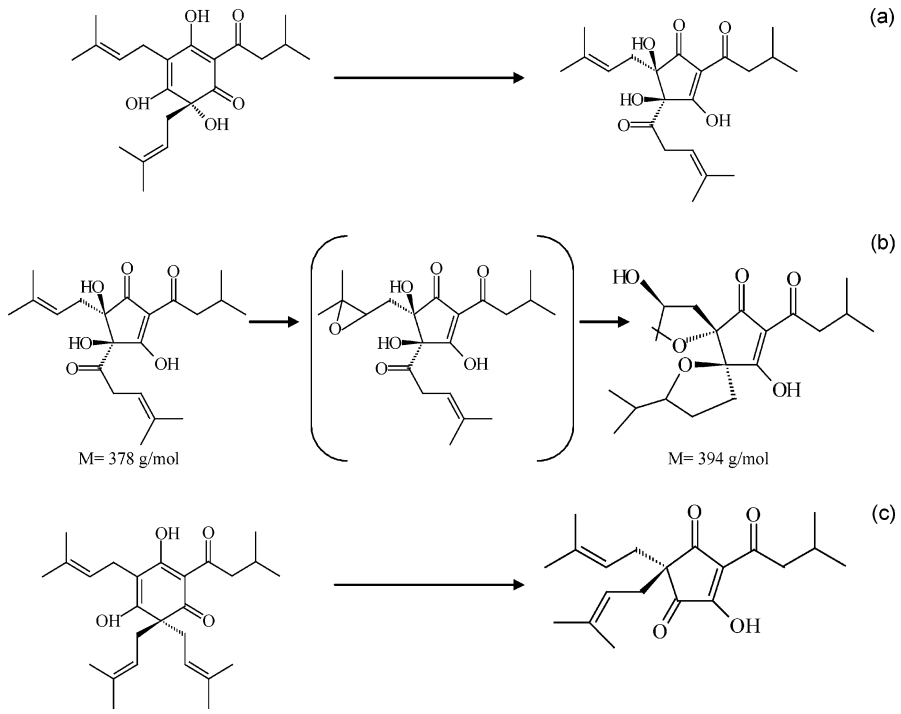

Figure 3. (**a**) Conversion of humulone into humulinone; (**b**) oxidation mechanism for the formation of the compound with molecular weight (M) 394 from humulinone; (**c**) conversion of lupulone into hulupone.

Table 1. Structures of Compounds Found in Different Varieties of Hops without Oxidation

	[M – H][a]			
analyte	Saaz	Nugget	Magnum	Columbus
posthumulone				303.5
cohulupone	317.2		317.2	317.2
hulupone/adhulupone	331.2		331.2	331.2
cohumulone	347.3	347.3	347.3	347.3
humulone/adhumulone	361.2	361.2	361.2	361.3
cohumulinone		363.2		363.2
prehumulone	375.2		375.2	
humulinone/adhumulinone	377.3	377.3	377.3	377.3
colupulone	399.3	399.3	399.3	399.3
lupulone/adlupulone	413.3	413.3	413.3	413.3

[a] [M – H] is the deprotonated ion.

Table 2. Structures of Compounds Found in Different Varieties of Hops with Forced Oxidation

	[M – H][a]			
analyte	Saaz	Nugget	Magnum	Columbus
posthumulone				
cohulupone	317.2	317.2	317.2	317.2
hulupone/adhulupone	331.2	331.2	331.2	331.2
cohumulone		347.3	347.3	347.3
humulone/adhumulone		361.2	361.2	361.2
cohumulinone	363.2	363.2	363.2	363.2
prehumulone	375.2		375.2	
humulinone/adhumulinone	377.3	377.3	377.3	377.3
colupulone		399.3	399.3	
lupulone/adlupulone		413.2	413.2	413.2

[a] [M – H] is the deprotonated ion.

composition and flow rate were optimized to increase the MS sensitivity of the compounds. Different sheath liquids were tested, that is, methanol/water and 2-propanol/water at different proportions, with 0.1% TEA and without TEA. With 2-propanol as organic solvent we obtained a better response than with methanol. Eight different percentages of sheath flow liquid were tested: 2-propanol/water at 40:60 v/v, 50:50 v/v, 60:40 v/v, and 70:30 v/v, with and without 0.1% v/v TEA in order to facilitate electrical contact when the negative mode was used. A 50:50 ratio of propanol/water with 0.1% v/v TEA as sheath liquid was judged to obtain the highest signal and best current stability. The choice of these variables represented a compromise between maintaining efficient electrophoretic separation and improving ionization performance. The influence of the sheath liquid flow rates of 1, 2, 3, 4, and 5 μL/min was also examined. We observed that the best results in terms of MS sensitivity were obtained when using a flow rate of 3 μL/min. The nebulizer pressure was then optimized by testing values of 2, 4, 6, 8, and 10 psi, the greatest sensitivity being obtained with 6 psi. The temperature of the interface was also optimized between 100 and 300 °C, the greatest sensitivity being obtained at 150 °C.

Table 3. Structures of Compounds Found in Naturally Oxidized Saaz and Nugget Hops

	[M – H][a]	
analyte	Saaz	Nugget
posthumulone		
cohulupone	317.2	317.2
hulupone/adhulupone	331.2	331.2
cohumulone	347.3	347.3
humulone/adhumulone		
cohumulinone	363.2	363.2
prehumulone		
humulinone/adhumulinone	377.3	377.3
colupulone		
lupulone/adlupulone		

[a] [M – H] is the deprotonated ion.

Another important parameter of the interface was the stability of the compound, which was studied between 25 and 100%. The MS signal decreased concomitantly with higher percentages because the number of molecules transferred into MS was low, whereas with lower percentages the majority of the compounds

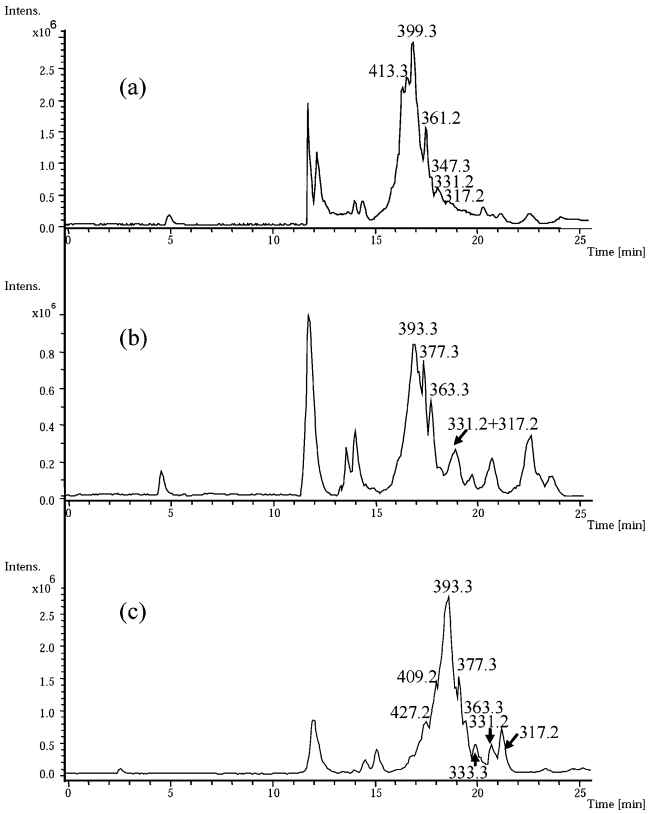

Figure 4. Differences among samples of Saaz hops: (**a**) without oxidation; (**b**) with forced oxidation; (**c**) naturally oxidized. The separation conditions are shown in **Figure 2**.

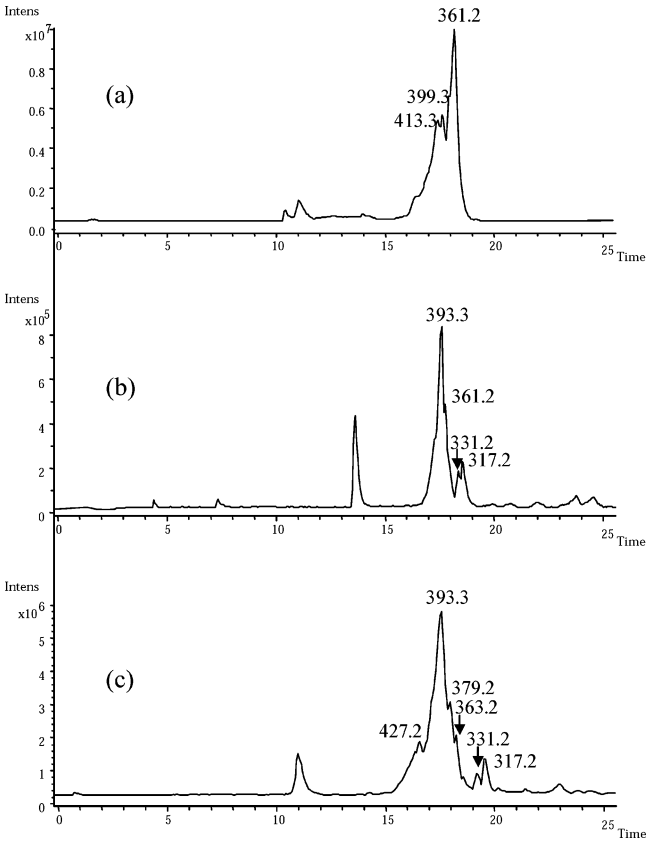

Figure 5. Differences among samples of Nugget hops: (**a**) without oxidation; (**b**) with forced oxidation; (**c**) naturally oxidized. The separation conditions are shown in **Figure 2**.

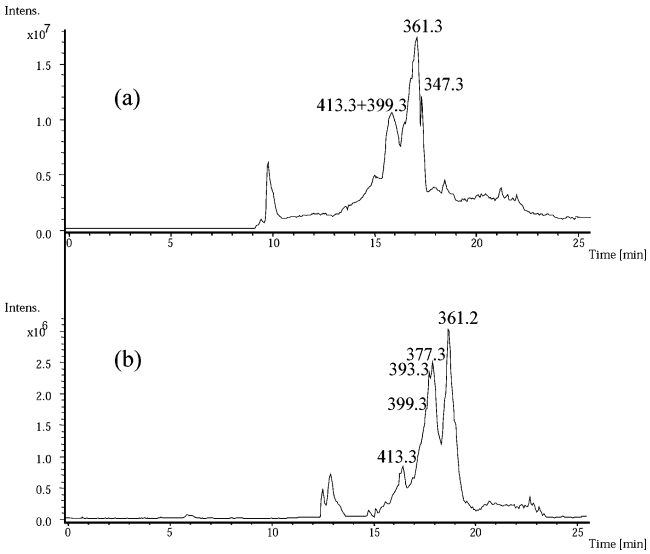

Figure 6. Differences among samples of Magnum hops: (**a**) without oxidation; (**b**) with forced oxidation. The separation conditions are shown in **Figure 2**.

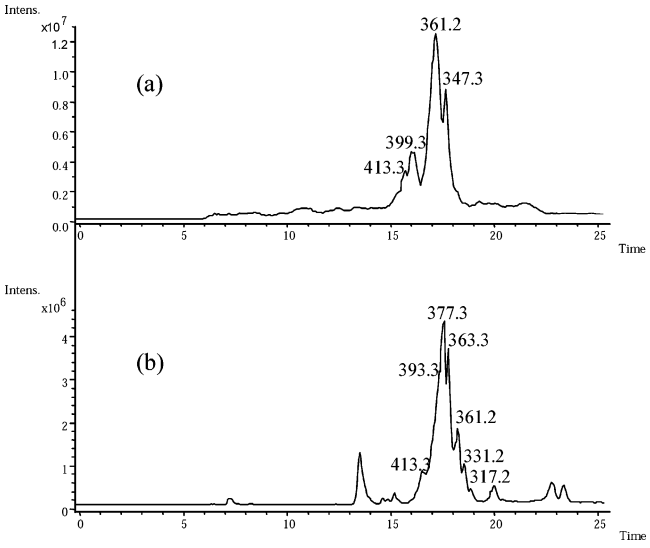

Figure 7. Differences among samples of Columbus hops: (**a**) without oxidation; (**b**) with forced oxidation. The separation conditions are shown in **Figure 2**.

became more stable, as indicated by an increase in the MS signal. This parameter is related to the voltage used in the capillary placed at the MS entrance; thus, the higher this parameter, the higher the voltage applied by the MS instrument and therefore the higher the solute fragmentation that can take place at that point. Thus, we chose 25% compound stability.

Figure 2 shows the base peak electropherogram and the extracted ion electropherogram obtained under the CE-ESI-MS conditions chosen for an extract of oxidized Saaz hops. Fifteen different compounds can be recognized: *m*/*z* 377.3 corresponds to humulinone/adhumulinone (overlap), oxidation products of humulone/adhumulone. Oxidation occurs leading to the creation of a double acyloin entity. The acyloin rearrangement with concurrent ring contraction may take place at both C-4 and C-6. This reaction is totally analogous to the important isomerization reaction of humulone to the isohumulones (**Figure 3a**); an ion with a ratio *m*/*z* of 393.3 indicates that two oxygen atoms have been incorporated into humulone/adhumulone (overlap). This

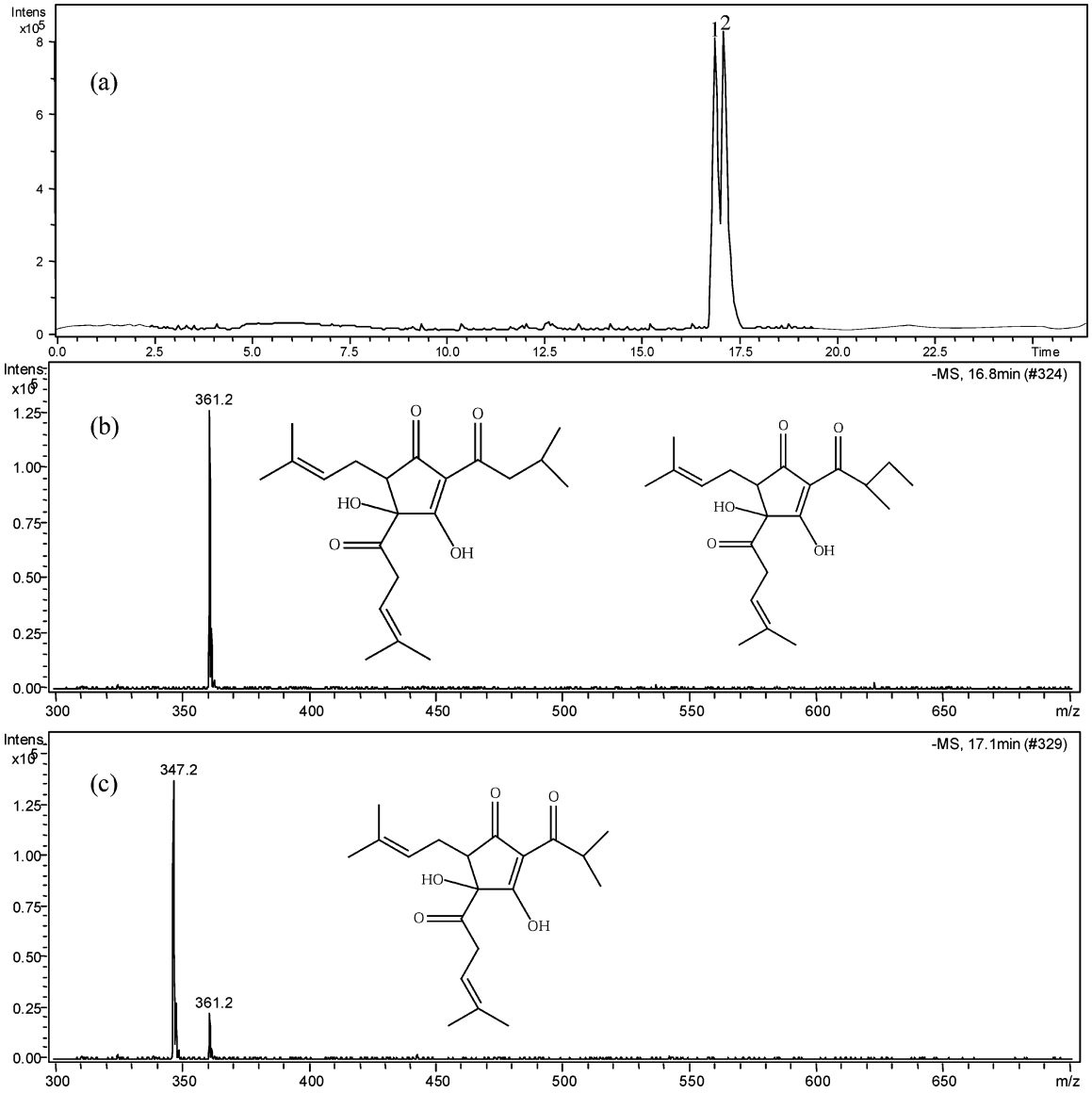

Figure 8. (**a**) Electropherogram of a mixture of three trans iso-α-acid standards: 1, *trans*-iso-humulone and *trans*-iso-adhumulone; 2, iso-cohumulone. (**b**) Mass spectra of isohumulone and iso-adhumulone with a *m*/*z* ratio of 361.2. (**c**) Mass spectra of iso-cohumulone with a *m*/*z* ratio of 347.2.

oxidation product belongs to the abeo-isohumulone group, which is derived from isohumulones but obtained directly through the oxidation of humulone. It is very likely that humulinone represents the first step in the reaction sequence. These oxidized compounds have a five-membered structure. The reaction mechanism of these compounds (**Figure 3b**) proceeds via the oxidation of the 3-methyl-2-butenyl side chains in humulinone, followed by cyclization, via either intramolecular dehydration or nucleophilic cleavage of the intermediate oxirane ring (*1*). The ions with *m*/*z* ratios of 409.2 and 425.2 are thought to be more highly oxidized compounds, thus indicating that three and four oxygen atoms, respectively, have been incorporated into humulone/adhumulone, but their structure and the formation mechanism are still unknown; *m*/*z* 363.3 corresponds to cohumulinone, an oxidation product of cohumulone, with a structure and oxidation mechanism similar to that of humulinone, and the *m*/*z* of 379.2 could be due to the incorporation of two oxygen atoms into cohumulone, in the same way as with humulone; *m*/*z* 331.2, hulupone/adhulupone (overlap), and *m*/*z* 317.2, cohulupone, correspond to the oxidation of the *β*-acids lupulone/adlupulone and colupulone. The structures of the oxidation products indicate that the lengths of the side chains in these compounds, together with the double bonds and hydroxyl groups, easily give rise to oxidation cyclizations, leading to five derivatives (**Figure 3c**) (*m*/*z* 375.2, prehumulone). Other compounds have been found in this extract: *m*/*z* 341.1, maltose; *m*/*z* 577.2, procyanidin; *m*/*z* 609.2, hesperedin (18.6 min) and rutin (19.5 min); *m*/*z* 447.1, luteolin-7-*O*-glucoside (17.2 min) and kaempferol-3-*O*-glucoside (20.5 min); *m*/*z* 463.1, quercetin-4′-*O*-glucoside; and *m*/*z* 337.1, desmethylxanthohumol. Some of them have easily been identified and confirmed using the hop acid standard.

The reproducibility of the CE-ESI-MS analysis, expressed by the relative standard deviation (RSD) of six consecutive injections, was 3.6% for the retention time and 6.9% for the peak area, both quite suitable for the intentions of this work.

Analysis of Acids in Different Hop Varieties. To demonstrate the capacity of the CE-ESI-MS method for the analysis of this type of compound in hop samples, we applied the method to different varieties. Four varieties were studied: Saaz (a classic variety with good aroma but poor storage stability); Nugget and Magnum (with similar properties of high α-acid, acceptable aroma, and good storage stability); and Columbus (with high α-acid, a strong but pleasant aroma, but poor storage stability).

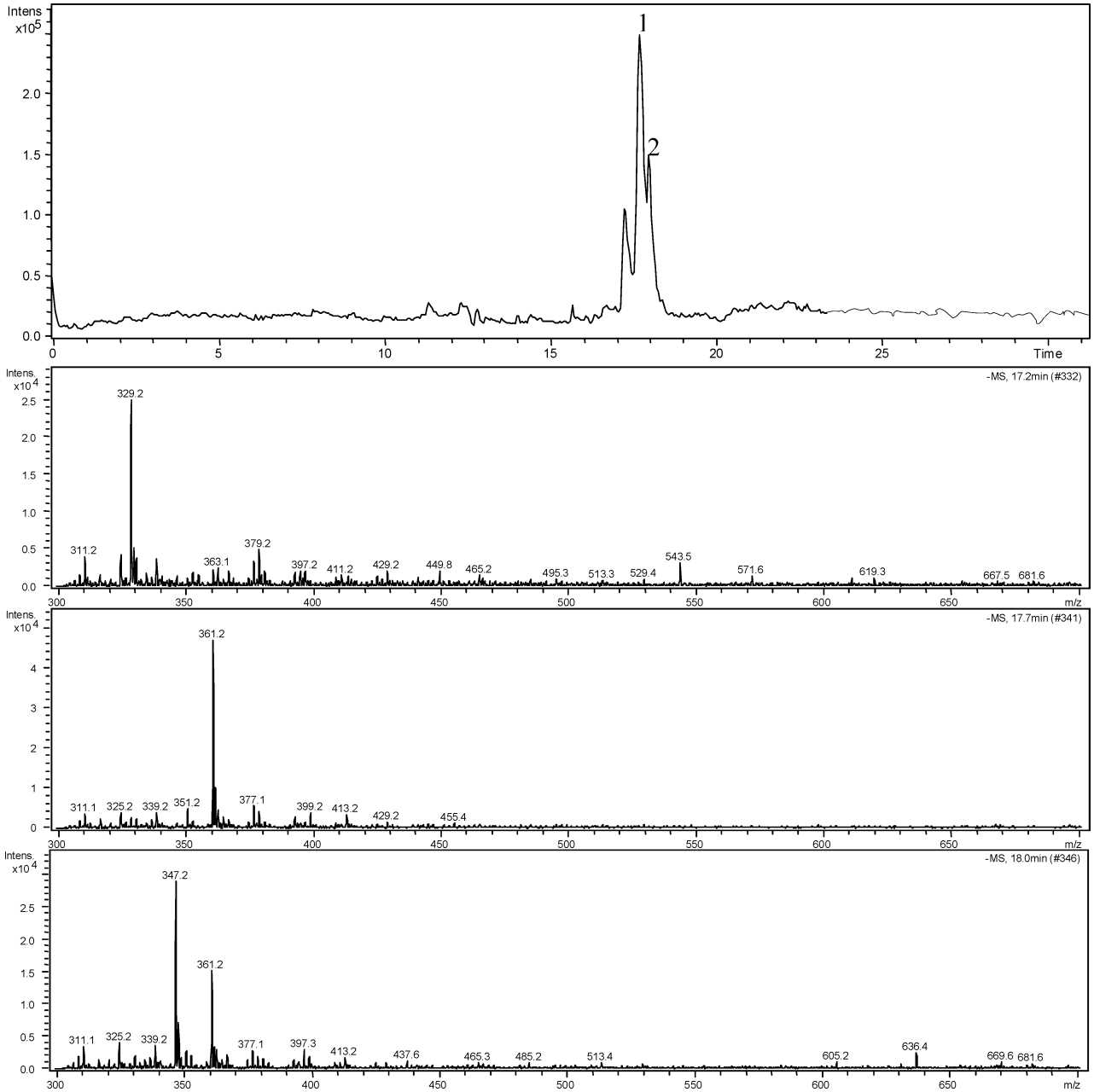

Figure 9. Electropherogram and mass spectra of an "extra" beer. The separation conditions are shown in **Figure 2** and the peak numbers in **Figure 8**.

Table 1 shows information about the structures of the compounds found in the four varieties of hop with no oxidation. As can be seen in this table, *m*/*z* 377.3, which corresponds to humulinone/adhumulinone, appears in all varieties. Humulinone is perhaps the best known nonvolatile oxidation product derived from humulone. Nugget hop is the variety with the best result because it contains fewer oxidized compounds, only cohumulinone (*m*/*z* 363.2), humulinone/adhumulinone (*m*/*z* 377.3), and another oxidation product of humulone with *m*/*z* 409.3. Columbus is the variety with the poorest storage stability, and so it contains more oxidized compounds; in addition to some of the components present in Nugget we also found cohulupone (*m*/*z* 317.2) and hulupone/adhulupone (*m*/*z* 331.2). These oxidation derivatives also appear in the varieties Saaz and Magnum, although the oxidation compound of cohumulone (*m*/*z* 363.2) does not appear in these varieties.

Analysis of Oxidation Derivatives in Different Varieties of Hops. The oxidation of hops was forced by heating 10 g to 80 °C for 2 h. We also studied two varieties, Nugget and Saaz, oxidized at room temperature for about 2 years. **Table 2** shows information about the structures of the compounds found in the four varieties of hops subjected to forced oxidation. The presence of characteristic hop compounds such as humulone/adhumulone (*m*/*z* 361.2) can be seen in the varieties Nugget, Magnum, and Columbus together with cohumulone (*m*/*z* 347.3) and lupulone/adlupulone (*m*/*z* 413.2). Colupulone (*m*/*z* 399.3) is to be found in only Nugget and Magnum. Apart from this, the typical oxidized compounds of α-acids [humulinone/adhumulinone (*m*/*z* 377.3) and cohumulinone (*m*/*z* 363.2)] and *β*-acids [hulupone/adhulupone (*m*/*z* 331.2) and cohulupone (*m*/*z* 317.2)] were to be found in all of the varieties. It can also be seen that the majority of α- and *β*-acids disappeared when we forced the oxidation in Saaz. Colupulone disappeared in Columbus. Other oxidation products have been found (*m*/*z* 379.2, 393.3, 409.2, 425.2, and 427.2), but they are not shown in the table.

For naturally oxidized Saaz and Nugget hops, only oxidation compounds appear, except for cohumulone, due to oxidation being more extensive over a longer period of time (**Table 3**).

Figure 4 shows the differences between samples of Saaz hops. We found that in the Saaz hops subjected to both forced and natural oxidation the maximum peak was at 393.3, corresponding to the gain of two oxygens into humulone. **Figure 5** shows the differences among hop Nugget without oxidation, hop Nugget with forced oxidation, and naturally oxidized hop Nugget. The same applied to the Nugget variety where both oxidized forms showed a maximum peak at 393.3.

Figure 6 shows the electropherogram of the samples of hop Magnum: hop Magnum without oxidation and hop Magnum with forced oxidation. With the Magnum variety we can detect the presence of peaks with *m*/*z* ratios of 361.2, corresponding to humulone/adhumulone, 399.3, corresponding to colupulone, and 413.3, corresponding to lupulone/adlupulone in both oxidized and nonoxidized hops.

Figure 7 shows the electropherogram of the samples of hop Columbus: hop Columbus without oxidation and hop Columbus with forced oxidation. In this variety, the most characteristic oxidation compounds found in Columbus are humulinone/adhumulinone (*m*/*z* 377.3) and cohumulinone (*m*/*z* 363.3).

Comparing the results for the four samples, we can deduce that the Saaz variety contains fewer hop acids than the other varieties. Nugget samples with no oxidation show the least number of oxidized derivatives, and thus this variety has the best storage stability. Magnum has the highest α- and *β*-acid contents.

Determination of Iso-α-acids in "Extra" Beer. Iso-α-acids, hop-derived compounds present in low concentrations in beers, are primary flavor constituents. The bitterness of beer is largely attributable to iso-α-acids, which are formed by isomerization during the wort-boiling stage of beer production (*3*).

The identification and quantification of iso-α-acids in an "extra" beer was carried out using the CE-ESI-MS method proposed, after preconcentrating 100 mL of the beer sample using a DSC-C18 column. The sample was recovered by passing it through four portions of 5 mL of acetone/water (75:25 v/v). The extract was dried, reconstituted in 2 mL of acetone/water (50:50 v/v), and filtered before analysis. The recovery percentage was ≈70% for the iso-α-acids using this extraction system. **Figure 8a** shows the electropherogram of a mixture of three iso-α-acid standards, **Figure 8b** the mass spectra of isohumulone and iso-adhumulone, which have an *m*/*z* ratio of 361.2, and **Figure 8c** the mass spectra of iso-cohumulone, which has an *m*/*z* ratio of 347.2. **Figure 9** shows the electropherogram and the mass spectra of an "extra" beer. As can be seen in this figure an unknown peak appears at *m*/*z* 329.2, and the iso-α-acids appear at *m*/*z* 361.2 and 347.2.

The quantification was carried out using calibration graphs studied between 25 and 500 mg L^{-1}. The correlation coefficients (r^2) obtained for the regression lines of the CZE plots of peak area versus concentration were all >0.998.

Detection limits for the analytes were 2.00 mg L^{-1} for isohumulone + iso-adhumulone and 2.22 mg L^{-1} for iso-cohumulone, and the limits of quantification were 6.67 mg L^{-1} for iso-humulone + iso-adhumulone and 7.41 mg L^{-1} for iso-cohumulone, all of which were calculated by the IUPAC (*27*).

The precision of the method was evaluated by determining the repeatability of the peak areas. The repeatability values obtained for three successive injections of a standard solution of 300 mg L^{-1} for each analyte were ≈5.7%.

The concentration found for isohumulone + iso-adhumulone was 2.26 mg L^{-1} and that for iso-cohumulone, 1.05 mg L^{-1}, in the initial beer sample, which was with Saaz hops. This variety is characterized by its high aroma and its low α-acid content, which in turn implies a low iso-α-acid content.

ACKNOWLEDGMENT

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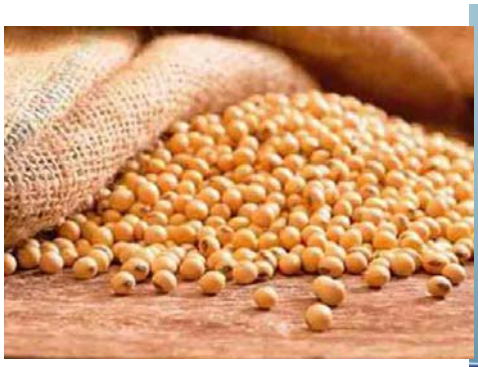

BLOQUE II:
SOJA

BLOQUE II. SOJA

La soja o soya (*Glycine max*) originaria de China es una planta de la familia de las leguminosas (*Fabaceae*), primeras plantas junto con los cereales cultivadas con fines alimenticios. El grano de soja y sus subproductos (principalmente, aceite y harina de soja) se utilizan en la alimentación humana y del ganado y se comercializa en todo el mundo, debido a sus múltiples usos.

1. Composición de la soja

Tiene un enorme interés desde el punto de vista nutricional pues es un buen alimento para el ser humano y un pienso excelente para el ganado, ya que contiene todos los nutrientes necesarios para la vida (Fig. 1): elevado porcentaje de proteínas (del orden del 35%) y aminoácidos esenciales, grasas (lípidos), fibra dietética, minerales (principalmente potasio y fósforo), vitaminas hidrosolubles e hidratos de carbono. Tiene también importantes cantidades de compuestos polifenólicos, principalmente flavonoides y antocianos. En el grupo de los flavonoides destacan las isoflavonas genistina y daidzina (precursores glicosilados de la genisteína y daidzeína, respectivamente) [103].

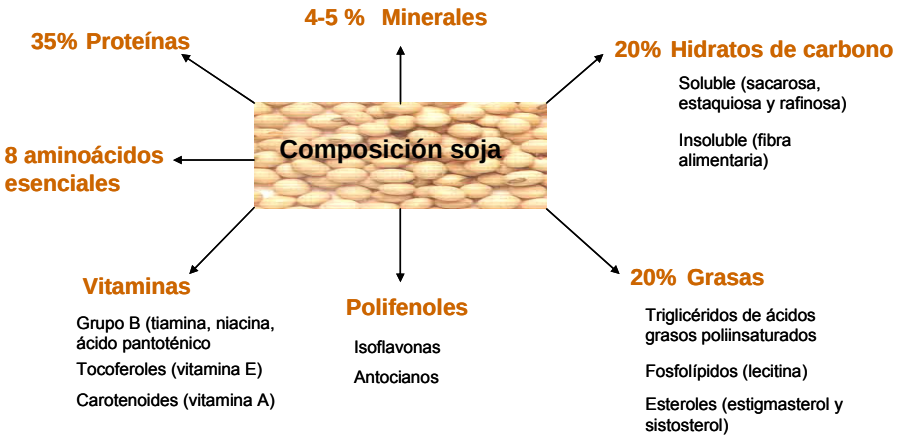

Figura 1. Composición química del grano de soja

[103] E. Herrera- Castillón, R. Jiménez-Fernández. "Componentes de la soja de interés en alimentación, nutrición y salud humana" en "La Salud y la Soja" V. Pastor, A. Perote (Eds). Edimsa, Madrid (2006)

Las isoflavonas son conocidas como fitoestrógenos, pues presentan una estructura química similar a los estrógenos, un tipo de hormonas sexuales femeninas, lo que hace que la soja se utilice en la prevención y tratamiento de los síntomas asociados a las carencias de estos estrógeno durante la menopausia. Además, se han investigado otros efectos beneficiosos para la salud, tales como su acción antioxidante, antiinflamatoria, antitrombótica, anticancerígena, etc. [104]

2. Alimentos transgénicos

Los alimentos sometidos a ingeniería genética o alimentos transgénicos son aquellos que se producen a partir de un organismo modificado genéticamente (GMO), "organismo, con la excepción de los seres humanos, en el que el material genético ha sido modificado de una manera que no se produce naturalmente en el apareamiento ni en la recombinación natural" (Directiva UE 2001/18/CEE). En el caso concreto de los transgénicos esta alteración en el material genético se debe a la introducción de un gen exógeno, esto es, proveniente de otro organismo completamente diferente.

2.1. Técnicas de ingeniería genética

Las técnicas de ingeniería genética que se usan consisten en aislar segmentos del ADN (material genético) de un organismo para introducirlos en el genoma (material hereditario) de otro, utilizando como vector otro ser vivo capaz de inocular fragmentos de ADN (*Agrobacterium tumefaciens*, virus), bombardeando las células con micropartículas de oro o tungsteno recubiertas del ADN que se pretenda introducir o utilizando métodos físicos como descargas eléctricas que permitan penetrar los fragmentos de ADN hasta el interior del núcleo, a través de las membranas celulares [105].

[104] A. O. Omoni, R. E. Aluko. "Soybean foods and their benefits: potential mechanism of action". *Nutr. Rev*. 63 (2005) 272-283.
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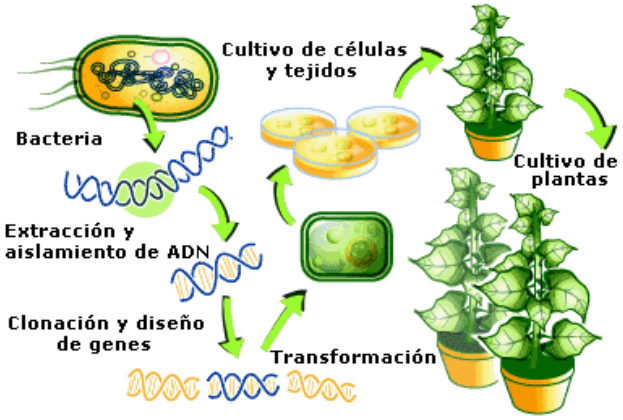

Figura.2. Proceso para la obtención de un transgénico

2.2. Mejoras introducidas

Los objetivos y mejoras principales introducidos por la ingeniería genética son:

- *Mejoras agronómicas de la planta*: resistentes a condiciones ambientales más agresivas (heladas, sequías, distintos tipos de suelos), resistencia a plagas y patógenos, crecimiento acelerado, tolerancia a herbicidas.
- *Mejora de calidad del producto*: incremento del valor nutritivo, mejora de caracteres organolépticos, fibras más resistentes, productos de mayor vida comercial.
- *Producción de nuevos compuestos*: alimentos medicinales que se podrían utilizar como vacunas u otros medicamentos, hormonas, nuevos productos del metabolismo secundario.

Carácter	Porcentaje
Tolerancia herbicidas	27%
Resistencia virus	18%
Resistencia insectos	14%
Retraso maduración	11%
Expresión enzimática	8%
Resistencia insectos + tolerancia herbicidas	7%
Androesterilidad	7%
Síntesis almidón	4%
St. Contención biológica	3%
Resistencia sequía	1%

Tabla 1. Modificaciones genéticas más comunes

En la actualidad, tienen mayor presencia alimentos procedentes de plantas transgénicas como el maíz, la cebada o la soja y una modificación empleada con frecuencia es la resistencia a herbicidas o plagas de insectos, puesto que de este modo es posible emplearlos afectando sólo a la flora ajena al cultivo. En el año 2007, los cultivos de transgénicos se extienden en 114,3 millones de hectárea, siendo España una de los 13 países con mayor número de hectáreas cultivadas, especialmente de maíz transgénico. En el año 2007 en Estados Unidos (uno de los principales productores) el 89% de plantaciones de soja lo eran de variedades transgénicas, así como el 83% del algodón y el 61% del maíz [106].

2.3. Inconvenientes

No todos acogen las posibilidades de la ingeniería genética con entusiasmo y sus oponentes, especialmente asociaciones ecologistas, insisten en que estas técnicas son peligrosas porque:

- Alteran los organismos, sin que sepamos muy bien las consecuencias que esto puede traer.
- Podrían causar alteraciones genéticas o reacciones alérgicas en los que los consumen.
- Las plantas tratadas genéticamente podrían alterar el equilibrio natural.

2.4. Legislación sobre transgénicos

Debido a la sensibilización del público en este campo, y para cumplir el derecho que tienen los consumidores a saber lo que consumen, algunos países comenzaron a establecer leyes que regularan el desarrollo, crecimiento y comercialización de los productos modificados genéticamente. Con respecto a la Unión Europea hasta el año 2003 la entrada en circulación de los GMOs era regido a través del Reglamento (CE) 285/ 97 [107] sobre alimentos nuevos. Con la entrada en vigencia de los Reglamentos 1829/2003 y 1830/2003 [108 , 109] se regulan exclusivamente los productos

[106] C. James. Global Status of Commercialized Biotech/GM Crops, 2007. International Service for the Acquisition of Agri-Biotech.
[107] Reglamento (CE) Nº 258/97 del Parlamento Europeo y del Consejo sobre nuevos alimentos y nuevos ingredientes alimentarios
[108] Reglamento (CE) Nº 1829/2003 sobre los alimentos e ingredientes alimentarios nuevos genéticamente modificados para el consumo humano y para el consumo animal

alimentarios provenientes de (o que contengan) GMOs. Esta nueva reglamentación contempla la indicación obligatoria en el etiquetado de la presencia de OGMs en un producto si contiene al menos un 0.9% de los mismos, sin importar si los casos de contaminación se dieron de forma casual o si técnicamente no pudo ser evitada.

2.5. Soja transgénica

Por su repercusión, los casos de la soja y el maíz transgénicos resultan de especial relevancia. La soja transgénica es mejorada por Ingeniería Genética para tolerar aplicaciones de herbicidas a base de glifosato, un inhibidor de la enzima *5-enolpiruvil-shikimato-3-fosfato sintetasa* (EPSPS) responsable de la síntesis de aminoácidos aromáticos en las plantas. Estos aminoácidos son necesarios en la fotosíntesis y por ello las plantas al no poder sintetizarlos mueren o frenan considerablemente su crecimiento. Para ello se utiliza un gen tolerante a este herbicida proveniente de una bacteria que habita comúnmente el suelo (Agrobacterium) que también sintetiza aminoácidos aromáticos. Este gen CP4 EPSPS fue clonado y transfectado a soja, y en 1996 se comenzó la comercialización de la soja transgénica. La introducción de las variedades de soja transgénica a partir de 1996 en diferentes países, ha reducido el número y costo de aplicaciones de herbicidas y ofrece considerables beneficios ya que facilita la agricultura de conservación. Sin embargo la modificación genética de la soja, como la de cualquier alimento transgénico, está siendo resistida por entidades ambientalistas que promueven el cultivo de soja "orgánica" libre de modificaciones genéticas.

2.6. Análisis de transgénicos: técnicas analíticas más utilizadas

La introducción de leyes obligando a etiquetar explícitamente los alimentos en cuya composición se incluyen transgénicos, llevó a la necesidad de desarrollar métodos rápidos y sensibles para evaluar la presencia de los OGMs en diferentes alimentos, especialmente maíz y soja. Los métodos analíticos más ampliamente utilizados se basan en la medida de las macromoléculas directamente implicadas en la modificación genética: nuevas proteínas expresadas por los genes y ADN [110, 111]. En líneas

[109] Reglamento (CE) Nº 1830/2003 sobre la indicación y etiquetado de la presencia de OGM y del seguimiento a los procesos de producción de alimentos e ingredientes
[110] E. Anklam, G. Ferruccio, P. Heinze, H. Pijnenburg, G. Van Den Eede. "Analytical methods for detection and determination of genetically modified organism in agricultural crops and plant-derived food products" *Eur. Food Res. Technol.* 214 (2002) 3-26.

generales, para la detección del las proteínas se han venido utilizando ensayos ELISA (Ensayo por inmunoabsorción ligado a enzimas), usando anticuerpos específicos para capturar las proteínas de interés, y más recientemente se han podido diferenciar cultivos transgénicos y no transgénicos basándose en su perfil proteico obtenido por CE-UV, CE-MS y HPLC [112].

Por otro lado, para la detección del ADN transgénico se utiliza la PCR [113] (reacción en cadena de la polimerasa), técnica que permite amplificar un fragmento de ADN pudiéndose obtener hasta cien mil copias del mismo. Para verificar que la PCR ha generado el fragmento de ADN previsto se emplean técnicas de electroforesis, que separan los fragmentos de ADN en función de su tamaño. Los métodos clásicos de electroforesis en gel de agarosa, debido a su falta de precisión y sensibilidad, se empezaron a sustituir por técnicas de electroforesis capilar en gel (CGE) con detección UV o LIF (Fluorescencia inducida por láser) y más recientemente por electroforesis capilar en microchip (MEC) y técnicas de microarray.

Debido a las exigencias de la Unión Europea en cuanto a contenido de los alimentos trangénicos que exigen medidas cuantitativas y no solo la presencia o no del GMO´s los métodos de PCR tradicionales evolucionaron hacia métodos cuantitativos: PCR en tiempo real, empleando marcadores fluorescentes o sondas moleculares para monitorizar los productos formados durante cada ciclo de la reacción de amplificación o PCR competitiva cualitativa (QC-PCR) donde se establece una relación entre el ADN transgénico y un competidor.

Hasta hace poco tiempo se ha dedicado un considerable esfuerzo para el desarrollo de técnicas analíticas a nivel genómico (ADN) y proteómico (proteínas) para la identificación y cuantificación de alimentos transgénicos, pero se ha prestado mucha menos atención al conocimiento de los productos finales de la expresión genética, los metabolitos. Dado que la información genética contenida en el ADN y transcrita a través del ARN, determina la síntesis de una proteína y ésta a su vez la de los

[111] M. Hernández, D. Rodríguez-Lázaro, A. Ferrando. "Current methodology for detection, identification and quantification of genetically modified organisms". Curr. Anal. Chem. 2 (2005) 203-221.
[112] J. M. Rodríguez- Nogales, A. Cifuentes, M. C. García, M. L. Marina. "Characterization of protein fractions from Bt-transgenic and non-transgenic Maite varieties using perfusion and monolithic RP-HPLC. Maize differentiation by multivariate analysis" *J. Agric. Food Chem.* 55 (2007) 3835-3842.
[113] V. García-Cañas, A. Cifuentes, R. González. "Detection of genetically modified organisms in foods by DNA amplification techniques". *Crit. Rev. Food Sci. Nutr*. 44 (2004) 425-436.

metabolitos presentes en un sistema biológico, una modificación a nivel genético, como en el caso de los alimentos trangénicos podría afectar al metaboloma (Fig 3).

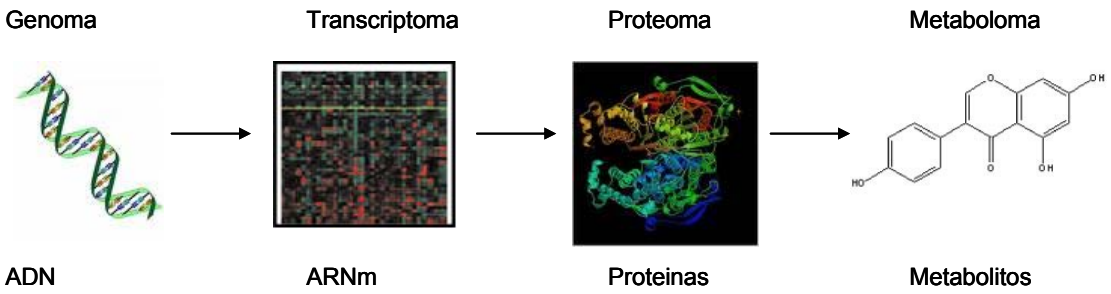

Figura 3. Esquema de las interrelaciones de las ómicas, en el estudio de los sistemas biológicos, entre ellos las plantas

Por lo tanto, estudiar los perfiles metabolómicos de alimentos transgénicos y no transgénicos puede conducirnos al descubrimiento de cambios significativos en metabolitos como polifenoles, ácidos grasos, vitaminas, aminoácidos etc… [114] que podrían ser utilizadas como biomarcadores para identificar la presencia de OMG´s en alimentos. Además, aporta información adicional sobre la composición de ese alimento.

Debida a la complejidad química del metaboloma (una planta puede contener del orden de 200.000 metabolitos, de muy diversa funcionalidad y polaridad, presentes en un amplio rango de concentraciones) se tienen que emplear técnicas analíticas de elevado poder de resolución y con alta sensibilidad y selectividad. La instrumentación típica en metabolómica se basa en técnicas de separación (Cromatografía de gases (GC), cromatografía de líquidos (LC)), técnicas espectroscópicas (RMN y espectrometría de masas) y los acoplamientos de ambas, seguidas en la mayoría de los casos de un tratamiento estadístico de los datos [115]. En lo que respecta a alimentos transgénicos encontramos en bibliografía estudios bastante recientes en los que se utilizan principalmente LC-MS [116], GC-MS [117,118] y RMN [119, 120] seguidos,

[114] Institute of Medicice and Nacional Research Council of the Nacional Academies. Safety of Genetically Engineered Foods; The National Academies press, Washington DC (2004).
[115] J. M. Cevallos-Cevallos, J. I. Reyes-De-Corcuera, E. Etxebarria, M. D. Danyluk, G. E. Rodrick "Metabolomic analysis in food science: a review" *Trends Food Sci. Technol.* 20 (2009) 557- 566.
[116] G. L. Gall, M. S. Dupont, F. A. Mellon, A. L. Davis, G. J. Collins, M. E. Verhoeyen, I. J. Colquhoun. "Characterization and content of flavonoids glycosides in genetically modified tomato (Lycopersicon esculentum) fruits" *J. Agric. Food Chem*. 51 (2003) 2438-2446.
[117] J. L. Bernal, M. J. Nozal, L. Toribio, C. Diego, R. Mayo, R. Maestre. "Use of supercritical fluid extraction and gas chromatography–mass spectrometry to obtain amino acid profiles from several genetically modified varieties of maize and soybean" *J. Chromatogr. A* 1192 (2008) 266–272.

en algunos casos, de técnicas de análisis multivariante para el análisis de diferentes tipos de alimentos sometidos a modificación genética: tomate, soja, arroz, trigo, maíz, etc. descubriendo en la mayoría de los casos diferencias significativas entre el perfil metabolómico de los alimentos modificados y los originales.

[118] J. Zhou, C. F. Ma, H. L. Xu, K. L. Yuan, X. Lu, Z. Zhu, Y. N. Wu, G. W. Xu. "Metabolic profiling of transgenic rice with cryIAc and sck genes: An evaluation of unintended effects at metabolic level by using GC-FID and GC–MS" *J. Chromatogr. B* 877 (2009) 725–732.
[119] C. Manetti, C. Bianchetti, M. Bizarri, L. Casciani, C. Castro, G. Dáscenzo, M. Delfini, M. E. Di Cocco, A. Laganá, A. Miccheli, M. Motto, F. Conti. "NMR-based metabonomic study of transgenic maize" *Phytochemistry* 65 (2004) 3187-3198.
[120] G. Le Gall, I. J. Colquhoun, A. L. Davis, G. J. Collins, M. E. Verhoeyen. "Metabolite profiling of tomato (Lycopersicon esculentum) using 1H NMR spectroscopy as a tool to detect potential unintended effects following a genetic modification" *J. Agric. Food Chem*. 51 (2003) 2447-2456.

CAPÍTULO 3:
Estudio metabólico comparativo entre soja convencional y soja modificada genéticamente mediante CE-ESI-TOF MS

Como veíamos en la introducción, diferentes metodologías analíticas han sido empleadas para el análisis de alimentos transgénicos a nivel del metaboloma. Sin embargo, las posibilidades de CE-MS no habían sido investigadas hasta el momento de comenzar este estudio, por lo que el tercer capitulo de la tesis tuvo como objetivo demostrar el potencial de la electroforesis capilar acoplada a espectrometría de masas con analizador de tiempo de vuelo (CE-ESI-TOF MS) para llevar a cabo una comparación a nivel metabólico entre soja convencional y soja modificada genéticamente, establecer las diferencias entre ellas y encontrar algún biomarcador que nos permitiera diferenciarlas. Para comparar los dos tipos de soja, fue imprescindible que crecieran en las mismas condiciones agronómicas ya que el metaboloma, al ser el resultado de la interacción entre factores genéticos y factores externos, es muy dinámico. Tanto el tratamiento de muestra, como la adquisición de datos estuvieron orientados a cubrir el mayor intervalo, en cuanto a tipo y número de compuestos presentes en la muestra.

Este trabajo de investigación se llevó a cabo durante una estancia en el Instituto de Fermentaciones Industriales (IFI) del Centro Superior de Investigaciones Científicas (CSIC) en Madrid, donde tuve la oportunidad de profundizar en el empleo de la técnica de CE.

Estimamos que sería también interesante mencionar trabajos posteriores al recogido en esta memoria de tesis, donde también se utiliza la plataforma analítica CE-ESI-TOF MS para comparar diferentes tipos de maíz transgénico con sus correspondientes líneas isogénicas [121 , 122]. Estos resultados confirman las variaciones en el metabolismo de los organismos transgénicos, que incluyen diferencias tanto en el número como en la cantidad de los compuestos identificados.

[121] T. Levando, C. Leon, M. Kaljurand, V. García-Cañas, A. Cifuentes. "Capillary electrophoresis time-of-flight mass spectrometry for comparative metabolomics of transgenic versus conventional maize". *Anal. Chem.* 80 (2008) 6329-6335.
[122] C. León, I. Rodríguez-Meizoso, M. Lucio, V. García-Cañas, E. Ibáñez, P. Schmitt-Kopplin, A. Cifuentes. "Metabolomics of transgenic maize combining Fourier transform-ion cyclotron resonant-mass spectrometry, capillary electrophoresis-mass spectrometry and pressurized liquid extraction" *J Chromatogr A* 1216 (2009) 7314-7323.

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Comparative metabolomic study of transgenic versus conventional soybean using capillary electrophoresis–time-of-flight mass spectrometry

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ABSTRACT

In this work, capillary electrophoresis–time-of-flight mass spectrometry (CE–TOF-MS) is proposed to identify and quantify the main metabolites found in transgenic soybean and its corresponding non-transgenic parental line both grown under identical conditions. The procedure includes optimization of metabolites extraction, separation by CE, on-line electrospray-TOF-MS analysis and data evaluation. A large number of extraction procedures and background electrolytes are tested in order to obtain a highly reproducible and sensitive analytical methodology. Using this approach, a large number of metabolites were tentatively identified based on the high mass accuracy provided by TOF-MS analyzer, together with the isotopic pattern and expected electrophoretic mobility of these compounds. In general, the same metabolites and in similar amounts were found in the conventional and transgenic variety. However, significant differences were also observed in some specific cases when the conventional variety was compared with its corresponding transgenic line. The selection of these metabolites as possible biomarkers of transgenic soybean is discussed, although a larger number of samples need to be analyzed in order to validate this point. It is concluded that metabolomic procedures based on CE-MS can open new perspectives in the study of transgenic foods in order to corroborate (or not) the equivalence with their conventional counterparts.

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1. Introduction

Genetic engineering has been applied to plants since the mid-1980s and since then the use of genetically modified organisms (GMOs) has seen a great increase in agriculture and food science [1]. As an example, in 2005 more than 900,000 km^2 of GM crops were cultivated worldwide. GM plants are obtained by inserting manipulated fragments of DNA from a different organism in order to improve some characteristics of the original crop, such as its resistance to plagues, pesticides or extreme environmental conditions, to provide better nutritional properties, etc. [2,3].

The transgenic soybean variety tolerant to glyphosate is one of the most extended GM crops in the world. In 2005, 87% of U.S. soybean fields were planted with glyphosate resistant varieties [4]. Glyphosate binds to and blocks the activity of 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS), an enzyme of the aromatic amino acid biosynthetic pathway (shikimate pathway) [5]. The glyphosate inhibition of EPSPS prevents the plant from producing the aromatic amino acids (phenylalanine, tyrosine, tryptophan) essential for protein synthesis. Some microorganisms have a version of 5-enolpyruvoylshikimate-3-phosphate synthetase that is resistant to glyphosate inhibition. The version used in genetically modified crops was isolated from *Agrobacterium tumefaciens* strain CP4 (CP4 EPSPS) that was resistant to glyphosate [6,7]. This CP4 EPSPS gene was cloned and transfected into soybeans, and in 1996, such genetically modified soybean was made commercially available. This greatly improved the ability to control weeds in soybean fields since glyphosate could be sprayed on fields without affecting the crop.

In the last decades, several aspects of GMOs have been criticized and scientific and public debate is open about their influence on the environment and their safety as food and feed. As a result, many countries have implemented regulations regarding the development, growing, and commercialization of these genetically modified products. The European Union (EU) has dedicated special attention to customer information, and food products containing more than 0.9% of genetically modified soybean and/or maize must be labelled as transgenic [8].

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Therefore, the development of fast, sensitive and informative analytical methods is of paramount importance not only to fulfil the labelling requirements but also to evaluate other possible alterations in GM grains and foods [9]. In this regard, there are two general approaches for the detection of GMOs, based on the detection of two types of macromolecules specific for the genetic modification: proteins and DNA. The new or modified proteins contained in novel foods and ingredients are mainly detected using immunoassay methods (enzyme-linked immunosorbent assays (ELISA) methods). However, protein detection by immunoassay requires the use of antibodies raised against the protein encoded by the transgene [10,11]. Capillary electrophoresis (CE) [12,13] and 2D electrophoresis, where mixtures of proteins are separated based on their isoelectric point and molecular weight, have also been used to study the different protein profiles between some transgenic and non-transgenic crops [14–16].

Regarding the detection of GMOs based on DNA analysis, the most frequent procedure is to apply polymerase chain reaction (PCR) methods, where DNA fragments are amplified, followed by agarose gel electrophoresis plus ethidium bromide staining of the amplification products [17]. To avoid the restrictions in sensitivity and resolution of the slab gel electrophoresis, capillary gel electrophoresis (CGE) has been recently used as an attractive alternative providing very high efficiency, resolution and sensitivity [18,19]. DNA separations are performed in capillaries using polymer solutions where the electroosmotic flow (EOF) is completely suppressed [20–24]. Other method like PCR and immunoassay kits (PCR–ELISA) has also been described in the field of GMO analysis [25,26]. One of the last challenges in GMO detection is the development of multiplex-PCR approaches able to detect multiple GMOs in a single analysis [27,28]. The separation and detection of multiplex-PCR products for the detection of GMOs were achieved by agarose gel electrophoresis (AGE) [29] and other innovative procedures like, capillary electrophoresis, microchip capillary electrophoresis (MCE) [30], capillary gel electrophoresis with laser-induced fluorescent detection (CGE-LIF) [31,32], and DNA arrays or biosensors [33]. One of the main deficiencies of the procedures based on DNA are related to the semiquantitative character of the PCR amplification, recently overcome by the development of real-time PCR (RT-PCR) [34,35] and competitive quantitative PCR (QC-PCR) techniques using capillary gel electrophoresis [36,37].

An alternative procedure is to investigate the substantial equivalence of a GMO and its isogenic counterpart using profiling (or shotgun) procedures. In this regard, the comparison of the content of secondary metabolites has been proposed as an interesting approach to carry out this type of comparative studies. In the case of soybean, the studies done so far have been focused on the analysis of a given family of compounds following a more targeted approach. Thus, isoflavones, the main secondary metabolites in soybean, have been determined by HPLC [38] and LC/MS [39] and the results did not show significant differences between GM and non-GM soybeans. In other publication, the content of tocopherols, sterols, and phospholipids in oils obtained from GM soybeans and non-GM soybeans were determined by normal- and reversed-phase HPLC and GC observing some difference in the phospholipids fraction [40]. Several methods for identifying secondary metabolites of soybean, mainly isoflavones, have been published based on HPLC and capillary electrophoresis techniques with UV [41,42] and mass spectrometry detection [43,44], however, these works were not dealing with GM versus non-GM comparisons.

The aim of this study was to develop a new analytical strategy based on comparative metabolomics able to determine differences between GM soybean and its isogenic wild variety both grown under the same conditions. The proposed method is based on the following steps: (i) soybean metabolites extraction, (ii) CE separation, (iii) on-line ESI–TOF-MS analysis and (iv) data evaluation.

2. Experimental

2.1. Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogencarbonate from Fluka (Buchs, Switzerland) ammonium acetate from Panreac (Barcelona, Spain) and ammonium hydroxide from Merck (Darmstadt, Germany) were used for the CE running buffers at different concentrations and pH values. Buffers were prepared by weighting the quantity indicated in doubly distilled water and adding ammonium hydroxide to adjust the pH. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). Triethylamine (TEA) (Sigma, St. Louis, MO, USA) and 2-propanol of HPLC grade (Scharlau, Barcelona, Spain) were used in the sheath liquid. For the extraction of the soybean compounds, methanol, ethanol, hexane, and acetonitrile from Lab-Scan (Dublin, Ireland) and ethyl acetate from Fluka (Buchs, Switzerland) were used.

The soybean used for the optimization was obtained from an herbalist's shop (Madrid, Spain). The isogenic and transgenic soybean seeds used for the comparative metabolomic study were supplied by Professor Giovanni Dinelli (University of Bologna). Isogenic and transgenic plants were grown under the same conditions in a growth chamber: at the end of growing cycle the seeds were collected and finely ground as flour. The transgenic and non-transgenic nature of all these soybean samples was confirmed based on their DNA using an analytical procedure developed in our laboratory and described elsewhere [21,23,24,32,36].

2.2. Extraction procedures

To extract the major number of compounds from the soybean seeds different organic solvents together with different water percentages were tested: methanol (60, 80, 90 and 100%, v/v), ethanol (60, 80, 90 and 100%, v/v), ethyl acetate (100%, v/v) and acetonitrile (70 and 100%, v/v). All the extracts profiles were compared using the same initial CE–UV conditions: running buffer 100 mM ammonium hydrogencarbonate at pH 9 + 5% (v/v) acetonitrile, voltage of 28 kV, 5 s injection time, and detection wavelength at 200 nm. With these initial conditions the electric current was around 80 μA.

The extraction protocol was as follows: soybean seeds were finely grounded at 5 °C using a mill and maintained in the fridge at 4 °C. After, 1 g of the milled samples was mixed with 15 mL of the different solvents and extracted during 30 min in the ultrasonic bath. The extracts were then centrifuged at 5750 × *g* for 15 min at 5 °C. The supernatants were separated in three aliquots of 4 mL and evaporated in a concentrator. The residue was dissolved in 500 μL of the solvent and analyzed by CE–UV. It is important to keep the temperature below 20 °C because it is known that this temperature is the most suitable for extracting the compounds (specially isoflavones) from seeds with little or no modifications of its composition [42].

An alternative extraction procedure was tested removing the fat (mainly phospholipids) with hexane previously to the extraction. Thus, once the soybean was milled, 1 g was extracted with 10 mL of hexane during 15 min in the ultrasonic bath. The extracts were centrifuged at 5750 × *g* for 10 min at 5 °C, the supernatant was eliminated and the solid was extracted with the solvent as described above. The use of hexane did not improve the results of the extraction.

2.3. CE–UV and CE–MS

Analyses were performed in a PACE/2100 apparatus (Beckman, Fullerton, CA, USA) equipped with a UV–Vis detector working at 200 nm and coupled to a Bruker Daltonik micrOTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray ionization (ESI) interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The CE instrument was controlled by a personal computer running the System Gold Software from Beckman. Bare fused-silica capillaries with 50 μm I.D. and 375 μm O.D. were purchased from Composite Metal Services (Worcester, England). The detection length to the UV detector was 84 cm and the total length was 90 cm (corresponding to the MS detection length). Injections were made at the anodic end using N_2 at pressure of 3570 Pa and the times indicated in each case.

Electrical contact at the electrospray needle tip was established via a sheath liquid pumped by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, IL, USA). The ESI-voltage of the micrOTOF is applied at the end cap of the transfer capillary to the MS (−4.2 kV) with the spray needle being grounded. The mass spectrometer was run in the negative mode. The micrOTOF was operated to acquire spectra in the range of 50–1000 *m*/*z*. Transfer parameters were optimized for high sensitivity while keeping the resolution to better 10,000. The accurate mass data of the molecular ions were processed by DataAnalysis 3.3. software (Bruker Daltonik). It provides a list of possible elemental formulae by means of the Generate Molecular Formula editor (GMF), which uses a CHNO algorithm. This provides information about elemental composition, sigma and *m*/*z* values. The calibration of the MS was performed using sodium formate.

All new capillaries were conditioned before their first use by rising for 30 min with 0.1 M sodium hydroxide followed by water for 10 min and then running buffer for 20 min. Capillary conditioning between runs was carried out by flushing for 10 min with 0.1 M sodium hydroxide, then for 5 min with water and finally for 10 min with running buffer. At the end of the day the capillary was rinsed with water for 15 min and dried with air for 5 min.

3. Results and discussion

3.1. Optimization of metabolites extraction from soybean samples

The possibility to obtain a good extraction procedure of metabolites from soybean was deeply explored. To do this, the different extraction procedures above described were investigated in order to determine the extraction conditions that led to a higher number of peaks in a reproducible way, assuming this would mean a higher number of metabolites extracted. CE–UV was initially employed to monitorize at 200 nm the results of the extraction. The soybean bought in a local market was selected for the extraction optimization. As the aim of this part was to extract the highest number of possible metabolites, extraction solutions able to cover a wide range of polarities were frequently used, including ethanol:water and methanol:water mixtures at different concentrations. The results obtained using the different extraction solvents are given in Fig. 1. The best extraction procedures were obtained with methanol and ethanol with different percentages of water.

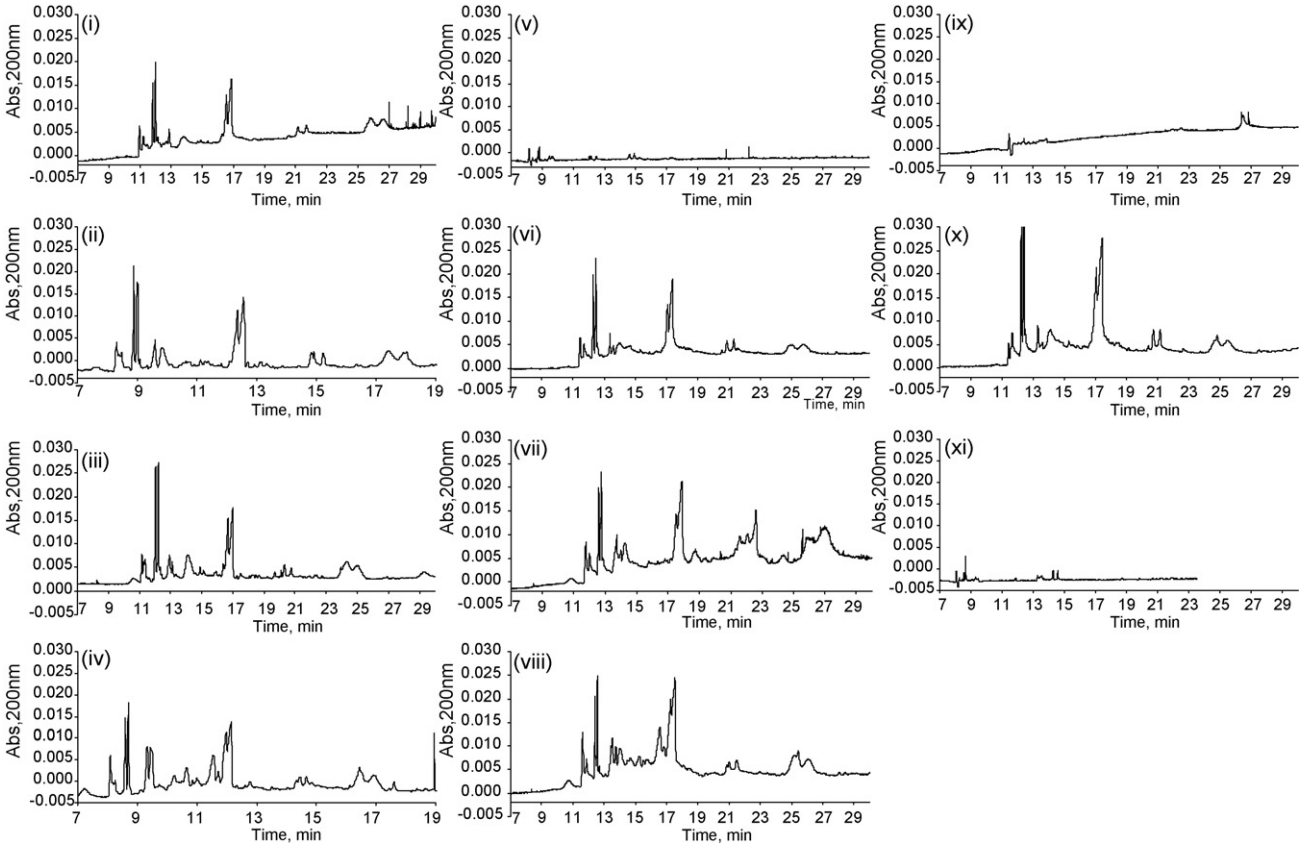

Fig. 1. CE–UV electropherograms of soybean extracts obtained using the following solvents: (i) methanol; (ii) methanol/water, 90/10; (iii) methanol/water, 80/20; (iv) methanol/water, 60/40; (v) ethanol, (vi) ethanol/water, 90/10; (vii) ethanol/water, 80/20; (viii) ethanol/water, 60/40; (ix) acetonitrile; (x) acetonitrile/water, 70/30; (xi) ethyl acetate. All the solvents were compared under the same initial conditions: fused-silica capillary with 50 μm I.D.,375 μm O.D., 90 cm total length, running buffer 100 mM ammonium hydrogencarbonate at pH 9 + 5% (v/v) acetonitrile, voltage: 28 kV, 5 s injection time, electrical current 80 μA. Detection wavelength: 200 nm.

The intensity of some peaks decreased at both higher (90%, v/v) and smaller (60%, v/v) percentages of organic solvent. Finally, the selected extract solvent consist of methanol/water (80/20, v/v) since it provided the highest number of metabolites extracted with high intensity as can be seen in Fig. 1(iii).

3.2. CE–UV analysis of soybean metabolites

The objective in this part of the work was to obtain a BGE compatible with the subsequent ESI–MS analysis and able to provide fast separation with high resolution and sensitivity. The effect of different separation parameters on resolution, sensitivity, analysis time, and peak shape was studied using a methanol/water (80/20, v/v) extract from commercial soybean. Preliminary experiments were carried out using CE with UV detection in order to find a suitable background electrolyte (BGE) compatible with CE–ESI–MS [45,46]. For this reason, in this work only volatile BGEs were tested at different pH values and ionic strengths. Initially, the conditions tested were type, concentration and pH of buffer. First, ammonium hydrogencarbonate and ammonium acetate at 100 mM and pH 10 were tested. In general the profiles obtained using carbonate-containing BGEs were better than the obtained with acetate. Finally ammonium hydrogencarbonate was selected because of the short analysis time provided by this BGE (data not shown).

Next, the effect of BGEs at different pH values (8, 9 and 10) adjusted by adding ammonium hydroxide was studied. A pH value of 9 was selected as optimum in term of efficiency, resolution and analysis speed. The effect of the BGE ionic strength was next studied testing concentrations of 25, 50 and 100 mM. The best results were obtained with 50 and 100 mM of ammonium hydrogencarbonate at pH 9 observing slightly better resolution with 100 mM while, as expected, the analysis time was shorter with 50 mM. To improve the resolution between peaks, different percentages of organic solvent (acetonitrile, ACN) were added to the two buffers (50 and 100 mM ammonium hydrogencarbonate). The best results in terms of peak shape and resolution were obtained with the following three BGEs: 50 mM ammonium hydrogencarbonate at pH 9, 50 mM ammonium hydrogencarbonate at pH 9 + 20% (v/v) ACN, and 100 mM ammonium hydrogencarbonate at pH 9. As no significant differences were found in CE–UV between these three buffers, they were selected for a posterior study by CE–ESI–TOF-MS choosing then the most appropriate BGE.

Based on these conditions different voltages: 20, 25, 28 and 30 kV were applied and we found that in general a voltage of 28 kV shortened the analysis time and also gave good resolution and acceptable electrical current values (around 35 μA with the 50 mM ammonium hydrogencarbonate buffer, 28 μA with 50 mM ammonium hydrogencarbonate + 20% (v/v) ACN and 65 μA with 100 mM ammonium hydrogencarbonate). The injection were made at the anodic end using N_2 pressure of 3570 Pa for 3, 5, 10 and 15 s, selecting 10 s as optimum injection in terms of sensitivity, resolution and stability of the separation.

The repeatability of the optimized CE–UV method, using 50 mM ammonium hydrogencarbonate at pH 9 as BGE, was then evaluated by carrying out three replicate determinations with the same sample and with three different extracts and was expressed by the relative standard deviation (RSD) for the migration time and for the peak area. The RSDs of analysis time and peak area were determined for three of the peaks present in the extracts. The results can be observed in Table 1 showing that the procedure is reproducible.

3.3. Optimization of ESI–MS parameters

In order to select the BGE most compatible with CE–ESI–TOF-MS the three mentioned buffers were tested using as sheath liquid isopropanol/water (50/50, v/v) + 0.1% (v/v) ammonium hydroxide (Fig. 2). The best results in terms of sensitivity were obtained with 50 mM of ammonium hydrogencarbonate (Fig. 2(i)). Therefore, these conditions were chosen for the subsequent ESI optimization.

Table 1
Mean, standard deviation (SD) and relative standard deviation (RSD) of three peaks in three consecutive injections of the same extract and with three different extracts

	Three different injections		Three different extracts	
	Peak area	Analysis time	Peak area	Analysis time
Mean				
Peak 1	0.151	6.63	0.130	6.61
Peak 2	0.130	10.60	0.111	10.59
Peak 3	0.560	12.01	0.490	11.96
SD				
Peak 1	0.0170	0.019	0.0191	0.035
Peak 2	0.0102	0.044	0.0182	0.076
Peak 3	0.0361	0.067	0.0804	0.100
RSD (%)				
Peak 1	11.3	0.29	14.6	0.53
Peak 2	7.88	0.42	16.4	0.72
Peak 3	6.45	0.56	16.4	0.84

Sheath liquid composition, sheath liquid flow rate, nebulizer pressure, dry gas flow rate and ESI chamber temperature were then optimized, selecting the MS intensity of several peaks as optimization criterion. The choice of these variables represented a compromise between maintaining efficient and well-resolved electrophoretic separation and improving ionization performance. Sheath liquid isopropanol/water (50/50) was tested together with different quantities (0.1 and 0.5% (v/v)) of TEA and ammonium hydroxide trying to improve the ionization yield. The highest MS signal was achieved using a sheath liquid containing 0.1% (v/v) ammonium hydroxide. Therefore, the sheat liquid selected was composed of water-2-propanol (50/50, v/v) and 0.1% (v/v) ammonium hydroxide. As optimum sheath liquid flow a value of 0.24 mL/h was selected because lower flows reduced the ionization yield due to the instability of the spray, while at higher flows dilution of the electrophoretic bands emerging from the capillary was too high and the intensity of the MS signal for these compounds was reduced. The other ESI parameters were chosen according to the sheath liquid flow of 0.24 mL/h and the most suitable ones were: nebulizer pressure of 0.4 bar, dry gas flow equal to 4 L/min and dry gas temperature 180 °C.

In summary, the best results were obtained by using the following CE–ESI–MS conditions: the running buffer was 50 mM ammonium hydrogencarbonate at pH 9, voltage 28 kV and 10 s of hydrodynamic injection at 3570 Pa. The sheath liquid consisted of water-2-propanol (50/50, v/v) with 0.1% (v/v) ammonium hydroxide pumped at 0.24 mL/h, used together with a nebulizer gas pressure at 0.4 bar and a dry gas flow rate of 4 L/min at 180 °C. With these optimum conditions the electric current was around 35 μA.

Fig. 3 shows the CE–MS base peak electropherogram of an extract of commercial soybean as well as the extracted ion electropherograms (in migration order) obtained under optimum CE–ESI–TOF-MS conditions. As can be seen, under these conditions a large number of metabolites could be detected.

3.4. Identification of compounds

The identification of the majority of metabolites can be performed by a careful interpretation of the mass spectra combined with the aid of their electrophoretic mobility. The assignment was later confirmed by soybean composition and metabolic pathways found in literature. The compounds tentatively identified are summarized in Table 2, including experimental *m*/*z* values,

Table 2
Metabolites detected by CE–ESI–TOF-MS in a soybean extract

	Mass	Migration time (min)	Fragments	Molecular formula	Error (ppm)	Sigma	Possible compounds	Pathways and references
1	173.1048	8.431	131.0822	$C_6H_{13}N_4O_2$	2.4	0.0579	Arginine	Arginine and proline metabolism [50]
2	845.2770	9.686	683.2248/665.2139/503.1617/341.1100	$C_{37}H_{49}O_{22}$	−5.9	0.2174	Dihydro tetramethoxyflavone triglucoside	
3	431.1429	9.787		$C_{22}H_{23}O_9$	−8.9	0.1435	Medicarpin 3-*O*-glucoside	Isoflavonoid biosynthesis
4	114.0557	9.937		$C_5H_8NO_2$	3.1	0.0420	Proline	Arginine and proline metabolism [50]
5	415.1056	10.004	253.0542	$C_{21}H_{19}O_9$	−5.3	0.0269	Daidzein 7-*O*-glucoside (daidzin)	Isoflavonoid biosynthesis [41,42,44]
6	134.0472	10.054		$C_4H_8NO_4$	−9.4	0.0290	4-Hydroxy-L-threonine	Vitamin B6 metabolism
7	431.0983	10.071	269.0455	$C_{21}H_{19}O_{10}$	0.1	0.0335	Genistein 7-*O*-glucoside (genistin)	Isoflavonoid biosynthesis [41,42,44]
8	130.0876	10.221		$C_6H_{12}NO_2$	−1.8	0.0057	Leucine	Valine leucine and isoleucine degradation [50]
9	203.0831	10.272		$C_{11}H_{11}N_2O_2$	−2.5	0.0068	Tryptophan	Tryptophan metabolism [50]
10	131.0826	10.339		$C_5H_{11}N_2O_2$	−0.4	0.0449	2.5-Diaminopentanoic acid(ornithine)	Urea cycle and metabolism of amino groups
11	173.0937	10.355		$C_7H_{13}N_2O_3$	−3.5	0.0498	*N*-acetylornithine	Urea cycle and metabolism of amino groups
12	180.0666	10.489		$C_9H_{10}NO_3$	0.2	0.0600	Tyrosine	Tyrosine metabolism [50]
13	154.0627	10.539		$C_6H_8N_3O_2$	−3.5	0.0549	Histidine	Histidine metabolism [50]
14	164.0719	10.573		$C_9H_{10}NO_2$	−1.6	0.0597	Phenylalanine	Phenylalanine metabolism [50]
15	131.0467	10.890		$C_4H_7N_2O_3$	−4.0	0.0372	Asparagine	Alanine and aspartate metabolism [50]
16	663.2668	12.028		$C_{33}H_{43}O_{14}$	−1.4	0.1898	$C_{33}H_{43}O_{14}$	
17	563.1622	12.429		$C_{30}H_{27}O_{11}$	−11.2	0.1703	Liquiritigenin 6-coumarylglucoside	Chalcone biosynthesis (isoflavone precursor)
18	443.1920	12.596		$C_{21}H_{31}O_{10}$	0.7	0.1384	$C_{21}H_{31}O_{10}$	
19	329.2343	12.847		$C_{18}H_{33}O_5$	−2.9	0.1169	Trihydroxyoctadec-11-enoic acid	Linoleic acid metabolism
20	449.1087	12.864		$C_{21}H_{21}O_{11}$	−1.6	0.1378	Taxifolin 3-rhamnoside	Flavonol biosynthesis
21	327.2160	12.880		$C_{18}H_{31}O_5$	5.3	0.4330	2.3-Dinor-8-iso prostaglandin F1alpha	Arachidonic acid metabolism
22	433.1132	12.897		$C_{21}H_{21}O_{10}$	4.2	0.1374	Naringenin 7-*O*-glucoside	Chalcone biosynthesis (isoflavone precursor)
23	501.1062	12.998	457.1149/269.0452	$C_{24}H_{21}O_{12}$	2.8	0.2790	Daidzein 7-*O*-malonylglucoside	Isoflavonoid biosynthesis [41,42,44]
24	517.1028	13.148	473.1084/269.0452	$C_{24}H_{21}O_{13}$	−0.3	0.1370	Genistein 7-*O*-malonylglucoside	Isoflavonoid biosynthesis [41,42,44]
25	295.2278	13.215		$C_{18}H_{31}O_3$	0.2	0.1160	Epoxyoctadecenoic acid	Linoleic acid metabolism
26	333.0605	13.516		$C_{16}H_{13}O_8$	3.1	0.1041	6-Methoxytaxifolin	Flavonol biosynthesis
27	521.2661	13.767		$C_{31}H_{37}O_7$	−12.3	0.1748	Kushenol M	Flavanone biosynthesis
28	491.2523	13.834		$C_{30}H_{35}O_6$	−7.1	0.1686	Sophora-iso-flavanone D	Flavanone biosynthesis
29	519.2488	13.867		$C_{31}H_{35}O_7$	−9.3	0.1747	Exiguaflavanone D	Flavanone biosynthesis
30	505.2527	13.901		$C_{31}H_{37}O_6$	13.6	0.1744	Kushenol B	Flavanone biosynthesis
31	267.0675	14.218		$C_{16}H_{11}O_4$	4.4	0.0845	Formononetin	Isoflavonoid biosynthesis
32	253.0494	14.671		$C_{15}H_9O_4$	4.9	0.0959	Daidzein	Isoflavonoid biosynthesis [41,42,44]
33	269.0446	14.855		$C_{15}H_9O_5$	3.4	0.0963	Genistein	Isoflavonoid biosynthesis [41,42,44]
34	309.1077	15.290	128.0340	$C_{14}H_{17}N_2O_6$	4.7	0.0143	γ-L-Glutamyl-L-tyrosine	Dipeptide (carboxypepetide) biosynthesis [57]
35	293.1136	15.491		$C_{14}H_{17}N_2O_5$	2.5	0.0187	γ-L-Glutamyl-L-phenylalanine	Dipeptide (carboxypepetide) biosynthesis [57]
36	195.0506	15.809		$C_6H_{11}O_7$	2.4	0.0408	Gluconic acid (L-gluconate)	Pentose phosphate pathway
37	179.0556	15.959		$C_6H_{11}O_6$	2.9	0.0404	Fuconate	Fructose and mannose metabolism
38	172.0613	16.277	128.0732	$C_7H_{10}NO_4$	1.1	0.0478	*N*-Acetyl–L-glutamate 5-semialdehyde	Urea cycle and metabolism of amino groups
39	176.0555	16.929	146.0458	$C_6H_{10}NO_5$	5.5	0.0420	4-Hydroxy-4-methyl glutamate	Glutamate metabolism
40	319.0822	16.962		$C_{16}H_{15}O_7$	0.3	0.1038	4-Coumaroylshikimate	Phenylpropanoid biosynthesis
41	149.0449	17.079		$C_5H_9O_5$	4.4	0.0336	Trihydroxypentanoic acid	Alkyl carboxylic acid biosynthesis
42	165.0396	17.197		$C_5H_9O_6$	5.3	0.0341	Tetrahydroxypentanoic acid (L-xylonate)	Pentose and glucuronate interconversion
43	146.0454	17.882	128.0340	$C_5H_8NO_4$	3.6	0.0352	Glutamic acid	Glutamate metabolism [50]
44	135.0296	18.016		$C_4H_7O_5$	2.3	0.0273	Trihydroxybutanoate (threonate)	Ascorbate and aldarate metabolism
45	132.0298	18.718		$C_4H_6NO_4$	3.0	0.0289	Aspartic acid	Alanine and aspartate metabolism [50]

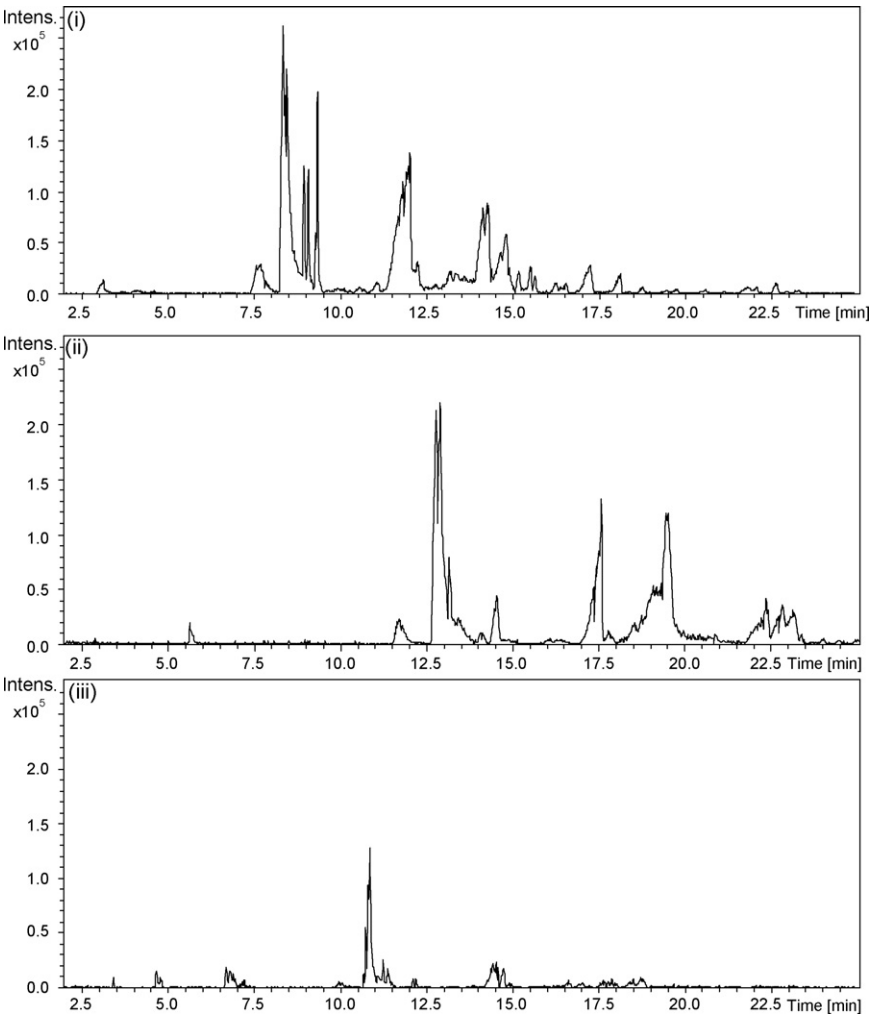

Fig. 2. CE–TOF-MS electropherograms using as BGE: (i) 50 mM ammonium hydrogencarbonate, (ii) 50 mM ammonium hydrogencarbonate + 20% (v/v) ACN, (iii) 100 mM ammonium hydrogencarbonate. The rest of CE–ESI–MS conditions were: voltage: 28 kV; injection time: 10 s; sheath liquid: 2-propanol/water 50:50 (v/v) containing 0.1% (v/v) ammonium hydroxide, flow rate 0.24 mL/h, drying gas: 4 L/min at 180 °C, nebulizing gas pressure: 4 bar. MS analyses were carried out using negative polarity. MS scan 50–1000 *m*/*z*.

fragments detected, the error and sigma value (comparison of the theoretical with the measured isotope pattern) a list of possible compounds and references about these including soybean pathways.

A reduced number of possible elemental compositions are obtained from the accurate mass of the suspected peak combined with the correct determination of the isotopic pattern applying ESI–TOF-MS analyzer. These elemental compositions can then be matched against available databases using the deduced molecular formula as a search criterion [47,48].

Additional proves that corroborate the adequacy of the compounds given in Table 2 can be found from the electrophoretic mobility of these compounds. The total migration time for highly charged small molecules is longer than that for molecules of smaller charge and greater size [49].

The first group of peaks migrated very close to the EOF and most of them were identified as aminoacids (essential aminoacids present in soybean and others produced via secondary metabolism [50]) as e.g., proline, 4-hydroxy-L-threonine, leucine, tyrosine, asparagine. They have a carboxylic group that at pH 9 will be fully ionized and also have a primary amine susceptible to bear positive charge at that pH, giving rise to a net charge near to zero that explains this behaviour.

The last migrating peaks (as e.g., glutamic acid, aspartic acid) correspond to compounds with carboxylic groups that are totally ionized at the separation pH 9, providing to the molecules the highest negative electrical charge/size ratio, the lowest apparent electrophoretic mobility and therefore high migration time.

Major isoflavones previously observed in several studies [41,42,44] were also detected in the present work. Soybean was revealed to be more abundant in malonyl derivatives of genistin and daidzin, followed by genistin, daidzin and their aglycones (genistin and daidzein). In spite of their abundance in soybean seeds, malonyl forms are thermally unstable and are easily converted into their corresponding glycosides forms.

Interestingly, the migration order of the isoflavones, previously studied in literature [41,42], is also in good agreement with our results. Thus, the glycosides (daidzin and genistin) were detected earlier because of their higher molecular weights; then the malonyl derivatives migrated due to increased electrophoretic mobility with the introduction of a negative charge (malonate) to the sugar that overcompensate the increase in molecular mass. The smaller aglycone molecules with higher mobility toward the anode and against the EOF were detected lattest. In all cases daidzein was detected before genistein because of the extra hydroxyl group of genistein [51].

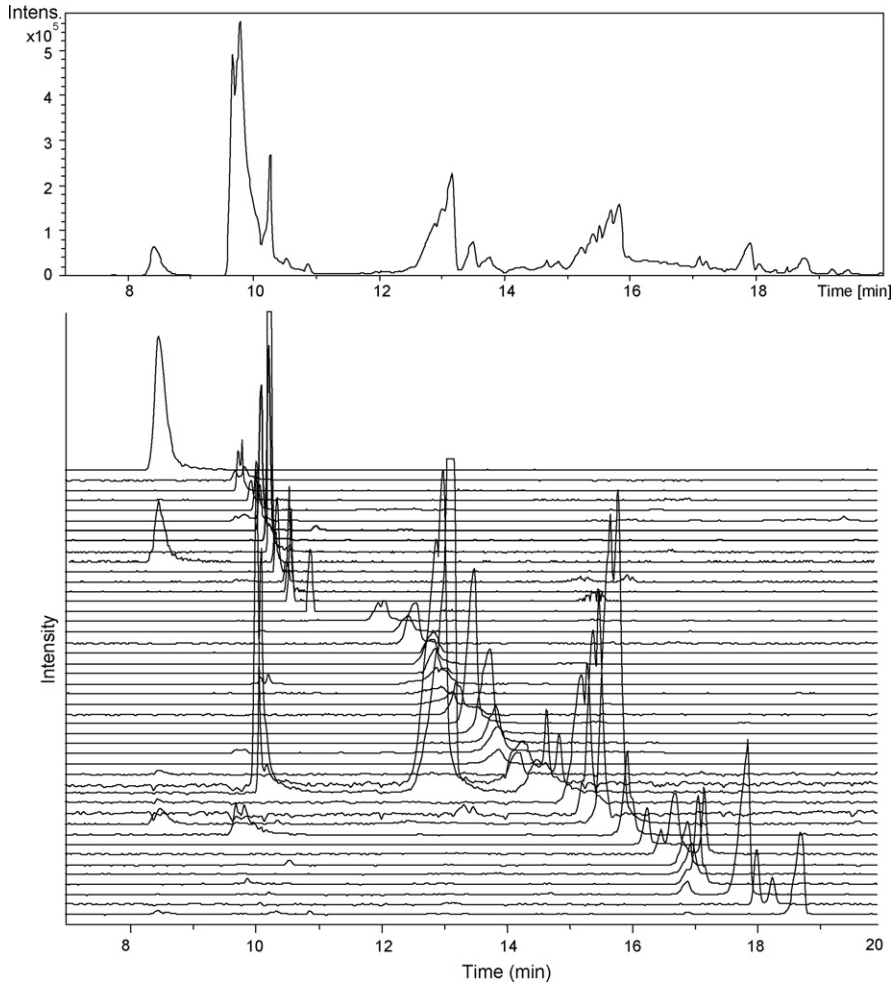

Fig. 3. CE–TOF-MS base peak electropherogram (BPE) of commercial soybean, using the optimal conditions and CE–TOF-MS extracted ion electropherograms (EIEs) of the detected compounds. CE–MS conditions: Buffer: 50 mM ammonium hydrogencarbonate at pH 9. Voltage: 28 kV. Injection time: 10 s. Sheath liquid: 2-propanol/water 50:50 (v/v) containing 0.1% (v/v) ammonium hydroxide, flow rate 0.24 mL/h. Drying gas: 4 L/min at 180 °C. Nebulizing gas pressure: 4 bar. Electrical current 35 μA. MS analyses were carried out using negative polarity. MS scan 50–1000 *m/z*.

The glycosides and their malonyl derivatives also contain the [M–H] ions corresponding to their aglycones forms produced in the electrospray (ESI–TOF) so, we can observe in the extracted ion electropherograms (EIEs) of daidzein (253,049) and genistein (269,044) three peaks: first two correspond to the fragments of the glycosides and malonyl derivatives and the last one is the aglycon.

Although these isoflavones are the predominant flavonoids in soybean seeds, other flavonoids have been tentatively identified in this work since flavonoids are one of the largest and most widespread groups of plant secondary metabolites [52]. The first two phenolic compounds tentatively identified as dihydro tetramethoxyflavone triglucoside and medicarpin 3-*O*-glucoside showed peaks migrating near the EOF, in good agreement with its low ionizable character. Daidzin and genistin migrated later than the EOF due to a partial ionization of the free –OH groups at the basic running buffer. Other flavonoids identified were: taxifolin 3-rhamnoside, naringenin 7-glucoside, 6-methoxytaxifolin and formononetin.

Besides it is very common to find in plant extracts flavonoid glycosided acylated with acids such as malonic, acetic, coumaric etc. [53,54] as e.g., liquiritigenin 6-coumarylglucoside, tentatively identified in soybean extract.

Small traces of other flavonoids in soybean extract were tentatively identified as: Kushenol M, Sophora-iso-flavanone D, Exiguaflavanone and Kushenol B that contain prenyl, geranyl, lavandulyl and similar groups. These results are in good agreement with the data found in the literature about plants belonging to the family of Fabacea [55,56] in which some isoflavones with prenyl groups or further *O*-heterocyclic rings were detected. Nevertheless, the high sigma and error values found for these compounds have to be also considered (see Table 2).

It was also possible to study other compounds present in this fraction of the soybean. Among them we found compounds from the linoleic acid metabolism as e.g., trihydroxyoctadec-11-enoic and epoxyoctadecenoic acid, other belonging to arachidonic acid metabolism as e.g. 2, 3-dinor-8-iso prostaglandin F1-alpha and dipeptides as γ-L-glutamyl-L-tyrosine and γ-L-glutamyl-L-phenylalanine [57]. These and other compounds are listed in Table 2.

3.5. Comparison between conventional and transgenic soybean

A comparison between the metabolomic profile obtained with the transgenic soybean and its isogenic wild variety was next carried. As can be seen in Table 3, comparing the two samples (conventional and GM soybean), certain compounds were detected in both soybeans, although there were interesting differences in the intensity of their signals. Thus, the GM

Table 3
Metabolites detected by CE–TOF-MS in conventional and GM soybeans

	Mass	Possible compounds	Conventional	GM
1	173.1048	Arginine	YES	YES
2	845.2770	Dihydro tetramethoxyflavone triglucoside	YES	YES
3	431.1429	Medicarpin 3-*O*-glucoside	YES	YES
4	114.0557	Proline	YES[a]	YES
5	415.1056	Daidzein 7-*O*-glucoside (daidzin)	YES	YES
6	134.0472	4-Hydroxy-L-threonine	YES	NO
7	431.0983	Genistein 7-*O*-glucoside (genistin)	YES	YES
8	130.0876	Leucine	YES	YES
9	203.0831	Tryptophan	YES	YES
10	131.0826	2,5-Diaminopentanoic acid(ornithine)	YES	YES
11	173.0937	*N*-acetylornithine	YES	YES
12	180.0666	Tyrosine	YES	YES
13	154.0627	Histidine	YES[a]	YES
14	164.0719	Phenylalanine	YES	YES
15	131.0467	Asparagine	YES[a]	YES
16	663.2668	$C_{33}H_{43}O_{14}$	YES	YES
17	563.1622	Liquiritigenin 6-coumaroylglucoside	YES	YES[b]
18	443.1920	$C_{21}H_{31}O_{10}$	YES	YES
19	329.2343	Trihydroxyoctadec-11-enoic acid	YES	YES
20	449.1087	Taxifolin 3-rhamnoside	YES	YES
21	327.2160	2,3-Dinor-8-iso prostaglandin F1alpha	YES	YES
22	433.1132	Naringenin 7-*O*-glucoside	YES	YES[b]
23	501.1062	Daidzein 7-*O*-malonylglucoside	YES	YES
24	517.1028	Genistein 7-*O*-malonylglucoside	YES	YES
25	295.2278	Epoxyoctadecenoic acid	YES	YES
26	333.0605	6-Methoxytaxifolin	YES	YES[b]
27	521.2661	Kushenol M	YES	YES
28	491.2523	Sophora-iso-flavanone D	YES	YES
29	519.2488	Exiguaflavanone D	YES	YES
30	505.2527	Kushenol B	YES	YES
31	267.0675	Formononetin	YES	YES
32	253.0494	Daidzein	YES	YES
33	269.0446	Genistein	YES	YES
34	309.1077	γ-L-Glutamyl-L-tyrosine	YES	YES
35	293.1136	γ-L-Glutamyl-L-phenylalanine	YES	YES
36	195.0506	Gluconic acid (L-gluconate)	YES[a]	YES
37	179.0556	Fuconate	YES	YES
38	172.0613	*N*-acetyl-L-glutamate 5-semialdehyde	YES	YES
39	176.0555	4-Hydroxy-4-methyl glutamate	YES	YES
40	319.0822	4-Coumaroylshikimate	YES	YES
41	149.0449	Trihydroxypentanoic acid	YES[a]	YES
42	165.0396	Tetrahydroxypentanoic acid (L-xylonate)	YES	YES
43	146.0454	Glutamic acid	YES	YES
44	135.0296	Trihydroxybutanoate (threonate)	YES	YES
45	132.0298	Aspartic acid	YES	YES

[a] Means peak area of the compound is significantly higher ($p > 0.95$) in conventional soybean than in transgenic soybean.
[b] Means peak area of the compound is significantly higher ($p > 0.95$) in transgenic soybean than in conventional soybean.

soybean produced higher amounts of some metabolites (as e.g., liquiritigenin 6-coumaroylglucoside, naringenin 7-*O*-glucoside and 6-methoxytaxifolin), while for other metabolites (as e.g., proline, histidine, asparagine, gluconic acid, and trihydroxypentanoic acid) the conventional soybean produced higher amounts. However, the main qualitative difference between GM and wild soybeans was found in the compound 6 tentatively identified as 4-hydroxy-L-threonine (*m*/*z* 134). Fig. 4 shows the comparison of the extracted ion electropherograms of some analytes identified by CE–ESI–TOF-MS in the transgenic and conventional soybeans. Comparing the results for the two samples, we could not find 4-hydroxy-L-threonine in the electropherogram obtained with transgenic soybean. We have represented this compound with an asterisk and have compared it with other peaks that as can be seen are unchanged what eliminates any possible analytical artifact. Before this compound can be assigned as a possible indicator of this genetic modification, a large number of samples should be analyzed.

On the basis of obtained data, some biochemical considerations can be drawn. By comparing conventional and GM metabolite profiles a different expression of three free amino acids (i.e. proline, histidine and asparagine) and of one amino acid derivative (i.e. 4-hydroxy-L-threonine) was observed. Although proline, asparagine and threonine are biosynthesized in different anabolic pathways (glutamine–proline, alanine–asparagine and threonine–methionine pathways, respectively), they are interconnected sharing a common precursor. The common precursor of proline and asparagine is the glutamic acid, while the precursor of threonine is the aspartic acid [5]. It is to underline that glyphosate resistant soybean expresses both endogenous and transgenic EPSPS [6,7]. As a consequence considering that EPSPS is a key enzyme of shikimate pathway, it is plausible that the synthesis of aromatic amino acids (phenylalanine, tyrosine, tryptophan) may be differently regulated in GM soybean and corresponding isogenic line. The different allosteric properties of eukaryote (endogenous soybean enzyme) and prokaryote (*A. tumefaciens* enzyme) EPSPS may be the basis of different biosynthetic regulatory systems. The presumable different regulation of shikimate pathways can explain the higher relative content of liquiritigenin, naringenin and taxifolin derivatives observed in GM soybean. In fact, these three compounds share common precursors: the aromatic amino acids phenylalanine and tyrosine for the flavanones naringenin and liquiritigenin, and narigenin for the majority of flavonoids (including the flavonol

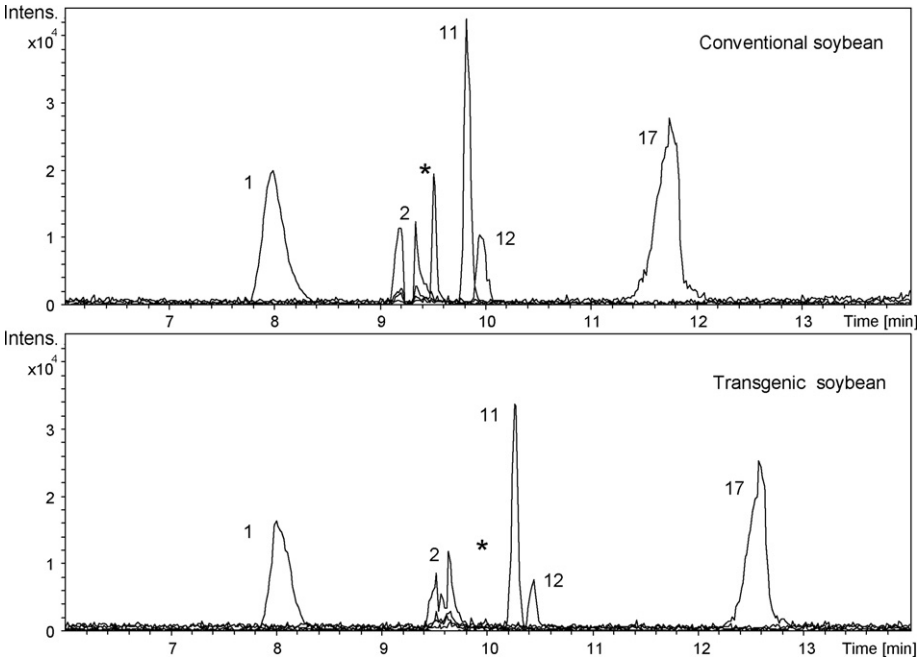

Fig. 4. Comparison of the CE–TOF-MS extracted ions electropherograms (EIEs) of some metabolites found in conventional and transgenic soybean. (*) compound that changes; 1, 2, 11, 12, 17 unmodified compounds. Peak numbers correspond to the compounds identified in Table 2. Conditions as in Fig. 3.

taxifolin) [58]. Further studies are in progress in order to confirm at transcriptional level the observed metabolomic differences between GM and conventional soybean.

4. Conclusions

In the present work, a complete analytical method (including an extraction protocol, CE–ESI–TOF-MS analysis and data evaluation) has been developed to comparatively study the metabolic profile of conventional and GM soybean. This method allows the tentative identification of more than 40 compounds, including, isoflavones, aminoacids, carboxylic acids, peptides and other analytes. The results show that some of the detected metabolites do not change, while other show significant quantitative differences in their intensities in the conventional and GM soybean. A compound tentatively identified as 4-hydroxy-L-threonine seems to disappear in the transgenic soybean compared to its parental non-transgenic line.

Acknowledgements

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BLOQUE III:
ACEITE DE OLIVA

BLOQUE III. ACEITE DE OLIVA

Según el Consejo Oleícola Internacional (COI), se considera *aceite de oliva* al aceite procedente exclusivamente del fruto del olivo (Olea europaea), con exclusión de los aceites obtenidos utilizando disolventes o por procedimientos de reesterificación y de toda mezcla con aceites de otra naturaleza.

1. Tipos de aceite de oliva

Independientemente de los factores propios de la materia prima utilizada, debemos distinguir entre varios tipos de aceite: aceite de oliva virgen (extra, virgen o lampante), aceite de oliva refinado y aceite de oliva [123].

El **aceite de oliva virgen** se obtiene del fruto del olivo únicamente por procedimientos mecánicos o por otros medios físicos, en condiciones, especialmente térmicas, que no produzcan la alteración del aceite, y que no haya tenido más tratamiento que el lavado, la decantación y el filtrado (Fig. 1 proceso de elaboración del aceite de oliva virgen).

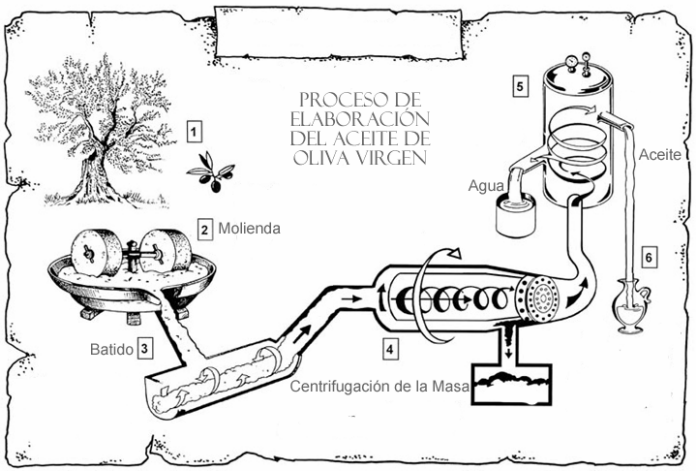


Figura 1. Proceso de elaboración del aceite de oliva virgen

Si el aceite se ha elaborado a partir de aceitunas sanas y en su estado de maduración óptimo, puede ser consumido directamente y, atendiendo a diversas características físico-químicas y organolépticas se clasifica en: *aceite de oliva virgen*

[123] Reglamento (CE) Nº 1989/2003 de la Comisión, de 6 de noviembre de 2003, que modifica el reglamento (CEE) nº 2568/91, relativo a las Características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis.

(acidez libre, expresada en ácido oleico, no supera 2 g por cada 100 g) y *aceite de oliva virgen extra* (acidez libre, expresada en ácido oleico, no supera 0,8 g por cada 100 g). Si el aceite obtenido directamente de las aceitunas, por cualquier circunstancia, no es apto para el consumo estamos ante un *aceite de oliva lampante (*acidez libre, expresada en ácido oleico, es superior a 2 g por cada 100 g). Este aceite es sometido a un proceso de refinación, donde pierde prácticamente la totalidad de su color y los olores y sabores desagradables que lo caracterizan, y se obtiene el **aceite de oliva refinado**, un aceite incoloro, sin olor e insípido (acidez libre, expresada en ácido oleico, no podrá ser superior a 0,3 g por 100 g). Este aceite de oliva refinado se mezcla con pequeñas cantidades de aceite de oliva virgen extra o aceite de oliva virgen para obtener una nueva categoría de aceite compuesta de aceite de oliva refinado y aceite de oliva virgen, generalmente conocido como **aceite de oliva** (acidez libre, expresada en ácido oleico, no podrá ser superior a 1 g por 100 g).

Dentro de la categoría de aceites de oliva virgen extra, sería posible hacer una sub-clasificación en:

- *Monovarietales*: Elaborados en base a una sola variedad de aceituna
- *Coupages*: Elaborados en base a diversas variedades de aceitunas, con el objetivo de obtener siempre los mismos estándares de sabor y aroma.
- *Denominación de Origen Protegida (DOP):* Elaborados en base a aceitunas procedentes de un área geográfica determinada y oficialmente reconocida.

Las aplicaciones de los métodos desarrollados en la presente memoria fueron llevadas a cabo utilizando aceites monovarietales.

2. Variedades de aceituna

En España, se cultivan casi un centenar de variedades distintas de aceitunas [124], aunque con sólo 4 de ellas (Picual, Arbequina. Hojiblanca y Cornicabra) se tiene más del 60% de la olivicultura y sólo la variedad Picual produce prácticamente la mitad del aceite español. Otras variedades españolas representativas y que se utilizan comercialmente son: Farga, Manzanilla, Gordal, Lechín de Sevilla, Empeltre, Picudo,

[124] D. Barranco, A. Cimato, P. Florino, L. Rallo, A. Touzani, C. Castañeda, F. Sefarini, I. Trujillo. "Catálogo mundial de variedades de olivo". Ed. Consejo Oleícola Internacional. (2001).

Verdial de Vélez-Málaga, Lechín de Granada, Verdial de Huévar, Morisca, Manzanilla Cacereña, Blanqueta… Cada variedad aporta unas características específicas al aceite resultante, además de que influyen otros factores como el proceso de elaboración, la madurez del fruto, el suelo, clima, edad de los olivos, etc.

3. Importancia económica del aceite de oliva

La importancia económica y la resultante social del sector del aceite de oliva son evidentes en los países mediterráneos, tanto por la posición que ocupa en términos del producto interior bruto agrícola nacional, como por el empleo y riqueza que genera.

Según datos proporcionados por el COI la producción mundial de aceite de oliva alcanzó en la campaña 2008-2009 los 2.665.000 toneladas, concentrándose principalmente en las siguientes regiones europeas y norteafricanas: España (41,3 %), Italia (23.4%), Grecia (13.6%), Portugal (1.46%), Siria (4.5%), Turquía (4.2%), Túnez (6.6%) y Marruecos (2.8%). Entre España, Italia y Grecia acaparan el 75% del total de la producción mundial. En cuanto al consumo de aceite de oliva, éste está prácticamente concentrado en las regiones productoras. Sólo entre Italia, España y Grecia suman más del 50% del consumo total mundial. En los últimos años, se está registrando un aumento del consumo en los países sin tradición productora como Estados Unidos con un consumo equivalente al 8% del total mundial [125].

4. Composición química del aceite de oliva

El aceite de oliva es una matriz compleja compuesta principalmente por triglicéridos, y en menor proporción por ácidos grasos libres y un 0.5-1.5% de constituyentes no glicerídicos. Su composición varía en función de múltiples factores, tales como la variedad de aceituna, la exposición solar, la localización geográfica, las características del olivar, la forma de extracción y la conservación del aceite, etc.

En general pueden distinguirse dos fracciones en la composición del aceite de oliva: una saponificable y otra insaponificable.

[125] www.internationaloliveoil.org

Figura 2. Esquema de la composición química del aceite de oliva

La fracción saponificable representa entre el 98.5% y el 99.5% del peso del aceite de oliva. Está formada principalmente por glicéridos, diferenciándose entre ellos por los ácidos grasos que los forman y por el número de grupos alcohol de la glicerina que se han unido a ácidos grasos. Los mono y diglicéridos se encuentran en pequeña cantidad, 0.2% y 1.3% sobre ácidos grasos totales respectivamente, así como fosfátidos y algunos ácidos libres, constituyendo los triglicéridos el grupo mayoritario [126,127].

Los ácidos grasos del aceite de oliva son básicamente monoinsaturados (AGMI). El ácido oleico es el ácido graso mayoritario y representa por término medio el 79% del total de ácidos grasos aunque puede variar entre 57-82%. Le siguen en cantidad los ácidos grasos saturados (AGS) (13-20%), fundamentalmente palmítico (11-17%) y esteárico, y los ácidos grasos poliinsaturados (AGPI) entre los que se encuentran los ácidos grasos esenciales linoleico (4-20%) y linolénico (0.9%).

La fracción insaponificable constituye entre el 0.5 y el 1.5% de los aceites y contiene un gran número de compuestos que se encuentran en concentraciones del orden de miligramos por kilo de aceite (ppm) por lo que también se denominan "componentes minoritarios". Esta fracción es, en parte, la responsable de la estabilidad oxidativa y las características organolépticas excepcionales de estos aceites.

[126] E. Fedeli. "Lipids of olives" en "Progress on Chemistry of fats and other lipids". E. Ralph, T. Holman (Ed). Pergamon Press. Paris (1997) 15-74.
[127] R. Aparicio, J. Harwood. "Manual del Aceite de Oliva". 1ª Edición. (2003).

Los componentes minoritarios de los aceites vegetales se eliminan mayoritariamente durante los procesos de refinación. Es por ello que el aceite de oliva virgen, al ser obtenido únicamente mediante los procesos de lavado, prensado, centrifugación y filtración, conserva todos sus componentes minoritarios, muchos de ellos con actividad antioxidante.

Este grupo de compuestos incluye a los hidrocarburos, esteroles, tocoferoles, pigmentos, alcoholes grasos, compuestos volátiles y aromáticos y compuestos fenólicos.

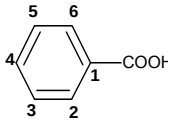
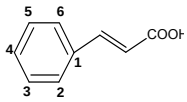
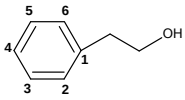
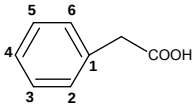
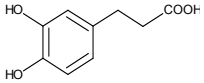
5. Compuestos fenólicos del aceite de oliva

Cuando se habla de los compuestos fenólicos del aceite de oliva, a menudo se usa el término de "polifenoles". "Compuestos fenólicos" o "polifenoles" es un término que se usa frecuentemente para designar a aquellas sustancias que poseen un anillo aromático con uno o más grupos hidroxilo unidos a él [128].

La fracción fenólica del aceite de oliva es una mezcla heterogénea de compuestos, cada uno de los cuales posee unas propiedades distintas y ejerce diversa influencia sobre la calidad del aceite.

La Tabla 1 contiene de modo esquemático las distintas categorías que podemos distinguir entre los compuestos fenólicos del aceite de oliva.

[128] A. Escarpa, M. C. González. "An overview of analytical chemistry of phenolic compounds in foods". *Crit. Rev. Ana. Chem.* 31 (2001) 57-139.

Compound Name	Substituent	Structure
Benzoic and derivatives acids		
3-hydroxybenzoic acid	3-OH	
p- hydroxybenzoic acid	4-OH	
3,4-dihydroxybenzoic acid	3,4-OH	
gentisic acid	2,5-OH	
vanillic acid	3-OCH_3, 4-OH	
gallic acid	3,4,5-OH	
siringic acid	3,5-OCH_3, 4-OH	
Cinnamic and derivatives acids		
o-coumaric acid	2-OH	
p-coumaric acid	4-OH	
caffeic acid	3,4-OH	
ferulic acid	3-OCH_3, 4-OH	
sinapinic acid	3,5-OCH_3, 4-OH	
Phenyl ethyl alcohols		
tyrosol (Ty) (*p*-hydroxyphenyl)ethanol]	4-OH	
Hydroxytyrosol (Hyty) [(*3,4* dihydroxyphenyl)ethanol]	3,4-OH	
Other phenolic acids and derivatives		
p-hydroxyphenylacetic acid	4-OH	
,4-dihydroxyphenylacetic acid	3,4-OH	
4-hydroxy-3-methoxyphenylacetic acid	3-OCH_3, 4-OH	
3-(3,4-dihydroxyphenyl)propanoic acid		

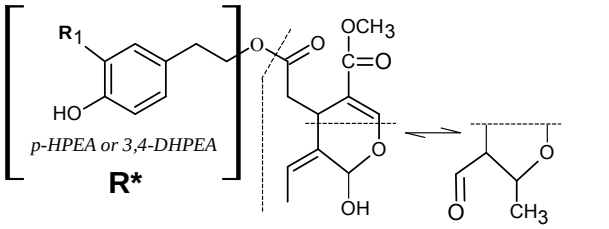
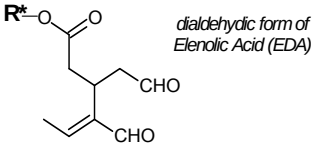
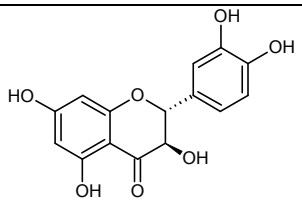
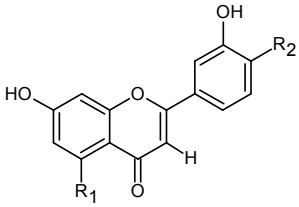
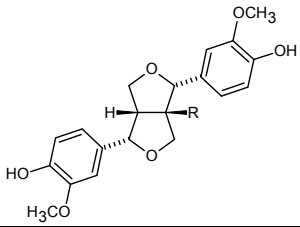
Compound Name	Substituent	Structure
Aglycons secoiridoids		
oleuropein aglycon or Hyty-EA	R_1-OH	
ligstroside aglycon or *Ty* -EA	R_1-H	
aldehydic form of oleuropein aglycon	R_1-OH	
aldehydic form ligstroside aglycon	R_1-H	
		p-HPEA or 3,4-DHPEA R* *Elenolic Acid (EA)* *aldehydic form of Elenolic Acid (EA)*
Dialdehydic forms of secoiridoids		
decarboxymethyl oleuropein aglycon (Hyty-EDA)	R_1-OH	
decarboxymethyl ligstroside aglycon (*Ty*-EDA)	R_1-H	*dialdehydic form of Elenolic Acid (EDA)*
Flavonols		
(+)-taxifolin		
apigenin	R_1-OH, R_2-H	
luteolin	R_1-OH, R_2-OH	
Lignans		
(+)-pinoresinol	R-H	
(+)-1-acetoxypinoresinol	R-$OCOCH_3$	
(+)-1-hydroxypinoresinol	R-OH	

Tabla 1. Compuestos fenólicos del aceite de oliva y estructura química de cada familia.

Los *ácidos fenólicos*, con la estructura básica C6-C1 (ácidos benzoicos) y C6-C3 (ácidos cinámicos), como los ácidos caféico, vanílico, siríngico, *p*-cumárico, *o*-cumárico, protocatecuico, sinápico y *p*-hidroxibenzoico, fueron el primer grupo de fenoles que se determinaron en aceite [129] confirmándose posteriormente su presencia como componentes minoritarios del aceite de oliva [130-131].

Los *alcoholes fenólicos* más importantes identificados en aceite de oliva son el di-hidroxifenil-etanol (hidroxitirosol, Hyty) y el hidroxifenil-etanol (tirosol, Ty), aunque también se pueden mencionar dentro de esta familia el hidroxitirosol acetato (Hyty-Acet), el tirosol acetato [132] y una forma glucosídica del Hyty [133].

Los *secoiridoides* son los compuestos fenólicos que están en una mayor concentración en el aceite. La mayor parte de esta fracción está compuesta por los derivados de las formas glucosiladas que naturalmente se forman en el fruto: oleuropeína y ligustrósido. La oleuropeína y el ligustrósido se definen como el éster heterosídico del ácido elenólico con el hidroxitirosol y con el tirosol respectivamente, ambos unidos a un residuo glucosídico. Durante el proceso de molienda, trituración, extracción y almacenamiento tiene lugar la hidrólisis del enlace glucosídico y la forma aglicona pasa al aceite [134]. Posteriormente se generan isoformas en la estructura elenólica, algunas de ellas reversibles, conservando el anillo fenólico. La formación de diferentes agliconas, algunas isoformas e isómeros hacen del grupo de los secoiridoides un grupo muy complejo y muchos de estos compuestos no han podido ser identificados completamente.

Así, los secoiridoides más abundantes en el aceite de oliva virgen son la forma dialdehídica del ácido elenólico (EDA) unida al hidroxitirosol o tirosol (Hyty-EDA) o

[129] G. F. Montedoro. "Phenolic substances present in virgin olive oil. Note I. Identification of phenolic acids and their antioxidant power" *Sci. Technol. Aliment*. 3 (1972) 177–186.
[130] G. P. Cartoni, F. Coccioli, R. Jasionowska, D. Ramires. "HPLC analysis of the benzoic and cinnamic acids in edible vegetable oils" *Ital. J. Food Sci.* 12 (2000) 163-173.
[131] A. Carrasco Pancorbo, C. Cruces-Blanco, A. Segura Carretero, A. Fernández Gutiérrez. "Sensitive determination of phenolic acids in extra-virgin olive oil by capillary zone electrophoresis". *J. Agric. Food Chem*. 52 (2004) 6687–6693.
[132] R. Mateos, J. L. Espartero, M. Trujillo, J. J. Ríos, M. León-Camacho, F. Alcudia, A. Cert. "Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection" *J. Agric. Food Chem.* 49 (2001) 2185-2192.
[133] A. D. Bianco, L. Muzzalupo, G. Romeo, M. L. Scarpati, A. Soriero, N. Uccella. "Microcomponents of olive oil. Note 3: glucosides of 2(3,4-dihydroxy-phenyl)ethanol" *Food Chem*. 63 (1998) 461–464.
[134] L. S. Artajo, M. P. Romero, M. Suárez, M. J. Motilva. "Partition of phenolic compounds during the virgin olive oil industrial extraction process". *Eur. Food Res. Technol* 225 (2007) 417-625.

(TY-EDA) y un isómero de la oleuropeína aglicona (Hyty unido al ácido elenólico). Estos compuestos fueron hallados por primera vez por Montedoro y sus colaboradores [135]. Posteriormente han sido también encontrados en aceite la oleuropeína aglicona (Ol Agl) y el ligustrósido aglicona (Lig Agl) con diferentes formas isoméricas y los derivados decarboxilados de ambos compuestos [136, 137]: deacetoxi* oleuropeina aglicona (DOA) y deacetoxi ligustrósido aglicona (D-Lig Agl).

En cuanto a los *flavonoides,* las flavonas apigenina (Apig) y luteolina (Lut), procedentes de la hidrólisis de los correspondientes glucósidos de la pulpa, fueron ya descritas en aceite de oliva en 1976 por Vázquez Roncero y col. [138] y posteriormente varios autores han corroborado su presencia en diferentes trabajos de investigación [139,140].

En el grupo de los *lignanos* el (+)-acetoxipinoresinol (Ac Pin), (+)-pinoresinol (Pin) y (+)-1-hidroxipinoresinol (H-Pin) son los que se han encontrado más frecuentemente en aceite [141,142]. Brenes *et al*., otorgó una importancia elevada a esta fracción cuando sugirió que la cantidad de lignanos podría utilizarse como marcador varietal [143].

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* En bibliografía se encuentra indistintamente "deacetoxi" o "forma decarboxilada" para referirse a los derivados de los secoiridoides sin – $COOCH_3$

[135] G. F. Montedoro, M. Servili, M. Baldioli, R. Selvaggini, E. Miniati, A. Macchioni "Simple and hydrolyzable compounds in virgin olive oil. 3. Spectroscopic characterizations of the secoiridoid derivatives" *J. Agric. Food Chem.* 41 (1993) 2228-2234.

[136] K. De la Torre-Carbot, O. Jauregui, E. Gimeno, A. I. Castellote, R. M. Lamuela-Raventos, M. C. Lopez-Sabater. "Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS". *J. Agric. Food Chem.* 53 (2005) 4331-4340.

[137] P. Rovellini, P. N. Cortesi. "Liquid chromatography-mass spectrometry in the study of oleuropein and ligstroside aglycons in virgin olive oils: aldehydic, dialdehydic forms and their oxidized products" *Riv. Ital. Sostanze Grasse* 69 (2002) 1–14S.

[138] A. Vázquez- Roncero, C. Janer, M. L. Janer. "Componentes fenólicos de la aceituna. III. Polifenoles del aceite". Grasas Aceites 27 (1976) 185-191.

139] M. J. Oliveras-López, M. Innocenti, C. Giaccherini, F. Ieri, A. Romani, N. Mulinacci. "Study of the phenolic composition of Spanish and Italian monocultivar extra virgin olive oils: distribution of lignans, secoiridoid, simple phenols and flavonoids" *Talanta* 73 (2007) 726-732.

[140] D. Ocakoglu, F. Tokatli, B. Ozen, F. Korel. "Distribution of simple phenols, phenolic acids and flavonoids in Turkish monovarietal extra virgin olive oils for two harvest years" *Food Chem.* 113 (2009) 401-410.

[141] R. W. Owen, W. Mier, A. Giacosa, W. E. Hull, B. Spiegelhalder, H. Bartsch. "Identification of lignans as major components in the phenolic fraction of olive oil" *Clin. Chem*. 46 (2000) 976–988.

[142] M. Brenes, F. J. Hidalgo, A. García, J. J. Ríos, P. García, R. Zamora, A. Garrido. "Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil" *J. Am. Oil Chem. Soc*. 77 (2000) 715–720.

[143] M. Brenes, A. García, J. J. Rios, P. García, A. Garrido. "Use of 1-acetoxypinoresinol to authenticate Picual olive oils" *Int. J. Food Sci. Technol*. 37 (2002) 615–625.

6. Importancia de los compuestos fenólicos

Una propiedad muy descrita para los compuestos polifenólicos del aceite de oliva es su actividad antioxidante, aunque estudios *in vitro* han demostrado que posee otras muchas propiedades biológicas que sugieren que podría tener efectos beneficiosos para la salud. Además, los polifenoles son los responsables de las características organolépticas excepcionales que presentan los aceites de oliva vírgenes [144].

6.1. Capacidad antioxidante

El potencial antioxidante de los compuestos fenólicos presentes en el aceite de oliva ha sido una cuestión que ha despertado gran interés, tanto por ser uno de los factores más importantes en lo que a estabilidad oxidativa (shelf-life) de los aceites se refiere como por su efecto quimio-protector en seres humanos.

Las grasas, los aceites y los alimentos con base lipídica, en general, se deterioran al sufrir diversas reacciones de degradación, tanto por calentamiento, como durante su almacenamiento prolongado. Los principales procesos de deterioro son las reacciones de oxidación y de descomposición de los productos de oxidación, que provocan una disminución del valor nutritivo y de la calidad sensorial. Cuando los lípidos se oxidan forman hidroperóxidos, los cuales son susceptibles de una posterior oxidación o descomposición en productos secundarios de la reacción, tales como aldehídos, cetonas, ácidos y alcoholes [145]. Este proceso oxidativo, se potencia por el efecto de fenómenos externos al propio aceite, tales como la presencia de luz, aire, calor y trazas metálicas, y también depende de características intrínsecas del aceite, tales como el grado de instauración y el contenido en antioxidantes naturales. El predominio de ácidos grasos monoinsaturados y la presencia de potentes antioxidantes hacen al aceite de oliva virgen más estable que el resto de aceites vegetales.

Numerosos autores han puesto en evidencia que aceites de oliva con alto contenido total en polifenoles presentan mejor estabilidad frente a la oxidación [146-

[144] A. Bendini, L. Cerretani, A. Carrasco-Pancorbo, A. M. Gómez-Caravaca, A. Segura- Carretero, A. Fernández- Gutiérrez, G. Lercker. "Phenolic molecules in virgin olive oil: a survey on their sensory properties, antioxidant activity and analytical methods to check them. An overview of the last decade" *Molecules* 12 (2007) 1679-1719.
[145] E. N. Frankel. "Lipid oxidation: Mechanism, products and flavor significance" *J. Am. Oil Chem. Soc*. 61 (1984) 1908-1916.
[146] M. Tsimidou, G. Papadopoulos, D. Boskou. "Phenolic compounds and stability of virgin olive oil" *Food Chem* 45 (1992) 141-144.

148]. También se ha investigado el papel de determinados compuestos fenólicos frente a la estabilidad oxidativa [149,150], poniendo de manifiesto su actividad antioxidante de forma individual. En la mayoría de los casos, la mejor correlación se ha observado para aquellos compuestos que presentan en su estructura grupos orto-difenólicos, como el Hyty y sus derivados secoiridoides (Hyty-EDA, Ol Agl and DOA).

La presencia de antioxidantes para mantener la estabilidad oxidativa del aceite se convierte en esencial cuando la grasa se utiliza en operaciones culinarias que usan tratamientos a altas temperaturas, tales como la cocción en horno y la fritura. En trabajos donde se estudió el perfil polifenólico del aceite tras someterlo a tratamientos térmicos a altas temperaturas [151] se observaron los cambios más significativos, de nuevo para el Hyty y sus derivados secoiridoides, que mostraron una reducción rápida de su concentración, lo que confirma su elevado poder antioxidante.

Estos antioxidantes también actúan a nivel celular ayudando a prevenir el daño causado por los radicales libres a los tejidos corporales. El cuerpo produce estos radicales libres porque necesita oxígeno, y la cantidad se incrementa a medida que envejecemos. Estos radicales libres se han relacionado con enfermedades del corazón, cáncer y envejecimiento. A pesar de que el cuerpo produce sus propios antioxidantes, los alimentos que comemos son una fuente importante. En varios estudios en los que se llevó a cabo el aislamiento y la purificación de varios de los componentes de esta fracción, se determinó que estos eran antioxidantes más potentes que los clásicos radicales libres "scavengers" *in vivo* e *in vitro*, vitamina E y dimetil sulfóxido [152].

La actividad antioxidante de los componentes del aceite de oliva virgen se ha relacionado con la protección frente a importantes enfermedades crónicas y

[147] F. Gutiérrez, T. Arnaud, A. Garrido "Contribution of polyphenols to the oxidative stability of virgin olive oil" *J. Sci. Food Agric.* 81 (2001) 1463-1470.
[148] B. Baccouri, W. Zarrouk, O. Baccouri, M. Guerfel, I. Nouairi, D. Krichene, D. Daoud, M. Zarrouk. "Composition, quality and oxidative stability of virgin olive oils from some selected wild olives (Olea europaea L. Subsp. Oleaster)" *Grasas Aceites* 59 (2008) 346-351.
[149] M. H. Gordon, F. Paiva-Martins, M. Almeida. "Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols" *J. Agric. Food Chem*. 49 (2001) 2480-2485.
[150] A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura-Carretero, M. Del Carlo, T. Gallina-Toschi, G. Lercker, D. Compagnone, A. Fernández-Gutiérrez. "Evaluation of the antioxidant capacity of individual phenolic compounds in virgin olive oil" *J. Agric. Food Chem*. 53 (2005) 8918-8925.
[151] A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura- Carretero, G. Lercker, A. Fernández-Gutiérrez. "Evaluation of the influence of thermal oxidation on the phenolic composition and on the antioxidant activity of extra-virgin olive oils" *J. Agric. Food Chem*. 12 (2007) 4771-4780.
[152] F. Visioli, C. Galli. "Free-radical-scavenging actions of olive oil phenolics". *Lipids* 34 (1999) S315-S315.

degenerativas tales como las enfermedades coronarias (CHD), las enfermedades de envejecimiento neuro-degenerativo y tumores localizados en diversas zonas [153].

6. 2. Propiedades saludables

Durante siglos, los pueblos mediterráneos han apreciado los beneficios nutricionales, médicos y cosméticos del aceite de oliva.

Actualmente un número creciente de indicios apuntan al papel crucial que desempeña el aceite de oliva como integrante básico de la dieta Mediterránea, por sus efectos beneficiosos sobre la salud, especialmente en la prevención de enfermedades coronarias y diferentes tipos de cáncer y en otras patologías como la obesidad, la diabetes, etc.

En un principio, dichos efectos se atribuyeron principalmente a su alto contenido en ácidos grasos monoinsaturados, sin embargo, poco a poco ha ido aumentando la hipótesis de que los efectos beneficiosos del aceite de oliva en la dieta Mediterránea podrían no ser enteramente debidos a su composición lipídica, y componentes minoritarios como los polifenoles podrían estar jugando un papel muy importante. Varios estudios han intentado dilucidar la contribución de los compuestos fenólicos a los efectos positivos en la salud atribuidos al aceite de oliva virgen.

- ***Enfermedad cardiovascular***

Las enfermedades cardiovasculares (ECV) constituyen la primera causa de mortalidad y morbilidad en los países desarrollados siendo las responsables de 16.7 millones de defunciones en todo el mundo lo que supone el 29.3% del total. La presencia de radicales libres y el estrés oxidativo juegan un papel muy importante en el desarrollo de la aterosclerosis. Comparando las tasas de mortalidad por ECV de España y otros países del norte de Europa se ha observado que, al igual que otros países del Mediterráneo, España posee unas tasas de mortalidad inferiores. Esta menor incidencia ha sido atribuida a los hábitos dietéticos de la región mediterránea, cuyas principales

[153] S. Cicerale, X. A. Conlan, A. J. Sinclair, R. S. J. Keast. "Chemistry and health of olive oil phenolics". *Crit. Rev. Food Sci. Nutr*. 49 (2009) 218-236.

características son el empleo del aceite de oliva como principal fuente de grasa de la dieta y el elevado consumo de frutas y verduras [154].

Numerosos estudios epidemiológicos y de intervención llevados a cabo en sujetos humanos han mostrado que la sustitución de las grasas saturadas en la dieta por el aceite de oliva dio lugar a un descenso de los niveles de colesterol-LDL (lipoproteínas de baja densidad) [155,156] que fue acompañado de un aumento de colesterol-HDL (lipoproteínas de alta densidad), que ejerce un papel protector, ya que transporta el "colesterol malo" (LDL) -depositado en las arterias- hasta el hígado para su eliminación, reduciendo los riesgos de trombosis arterial y de infarto. En un principio, tales efectos fueron atribuidos mayoritariamente a los AGMI, sin embargo, un elevado número de trabajos científicos posteriores han evidenciado el posible papel de las sustancias antioxidantes, en concreto de los polifenoles, en relación con la enfermedad cardiovascular y el desarrollo de la aterosclerosis [157].

Las LDL, son sólo realmente nocivas cuando se oxidan, desempeñando esta oxidación un papel crucial en el desarrollo de la aterosclerosis. Si esto ocurre, se forman unas partículas que crean una placa que se acumula y aumentan increíblemente las posibilidades de bloquear una arteria. Diversos estudios de intervención en humanos han mostrado un efecto protector de los polifenoles del aceite de oliva virgen frente a la oxidación de la LDL [158 - 160] gracias a su actividad antioxidante. Entre los compuestos fenólicos presentes en el aceite de oliva, tienen especial interés aquellos que

[154] M. I. Covas, V. Konstantinidou, M. Fito. "Olive oil and cardiovascular health" *J. Cardiovasc. Pharmacol*. 54 (2009) 477-482.
[155] P. M. Kris-Etherton, T. A. Pearson, Y. Wan, R. L. Hargrove, K. Moriarty, V. Fishell, T. D. Etherton. "High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations" *Am. J. Clin. Nutr*. 70 (1999)1009-1015.
[156] A. Trichopoulou, D. Corella, M. A. Martínez-González, F. Soriguer, J. M. Ordovas. "The mediterranean diet and cardiovascular epidemiology" *Nutr. Rev.* 64 (2006) S13-S19.
[157] C. Manach, A. Mazur, A. Scalbert. "Polyphenols and prevention of cardiovascular diseases" *Curr. Opin. Lipidology* 84 (2006) 77-84.
[158] M. C. Ramirez-Tortosa, G. Urbano, M. López-Jurado, T. Nestares, M. C. Gómez, A. Mir, E. Ros, J. Mataix, A. Gil. "Extra-virgin olive oil increases the resistance of LDL to oxidation more than refined olive oil in free-living men with peripheral vascular disease" *J. Nutr.*129 (1999) 2177-2183.
[159] R. Llenen, A. J. C. Roodenburg, M. N. Vissers, J. A. Schuubiers, K. P. A. M. Van Putte, S. A. Wiseman, F. H. M. M. Van de Put. "Supplemetation of plasma with olive oil phenols and extracts: influence on LDL oxidation" *J. Agric. Food Chem*. 50 (2002) 1290-1297.
[160] J. Marrugat, M. I. Covas, M. Fitó, H. Schroder, E. Miró-Casas, E. Gimeno, M. C. López- Sabater, R. de la Torre, M. Farre. "Effects of differing phenolic content in dietary olive oils on lipids and LDL oxidation-a randomized controlled trial" *Eur. J. Nutr*. 43 (2004) 140-147.

poseen grupos orto-difenólicos, principalmente la oleuropeína y el Hyty, por ser grandes inhibidores de la oxidación de la LDL *in vitro* [161].

- ***Cáncer***

El cáncer representa otra de las principales causas de muerte en los países desarrollados donde además su incidencia es creciente, constituyendo en la actualidad un problema sanitario de primera magnitud. En líneas generales se espera que una cuarta parte de los hombres y una quinta parte de las mujeres tenga un proceso relacionado con el cáncer a lo largo de su vida.

Varios estudios han mostrado una menor incidencia del cáncer en los países mediterráneos, en concreto España, Italia, Portugal y Grecia, en comparación con otros del norte de Europa y los Estados Unidos. Estos datos llevaron a pensar que la dieta mediterránea podría estar relacionada con la baja incidencia de esta enfermedad y estudios epidemiológicos demostraron la posibilidad de una moderada, pero significativa, reducción en el riesgo de padecer cáncer a través de la ingesta de uno de los ingredientes principales de la dieta mediterránea, el aceite de oliva, prestando especial atención a su posible papel en la prevención del cáncer de mama [162,163].

Los primeros estudios existentes en la literatura científica sobre aceite de oliva y cáncer estaban orientados a estudiar el posible efecto beneficioso de dicho aceite gracias a su alto contenido en ácidos grasos monoinsaturados que inhiben el crecimiento tumoral y potencian los mecanismos de apoptosis, sugiriendo un efecto favorable en la prevención y desarrollo de cáncer, principalmente de aquellos afectados por la dieta (mama, colon, próstata) [164-166].

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[161] F. Visioli, C. Galli. "Oleuropein protects low density lipoprotein from oxidation" *Life Sci*. 55 (1994) 1965-1971.
[162] J. M. Martin-Moreno, P. Willett, L. Gorgojo. "Dietary fat, olive oil intake and breast cancer risk" *Int. J. Cancer* 58 (1994) 774-780.
[163] C. La Vecchia, E. Negri, S. Franceschi. "Olive oil and other dietary fats, and the risk of breast cancer" *Cancer Causes Control* 6 (1995) 545-550.
[164] M. Hughes-Fulford, Y. Chen, R. R. Tjandrawinata. "Fatty acid regulates gene expression and growth of human prostate cancer PC.3 cells". *Carcinogenesis* 22 (2001) 701-707.
[165] T. Yamaki, T. Yano, H. Satoh, T. Endo, C. Matsuyama, H. Kumagai, M. Miyahara, H. Sakurai, J. Pokorny, S. J. Shin, K. Hagiwara. "High oleic acid oil suppresses lung tumorigenesis in mice through the modulation of extracellular signal-regulated kinase cascade" *Lipids* 37 (2002) 783-788.
[166] X. Llor, E. Pons, A. Roca, M. Alvarez, J. Mane, F. Fernández-Banares, M. A. Gassull. "The effects of fish oil, olive oil, oleic acid and linoleic acid on colorectal neoplastic processes" *Clin. Nutr.* 22 (2003) 71-79.

Recientes experimentos de laboratorio llevados a cabo por Menéndez y col. [167] sobre células de mama cancerosas demuestran que el ácido oleico reduce drásticamente los niveles del oncogen denominado Her-2/neu que se halla en grandes proporciones en más de la quinta parte de las pacientes con cáncer de mama y se le asocia con tumores altamente agresivos. El ácido oleico no sólo logró reprimir la actividad del gen, también impulsó la efectividad de un fármaco contra el cáncer de mama llamado herceptina, que ha sido vital en la prolongación de las vidas de muchas pacientes.

Hasta hace poco tiempo los estudios sobre el efecto beneficioso del aceite de oliva virgen habían sido dirigidos únicamente a la fracción saponificable (lípidos) y se había prestado muy poca atención a la fracción no saponificable, rica en sustancias con alta capacidad antioxidante. Evidencias científicas han demostrado que el estrés oxidativo y la generación de radicales libre juegan un importante papel en la iniciación y progresión del cáncer, por lo que la presencia de antioxidantes como los polifenoles podría tener la habilidad de destruir las sustancias que lideran la proliferación de células cancerígenas. De ahí que en la última década, hayan aumentado los estudios experimentales que analizan el efecto de estas sustancias en la promoción y progresión de la carcinogénesis [168,169]. Investigaciones epidemiológicas y ensayos clínicos en humanos [170, 171] han demostrado las propiedades antioxidantes de los polifenoles presentes en el aceite de oliva, midiendo biomarcadores de oxidación directamente relacionados con cualquier proceso cancerígeno como la 8-oxo-dG (8-Oxo-7,8-dihydro-2'-deoxyguanosine), que es la lesión oxidativa más frecuentemente encontrada en el ADN.

Además resulta interesante establecer el efecto de estos compuestos a nivel molecular por lo que en los últimos 5 años, de forma paralela a los trabajos recogidos en

[167] J. A. Menéndez, L. Vellon, R. Colomer, R. Lupu. "Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu oncogen amplification" *Ann. Oncol*. 16 (2005) 359-371.
[168] C. S Yang, J. M. Landau, M. T. Huang, H. L. Newmark. "Inhibition of carcinogenesis by dietary polyphenolics compounds" *Annu. Rev. Nutr*. 21 (2001) 381-406.
[169] R. W. Owen, A. Giacosa, W. E. Hull, R. Haubner, B. Spiegelhalder, H. Bartsch. "The antioxidant/anticancer potential of phenolic compounds isolated from olive oil" *Eur. J. Cancer* 36 (2000) 1235-1247.
[170] T. Weinbrenner, M. Fitó, R. De la Torre, G. T. Sáez, P. Rijken, C. Tormos, S. Coolen, M. Farré-Albadalejo, S. Abanades, H. Schroder, J. Marrugat, M. I. Covas. "Olive oils high in phenolic compounds modulate oxidative/antioxidative status in men" *J. Nutr*. 134 (2004) 2314-2321.
[171] T. G. Sotiroudis, S. A. Kyrtopoulos, A. Xenakis, G. T. Sotiroudis. "Chemopreventive potential of minor components of olive oil against cancer" *Ital. J. Food Sci.* 15 (2003) 169-185.

esta memoria de tesis, varios grupos han investigado la activada anticancerígena de los polifenoles del aceite de oliva en estudios in vitro con diferentes tipos de líneas cancerígenas : colon [172], mama [173], leucemia [174] etc.

Los resultados obtenidos muestran que los polifenoles son capaces de afectar al ciclo global de la carcinogéneis, y los mecanismos que pueden estar implicados son la modificación de la respuesta genética, modulación de la cascada inflamatoria oxidativa, control de la proliferación y diferenciación celular y cambios en las estructuras y función de las membranas celulares.

Todavía queda mucho por trabajar en este campo, sería importante profundizar y entender los mecanismos moleculares que explicasen por qué estos compuestos reducen la incidencia de determinados tipos de tumores.

- ***Otras propiedades saludables***

Se han descrito efectos de algunos de los compuestos polifenólicos del aceite de oliva en la *inflamación y la agregación plaquetaria*. El efecto anti-inflamatorio (similar al ibuprofeno) que exhibe un compuesto fenólico presente en el aceite de oliva llamado "oleocanthal" (D-Lig Agl) fue puesto de manifiesto por Beauchamp y col. [175]. En cuanto a la inhibición de la agregación plaquetaria, se puso de manifiesto en estudios *in vitro* donde el Hyty y la oleuropeína mostraron una actividad comparable a la del ácido acetilsalicílico (aspirina) con una concentración inhibitoria del 50%, [176].

Se han descrito, asimismo, propiedades antimicrobianas *in vitro* frente a varios agentes infecciosos del tracto gastrointestinal y respiratorio mostrando concentraciones

[172] Z. H Yumi, Y. Hashim, I. R. Rowland, H. McGlynn, M. Servili, R. Selvaggini, A. Taticchi, S. Esposto, G. F. Montedoro, L. Kaisalo, K. Wähälä and C. I. R. Gill. "Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro" *Int. J. Cancer* 122 (2008) 495–500.
[173] J. Han, T. P. N. Talorete, P. Yamada, H. Isoda. "Antiproliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells" *Cytotechnology* 59 (2009) 45-53.
[174] R. Fabiani, A. De Bartolomeo, P. Rosignoli, M. Servili, R. Selvaggini, G. F. Montedoro, C. Di Saverio, G. Morozzi. "Virgin olive oil phenols inhibit proliferation of human promyelocytic leukaemia cells (HL60) by inducing apoptosis and differentiation" *J. Nutr*. 136 (2006) 614–619.
[175] G. K. Beauchamp, R. S. J. Keast, D. Morel, J. Lin, J. Pika, Q. Han, C.H. Lee, A. B. Smith, P. A. S. Breslin. "Ibuprofen-like activity in extra-virgin olive oil". *Nature* 437 (2005) 45–46.
[176] A. Petroni, M. Blasevich, M. Salami, N. Napini, G. F. Montedoro, C. Galli. "Inhibition of platelet-aggregation and eicosanoid production by phenolic components of olive oil". *Thromb. Res.* 78 (1995) 151-160.

mínimas inhibitorias que, en algunos casos, fueron inferiores a las que presentaron antibióticos como la ampicilina [177].

La presencia de antioxidantes inhiben los radicales libres, responsables del deterioro dermatológico, por lo que previene contra el *envejecimiento* y además puede evitar el deterioro cognitivo relacionado con la edad y la demencia.

De igual modo, el aceite de oliva juega un importante papel en la *diabetes*. La investigación ha demostrado que las personas que en su dieta disfrutan del aceite de oliva, tienen un mejor control sobre su diabetes y niveles más bajos de algunas grasas en la sangre.

El aceite de oliva tiene un efecto protector definitivo en el metabolismo, las arterias, el estómago y bilis. Su consumo habitual favorece la absorción del calcio y la mineralización de los huesos**,** contribuyendo, de esta forma, al correcto desarrollo óseo en los más pequeños y a la prevención de la osteoporosis y otras enfermedades degenerativas. Tiene un efecto único sobre los lípidos del suero sanguíneo. Además, parece tener un efecto colagógico (expulsión de la bilis) y un efecto terapéutico sobre las úlceras pépticas.

6.3. Características sensoriales

Por otra parte, los polifenoles también contribuyen a las propiedades organolépticas de los aceites de oliva vírgenes y han sido descritos como "amargos" y "astringentes" [178,179], así como responsables de características organolépticas en general [180]. Menos conocida es su faceta "picante" asociada a sensaciones que "queman" al gusto [181,182].

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[177] G. Bisignano, A. Tomaino, R. Lo Cascio, G. Crisafi, N. Uccella, A. Saija. "On the invitro antimicrobial activity of oleuropein and hydroxytyrosol". *J. Pharm. Pharmacol.* 51 (1999) 971-974.

[178] F. Gutiérrez-Rosales, S. Perdiguero, R. Gutiérrez, J. M. Olías. "Evaluation of the bitter taste in virgin olive oil"*J. Am. Oil Chem. Soc*. 69 (1992) 394–395.
[179] F. Gutiérrez-Rosales, J. J. Ríos, M. L. Gómez-Rey. "Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry" *J. Agric. Food Chem.* 51 (2003) 6021–6025.
[180] D. Ryan, K. Robards. "Phenolic compounds in olives" *Analyst* 123 (1998) 31R–44R.
[181] M. Servili, G. Montedoro. "Contribution of phenolic compounds to virgin olive oil quality" *Eur. J. Lipid Sci. Technol*. 104 (2002) 602–613.
[182] P. Andrewes, J. L. H. C. Busch, T. de Joode, A. Groenewegen, H. Alexandre. "Sensory properties of virgin olive oil polyphenols: Identification of deacetoxy-ligstroside aglycon as a key contributor to pungency" *J. Agric. Food Chem*. 51 (2003) 1415–1420.

A pesar de todo esto, la relación exacta e inequívoca entre las características sensoriales y los fenoles más hidrofílicos del aceite de oliva está aún por definir. Varios autores han asociado la característica negativa de flavor "atrojado" a la presencia de ácidos fenólicos en el aceite [183], mientras que otros estudios no demostraban ninguna relación entre la sensación de "amargor" y el contenido en ácido fenólicos de un aceite [184].

Las relaciones existentes entre los derivados de los secoiridoides y el amargor han sido igualmente estudiadas; primero, el interés se focalizó en dos derivados de la oleuropeína y de la demetiloleuropeína, tales como Hyty-EDA y Ty-EDA [185,186]. En este caso, García y col. estudiaron la reducción del amargor del aceite mediante un tratamiento térmico de las aceitunas y encontraron una buena correlación entre el amargor del aceite y el contenido de derivados secoiridoides del Hyty.

En posteriores estudios, se observó que existe relación entre las propiedades sensoriales de amargor y picante y el contenido en derivados del ligustrósido [187] y el contenido de la forma aldehídica de la Ol Agl [188].

7. Análisis del perfil polifenólico: técnicas analíticas más utilizadas

La relación de los compuestos fenólicos del aceite de oliva virgen con su estabilidad oxidativa, propiedades saludables y características organolépticas despertaron el interés en su determinación, tanto desde un punto de vista cualitativo, como cuantitativo. Esta compleja fracción fenólica es muy heterogénea, al estar constituida por un elevado número de compuestos muy variados, por lo que los métodos y técnicas analíticas que se han empleado para identificarlos y cuantificarlos son también muy diversos.

[183] E. Graciani-Costante, A. Vázquez-Roncero. "Cromatografía líquida de alta eficacia (HPLC). III. Aplicación a diversos tipos de aceites vírgenes" *Grasas Aceites* 32 (1981) 365–371.
[184] N. Uccella. A. H. Spanier, F. Shahidi, T. H. Parliment, C. J. Mussinan, C. T. Ho, E. Tratas Conti, "Food Flavours and Chemistry: Advances of the New Millenium". The Royal Society of Chemistry Publishers, Cambridge, UK, (2001).
[185] A. K. Kiritsakis. "Flavor components of olive oil - A review" *J. Am. Oil Chem. Soc*. 75 (1998) 673–681.
[186] J. M. García, K. Yousfi, R. Mateos, M. Olmo, A. Cert. "Reduction of oil bitterness by heating of olive (Olea europaea) fruits" *J. Agric. Food. Chem*. 49 (2001) 4231–4235.
[187] M. J. Tovar, M. J. Motilva, M. P. Romero. "Changes in the phenolic composition of virgin olive oil from young trees (Olea europaea L. cv. Arbequina) grown under linear irrigation strategies" *J. Agric. Food. Chem*. 49 (2001) 5502–5508.
[188] R. Mateos, A. Cert, M. C. Pérez-Camino, J. M. García. "Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives" *J. Am. Oil Chem. Soc*. 81 (2004) 71–75.

Las técnicas analíticas empleadas en el análisis de fenoles del aceite de oliva incluyen desde las primeras determinaciones espectrofotométricas, con las que se determinaba el contenido total de polifenoles, hasta las más modernas técnicas separativas acopladas a distintos sistemas de detección, pasando por cromatografía en papel o en capa fina [189]. La transición desde los métodos espectrofotométricos hasta el empleo de métodos analíticos más específicos (HPLC, GC), vino motivada por la necesidad de separar e identificar los compuestos fenólicos individualmente. Además de las dos técnicas cromatográficas por excelencia, recientemente la electroforesis capilar se ha aplicado con gran éxito al análisis de fenoles en aceite de oliva

Como puede verse en la Tabla 1, sin lugar a dudas, HPLC acoplada con distintos sistemas de detección ha sido la técnica más empleada para llevar a cabo el análisi de estos compuestos.

Técnica analítica empleada	**Porcentaje respecto al total de artículos publicados (usando técnicas separativas)**	**Detectores empleados**
GC	≈15%	FID y MS
HPLC	>80%	UV, fluorescencia, detección electroquímica, biosensores, RMN y MS
CE	<5%	UV y MS

Tabla 2. Uso relativo de cada técnica separativa y distintos sistemas de detección para llevar a cabo el análisis de los compuestos fenólicos presentes en el aceite de oliva. Porcentajes calculados tras realizar búsquedas exhaustivas en ISI, Scopus y Scirus.

En HPLC, la correcta separación de los compuestos fenólicos del aceite de oliva se lleva a cabo en fase inversa, normalmente con columnas C18, utilizando como fase móvil un disolvente polar. En algunas ocasiones. la elución isocrática ha proporcionado una buena resolución, aunque en la mayoría de las aplicaciones se requiere la utilización de un gradiente consistente en. al menos. 2 disolventes: un ácido diluido y un disolvente orgánico. Como sistema de detección se comenzó empleando fundamentalmente la detección UV [190- 192], pero con la aparición de la MS, que permite obtener datos de

[189] A. Carrasco-Pancorbo, L. Cerretani, A. Bendini, A. Segura- Carretero, T. Gallina- Toschi, A. Fernández- Gutiérrez. "Analytical determination of polyphenols in olive oil" *J. Sep. Sci.* 28 (2005) 837-858.
[190] M. Tsimidou, G. Papadopoulos, D. Boskou. "Determination of phenolic-compounds in virgin olive oil by reversed-phase HPLC with emphasis on UV-detection". *Food Chem*. 44 (1992) 53-60.
[191] R. Mateos, J. L. Espartero, M. Trujillo, J. J. Ríos, M. León-Camacho, F. Alcudia, A. Cert. "Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-

masa molecular, facilitando la identificación de estos compuestos, el número de aplicaciones con este detector ha ido en aumento [193- 195]. Se han utilizado diferentes tipos de interfases, aunque la mayoría de las aplicaciones emplean ESI.

La determinación cualitativa y cuantitativa de los compuestos fenólicos del aceite de oliva utilizando GC está mucho menos extendida, probablemente debido a la necesidad de la derivatización previa de los compuestos antes del análisis. Sin embargo, como técnica complementaria a la cromatografía líquida, puede aportar información muy interesante sobre el complejo perfil polifenólico del aceite de oliva. En los métodos analíticos estandarizados, el sistema de detección más ampliamente usado es la ionización de llama (FID) pero el uso de la espectrometría de masas como sistema de detección, al igual que ocurría en HPLC, ha ganado mucho interés en los últimos años [196,197]. Las interfases más ampliamente utilizadas han sido ionización química y especialmente el impacto electrónico.

En cuanto a la electroforesis capilar tan sólo tres grupos de investigación, dos italianos y el nuestro perteneciente a la Universidad de Granada se han ocupado del estudio de los polifenoles del aceite utilizando esta técnica. Se ha usado principalmente la modalidad de electroforesis capilar en zona con detección ultravioleta [198] o acoplada a espectrometría de masas [199], obteniendo resultados muy satisfactorios y

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performance liquid chromatography with diode array ultraviolet detection" *J. Agric. Food Chem.* 49 (2001) 2185-2192

[192] M. Tasioula-Margari, O. Okogeri. "Simultaneous determination of phenolic compounds and tocopherols in virgin olive oil using HPLC and UV detection" *Food Chem.* 74 (2001) 377-383.

[193] F. Gutiérrez-Rosales, J. J. Ríos, M. L. Gómez-Rey. "Main polyphenols in the bitter taste of virgin olive oil. Structural confirmation by on-line high-performance liquid chromatography electrospray ionization mass spectrometry" J. *Agric. Food Chem* 51 (2003) 6021-6025.

[194] M. Bonoli , A. Bendini , L. Cerretani, G. Lercker , T. G. Toschi . "Qualitative and semiquantitative analysis of phenolic compounds in extra virgin olive of as a function of the ripening degree of olive fruits by different analytical techniques" J. *Agric. Food Chem.* 52 (2004) 7026-7032.

[195] K. De la Torre, O. Jáuregui, E. Gimeno, A. I. Castellonte, R. M. Lamuela-Raventós, M. C. López-Sabater. "Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD and HPLC-MS/MS" J. *Agric. Food Chem.* 53 (2005) 4331-4340.

[196] L. Liberatore, G. Procida, N. D'Alessandro, A. Cichelli. "Solid-phase extraction and gas chromatographic analysis of phenolic compounds in virgin olive oil" *Food Chem.* 73 (2001) 119-124.

[197] J. J. Ríos, M. J. Gil, F. Gutiérrez-Rosales. "Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products" *J. Chromatogr. A* 1093 (2005) 167-176.

[198] A. Carrasco-Pancorbo, A. M. Gómez-Caravaca, L. Cerretani, A. Bendini, A. Segura-Carretero, A. Fernández-Gutiérrez; "A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra virgin olive oil" *J. Sep. Sci.* 29 (2006) 2221–2233.

[199] A. Carrasco-Pancorbo, C. Neususs, M. Pelzing, A. Segura-Carretero, A. Fernández-Gutiérrez. "CE- and HPLC-TOF-MS for the characterization of phenolic compounds in olive oil" *Electrophoresis* 28 (2007) 806-821.

permitiendo la identificación y cuantificación de diferentes familias de compuestos fenólicos.

En los últimos diez años, MS y RMN se han convertido en técnicas indispensables para estudiar los perfiles fenólicos debido a la necesidad de emplear detectores con la capacidad de poder dar información estructural acerca de las moléculas bajo estudio e incluso identificarlas.

Como se ha indicado anteriormente, la espectrometría de masas se ha aplicado al análisis de compuestos fenólicos del aceite de oliva principalmente como sistema de detección después de la separación mediante otras técnicas como HPLC, GC o CE, usando distintos métodos de ionización (APCI, ESI, EI, CI, etc.) que transferían los iones a diferentes analizadores (Q, IT, TOF, etc.). Sin embargo, algunos estudios recientes emplean la infusión directa con ionización por electrospray (ESI-MS) seguida de técnicas de análisis multivariante para la clasificación de muestras de aceites, como una alternativa a las técnicas utilizadas tradicionalmente reduciendo el tiempo de análisis [200] al prescindir de la separación cromatográfica o electroforética.

La resonancia magnética nuclear (RMN) de alta resolución mono o bidimensional ha sido utilizada en el análisis de mezclas complejas de varios extractos de alimentos que contienen fenoles. Christophoridou y col. [201] han publicado la primera aplicación del acoplamiento LC-SPE-RMN usando extracción en fase sólida post-columna para el análisis directo de compuestos fenólicos presentes en la fracción polar del aceite de oliva. Este grupo de investigación utilizó el mencionado acoplamiento para identificar nuevos compuestos en la fracción estudiada.

8. Biodisponibilidad de los polifenoles del aceite de oliva

Existen evidencias claras que correlacionan el consumo de aceite de oliva con la prevención de ciertas enfermedades, siendo los polifenoles uno de los principales responsables de este efecto promotor de la salud. Sin embargo, la posibilidad de que

[200] M. J. Lerma- García, J. M. Herreo-Martínez, G. Ramis-Ramos, E. F. Simo-Alfonso. "Prediction of the genetic variety of Spanish extra virgin olive oils using fatty acid and phenolic compound profiles established by direct infusión mass spectrometry" *Food Chem*. 108 (2008) 1142-1148.
[201] S. Christophoridou, P. Dais, L. H. Tseng, M. Spraul. "Separation and identification of phenolic compounds in olive oil by coupling high-performance liquid chromatography with postcolumn solid-phase extraction to nuclear magnetic resonance spectroscopy (LC-SPE-NMR)" *J. Agric. Food Chem.* 53 (2005) 4667-4679

sustancias antioxidantes, como los polifenoles, ejerzan efectos positivos en la salud va a depender de su **biodisponibilidad**, es decir, de que se absorban pasando al torrente sanguíneo y de que alcancen de forma activa los tejidos implicados. El término biodisponibilidad ha sido tomado del campo de la farmacología, donde fue definido originariamente como "proporción y extensión de un principio activo determinado que alcanza el sitio de acción". Este concepto es generalmente física y éticamente inalcanzable en humanos y como resultado, la definición farmacológica ha sido reformulada como "la fracción de una determinada dosis oral (compuesto original o sus metabolitos) de una preparación determinada que alcanza la circulación sistémica".

Aunque para la mayoría de los polifenoles presentes en alimentos existe información limitada sobre los mecanismos involucrados en su biodisponibilidad, en los últimos años se ha realizado un importante esfuerzo en la comprensión de la absorción y el metabolismo de estos compuestos. Manach y sus col [202] efectuaron una revisión interesante de los estudios publicados sobre la absorción, metabolismo, distribución tisular y eliminación de diferentes familias de compuestos fenólicos. Revisiones similares se han llevado a cabo para los polifenoles del aceite de oliva [203,204]. En líneas generales, hay evidencias de que agliconas y compuestos fenólicos simples, son absorbidos directamente a través de la pared intestinal y conjugados en el propio epitelio intestinal por metilación, sulfatación y/ o glucuronidación. Una vez cruzada la barrera intestinal, algunos compuestos sufren un proceso adicional de metabolización en el hígado. Los compuestos polifenólicos no absorbidos en el intestino delgado pasan al colon donde pueden ser metabolizados por la flora bacteriana. Además, los compuestos absorbidos y metabolizados en el hígado pueden regresar al intestino vía circulación enterohepática y alcanzar, así, el colon en una forma química diferente. El colon posee un enorme potencial catalítico e hidrolítico. Las reacciones de desconjugación de los enlaces glicosídicos tienen lugar rápidamente liberando los agliconas, aunque también hay evidencias de que algunos glicosídicos pueden ser absorbidos directamente por el intestino delgado, e incluso algunas bacterias son capaces de hidrolizar los agliconas hacia compuestos fenólicos más sencillos, como ácidos fenilacéticos y fenilpropiónicos,

[202] C. Manach, A. Scalbert, C. Morand, C. Rémesy, L. Jiménez. "Polyphenols: food sources and bioavailability" *Am. J. Clin. Nutr.* 79 (2004) 727-747.
[203] M. N. Vissers, P. L. Zock, M. B. Katan. "Bioavailability and antioxidant effects of olive oil phenols in humans: a review" *Eur. J. Clin. Nutr*. 58 (2004) 955-965.
[204] G. Corona, J. P. E. Spencer, M. A. Dessi. "Extra virgin olive oil phenolics: absorption, metabolism, and biological activities in the GI tract" *Toxicol. Ind. Health* 25 (2009) 285-293.

que pueden ser absorbidos y metabolizados. En el caso concreto de los polifenoles del aceite de oliva estudios llevados a cabo con sujetos sanos y pacientes con ileostomía (extirpación del colon) indicaron que la mayor parte de la absorción de los polifenoles tiene lugar en el intestino delgado [205]. Poco se sabe sobre el metabolismo de estos polifenoles a nivel del colon, aunque algunos autores piensan que los secoiridoides no absorbidos en el intestino delgado pueden sufrir una degradación por la microflora del colon dando lugar al Hyty en forma libre [206]. En cuanto a las formas agliconas de estos compuestos, se piensa que parte pueden ser hidrolizados a sus formas más simples por los ácidos del estómago y otra parte absorbidas intactas en el intestino delgado, aunque no existen estudios que lo confirmen. Una vez metabolizados en el hígado los compuestos pasan a la sangre y se distribuyen a otros órganos y tejidos, alcanzando por último el riñón donde son excretados con la orina. Tanto el contenido plasmático como el urinario de un compuesto determinado pueden ser usados para reflejar su absorción en el tracto gastrointestinal

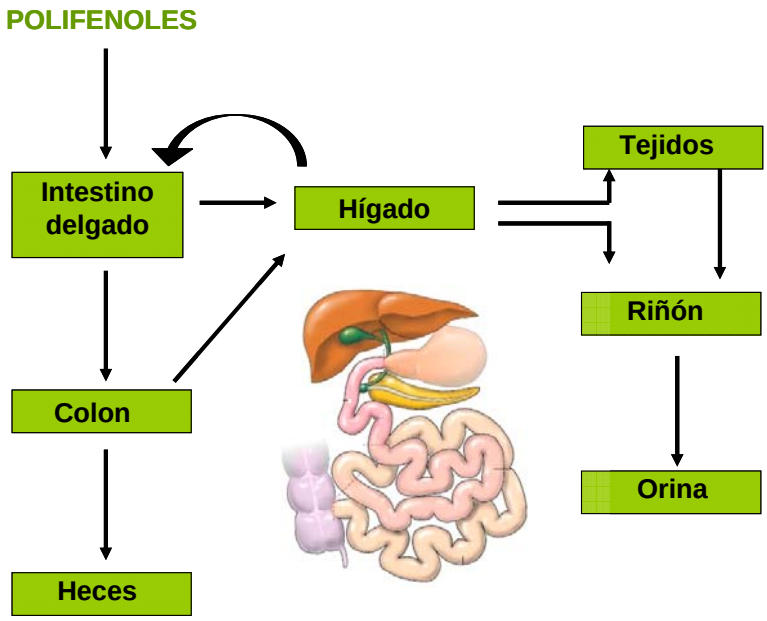

Figura 3. Posibles rutas de los polifenoles ingeridos en la dieta en humanos

[205] M. N. Vissers, P. L. Zock, A. J. C. Roodenburg, R. Leenen, M. B. Katan. "Olive oil phenols are absorbed in humans" *J. Nutr.* 132 (2002) 409-417.
[206] G. Corona, X. Tzounis, M. A. Dessi, M. Deiana, E. S. Debnam, F. Visioli, J. P. E. Spencer "The fate of olive oil polyphenols in the gastrointestinal tract: implications of gastric and colonic microflora-dependent biotransformation" *Free Radic. Res.* 40 (2006) 647-658.

Los factores bioquímicos que afectan a la biodisponibilidad de los polifenoles y que dan lugar a resultados a veces contradictorios, podrían ser:

- La absorción intestinal, que depende de muchos factores, como el pH o la estructura del polifenol y que se puede ver modificada si el compuesto está glicosilado.
- Las reacciones metabólicas que sufren las formas libres o agliconas en intestino, hígado o riñón. Estas reacciones de metilación, sulfatación o combinación con otros compuestos, como glucurónido están mediadas por enzimas del metabolismo.
- La formación de importantes metabolitos por la flora intestinal del colon.

La biodisponibilidad de los polifenoles puede ser muy diferente dependiendo del tipo de compuesto y de su estructura concreta.

Con respecto a los polifenoles del aceite de oliva encontramos en bibliografía estudios de biodisponibilidad en humanos y animales, principalmente de los fenoles simples Hyty y Ty. Los estudios llevados a cabo en animales de experimentación con compuestos aislados han permitido sugerir vías de metabolización para estos compuestos. En 1998 Bai y col [207] detectaron e identificaron por primera vez hyty en plasma de ratas, 10 min después de la administración oral del compuesto puro, resultados que fueron corroborados por Ruiz-Gutierrez y col [208]. En estudios posteriores se analizó la excreción urinaria de Hyty y Ty sintetizados y marcados radioisotópicamente previa administración por vía oral e intravenosa, empleando ratones como biomodelo [209]. Las recuperaciones del Hyty y Ty (tanto del compuesto original como los metabolitos) en orina recogida 24 h después de la administración fueron diferentes según si los compuestos eran administrados en agua (75% y 71% respectivamente) o en la propia matriz del aceite de oliva (99% y 98% respectivamente), poniendo en evidencia la importancia de la matriz en la biodisponibilidad de los compuestos. De acuerdo con estos trabajos parte del Hyty es eliminado sin sufrir

[207] C. Bai, X. Yan, M. Takenanka, K. Sekiya, T. Nagata. "Determination of synthetic hydroxytyrosol in rat plasma by GC-MS" *J. Agric. Food Chem*. 46 (1998) 3998-4001.
[208] V. Ruiz-Gutiérrez, M. E. Juan, A. Cert, J. M. Planas. "Determination of hydroxytyrosol in plasma by HPLC" *Anal. Chem*. 72 (2000) 4458-4461.
[209] K. L. Tuck, M. P. Freeman, P. J. Hayball, G. L. Strecht, I. Stupans. "The in vivo fate of hydroxytyrosol and tyrosol, antioxidant phenolic constituents of olive oil, after intravenous and oral dosing of labeled compounds to rats" *J. Nutr*. 131 (2001) 1993-1996.

cambios y otra parte se metaboliza, identificando como posibles metabolitos los derivados monosulfato, glucurónido y el ácido homovanílico [210]. En otro trabajo, empleando también Hyty marcado radioisotópicamente se estudió la distribución, absorción y metabolismo de este compuesto en diferentes órganos y tejidos (cerebro, corazón, riñones, hígado, pulmón…) así como su presencia en orina y plasma de rata. Se observó una rápida distribución (máxima radioactividad 5 min después de la administración) y se pudieron identificar derivados oxidados y metilados del Hyty (DOPET, DOPAL, DOPAC, MOPET, HVA), además de la presencia de sulfoconjugados, especialmente en orina [211]. Otros estudios con ratas que recibieron soluciones orales de oleuropeína [212] demuestran que parte de este compuesto se absorbe y aparece en plasma como tal e hidrolizado y convertido en Hyty, mientras que en orina ambos compuestos aparecen como derivados glucurónidos.

Aun cuando la información obtenida en estos estudios con animales es sumamente útil, un enfoque más adecuado desde un punto de vista nutritivo, es la administración a humanos y su identificación posterior en plasma u orina. Algunos estudios han demostrado la biodisponibilidad del Hyty y Ty del aceite de oliva en humanos, después de una dieta suplementada y a partir de la ingestión de aceite de oliva en su forma natural. Los primeros estudios llevados a cabo por Visioli y col [213] demuestran que Hyty y Ty del aceite de oliva son absorbidos de forma dosis-dependiente y excretados en orina principalmente en forma conjugada. La concentración de estos compuestos fenólicos aumenta rápidamente tras la ingesta de aceite de oliva y alcanza un pico en plasma aproximadamente 1 hora después de la ingesta [214] y entre 0-2 horas en orina, lo que vuelve a corroborar que el principal lugar de absorción de los polifenoles del aceite es el intestino delgado [215,216]. Más

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recientemente, Visioli y col [217] administraron soluciones de polifenoles vía oral, empleando distintos vehículos, poniendo de nuevo de manifiesto, que la absorción de Hyty es mucho mayor cuando el vehículo es lo más similar posible a la matriz natural. Aproximadamente el 98% del Hyty y Ty están presentes en plasma y orina en su forma conjugada, principalmente glucuronoconjugados, aunque también se han encontrado derivados metilados como el 3-O-methyl hydroxytyrosol, ácido homovanílico y el alcohol homovanílico [214, 218]. La presencia de estos metabolitos sugiere que las formas primarias ingeridas han sufrido metabolismo intestinal y/o hepático. Para estudiar ambos metabolismos sin necesidad de utilizar modelos animales y ensayos clínicos, más caros, se utilizan modelos de cultivo celulares de enterocitos (CaCo2) [219] y de hepatocitos (HepG2) [220], incubados con los polifenoles bajo estudio. Los metil-conjugados fueron los principales metabolitos detectados a nivel intestinal mientras que en las células hepáticas glucuronidos y metilglucurónidos, principalmente del Hyty, estuvieron también presentes.

De los resultados obtenidos en bibliografía se puede concluir que los compuestos fenólicos del aceite de oliva estudiados (Hyty y Ty) se absorben moderadamente bien y se han podido determinar sus metabolitos en fluidos biológicos (sangre y orina). Sin embargo la información disponible es todavía insuficiente y la biodisponibilidad de otros compuestos presentes en la fracción fenólica todavía no ha sido estudiada. Además no está claro el papel de los microorganismos intestinales en la hidrólisis de los conjugados y aún no se conoce hasta qué punto se producen pérdidas en heces, destrucción en intestino, depósitos en órganos o células sanguíneas, por lo que todavía queda mucho por trabajar en la determinación del destino metabólico y la biodisponibilidad de los polifenoles del aceite de oliva.

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CAPÍTULO 4
Caracterización de la fracción fenólica del aceite de oliva mediante un estudio bidimensional empleando HPLC-CE-ESI-TOF MS

Dado que una gran parte del trabajo experimental desarrollado en esta tesis está relacionado con el aceite de oliva, nos pareció interesante comenzar llevando a cabo una caracterización lo más profunda posible del perfil polifenólico de esta compleja matriz.

A pesar de la abundante bibliografía existente acerca de los fenoles del aceite de oliva, todavía existen muchos compuestos "desconocidos" en este interesante conjunto de antioxidantes y algunas familias de fenoles, como los secoiridoides, no han sido aún completamente estudiadas a causa de la complejidad de su estructura química y la de la matriz en la que están presentes. Por lo tanto, con objeto de conocer más acerca de la fracción más polar del aceite de oliva, nos propusimos llevar a cabo un estudio bidimensional (HPLC-CE-ESI-TOF MS) en el que se unieran el poder de separación de las técnicas de HPLC y CE, con la capacidad de ESI-TOF MS para obtener información estructural. La idea era aislar diferentes fracciones polifenólicas de extractos de una mezcla de aceites de oliva comerciales empleando HPLC semipreparativa como primera dimensión, y posteriormente analizar estas fracciones aisladas en una segunda dimensión utilizando CE acoplada a espectrometría de masas con analizador de tiempo de vuelo (ESI-TOF MS). Se utilizó la interfase ESI por ser el modo de ionización más versátil y el que se emplea normalmente para la detección de los iones separados mediante CE.

Pensamos que al utilizar estas dos técnicas, concebidas como complementarias, podríamos mejorar la caracterización de la fracción polifenólica del aceite de oliva, identificando no sólo los compuestos ya conocidos que habían sido descritos con anterioridad sino también nuevos compuestos presentes en esta fracción que no habían sido mencionados antes en bibliografía. Además, el uso del TOF como sistema de detección ayudaría a la identificación, combinando la determinación de masas exactas con el análisis de la distribución isotópica.

Por otra parte, hay que destacar la importancia que posee el hecho de disponer de fracciones polifenólicas aisladas (algunas de ellas puras), ya que muchos de los compuestos más importantes de esta fracción fenólica del aceite, no existen como estándares comerciales (es el caso de los secoiridoides y algunos lignanos). El estudio de las fracciones aisladas (su caracterización y evaluación de algunas propiedades) podría dar algo de luz y representar un primer paso para comprender las propiedades saludables que exhibe el aceite, ayudar a distinguir cuáles son las moléculas

responsables de sus características organolépticas y a evaluar su actividad antioxidante.

En concreto, en el trabajo desarrollado en este capítulo se pretendía utilizar las fracciones polifenólicas aisladas con dos objetivos:

1) Llevar a cabo una cuantificación más veraz de algunos fenoles del aceite de oliva empleando los estándares aislados identificados como puros y comparar esta cuantificación con respecto a la que se ha hecho tradicionalmente usando estándares externos (Dopac para Hyty, Ty y Hyty-Acet y Oleuropeína-glucósido para los lignanos y secoiridoides).

2) En colaboración con el Instituto Catalán de Oncología de Gerona, evaluar la actividad anticancerígena *in vitro* de algunas de estas fracciones aisladas.

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Research Article

A 2-D-HPLC-CE platform coupled to ESI-TOF-MS to characterize the phenolic fraction in olive oil

A 2-D-HPLC/CE method was developed to separate and characterize more in depth the phenolic fraction of olive oil samples. The method involves the use of semi-preparative HPLC (C18 column 250 × 10 mm, 5 μm) as a first dimension of separation to isolate phenolic fractions from commercial extra-virgin olive oils and CE coupled to TOF-MS (CE-TOF-MS) as a second dimension, to analyze the composition of the isolated fractions. Using this method, a large number of compounds were tentatively identified, some of them by first time, based on the information concerning high mass accuracy and the isotopic pattern provided by TOF-MS analyzer together with the chemical knowledge and the behavior of the compounds in HPLC and CE. From these results it can be concluded that 2-D-HPLC-CE-MS provides enough resolving power to separate hundreds of compounds from highly complex samples, such as olive oil. Furthermore, in this paper, the isolated phenolic fractions have been used for two specific applications: quantification of some components of extra-virgin olive oil samples in terms of pure fractions, and *in vitro* studies of its anti-carcinogenic capacity.

Keywords:
CE / Electrospray-TOF-MS / HPLC-CE-ESI-MS / Olive oil / Phenolic compounds
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1 Introduction

Extra-virgin olive oil (EVOO) is obtained from the olive fruit (*Olea europaea L.*) solely by mechanical means, without further treatment other than washing, filtration, decantation, or centrifugation [1]. Its chemical composition consists of major components that represent 98% of the total weight and includes mainly glycerols [2–4], but what makes EVOO unique among other vegetable oils are minor components (about 2% of the total oil weight), specially its high level of phenolic compounds [5, 6]. The phenolic fraction of olive oil is a heterogeneous and very complex mixture of compounds. A wide number of phenols have been already identified in olive oil, but even if this fraction has been studied over decades, it is still quite unknown, as many compounds remain unidentified. Simple phenols [7–10], lignans [11, 12], flavonoids [13] and secoiridoids [7, 14, 15] are important well-known categories of phenolic compounds, which can be found in olive oil. There is evidence that phenolic compounds from oil have different properties and exert diverse influence on the quality of olive oil [16, 17]. One of their most described characteristics is their anti-oxidant activity [18–22], although *in vitro* studies have demonstrated other biological properties, suggesting beneficial effects on health disease and pathologies prevention such as cancer, obesity, diabetes, or diseases heart, among others [23–26]. In addition, polyphenols are responsible of the exceptional organoleptic characteristics that EVOOs have [27–32].

A huge amount of literature is available on the development of methods for the determination of phenols in olives and virgin olive oils (VOOs). The most used analytical techniques for the determination of individual phenolic compounds in VOO are those based on spectrophotometric methods, as well as analytical separation (GC [33, 34], HPLC [35–37], and CE [9, 38, 39] coupled to different detectors [40]. However, a considerable number of phenolic compounds have still not been completely characterized and many problems remain to be resolved. One of the reasons lying

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Abbreviations: Ac, acetate; **Ac Pin,** (+)-1-acetoxypinoresinol; **D-Lig Agl,** decarboxilated or deacetoxy derivatives of Lig Agl; **DOA,** decarboxilated derivatives of Ol Agl; **EA,** elenolic acid; **EVOO,** extra-virgin olive oil; **FASN,** fatty acid synthase; **HYTY,** hydroxytyrosol; **HYTY-Ac,** hydroxytyrosol acetate; **ISCID,** internal source collision induced dissociation; **Lig Agl,** ligstroside aglycon; **m/z,** mass/charge ratio; **Ol Agl,** oleuropein aglycon; **Pin,** pinoresinol; **TY,** tyrosol; **Voo,** virgin olive oil

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behind these difficulties is the absence of suitable pure standards, in particular secoiridoid molecules and lignans. Moreover, phenolic fraction of oil is quite heterogeneous and complex and the matrix in which phenols are found (*i.e.* olive oil) is also rather complicated; these two facts cannot facilitate their analysis. Therefore, mono-dimensional systems are sometimes inadequate to achieve the complete separation of those compounds and an alternative approach could be to use HPLC with CE-ESI-TOF-MS afterwards, *i.e.* a 2-D-system. It has been demonstrated that in instances in which an HPLC method does not provide enough resolution, CE with its flexible experimental conditions should be assayed as a complementary second choice technique. With the CE analyses we added a new dimension of separation (based on completely different principles) after HPLC separation; moreover, MS will separate the analytes depending on the mass/charge ratio (m/z). The resolving power of a 2-D separation, measured as peak capacity, will be the product of the individual peak capacity in each dimension [41]. Several groups have explored automated, comprehensive multidimensional systems that couple chromatographic techniques (GC-GC, LC-LC, LC-GC) [42] or even chromatographic and electrophoretic separations (LC-CE) on-line [43, 44] and off-line [45, 46] to carry out different applications, but mainly to separate peptides and protein mixtures.

The aim of this study was to develop a new analytical strategy based on 2-D-HPLC-CE-ESI-MS to characterize the phenolic profile of olive oil. Fractions from the effluent from the HPLC system were collected and dried, redissolved and afterwards, analyzed by CE-ESI-MS. Apart from being useful to characterize the highly complex phenolic fraction from olive oil more in depth and perhaps to identify tentatively new components; the collected fractions could be an interesting tool used to quantify more properly the phenols present in the mentioned matrix, and to elucidate the contribution of phenols to the positive effects on health attributed to VOO. As far as an appropriate quantification is concerned, it has been previously described that direct comparison between the concentrations of olive oil phenols reported in the literature is quite difficult, as the reported concentrations often greatly differ (sometimes even in orders of magnitude) due to the lack of standards. Moreover, concerning their potential healthy effects, it is still unclear which component or combination of components of olive oil is responsible for this protective effect. The phenolic compounds, both known as unknown, deserve to be analyzed and studied in detail because they are good candidates to explain a substantial part of the benefits of consumption of olive oil, as well as to justify their anti-oxidant and sensory properties.

2 Materials and methods

2.1 Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Acetonitrile, from Lab-Scan (Dublin, Ireland), and acetic acid, from Panreac (Barcelona, Spain), were used in the mobile phase; whereas ammonium acetate from Panreac and ammonium hydroxide from Merck (Darmstadt, Germany) were used for the CE running buffers. Buffers were prepared by weighting the quantity indicated in doubly distilled water and adding ammonium hydroxide to adjust the pH. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). 2-Propanol of HPLC grade from Lab-Scan was used in the sheath liquid, and sodium hydroxide solution (1 M) from Panreac was used for capillary cleaning procedures before each analysis.

For the extraction of the polar fraction from olive oil, methanol and hexane from Panreac were used. Spanish EVOO samples used in the preliminary studies were obtained from a unique variety of olive fruit called Picual, Lechín, Cornicabra, Arbequina, Hojiblanca, and Picudo (January 2006). Two varieties were chosen for being used along the study in order to isolate the different phenolic fractions; they were Picual and Arbequina. The reason for choosing these two varieties were based on the high concentration of phenols and the number of peaks present in their profiles. We prepared a mixture EVOO Picual/EVOO Arbequina (1/1 v/v) to facilitate the isolation of the phenols.

2.2 Extraction procedure

Several previous publications have described different methods for the extraction of the polar phenolic fraction from the olive oil matrix. In general, we can say that those methods use two basic extraction techniques liquid-liquid and solid phase extraction (SPE) with different types and proportions of eluent [47, 40]. In a study carried out by Gómez-Caravaca *et al.* [38], different extraction systems both liquid-liquid and SPE (C-18, SAX, and DIOL) were compared in order to obtain the best results with regards to the number of compounds extracted and the level of preconcentration reached. Taking into account the results obtained in that paper, we chose SPE with Diol-cartridges to isolate the phenolic fraction from EVOO.

Briefly, the cartridge was placed in a vacuum elution apparatus and pre-conditioned passing 10 mL of methanol and then 10 mL of hexane. About 60 g of EVOO were thoroughly mixed with 60 mL of hexane and carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase. After a wash with hexane (15 mL) to remove the non-polar fraction of the oil, the sample was eluted with methanol (40 mL). The methanolic extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 35°C. The dried residue was then redissolved in 2 mL or 500 μL (depending on its use) of methanol/water (50/50 v/v) and filtered through a 0.25 μm filter before the analysis. We redissolved the residue in 2 mL when the phenolic extract had to be analyzed by analytical HPLC; while the residue was dissolved in 500 μL for carrying out the analysis and isolation by using semi-preparative HPLC.

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2.3 HPLC analyses

Analyses were carried out operating at room temperature on a System Gold HPLC (Beckman Coulter, Fullerton, CA, USA), including a 126 solvent module, a 168 diode array detector module and a manual sample valve injector with a 20 and 500 μL loop (Rheodyne, Cotati, CA, USA) for analytical or semi-preparative LC, respectively.

The semi-preparative HPLC column used for the isolation of the fractions was a Phenomenex Gemini column, 25 cm × 10 mm filled with C18 reversed-phase packing (5 μm average particle size) and the flow rate was 3 mL/min. However, the analytical HPLC column used for the characterization of the olive oils in the preliminary studies was a C_{18} Gemini column, 5 μm id, 25 cm × 3.0 mm (Phenomenex, Torrance, CA, USA), equipped with a pre-column (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min and the loop was of 20 μL. DAD detector was always set at 240 and 280 nm, because these wavelengths are the most appropriate for the detection of olive oil polyphenols. For most of these compounds, 280 nm is the most specific value of wavelength, although in the case of EA and derivatives, 240 nm would be the optimum value.

The mobile phases consisted of water plus 0.5% acetic acid (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: from 0 to 30 min, 95% (A): 5% (B) to 80% (A): 20% (B); from 30 to 40 min, 80% (A): 20% (B) to 70% (A): 30% (B); from 40 to 50 min, 70% (A): 30% (B) to 65% (A): 35% (B); from 50 to 60 min, 65% (A): 35% (B) to 50% (A): 50% (B); from 60 to 70 min, 50% (A): 50% (B) to 5% (A): 95% (B); from 70 to 75 min, 5% (A): 95% (B) to 95% (A): 5% (B). This last value was maintained for 5 min, and the run ended. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA) prior to degassification by ultrasonication. The same chromatographic gradient was used for analytical and semi-preparative separations.

2.4 CE analyses

Analyses were performed in a PACE System MDQ (Beckman Coulter) coupled to the mass detector using an orthogonal electrospray interface from Agilent (see below). The CE instrument was controlled by a personal computer running 32 Karat System Software from Beckman Coulter. Bare fused-silica capillaries with 50 μm id and total length 85 cm from Beckman Coulter were used. The running buffer was 40mM ammonium acetate at pH 9.5, voltage was set at 25 kV and 24 s hydrodynamic injections were made at the anodic end using N_2 at pressure of 0.5 psi.

Before their first use all new capillaries were conditioned by rising with 1 M sodium hydroxide for 10 min followed by a rinse with water for 5 min and then running buffer for 15 min. Capillary conditioning between runs consisted of 2 min with 1 M sodium hydroxide, then 2 min with water and finally 15 min with running buffer. At the end of the day the capillary was rinsed with water for 15 min and dried with air for 5 min.

2.5 MS

MS was performed using the microTOF^{TM} (Bruker Daltonik, Bremen, Germany), equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). MS/MS analyses were made by using a microTOF-Q (Bruker Daltonik). For CE-MS analysis, electrical contact at the electrospray needle tip was established *via* a sheath liquid pumped by a syringe pump (74900-00-05, Cole Palmer, Vernon Hills, IL, USA).

The parameters of the mass spectrometer were optimized by direct infusion experiments with EVOO extracts, as well as with several of the most important compounds belonging to this polar fraction of olive oil that exist as available commercially standards. We varied the parameters of the mass spectrometer, to achieve good sensitivity with reasonable resolution (5000–10 000) in the range of masses of interest (50–600 *m/z*). The optimization of the transfer parameters (radio frequencies and voltages) in the different skimmers, hexapoles and lenses was carried out in the direction of the entry of ions. As a general rule we can say that ions with high *m/z* values require high voltages to be transmitted.

The mass spectrometer was run in the negative mode and was operated to acquire spectra in the range of 50–600 *m/z*. The sheath liquid consisted of isopropanol-water (50/50 v/v) pumped at 0.24 mL/h and we used nebulizer gas pressure of 0.5 bar and a dry gas at flow rate of 5 L/min at 180°C.

The accurate mass data of the molecular ions were processed by DataAnalysis 4.0 software (Bruker Daltonik GmbH) that provides a list of possible elemental formulae by means of the Smart Formula editor, which uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents. Besides, an isotopic abundance pattern filter is required to reduce the number of candidates for an appropriate molecular formula (SigmaFit). For this purpose, the Generate Molecular Formula tool (Bruker Daltonik GmbH) creates robust statistical models using the masses and intensities of each isotope to do a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}) for increased confidence in the suggested molecular formula. The smaller the sigma value the better the fit, therefore for routine screening a threshold sigma value of 0.03 is generally considered appropriate.

The calibration of the MS was performed using litium formate clusters by switching the sheath liquid to a solution containing litium hydroxide in the sheath liquid of some formic acid in water:isopropanol 1:1 v/v. Due to the compensation of temperature drift in the MicroTOF, this external calibration provided accurate mass values (better

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5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

When we had doubts about the identity of some peaks present in the different isolated fractions, we carried out further fragmentation by using the MS/MS fragmentation achieved by high-resolution MS/MS (in the microTOF-Q used). MS^2 experiments were performed in the collision cell q on the isotopically pure (^{12}C) peak of the selected precursor ions by keeping the first quadrupole analyzer at 20 V relative to ground and operating at unit resolution, and scanning the TOF analyzer. The collision energy was set from 15 to 35 eV. All the acquisitions were averaged over 60 scans at a TOF resolving power of ~8000.

3 Results and discussion

3.1 Analysis of polyphenols in different EVOO samples

As a first step in the current work, different varieties of olive oil were studied to make a previous screening and choose the variety (or mix of varieties) that provided the highest number of compounds and amount of all of them.

EVOO of six different varieties of olive fruit-Picual, Lechín de Sevilla, Cornicabra, Arbequina, Hojiblanca and Picudo – were analyzed using HPLC with UV detection (Fig. 1) in order to select an olive oil rich in the phenols of interest. The use of an appropriate olive oil or mixture of olive oils for the isolation will allow obtaining higher amount of each compound, requiring less number of injections and therefore, of extractions. All the samples were prepared using the SPE protocol described in Section 2.2 and were analyzed with the optima conditions already described in Section 2. All the chromatograms are represented in the same absorbance scale in order to be able to carry out a proper comparison among the different varieties analyzed.

We could observe significant differences concerning the amount of polyphenols present in the different varieties. Picual was characterized because it was the only one where we detected significant amounts of ligstroside aglycon (Lig Agl). It was the richest variety in terms of oleuropein aglycon (Ol Agl) and it had important quantities of simple phenols (hydroxytyrosol (HYTY) and tyrosol (TY)) as well. However, it was one of the varieties with less amount of (+)-1-acetoxypinoresinol (Ac Pin), as previously reported by Brenes *et al*. [48]. As far as Lechín de Sevilla is concerned, the most representative compound in this variety was decarboxilated derivatives of Ol Agl (DOA). Lechín was quite poor in pinoresinol (Pin). EVOO Cornicabra was the one with less quantity of simple phenols and Lig Agl. However, it was richer than Lechín, Picudo and Hojiblanca in Ol Agl, but its concentration was much less than in Picual EVOO. Arbequina contained the largest amounts of lignans (Pin and Ac Pin) and it was outstanding the low presence of TY in that variety. Picudo variety was quite rich concerning simple phenols, mostly HYTY, but the concentration of DOA was very low. Hojiblanca showed a chromatographic profile similar to Picudo oil, except from the fact that it was a little richer in simple phenols and also rich in terms of EA (this fact can be observed better at 240 nm).

In general, the varieties very rich in some compounds had small amounts of others, or even lacked them, being very difficult to choose a variety in particular to proceed with the isolation step. Finally, a mixture of two varieties in 50/50 proportion was selected: Picual, with notable concentrations of all compounds and the richest oil in terms of secoiridoids. However, as Ac Pin was almost absent in that oil, we included in the mixture Arbequina EVOO, especially rich in lignans. In that way the two varieties complemented each other and an olive oil rich in the main phenolic compounds could be used for the isolation.

3.2 Bidimensional HPLC-CE analysis of phenols from EVOO

3.2.1 Isolation of phenolic fractions

Once the mixture of olive oils rich in the phenolic compounds of interest was chosen, 17 phenolic fractions corresponding to different peaks in the UV chromatogram at 240 and 280 nm were selected for the isolation (Fig. 2).

Semi-preparative reverse phase-HPLC was used with the same chromatographic conditions as those used in the analytical column, with the only difference of the flow, increased to 3 mL/min and the loop of 500 μL in order to increase the amount of sample injected into the column. The quantity of extract injected into the column was optimized in order to obtain concentrated profiles but with good resolution, and finally an injection of 200 μL was selected as optimum. The isolation of the compounds was carried out from Diol-SPE extracts of the mixture of EVOO.

The isolation was done trying to obtain the fractions as pure as possible avoiding other potential interferences. We needed more than 100 injections to get quantities of the isolated fractions that could be weighed. These compounds were manually collected and kept at −20°C. Once enough quantity was collected it was led to dryness in a rotary evaporator at 35°C and the difference in weight between the empty flask and the flask after the evaporation of the solvent, gave us the amount of solute collected.

3.2.2 Optimization of CE-ESI-TOF-MS and CE-ESI-qTOF-MS conditions

The fractions isolated with semi-preparative reverse phase-HPLC were analyzed using a complementary technique: CE coupled to MS-TOF in order to study their composition. The use of these techniques will improve the characterization of the isolated fractions due to the use of two techniques based on principles of separation completely different. Moreover, the use of TOF detection system can give us an excellent

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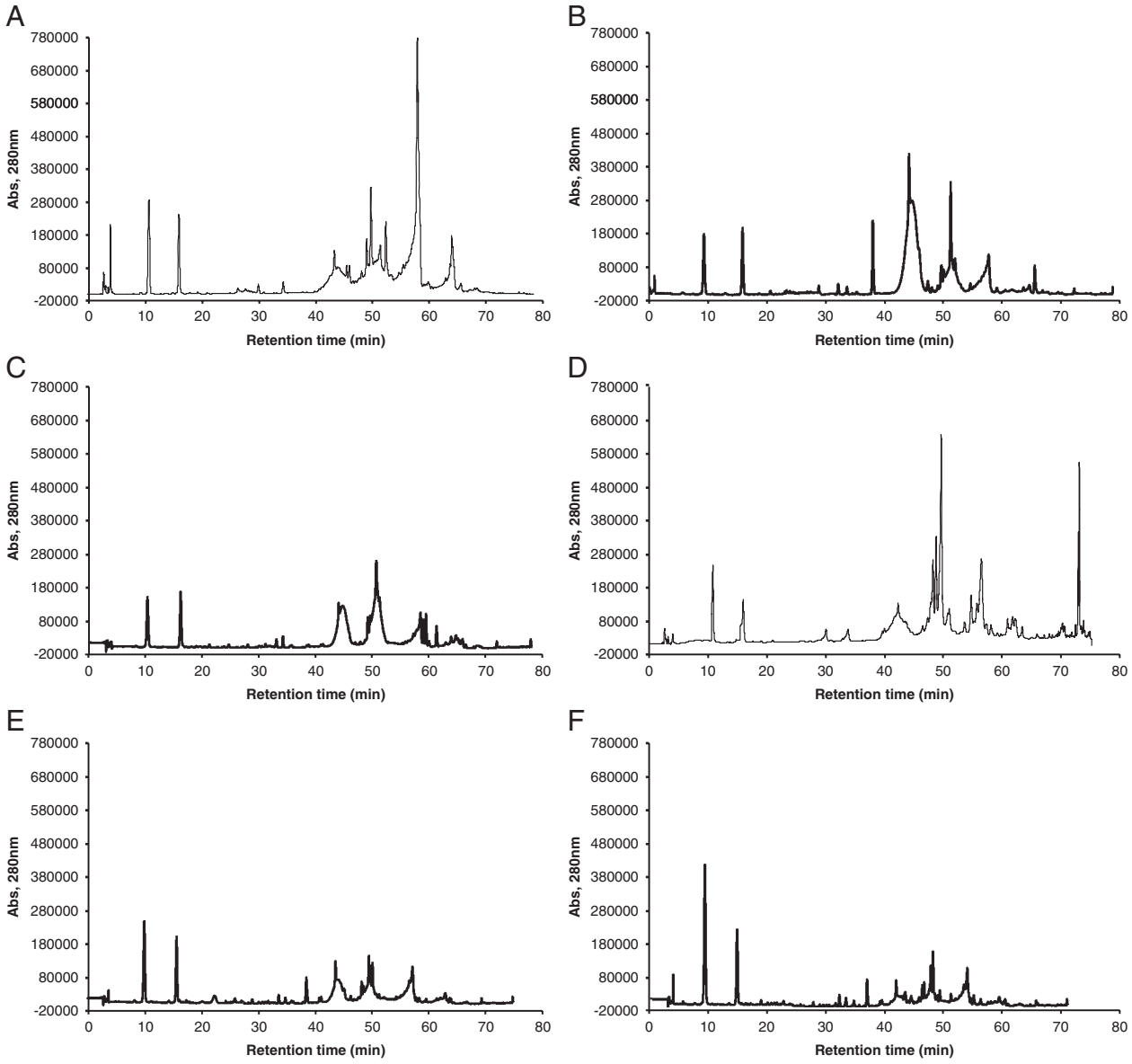

Figure 1. Chromatograms of the six different varieties used during the preliminary studies in order to select the most appropriate EVOO to proceed with the isolation collecting several fractions. (A) Picual, (B) Lechín, (C), Cornicabra, (D) Arbequina, (E) Hojiblanca and (F) Picudo. Chromatographic conditions were those described in Section 2, the analytical column was a C_{18} Gemini column, 5 μm id, 25 cm × 3.0 mm, and the detection was made at 280 nm.

accuracy in the determination of the mass, even allowing the measurement of the correct isotopic distribution pattern, which provides valuable additional information for determining the elementary composition.

The effect of different separation parameters on resolution, sensitivity, analysis time, and peak shape was studied using SPE extracts of olive oil samples. Initial electrophoretic conditions were chosen based on parameters previously described in literature [49]. Bare fused-silica capillaries with 50 μm id and 85 cm total length were used and a suitable BGE compatible with CE-ESI-MS, ammonium acetate, was chosen. With this buffer different pH values and different concentrations were tested obtaining the best results in terms of resolution, in the shortest analysis time, with 40 mM ammonium acetate at pH 9.5. Based on these conditions the voltage applied was varied between 20 and 30 kV, and we found that in general a voltage of 25 kV shortened the analysis time and also gave good resolution and acceptable current. The injections were made at the anodic end using N_2 pressure of 0.5 psi for 24 s.

Concerning the mass spectrometer, as commented before, the transfer parameters were optimized by direct infusion experiments with EVOO extracts, as well as with several of the most important compounds belonging to this polar fraction of olive oil that exist as available commercially standards. In the CE-ESI-MS coupling, the incompatibility of

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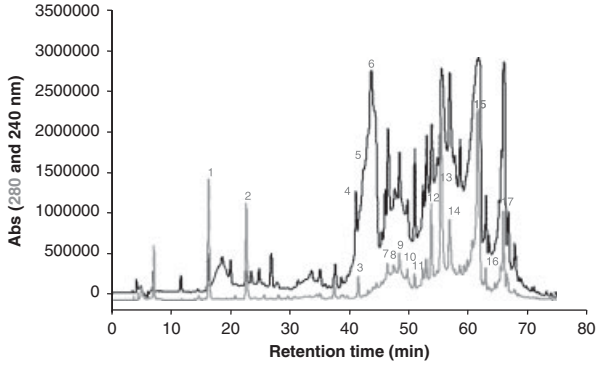

Figure 2. Chromatograms (at 240 and 280 nm) of the mixture of EVOOs selected for carrying out the isolation of several fractions analyzed by semi-preparative HPLC and the different fractions collected (with a number).

capillary flow (100 nL/min), with the flow necessary to the formation of a stable electrospray (1–200 μL/min) was solved using an additional liquid isopropanol/water (50/50) at a flow of 0.24 mL/h. The other ESI parameters were chosen according to the sheath liquid flow of 0.24 mL/h and the most suitable ones were: nebulizer pressure of 0.5 bar, dry gas flow equal to 5 L/min and dry gas temperature 180°C.

As commented before, MS^2 experiments were performed in the collision cell by keeping the first quadrupole analyzer at 20 V relative to ground and operating at unit resolution, and scanning the TOF analyzer. Particular attention was paid to the collision energy, since its influence was quite drastic concerning the effective energy applied for the fragmentation of the molecules. It was varied from 15 to 35 eV, checking the fragmentation patterns at diverse values and considering all the MS/MS information generated. All the acquisitions were averaged over 60 scans at a TOF resolving power of ~8000.

These CE-ESI-TOF-MS and CE-ESI-qTOF-MS optimized conditions were used to analyze the 17 different fractions isolated by semi-preparative HPLC.

3.2.3 Characterization of the isolated fractions by CE-ESI-TOF-MS and CE-ESI-qTOF-MS

Figure 3 shows the extracted ion electropherograms of the different isolated fractions and the compounds tentatively identified are summarized in Table 1, including experimental *m/z* values, fragments detected by ISCID (internal source collision induced dissociation) in the TOF that provides information to identify the compounds, fragment achieved after doing MS/MS experiments (in a qTOF instrument), the error and sigma value (comparison of the theoretical with the measured isotope pattern) and a list of possible compounds.

The identification of the compounds was performed by a careful interpretation of the MS spectra combined with information about polarity and electrophoretic mobility provided by the two complementary techniques used. Compounds isolated in the same fraction should have similar polarity and then these compounds are separated taking into account their electrophoretic mobility. Furthermore, ESI-TOF-MS analyzer provides information about accurate mass and isotopic pattern that allow obtaining a reduced number of possible elemental compositions that then can be matched against available databases. Some of the possible elemental compositions calculated seem to be not chemically coherent reducing the number of possibilities. With the help of the fragmentation pattern obtained with ISCID, the potential difference between capillary exit and the skimmer, a reliable identification of the compounds is possible, especially in such cases where other techniques (MS/MS and NMR) cannot be utilized due to the low intensity of the analytes. When the intensity of the analytes was enough, MS/MS experiments were carried out to confirm the identification of the compounds. The micro-TOF-Q offers three dimensions of identification information simultaneously on all results: precise mass, MS/MS and SigmaFit™ isotopic analysis; we took advantage of the three mentioned dimensions to carry out the tentative identification of compounds under study.

Some of the polyphenols mentioned in previous studies were isolated as pure fractions, that was the case of fraction 1 identified as HYTY, fraction 2, TY, fraction 6, EA, and fraction 17, Lig Agl. These pure fractions were later on used to quantify some compounds in the olive oil extract. Other LC fractions that had been previously assigned, with UV detection, to a single compound, were actually several compounds with similar polarity and therefore with the same retention time.

As the method used for isolating the phenols was a reversed phase LC method, the sixth first fractions contained highly polar compounds. So HYTY, TY and EA appeared as pure substances, while in the other fractions we can find a mixture of compounds separated on the basis of their size to charge ratio. In fraction 3 the main compound (with *m/z* 195.0663) identified as HYTY-acetate (HYTY-Ac) appeared accompanied by another compound with *m/z* 257.0664, identified by first time as a derivative of elenolic acid (EA): *hydroxy-EA*. This compound shows up before EA because of its extra hydroxyl group that increase its polarity; the fragments detected with the same losses as EA, $[M\text{-}H\text{-}32]^-$, $[M\text{-}H\text{-}76]^-$ and $[M\text{-}H\text{-}102\text{-}H_2O]^-$ confirmed its identity. Other compounds with very low intensity have been tentatively identified and can be found in Table 1, such as licodione (*m/z* 271.0601), a metabolite of flavone pathway and glepidotin C, a phytochemical compound (stilbenoid) previously identified in Glycyrrhiza glepidota with anti-viral activity. Last peaks correspond to compounds with carboxylic groups; quinic acid (*m/z* 191.0567) and succinic acid (*m/z* 191.0199), with the lowest electrophoretic mobility and therefore high migration time. Fraction 4 is composed by HYTY-Ac, EA and probably small quantities of other acids, such as xanthoxic (*m/z* 265.1465) and capric acid (*m/z* 171.1380), but the most abundant compound with *m/z* 419.1853 has been identified as a *dihydroxymethoxy dipre-*

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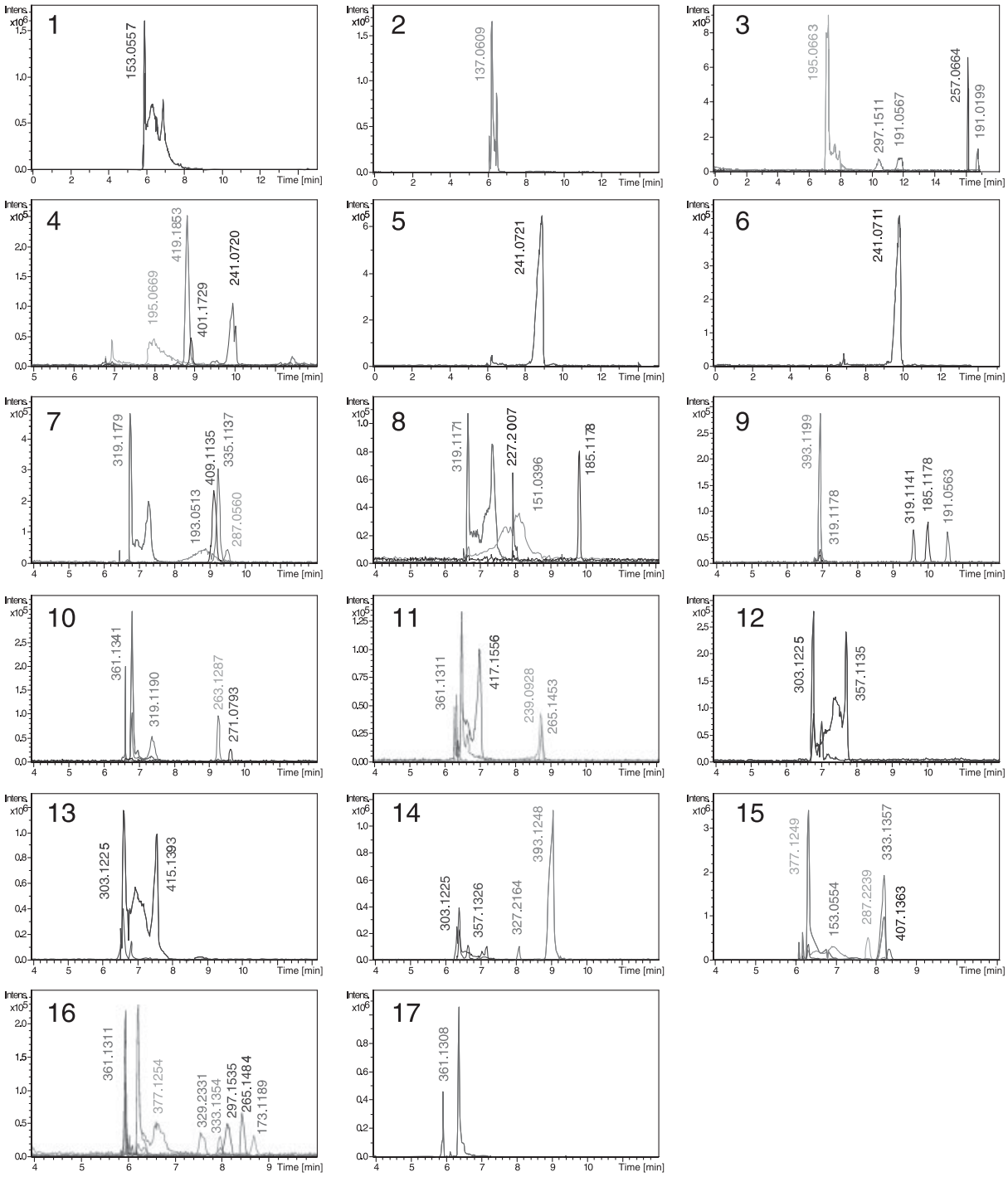

Figure 3. Extracted ion electropherograms of the isolated fractions together with information concerning the experimental *m/z* signal for every compound detected in all the fractions. Well-known phenolic compounds identified in some of the fractions: (1) HYTY, (2) TY, (3) HYTY-Ac, (5) EA, (8) DOA, (12) Pin, (13) Ac-Pin, (14) 10-H-OI AgI, (16) OI AgI, and (17) Lig AgI.

nylisoflavone, an isoflavone with prenyl groups found very commonly in plant extracts. Close to this peak other compound with *m/z* 401.1729 could correspond to the loss of a H_2O moiety (−18) from 419 and the closure of the ring resulting in an *alpinumisoflavone derivative* also with methoxy and prenyl groups. With the techniques used in the current study, we cannot predict the positions of the substituents in the chemical structure of these substances. For both compounds the presence of ions fragments at *m/z* 387 and 369 indicates the loss of 32. A small trace of other flavonoid with prenyl groups was tentatively identified as 8-prenyldihydro-kaempferol-7-glucoside.

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Table 1. *m/z* experimental, ISCID and MS/MS fragments, formula, error, sigma and possible identification for every compound found in the different fractions

Fractions	*m/z* experimental	ISCID fragments[a)]	MS/MS fragments[a)]	Formula (smart editor)	Error (ppm)	Sigma	Possible compounds
1	*153.0557*[b)]			C8H9O3	0.33	0.0020	HYTY
2	*137.0609*[b)]			C8H9O2	−0.96	0.0065	TY
3	*195.0663*[b)]			C10H11O4	0.01	0.0106	HYTY-Ac
	271.0601[c)]			C15H11O5	4.00	0.0141	Licodione
	297.1511			C19H21O3	5.00	0.0355	Glepidotin C
	191.0567			C7H11O6	−3.30	0.0166	Quinic acid
	257.0664	225,213,181,137	241,225,213,195,181,137	C11H13O7	1.25	0.0057	Hydroxy-EA
	191.0199			C6H7O7	−1.05	0.0026	Succinic acid
4	195.0669			C10H11O4	−3.24	0.0097	HYTY-Ac
	517.1609[c)]			C26H29O11	19.01	0.0829	8-Prenyldihydrokaempferol 7-glucosi
	419.1853[b)]	387		C26H27O5	2.66	0.0407	Diprenylisoflavone derivative
	401.1729	369		C26H25O4	7.40	0.0224	Alpinumisoflavone derivative
	355.1666[c)]			C14H27O10	−15.71	0.0468	Unknown
	241.0720	209,165,139,127	209,181,171,165,139,127	C11H13O6	−0.91	0.0029	EA
	265.1465[c)]			C15H21O4	−7.31	0.0967	Xanthonic acid
	171.1380[c)]			C10H19O2	5.11	0.0081	Capric acid
5	*241.0721*[b)]	209,165,139,127	209,181,171,165,139,127	C11H13O6	−1.31	0.0038	EA
	265.1471[c)]			C15H21O4	−9.60	0.0248	Xanthonic acid
	183.1001[c)]			C10H15O3	14.10	0.0306	Oleuropeic acid
	257.0668[c)]			C11H13O7	0.65	0.0204	Hydroxy-EA
	187.0971[c)]	169,125		C9H15O4	2.49	0.0074	Azelaic acid
6	*241.0711*[b)]	209,165,139,127	209,181,171,165,139,127	C11H13O6	2.71	0.0365	EA
7	*319.1179*[b)]	183	275,249,183,165	C17H19O6	2.59	0.0025	DOA
	193.0513			C10H9O4	−3.39	0.0140	Methyl caffeate
	409.1135	345,275	377,345,327,307,275,241,195,149,139,111	C19H21O10	1.35	0.0059	Dihydroxy-OI AgI
	243.0865[c)]	211,167		C11H15O6	−0.91	0.0132	Dihydroxy-EA
	335.1137	199	275,249,199,183,165	C17H19O7	−0.09	0.0037	Hydroxy D-OI AgI
	287.0560			C15H11O6	0.41	0.0092	Tetrahydroxy–isoflavanone
8	*319.1171*[b)]	183	275,249,183,165	C17H19O6	4.93	0.0035	DOA
	151.0396			C8H7O3	2.95	0.0331	Vanillin
	227.2007			C14H27O2	4.12	0.0220	Myristic acid
	185.1176			C10H17O3	4.13	0.0113	Oxodecanoic acid
9	*393.1199*[b)]		345,327,321,307,275,213,181,149,139,111	C19H21O9	2.04	0.0120	Hydroxy-OI AgI
	319.1178	183	275,249,183,165	C17H19O6	2.85	0.0190	DOA
	319.1141	199	285,259,233,199,183,165	C17H19O6	11.21	0.0029	Hydroxy D-Lig AgI
	185.1178			C10H17O3	2.74	0.0106	Oxodecanoic acid
	191.0563			C7H11O6	−1.22	0.0177	Quinic acid
10	*361.1341*[b)]			C19H21O7	−4.90	0.0129	Lig AgI
	319.1190	183	275,249,183,165	C17H19O6	−0.97	0.0138	DOA
	153.0550[c)]			C8H9O3	4.71	0.0116	Vanillyl alcohol
	263.1287	219,201,153		C15H19O4	0.56	0.0549	Abscisic acid

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Table 1. Continued

Fractions	*m/z* experimental	ISCID fragments[a)]	MS/MS fragments[a)]	Formula (smart editor)	Error (ppm)	Sigma	Possible compounds

Table 1. Continued.

Fractions	*m/z* experimental	ISCID fragments[a)]	MS/MS fragments[a)]	Formula (smart editor)	Error (ppm)	Sigma	Possible compounds
	271.0793	227,153		C12H15O7	−1.38	0.0123	Pentamethoxy benzoic acid
11	361.1311			C19H21O7	−5.10	0.0193	Lig Agl
	417.1556[b)]			C22H25O8	−0.25	0.0129	Syringaresinol
	153.0557[c)]	123		C8H9O3	3.35	0.0347	Vanillyl alcohol
	239.0928	195,121		C12H15O5	−1.29	0.0207	Trimethoxyhydrocinnamic acid
	265.1453			C15H21O4	−2.79	0.0967	Zinniol
12	361.1293[c)]			C19H21O7	−5.51	0.0329	Lig Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	357.1135[b)]			C20H21O6	2.50	0.0064	Pin
13	361.1281[c)]			C19H21O7	−3.32	0.0088	Lig Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	415.1393[b)]			C22H23O8	1.37	0.0051	Ac Pin
14	361.1306[c)]			C19H21O7	3.60	0.0125	Lig Agl
	377.1242[c)]			C19H21O8	−4.70	0.0035	Ol Agl
	303.1225	183	285,259,233,199,183	C17H19O5	4.36	0.0234	D-Lig Agl
	357.1326			C20H21O6	−5.01	0.0180	Matairesinol
	327.2164			C18H31O5	4.00	0.0135	2,3-Dinor-8-iso prostaglandin F1-α
	393.1248[b)]		377,345,327,321,307,291,275,241,149,139,111	C19H21O9	−14.06	0.0428	10-H-Ol Agl
15	*377.1249*[b)]	345,307,275	345,327,307,275,239,195,149,139,111	C19H21O8	−1.93	0.0105	Ol Agl
	153.0554	123		C8H9O3	0.90	0.0004	Dimethoxy phenol
	287.2239			C16H31O4	−3.84	0.0047	Dihydroxy-palmitic acid
	503.2037[c)]			C30H31O7	7.50	0.0353	Prenylflavones derivative
	471.1784[c)]			C29H27O6	6.20	0.0262	Prenylflavones derivative
	519.2063[c)]			C30H31O8	7.60	0.0449	Prenylflavones derivative
	333.1357	213,181	213,181,137	C18H21O6	−4.03	0.0070	Unknown
	407.1363	363,317		C20H23O9	−3.88	0.0047	Methyl aloeresin
16	361.1311	291,241	291,259,171,127,111,101	C19H21O7	−5.00	0.0144	Lig Agl
	377.1254[b)]	345,307,275	345,327,307,275,239,195,149,139,111	C19H21O8	−3.10	0.023	Ol Agl
	329.2331			C18H33O5	0.61	0.0033	Trihydroxy-octadecenoic acid
	333.1354	213,181		C18H21O6	−3.80	0.0189	Unknown
	297.1535			C19H21O3	−11.81	0.0484	Ostruthin
	265.1484			C15H21O4	−14.51	0.0408	Vanillic acid, heptyl ester
	173.1189			C9H17O3	−3.10	0.0293	Unknown
17	*361.1308*[b)]	291,241	291,259,171,127,111,101	C19H21O7	−4.76	0.0211	Lig Agl

a) In columns for ISCID and MS/MS fragments we do not include decimal figures to contain the size of the table. Anyway, mass accuracy information and isotopic pattern were used to carry out the tentative identification of compounds under study.

b) We represent in italics the main compound in each fraction.

c) Compounds in small traces that are not represented in Fig. 1.

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In fractions 5 and 6 the most important compound is EA, although in fraction 5 other acids in traces like xanthoxic acid, oleuropeic acid, azelaic acid, *etc* are also detected. In this fraction a group of different glycosilated flavonoids have also been identified but in very small traces because glycosilated compounds are usually lost during olive oil production and remain in vegetation water and/or solid residues resulting from olive processing.

Fractions from 7 to 17 are mainly lignans and secoiridoids, together with other compounds at very low concentration. In fraction 7, different oleuropein derivatives are detected among other compounds. With the highest intensity the DOA (*m/z* 319.1179) is detected with its main fragment *m/z* 183 $[M\text{-}H\text{-}136]^-$. A derivative of this compound, with *m/z* 335.1137, was found and identified as *hydroxy-decarboxilated Ol Agl* what is in good agreement with the migration time (higher in this case because of its extra hydroxyl group) and with its main fragment *m/z* 199 with the same loss $[M\text{-}H\text{-}136]^-$ than DOA. In order to corroborate the results obtained and because of the intensity of the compounds was enough, MS/MS experiments were performed using QTOF (collision energy 17 eV). Figure 4 shows the fragmentation patterns obtained for these two decarboxilated-oleuropein derivatives.

An ion with *m/z* signal of 409.1135 indicates that two hydroxyl groups have been incorporated into Ol Agl molecule and this compound was identified as *dihydroxy-Ol Agl*. It is eluted in LC before Ol Agl and hydroxy-Ol Agl because of its higher polarity, and in CE, it migrates after DOA due to its high mobility with the introduction of more negative charge. Moreover, it shows the same fragmentation patterns of the Ol Agl with the following fragmentation ions *m/z* 345, 307, 275, 195, 149, 139, 111 obtained in the MS/MS experiments (see Fig. 5). A compound with *m/z* 243.0865, very low intensity and with the same fragments as EA $[M\text{-}H\text{-}32]^-$ and $[M\text{-}H\text{-}76]^-$ was identified as *dihydro-EA*. Besides, other phenolic compounds like *methyl caffeate* and *tetrahydroxy-isoflavanone* were also found. We found that Fraction 8 was mainly composed by DOA (*m/z* 319.1171) accompanied by other small molecules that can be seen in the Table 1. In fraction 9, it was possible to identify one of the isomers of hydroxy-Ol Agl (*m/z* 393.1199) with almost the same fragmentation pattern and small quantities of DOA; but the most interesting fact was that, in this fraction, a MS signal with 319.1141 *m/z* (the same signal as DOA and the same loss $[M\text{-}H\text{-}120]^-$ as decarboxilated or deacetoxy derivatives of Lig Agl (D-Lig Agl) which produces a fragment of 199) was identified as *hydroxy-D-Lig Agl*. In Fig. 4 we can see the fragmentation pattern for this compound that corroborates its identification.

In Fraction 10 small quantities of Lig Agl, that will accompany the remaining fractions, start to appear due to this compound elutes from the LC column for a long time. We can also find DOA remaining and other compounds.

Fractions 11, 12, 13 correspond to the collection of lignans. The most important compound in fraction 11, with *m/z* 417.1556, was a lignan identified as *syringaresinol*. This compound has been recently described by Christophoridou *et al*. [50] in olive oil studies carried out with LC-SPE-NMR. This lignan was not completely pure, since was eluted with small amounts of other compounds, such as Lig Agl, *vanillyl alcohol*, *trimethoxyhidrocinnamic acid* and *zinniol*.

Fractions 12 and 13 so far identified in UV as Pin and Ac Pin appeared together with a compound of *m/z* 303.1225 identified as D-Lig Agl with the same fragmentation ion *m/z* 183 as DOA and small amounts of Lig Agl. Figure 4 shows the fragmentation of the mentioned compound. Fraction 14 was identified as *10-hydroxy-Ol Agl* (*m/z* 393.1248); that phenolic substance had the same fragmentation ions (*m/z* 345, 307, 275, 195, 149, 139, 111 obtained in the MS/MS experiments) as Ol Agl and its derivatives (see Fig. 5). In that fraction we found as well small proportions of other secoiridoids like D-Lig Agl (*m/z* 303.1225), Ol Agl and Lig Agl. A compound with *m/z* 357.1326 was tentatively identified as a new lignan named *matairesinol*.

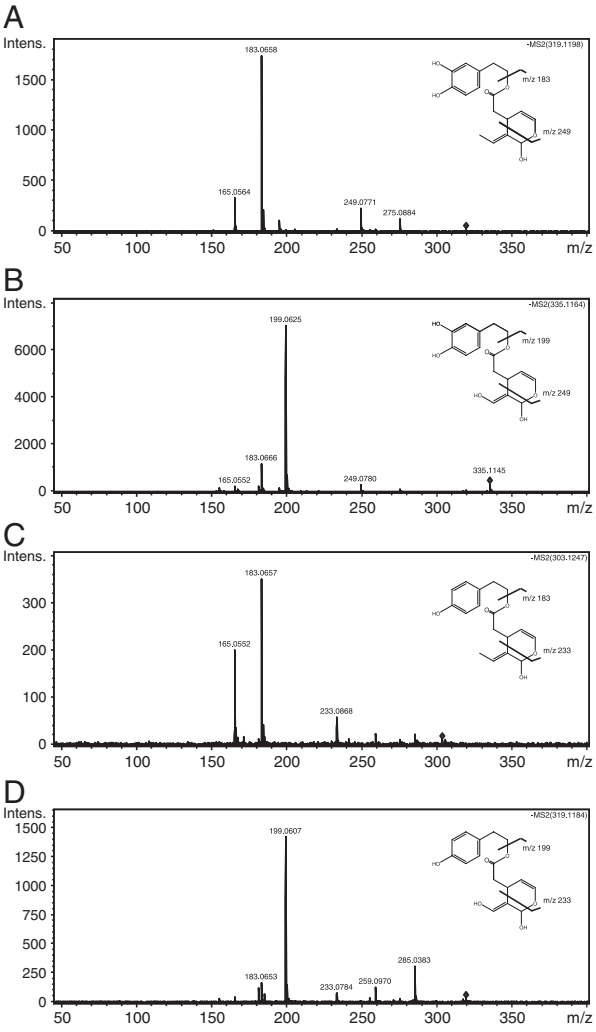

Figure 4. MS/MS spectra for (A) 319.1198, (B) 335.1164, (C) 303.1247 and (D) 319.1184 showing, in the structure of each compound, the possible fragmentation patterns.

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Fraction 15 so far identified as Ol Agl, also contains other compounds with low polarity, as for example a compound of *m/z* 333.1354 that could be a derivative of Lig Agl [361-CO]$^-$, since it shows some of the same losses [M-H-120] y [M-H-152] and also a fragment with *m/z* 137 equal to the Lig Agl. That tentative identification has to be still corroborated, that is the reason we do not identify it in Table 1. Moreover, a group of three compounds with very low intensities and similar migration time have been identified as three isoprenylated flavones with O-heterocyclic rings (closed furano and pyrano derivatives) very common in plant extracts. The introduction of these groups into the flavonoids converts them into more lipophilic substances [51]. It is also possible to find other compounds tentatively identified in Table 1.

Fraction 16 is mainly composed by a mixture of Ol Agl and Lig Agl together with other compounds in small quantities some of them still unidentified and fraction 17 is pure Lig Agl.

3.3 Application of the isolated and characterized fractions

As commented before, the isolated phenolic fractions offer an important potential because their individual study could help us to understand their importance not only concerning the health beneficial effects of the EVOO that contains them, but also regarding its sensory characteristics (bitterness, burning, astrigentness) or its antioxidant capacity. In this paper, the phenolic fractions have been used for two specific applications: quantification of some components of EVOO samples in terms of pure fractions, and *in vitro* studies of its anti-carcinogenic capacity.

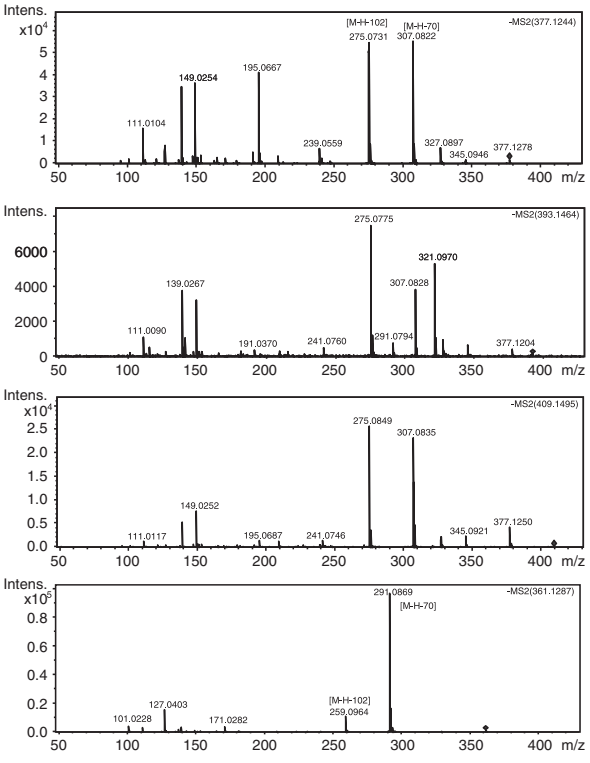

Figure 5. MS/MS spectra belonging to 377.1244, 393.1464, 409.1495 and 361.1287.

3.3.1 Quantification of some components of EVOO samples in terms of pure fractions

When the analyst has no commercially available standards, the three following approaches can be used: to quantify considering an external standard with a similar structure to the compound under study; to use an internal standard added to the extract that we wanted to analyze; or even to take into account external standards (with structure completely different from the analytes under study) in order to compare with other data found in literature [40, 47]. In the current work the quantification of several phenols from olive oil was done using the isolated standards in our laboratory (after checking their purity). We could have used every isolated fraction to quantify the different phenols present in oil getting results closer to the real concentrations and avoiding the traditional approaches, but since we figured out that some fractions were not 100% pure, we decided to show here just the results achieved for the quantification in terms of the highly pure fractions, that is HYTY, TY, EA and Lig Agl. Standard calibration graphs were prepared for each compound by using both UV detection (280 nm and 240 nm) (Table 2) and MS detection (Table 3). Both tables contain information regarding DL, calibration range and r^2. The calibration plots indicate good correlation between peak areas and analyte concentrations. We include the calibration curves and the other analytical parameters for Dopac and Ol Gluc (at different wavelengths). They have been widely used to quantify HYTY, TY and HYTY-Ac (Dopac) and lignans and secoiridoids (Ol Glu).

At the moment we used the isolated fractions as standards for quantification, HYTY and TY were commercially available; it is important to highlight that the results achieved by using our standards and those with commercial standards were not statistically significantly different. The quantitative results from HPLC-UV and HPLC-TOF are presented in Table 4 where the units are mg analyte/kg olive oil. We can easily observe the differences found in the quantification results when we compare the values achieved by using the isolated standards or when we use the traditional external ones. The results obtained by using Dopac as external standard to quantify HYTY and TY were quite different from those obtained when isolated standards were used (regardless the detection system used). The same happened when Ol Glu was used in UV to quantify EA and Lig Agl. The greatest differences were found when we used Ol Glu as external standard with TOF because of the unefficient response that this compound shows in MS probably due to a poor nebulization.

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Table 2. Analytical parameters for the HPLC-UV method[a)]

Compounds	LD (μg/mL)	Calibration range	Calibration curves	r^2
HYTY	0.032	QL-100	$y = 10045.61x–6481.98$	0.9932
TY	0.021	QL-100	$y = 15368.82x–5226.01$	0.9933
EA[b)]	0.019	QL-100	$y = 38072.54x+349715.80$	0.9991
Lig Agl	0.076	QL-200	$y = 4183.30x+18142.70$	0.9563
Dopac	0.013	QL-50	$y = 24868.02x–19085.33$	0.9990
Oleuropein glucoside	0.045	QL-250	$y = 7126.74x+22082.33$	0.9886
Oleuropein glucoside[b)]	0.010	QL-150	$y = 34193.45x+154568.17$	0.9761

a) $y = bx \pm a$, where y is the peak area (AU), x is the concentration (μg/mL), a is the y intercept, and r^2 is the correlation coefficient.
b) 240 nm LD = $3\sigma_b/b$ ($\sigma_b = 106.5668$, calculated using 100 data); LQ = $10\sigma_b/b$.

Table 3. Analytical parameters for the HPLC-ESI-TOF MS method[a)]

Compounds	LD (μg/mL)[b)]	Calibration range	Calibration curves	r^2
HYTY	0.060	QL-100	$y = 1312837.35x+2888083.52$	0.9881
TY	0.094	QL-100	$y = 1069088.26x+576012.73$	0.9831
EA	1.850	QL-200	$y = 176399.71x+471557.17$	0.9915
Lig Agl	0.294	QL-200	$y = 188216.09x+2113758.02$	0.9513
Dopac	0.078	QL-50	$y = 3096341.03x–9916830.16$	0.9878
Ol europein glucoside	5.921	QL-200	$y = 5643.60x+32943.33$	0.9607

a) $y = bx \pm a$, where y is the peak area, x is the concentration (μg/mL), a is the y intercept, and r^2 is the correlation coefficient.
b) LD was calculated considering $S/N = 3$.

Table 4. Quantitative results achieved by using HPLC-UV and HPLC-ESI-TOF MS methods[a)]

Compounds	mg Analyte/kg olive oil (TOF)	mg Analyte/kg olive oil (TOF)[b)]	mg Analyte/kg olive oil (UV)	mg Analyte/kg olive oil (UV)[c)]
HYTY	15.73 ± 0.97	8.59 ± 0.04	14.34 ± 0.23	6.25 ± 0.95
TY	9.07 ± 0.52	4.76 ± 0.02	8.90 ± 0.32	5.77 ± 0.20
EA[c)]	64.91 ± 3.93	2123.47 ± 128.82	41.11 ± 0.73	44.03 ± 0.82
Lig Agl	38.53 ± 3.84	1430.41 ± 128.05	44.77 ± 0.95	26.06 ± 0.56

a) (Value = X ± SD) ($n = 5$).
b) Quantified in terms of external standards widely used in literature (Dopac (for HYTY, TY and HYTY-Ac) and Oleuropein glucoside (for lignans and secoiridoids)).
c) 240 nm.

3.3.2 *In vitro* studies of its anti-carcinogenic capacity

Moreover, some of the isolated fractions were studied to evaluate the anti-proliferative and the pro-apoptotic effects of EVOO phenolic compounds, concluding that Ol Agl is among the first examples of how selected nutrients from an EVOO-rich ''Mediterranean diet'' directly regulate HER2-driven breast cancer disease [52]. After those findings, we kept working in the same direction and we tested the effects of phenolic fractions from EVOO on the expression of fatty acid synthase (FASN), a key enzyme involved in the anabolic conversion of dietary carbohydrates to fat in mammal's protein expression. EVOO lignans and secoiridoids were found to drastically suppress FASN protein expression in HER2 gene-amplified SKBR3 breast cancer cells, revealing that phenolic fractions, directly extracted from EVOO, may induce anti-cancer effects by suppressing the expression of the lipogenic enzyme FASN in HER2-overexpressing breast carcinoma cells, offering a previously unrecognized mechanism for EVOO-related cancer preventive effects [53].

More recently, the ability of EVOO polyphenols to modulate HER2 tyrosine kinase receptor-induced *in vitro* transformed phenotype in human breast epithelial cells was checked, identifying novel roles for naturally occurring EVOO-derived polyphenols in human breast cancer cell growth and HER2-regulated malignant transformation and providing new insights into the molecular mechanisms underlying the protective effects of naturally occurring EVOO biocompounds on breast cancer risk [54].

To the best of our knowledge, isolated fractions from EVOO have been used by first time in these kinds of

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biomedical studies, proving our point concerning the usefulness of the mentioned fractions for other purposes.

4 Concluding remarks

In the present work a 2-D-HPLC-CE method has been developed to isolate and characterize different phenolic fractions from EVOO. The use of two complementary techniques with different separation principles allows a more complete characterization of the olive oil profile. The mentioned techniques together with the potential of TOF-MS and qTOF-MS helped us to find some compounds, which have never been described before in this fraction of the olive oil. That opens new possibilities for future works, such as carrying out studies with NMR in order to elucidate the structures of tentatively identified compounds.

Furthermore, the importance of the isolated fractions is remarkable, since most of the compounds cannot be purchased as commercial standards and could facilitate their future use to study some characteristics of each individual fraction. In this work some of the isolated fractions have been used to quantify several compounds in the polyphenolic profile of olive oil and to make *in vitro* studies of their anti-cancer properties.

The authors have declared no conflict of interest.

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Apéndice

Como complemento a lo que se ha descrito en el artículo incluido en el capítulo 4, consideramos relevante hacer algo más de hincapié en explicar por qué resultó tan interesante disponer de las fracciones fenólicas aisladas para usarlas en los estudios llevados a cabo con células cancerígenas.

Como ya indicábamos en la introducción del bloque III los efectos de la fracción triglicérida del aceite de oliva, rica en ácidos grasos monoinsaturados, en la etiología y progresión de diferentes cánceres han sido ampliamente estudiados. Sin embargo, hasta el momento en el que se desarrolló este trabajo, el posible efecto anticancerígeno de los compuestos polifenólicos no había sido evaluado. Para poder determinar de manera individual la capacidad anticancerígena de cada compuesto fenólico presente en el aceite de oliva, habría que sintetizarlos o aislarlos. De ahí la importancia de disponer de las fracciones polifenólicas aisladas que permitieron estudiar la capacidad anticancerígena de cada compuesto o pequeño grupo de compuestos de forma individual en diferentes líneas de cáncer de mama, pudiendo establecer qué componentes (o combinación de componentes) del aceite de oliva podrían ser los responsables de su efecto protector.

Estos estudios *in vitro* llevados a cabo por el grupo de investigación del Doctor Javier Abel Menéndez del Instituto Catalán de Oncología de Gerona trajeron consigo la publicación de 4 trabajos de investigación.

Antes de comentar de modo muy resumido los aspectos más importantes de los resultados alcanzados en estos trabajos, parece conveniente resaltar el hecho de que en todos los estudios se emplearon líneas celulares de cáncer de mama tipo HER2. Entre el 25 y el 30% de las mujeres con cáncer de mama presentan una alteración del gen HER2. El HER2/neu es un oncogén que produce una proteína denominada HER2 (Receptor 2 del factor de crecimiento Epidérmico Humano erb-B2), que se encuentra en la superficie de numerosas células y que es un receptor de membrana con actividad tirosina-quinasa involucrada en la regulación de la proliferación celular. Cuando el gen HER2 está alterado, produce más cantidad de proteína de lo normal, se dice entonces que está incrementada su expresión o que la proteína está sobreexpresada. Como resultado de ello, estas células de cáncer de mama crecen con mayor rapidez y más agresivamente que aquellas que no tienen proteína HER2 adicional. Las células de cáncer de mama con HER2 adicional se conocen como HER2 +.

Dicho esto, pasamos a enumerar los trabajos publicados junto con un pequeño resumen de los mismos.

1. "**Olive oil´s bitter principle reverses acquired autoresistance to trastuzumab (Herceptin™) in HER2-overexpressing breast cancer cells".** J. A. Menendez, A. Vázquez- Martin, R. Colomer, J. Brunet, A. Carrasco-Pancorbo, R. García- Villalba, A. Fernández-Gutiérrez, A. Segura-Carretero. BMC Cancer 7 (2007) 80-99.

En este trabajo se evaluó el efecto anti-proliferativo y pro-apoptótico de varios compuestos fenólicos (Hyty, Ty Oleuropeína glucósido y Ol Agl) frente a células de cáncer de mama tipo HER2, siendo los resultados especialmente destacables para la Ol Agl. Además, en presencia de Ol Agl se aumentó unas 50 veces la eficacia del anticuerpo monoclonal anti-HER2 trastuzumab (medicamento que se prescribe a las mujeres con cáncer de mama conjuntamente a la quimioterapia) y células que habían adquirido autoresistencia frente a ese medicamento, se volvían a hacer sensibles al mismo cuando se les distribuía junto con la Ol Agl.

2. "tabAnti-HER2 (erbB-2) oncogen effects of phenolic compounds directly isolated from commercial Extra-Virgin Olive oil (EVOO)" J. A. Menendez, A. Vázquez- Martin, R. García- Villalba, A. Carrasco-Pancorbo, C. Oliveras-Ferraros, A. Fernández-Gutiérrez, A. Segura-Carretero. BMC Cancer 8 (2008) 377-400.

En este segundo trabajo se evaluaron los efectos anti-tumorales de las fracciones fenólicas aisladas que contenían fenoles simples (Hyty, Ty), ácido elenólico (EA), lignanos (Pin y Ac Pin) y secoiridoides (DOA, Ol Agl y Lig Agl) en células de cáncer de mama SKBR3 y MCF7/HER2. Los resultados mostraron que las fracciones aisladas ricas en lignanos (Ac Pin) y, sobre todo, en secoiridoides (Ol Agl y Lig Agl) fueron las más efectivas en la disminución de la proliferación celular, la inducción de muerte celular y en la reducción de los niveles de expresión y actividad del oncogén HER2.

3. "Analyzing effects of extra-virgin olive oil polyphenols on breast cancer-associated fatty acid synthase protein expression using reverse-phase protein microarrays" J. A. Menendez, A. Vázquez- Martin, C. Oliveras-Ferraros R. García-

Villalba, A. Carrasco-Pancorbo, A. Fernández-Gutiérrez, A. Segura-Carretero. International Journal of Molecular Medicine 22 (2008) 433-439.

La sintasa de ácidos grasos (FASN), enzima que produce la síntesis de novo de ácidos grasos, está sobreexpresada e hiperactivada en la mayoría de los carcinomas humanos. Por eso, la FASN y el metabolismo de los ácidos grasos está resultando de especial interés para el diagnóstico y el tratamiento del cáncer. En este caso se analizó el efecto de diferentes fracciones fenólicas aisladas y patrones comerciales de ácidos fenólicos y flavonoides en la expresión de FASN en células de cáncer de mama SKBR3 y MCF7/HER2 (HER2-positivas y que sobre-expresan FASN). Los flavonoides y las fracciones fenólicas ricas en secoiridoides y lignanos fueron potentes inhibidores de la expresión del FASN. De nuevo, como en el caso de HER2 (y probablemente de una manera relacionada, pues HER2 es un regulador positivo de FASN), es necesaria una estructura polifenólica para que puedan observarse efectos anti-tumorales.

4. "Extra-virgin olive oil polyphenols inhibit HER2 (erbB-2)-induced malignant transformation in human breast epithelial cells : Relationship between the chemical structures of extra-virgin olive oil secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2" J. A. Menendez, A. Vázquez- Martin, C. Oliveras-Ferraros R. García- Villalba, A. Carrasco-Pancorbo, A. Fernández-Gutiérrez, A. Segura-Carretero. International Journal of Oncology 34 (2009) 43- 51

En este último trabajo se muestran los efectos de las diferentes fracciones fenólicas en la actividad oncogénica de HER2. Para ello se evalúa el grado de fosforilación del residuo tirosina 1248 del dominio tirosina kinasa de la oncoproteína HER2. Este residuo es el principal sitio de autofosforilación de la oncoproteína HER2 y, por tanto, representa una medida indirecta de la capacidad de la proteína HER2 para transmitir señales oncogénicas. Significativamente todos lo fenoles del aceite de oliva virgen extra fueron capaces de reducir, en una manera dosis-dependiente, el status activo de la oncoproteína HER2. Estos descensos en la capacidad de transmitir señales

fueron especialmente dramáticos en el caso del lignano Ac Pin y los secoirioides. El estudio de la estequiometría de estos polifenoles podría proporcionar una excelente plataforma para el diseño de nuevos medicamentos anti-tumorales.

CAPÍTULO 5:
Caracterización y cuantificación del perfil polifenólico de aceites de oliva virgen extra con probada actividad anticancerígena mediante RRLC-ESI-TOF MS

Una vez estudiada la actividad anticancerígena de diferentes fracciones fenólicas aisladas de extractos de aceite de oliva, parecía lógico evaluar el extracto completo de fenoles con objeto de considerar los posibles efectos sinérgicos y antagónicos entre los diferentes compuestos. Se prepararon extractos de 8 aceites de oliva comerciales de 3 variedades diferentes de aceituna (Picual, Hojiblanca y Arbequina) mediante extracción en fase sólida con cartuchos DIOL y, en colaboración con el Instituto Catalán de Oncología de Gerona, se evaluó su actividad anti-proliferativa en diferentes líneas cancerígenas (SKBR3, MCF-7/HER2 y JIMT-1). En la mayoría de los casos se observó un importante descenso en la viabilidad celular cuando un 0.1% del extracto de aceite de oliva se añadía al medio de cultivo de las células, siendo los resultados especialmente buenos para el aceite de la variedad Picual. A la vista de estos resultados, pareció interesante tratar de encontrar una relación entre la composición polifenólica de los diferentes aceites de oliva y su actividad anticancerígena, con objeto de establecer qué compuesto o combinación de compuestos podrían ser los responsables de los efectos anti-cancerígenos que presenta esta matriz.

Para evaluar la composición polifenólica de los diferentes aceites en este quinto capítulo de la tesis nos propusimos desarrollar una nueva metodología analítica que fuese rápida, eficaz y sensible empleando cromatografía líquida acoplada a espectrometría de masas con analizador de tiempo de vuelo (LC ESI-TOF MS). Se utilizó un equipo de RRLC (Rapid Resolution Liquid Chromatography) que, sin llegar a ser un UPLC (Ultra Performance Liquid Chromatography), es capaz de soportar presiones más elevadas (hasta 600 bar) que un LC convencional, por lo que se puede trabajar con columnas de tamaño de partícula muy pequeño (1.8 µm) y flujos elevados, obteniendo separaciones rápidas, con alta eficacia y buena resolución y reproducibilidad. El objetivo era identificar el mayor número posible de compuestos polifenólicos en el perfil, utilizando la información de trabajos previos (como el estudio exhaustivo desarrollado en el capítulo 4) y la herramienta tan potente que es el TOF, e intentar llevar a cabo una cuantificación veraz de los compuestos identificados en los 8 aceites.

Debido a la ausencia de patrones comerciales de la mayoría de los fenoles del aceite de oliva, en los métodos desarrollados en bibliografía donde se cuantifican estos compuestos se suelen emplear patrones externos o estándares internos con estructura similar a los compuestos fenólicos que se quieren cuantificar, asumiendo que sus

factores de respuesta en el detector utilizado son iguales. Las diferencias a la hora de elegir los estándares para la cuantificación, junto con los numerosos factores que afectan a la composición polifenólica de un aceite (características genéticas de la variedad de aceituna cultivada, parámetros tecnológicos empleados durante el procesamiento de las aceitunas…), hacen que no sea raro encontrar en bibliografía datos de cuantificación muy diferentes, incluso en órdenes de magnitud. En el trabajo desarrollado en este capítulo se propone llevar a cabo la cuantificación teniendo en cuenta el contenido total de polifenoles (determinado por el método espectrofotométrico de Folin Cicolteau) y el porcentaje de cada compuesto (expresado como área relativa con respecto al área total del cromatograma) corregido con el factor de respuesta. Cada compuesto, en función de su estructura, presenta una respuesta diferente en el espectrómetro de masas, que depende en gran medida de su eficacia de ionización en la interfase. La determinación de los factores de respuesta de todos los compuestos en una muestra compleja es un trabajo bastante tedioso, que requiere la disponibilidad de patrones. Para llevar a cabo este trabajo nosotros disponíamos de patrones comerciales, (algunos de ellos, como el pinoresinol, puestos a la venta hacía relativamente poco tiempo), y otros compuestos aislados y caracterizados como puros en el trabajo desarrollado en el capítulo 4. Por lo tanto, podríamos establecer factores de respuesta para compuestos pertenecientes a diferentes familias y utilizarlos para llevar a cabo una cuantificación veraz.

Los datos que se obtuvieran acerca de la composición fenólica de cada uno de los aceites podrían ser tratados mediante técnicas exploratorias multivariantes, en particular análisis de componentes principales para establecer la relación entre cada compuesto fenólico determinado y la variedad del aceite, llegando incluso a identificar los fenoles decisivos a la hora discriminar entre los diferentes tipos de aceite, que como indicábamos antes, en estudios previos habían demostrado diferente actividad anticancerígena. De esta forma, se pretendía llegar al objetivo final de establecer una correlación entre la composición fenólica de los extractos de aceite de oliva y su capacidad antiproliferativa.

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Characterization and quantification of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method

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ABSTRACT

The characterization and quantification of extra-virgin olive oil (EVOO) phenolic compounds by a rapid resolution liquid chromatography (RRLC) method coupled to diode-array and time of flight mass spectrometry (TOF) detection systems was developed. The RRLC method transferred from a conventional HPLC one achieved better performance with shorter analysis times. The phenolic compounds were separated with a C18 column (150 mm × 4.6 mm, 1.8 μm) using water with 0.5% acetic acid and acetonitrile as mobile phases. Good peak resolution was obtained and 19 different phenols were identified in less than 20 min providing a new level of information about the samples in shorter time. The applicability of this analytical approach was confirmed by the successful analysis of three different EVOO varieties (Picual, Hojiblanca, and Arbequina) obtained from different trademarks. Besides identification of the most important phenolic compounds and their quantification in three different ways (RRLC-UV, RRLC-MS and a new approach using the total polyphenol content obtained with Folin Ciocalteau, the relative areas and the response factors), we also described the occurrence of correlations between the phenolic composition of EVOO-derived crude phenolic extracts and their anti-proliferative abilities toward human breast cancer-derived cell lines. When compared with lignans-rich EVOO varieties, secoiridoids-rich EVOO had a significantly strong ability to alter cell viability in four different types of human breast carcinoma cells.

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1. Introduction

For centuries, Mediterranean people have appreciated the nutritional, medical and cosmetics benefits of olive oil. Nowadays, a growing number of evidences point to the important role that extra-virgin olive oil (EVOO) plays as a crucial ingredient of the Mediterranean diet regarding their beneficial effects on health [1]. The hypothesis that minor components such as phenolic compounds could play a major role in the healthy effects of EVOO – including the prevention of chronic diseases such as cancer, obesity, diabetes, or coronary diseases – has gradually been increasing and several studies have attempted to elucidate the ultimate mechanisms through which EVOO-derived phenols might contribute to these healthy properties [2–8]. On the other hand, phenolic compounds also affect the organoleptic properties (flavour, astringency, ...) [9–11] and oxidative stability of EVOO [12,13]. Considering the importance of this class of analytes, it would be very interesting to develop fast and powerful analytical methods for the characterization and quantification of this important family of EVOO compounds.

The development of methodologies for the determination of phenols in EVOO has been discussed extensively in literature. Due to the need to carry out an individual identification of each phenolic compound present in the extracts, the traditional methods

Abbreviations: Ac Pin, (+)-1-acetoxypinoresinol; Apig, apigenin; D-Lig Agl, decarboxymethyl ligstroside aglycon; DOA, decarboxymethyl oleuropein aglycon; EA, elenolic acid; EVOO, extra-virgin olive oil; Hyty, hydroxytyrosol; Hyty-Acet, hydroxytyrosol acetate; H-EA, hydroxy elenolic acid; H-Ol Agl, hydroxy oleuropein aglycon; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycon; H-DOA, hydroxy decarboxymethyl oleuropein aglycon; Lig Agl, ligstroside aglycon; LOD, limit of detection; LOQ, limit of quantification; Lut, luteolin; Ol Agl, oleuropein aglycon; Pin, (+)-pinoresinol; RSD%, relative standard deviation; Syri, syringaresinol; Ty, tyrosol; TPC, total polyphenol content.

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were replaced with separative techniques [*e.g.* gas chromatography (GC) [14], high-performance liquid chromatography (HPLC) [15,16], and capillary electrophoresis (CE) [17] coupled to different detectors [18]. However, most of the discussions have been focused on the optimization of high-performance liquid chromatography (HPLC) methods, mainly with reversed phase C18 columns and different mobile phases and gradients, followed by ultraviolet (UV) [19], electrochemical [20], fluorescence [21] or mass spectrometric (MS) detection [17,22]. Recently, an improvement in chromatographic performance has been achieved by the introduction of rapid-resolution LC (RRLC) and ultra-performance LC (UPLC) [23]. These approaches use narrow-bore columns packed with very small particles (1.8 μm) and high flow rate with delivery systems operating at high back-pressures. The major advantages of RRLC over conventional HPLC are improved resolution, shorter retention times, higher sensitivity, and better performance. Coupling RRLC with MS further offers a potent analytical alternative, which has been applied in recent publications characterizing food products [24–28].

Our first goal herein was to develop a RRLC-ESI-TOF MS-based alternative method for the rapid identification and quantitation of the most representative phenolic compounds present in different EVOOs. Quantitative or semi-quantitative information on olive oil phenols is of great interest to find the compounds responsible of the olive oil benefits and to distinguish EVOOs with different anticancer properties. Pure reference standards for each analyte are required to get an accurate quantitation based on the construction of calibration curves; however, due to the lack of commercial standards, only a few compounds have been quantified in this way and different approaches have been followed trying to quantify as many compounds as possible in this complex matrix: considering external standards with similar structure or even with structure completely different to the compound under study and using internal standards added to the extract that we wanted to analyze. One of the limitations of these approaches is that the relative response of the different compounds in mass spectrometry (and also in other detection systems) is very sensitive to the variations in chemical structure and the error in the quantification may be high. In the current work, we propose a new approach for direct and reliable quantitation of olive oil phenolic compounds taking into account the total polyphenol content determined by Folin Ciocalteau method and the response factors of the phenolic compounds in MS. The quantitative results were compared with those obtained using RRLC-UV and RRLC-ESI-TOF MS.

On the other hand, in previous studies we have demonstrated that individual phenolic fractions obtained from a 50/50 mixture of two commercial EVOO samples induced cytotoxic effects toward cultured human breast cancer cells [4,5,7]. Importantly, EVOO polyphenols (*i.e.* lignans and secoiridoids) – but not monophenols and phenolic acids – strongly suppressed the growth of breast cancer cells bearing high levels of HER2 (*erb*B2) – one of the most commonly analyzed oncogenes that plays a decisive role in malignant transformation, tumorigenesis and metastasis in a biologically aggressive subset of human breast carcinomas. However, it is necessary to consider that because of the biological effects of phenolic compounds – including tumoricidal actions – are varied and compound specific, combinatorial effects (*i.e.* addition, antagonism or synergism) can occur in EVOO naturally exhibiting enriched or low levels of specific phenolics.

Therefore the second aim of this study was to investigate the anti-breast cancer effects of whole crude EVOO phenolic extracts containing significantly different phenolic compositions and to determine the relationship between the chemical nature and/or the concentration of individual phenolic compounds and the ability of each crude EVOO phenolic extract to decrease breast cancer cell viability.

2. Materials and methods

2.1. Chemicals and samples

Methanol and *n*-hexane of HPLC grade used for the extraction of the phenolic compounds from the olive oil samples were supplied from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used for preparing mobile phase. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA).

Standards of hydroxytyrosol, tyrosol, luteolin and apigenin were purchased by Sigma–Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Other phenolic compounds used as pure standard samples, elenolic acid and ligstroside aglycon, were isolated from EVOOs by semipreparative HPLC [29]. Stock solutions at concentration of 500 mg/L for each phenol were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations.

Eight EVOO samples used for the study were acquired from a supermarket (Granada, Spain). EVOOs of three different olive fruit varieties so-called Picual, Hojiblanca and Arbequina and from different trademarks (Carbonell, Borges, Hojiblanca and Coosur) were chosen for the analysis. As it is considered in literature EVOOs are relatively constant in terms of lipid composition, but the micronutrient contents (*e.g.* α-tocopherol, carotenoids, sterols and phenolic compounds) significantly vary based upon the localization of cultivation, climate, olive variety and production techniques [30,31].

2.2. Breast cancer cell lines and culture conditions

MCF-7 and SKBR3 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and they were routinely grown in Improved MEM supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine. Construction of pBABE/HER2, retroviral infection of MCF-7 and stable selection of MCF-7/HER2 cells were performed as described elsewhere. JIMT-1 cells were established at Tampere University and are available from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/). JIMT-1 cells were grown in F-12/DEMEM (1:1) supplemented with 10% FBS and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Sample extraction

Specific solid phase extraction (SPE) method with Diol-cartridges, previously described elsewhere [32], was used with the aim of obtaining the major number of phenolic compounds at the highest concentration from each EVOO matrix. Briefly, the extraction consisted of passing through a column, previously conditioned with 10 mL of methanol and 10 mL of hexane, 60 g of EVOO dissolved in 60 mL of hexane. After removing the non-polar fraction with 15 ml of hexane, the phenolic compounds were recovered with methanol (40 ml). The final volume was dried in a rotary evaporator under reduced pressure at 35 °C and the residue was reconstituted in 2 mL of methanol.

2.4. Determination of total phenols

Total phenol content of different EVOO extracts was determined using Folin-Ciocalteu technique [33]. Briefly, 50 μL of the 1:10 diluted methanolic extracts of EVOOs was assayed with 250 μL of Folin reagent and 500 μL of saturated solution of sodium carbonate. The mixture was diluted with water to a final volume of

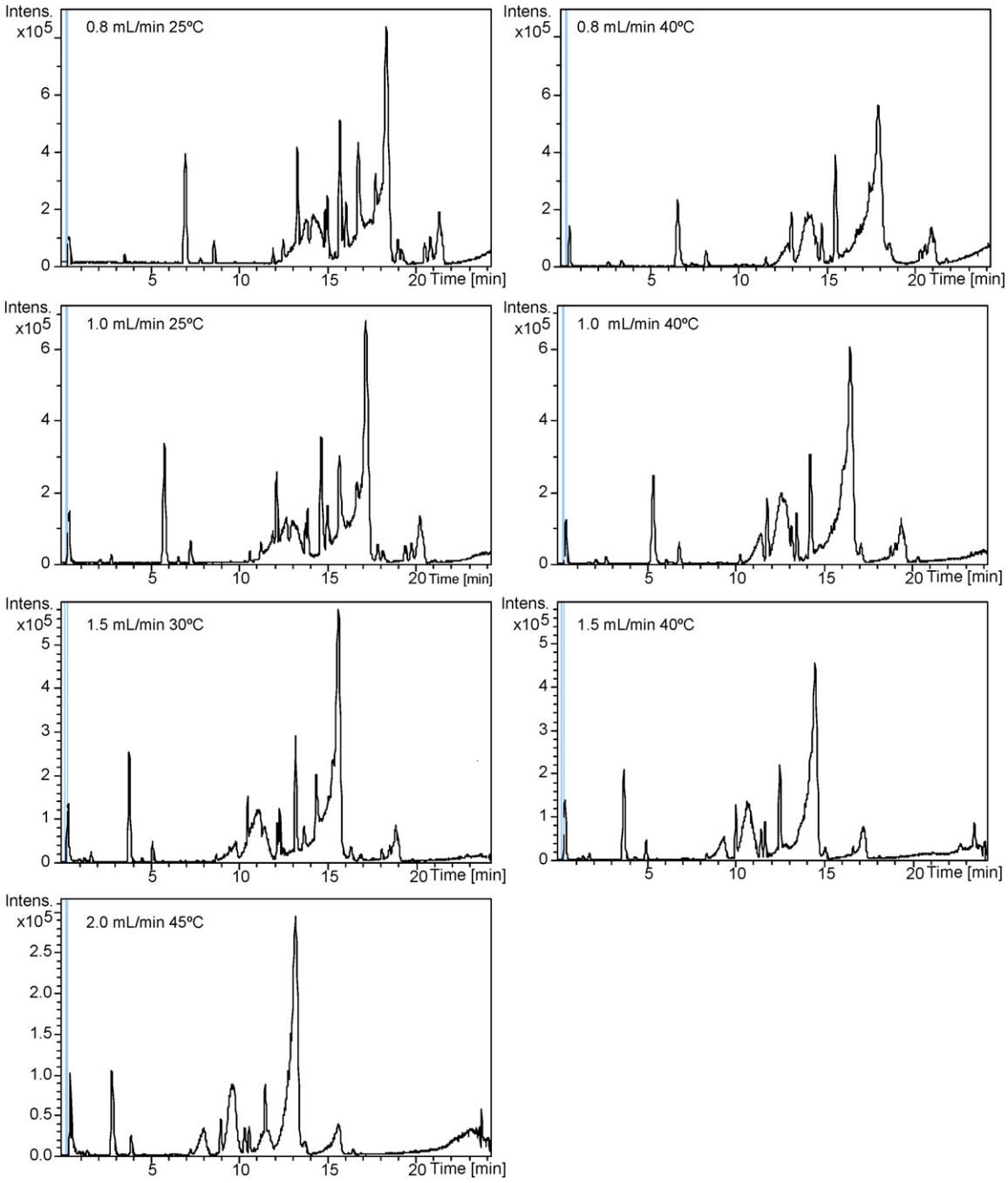

Fig. 1. RRLC-ESI-TOF Base peak chromatograms (BPC) of an olive oil extract (Picual Borges) using different mobile phase flow rates and temperatures. The rest of chromatographic conditions were those described in Section 2, the analytical column was a C_{18} Zorbax column, 1.8 μm i.d., 4.6 mm × 150 mm and the mobile phase: water + 0.5% acetic acid (A) and ACN (B).

5 mL. The absorbance relative to that of the blank was measured using Spectronic Genesys 5 (Rochester**,** NY, USA) at 725 nm after incubation for 1 h at room temperature. The total polyphenol content was expressed as mg/kg olive oil of caffeic acid. For the caffeic acid, the curve absorbance versus concentrations is described by the equation $y = 28.773x - 0.2104$ ($R^2 = 0.998$).

2.5. Rapid resolution liquid chromatography analyses

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. Polyphenolic compounds were separated by using a Zorbax C18 analytical column (4.6 × 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at 30 °C and a flow rate of 1.5 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: 0–10 min, 5–30% B; 10–12 min, 30–33% B; 12–17 min, 33–38% B; 17–20 min, 38–50% B; 20–23 min, 50–95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 10 min. A volume of 10 μL of the 1:10 diluted methanolic extracts of olive oil was injected. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.

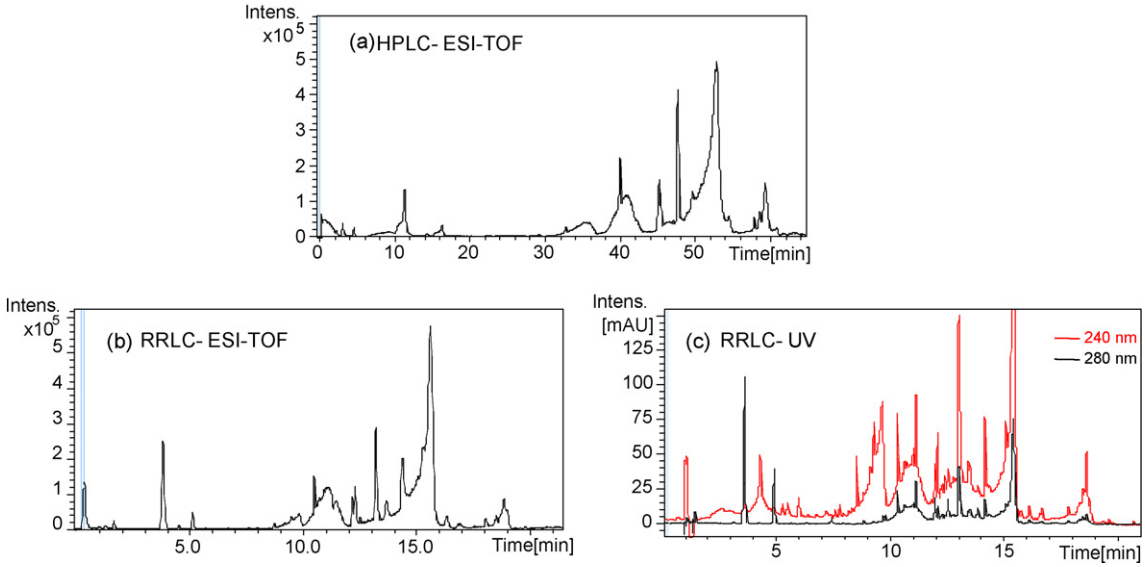

Fig. 2. Comparison of the chromatograms obtained with the previous HPLC-ESI-TOF method (a) and the new optimized RRLC method with both detectors: TOF (b) and UV (280 and 240 nm) (c). Chromatographic conditions were described in Section 2.

2.6. Mass spectrometry

The RRLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). TOF analyzers provide greatly improved mass resolution (8000–10,000 at 250 *m/z*) and significantly higher sensitivity and accuracy when acquiring full-fragment spectra compared with traditional instruments. The parameters of the mass spectrometer were similar to those previously optimized in recent works with the same EVOO matrix [29] acquiring spectra in the range of 50–800 *m/z* in the negative mode.The flow rate used in the new RRLC method 1.5 ml/min was too high for achieving a stable electrospray ionization (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 0.21 mL/min, low enough to avoid the introduction of humidity in the system. According to this inflow the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190 °C.

SmartFormula™ tool within DataAnalysis was used for the calculation of elemental composition of compounds; it lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulas, the TIP adds a second dimension to the analysis, using the masses and intensities of each isotope to do a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue™). 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.

2.7. Metabolic status assessment (MTT-based cell viability assays)

Breast cancer cells were seeded at a density of 3000 cells per well in a 96-well plate. The next day, cells were treated with concentrations ranging from 0% to 0.1% (v/v) of crude EVOO phenolic extracts dissolved in ethanol. An appropriate amount of ethanol (v/v) was also added to control cells. After 5 days of treatment (extracts were not renewed during the entire period of cell exposure), the cells

Table 1
Main phenolic compounds identified in an olive oil extract (Picual Borges) by RRLC-ESI-TOF including: retention time, ISCID (Internal source collision induced dissociation), fragments, *m/z* experimental and calculated, molecular formula, error and sigma.

Compounds	Retention time (min)	Fragments	*m/z* experimental	*m/z* calculated	Molecular formula	Error	Sigma
Hydroxytyrosol	3.9	123	153.0559	153.0557	$C_8H_{10}O_3$	−0.9	0.013
Tyrosol	5.2		137.0606	137.0608	$C_8H_9O_2$	1.7	0.059
Hydroxy elenolic acid	8.7	181, 137	257.0651	257.0667	$C_{11}H_{13}O_7$	6.0	0.026
Hydroxytyrosol acetate	9.0		195.0670	195.0662	$C_{10}H_{11}O_4$	−3.8	0.036
Elenolic acid	9.8	139	241.0713	241.0718	$C_{11}H_{13}O_6$	2.0	0.042
Hydroxy D-oleuropein aglycon	10.5	199	335.1150	335.1136	$C_{17}H_{19}O_7$	−4.2	0.053
Decarboxymethyl oleuropein aglycon	11.1	183	319.1190	319.1187	$C_{17}H_{19}O_6$	−1.0	0.031
Luteolin	12.1		285.0407	285.0405	$C_{15}H_{19}O_6$	−0.8	0.006
Syringaresinol	12.2		417.1537	417.1555	$C_{22}H_{25}O_8$	4.3	0.034
Hydroxy D-ligstroside aglycon	12.3	199	319.1202	319.1187	$C_{17}H_{19}O_6$	−4.6	0.045
Pinoresinol	12.7		357.1347	357.1344	$C_{20}H_{21}O_6$	−0.9	0.017
Acetoxypinoresinol	13.1		415.1389	415.1398	$C_{22}H_{24}O_8$	0.9	0.013
10-Hydroxy oleuropein aglycon	13.2		393.1205	393.1191	$C_{19}H_{21}O_9$	−3.5	0.044
Decarboxymethyl ligstroside aglycon	13.3	183	303.1245	303.1238	$C_{17}H_{19}O_5$	−2.4	0.014
Apigenin	14.0		269.0452	269.0455	$C_{15}H_{19}O_5$	1.4	0.030
Methyl D-oleuropein aglycon	15.2		333.1357	333.1344	$C_{18}H_{21}O_6$	−3.9	0.052
Oleuropein aglycon	15.6	345, 307, 275	377.1253	377.1242	$C_{19}H_{21}O_8$	−2.8	0.044
Methyl oleuropein aglycon	18.5	345, 275	391.1405	391.1398	$C_{20}H_{23}O_8$	−1.8	0.079
Ligstroside aglycon	18.7	291, 241	361.1303	361.1293	$C_{19}H_{21}O_7$	−2.8	0.046

Table 2
Analytical parameters for the RRLC-ESI-TOF and RRLC-UV methods: relative standard deviation (RSD%), limit of detection (LOD) and quantitation (LOQ), linearity, calibration curves and r^2.

Analytes	RSD%	LOD (μg/ml)	LQ (μg/ml)	Linearity (μg/ml)	Calibration curves	r^2
Hyty						
UV	1.4	0.15	0.50	LQ-100	$y = 6.533x + 11.177$	0.996
TOF	4.6	0.09	0.30	LQ-50	$y = 39934x + 42004$	0.993
Ty						
UV	1.5	0.35	0.66	LQ-100	$y = 4.197x + 2.417$	0.999
TOF	2.1	0.31	1.03	LQ-50	$y = 12596x + 26635$	0.991
EA						
UV	1.1	3.50	11.67	LQ-300	$y = 10.665x - 25.26$	0.998
TOF	3.4	1.44	4.80	LQ-300	$y = 6688x + 76261$	0.991
Pin						
UV	1.8	0.08	0.26	LQ-100	$y = 5.632x + 5.094$	0.999
TOF	3.3	0.06	0.20	LQ-50	$y = 37578x + 53556$	0.991
Lut						
UV	0.8	0.04	0.13	LQ-100	$y = 11.131x + 7.926$	0.999
TOF	2.8	0.02	0.06	LQ-25	$y = 114566x + 59826$	0.994
Apig						
UV	0.8	0.04	0.13	LQ-100	$y = 17.292x + 4.996$	0.999
TOF	2.0	0.02	0.06	LQ-25	$y = 150131x + 118916$	0.991
Lig Agl						
UV	1.6	1.50	5.00	LQ-100	$y = 0.853x + 3.851$	0.991
TOF	3.0	0.43	1.43	LQ-300	$y = 9019x + 59184$	0.993

Every compound was quantified in UV at 280 nm except EA at 240 nm.
Hyty: hydroxytyrosol; Ty: tyrosol; EA: elenolic acid; Pin: pinoresinol; Lut: luteolin; Apig: apigenin; Lig Agl: ligstroside aglycon.

Table 3
Quantitative results (mg/kg) achieved by RRLC-UV, RRLC-ESI-TOF MS and the approach using the results obtained with Folin Ciocalteau method, the relative areas and the response factors.

Olive oils	Hyty	Ty	EA	Lut	Pin	Apig	Lig Agl
P. Carbonell							
UV	11.23 (a)	10.98 (a)	62.73 (a)	2.94 (a)	5.26 (a)	0.93 (a)	45.54 (a)
TOF	10.67 (a)	9.24 (b)	48.15 (b)	3.28 (a)	1.54 (b)	1.00 (a)	48.30 (a)
RF	9.25 (b)	7.89 (c)	47.81 (b)	3.45 (a)	1.80 (b)	1.28 (a)	47.67 (a)
P. Borges							
UV	22.37 (a)	12.43 (a)	78.34 (a)	1.59 (a)	3.37 (a)	0.47 (a)	51.01 (a)
TOF	20.20 (a)	11.84 (a)	68.18 (b)	1.84 (b)	0.77 (b)	0.43 (a)	64.78 (b)
RF	17.90 (a)	10.31 (a)	67.48 (b)	2.11 (b)	1.22 (b)	0.74 (a)	65.83 (b)
A. Carbonell							
UV	4.32 (a)	3.84 (a)	27.17 (a)	4.27 (a)	5.61 (a)	1.34 (a)	6.08 (a)
TOF	3.64 (a)	2.74 (b)	8.61 (b)	4.17 (a)	2.56 (b)	1.56 (a)	6.98 (a)
RF	3.80 (a)	3.22 (b)	13.17 (b)	4.82 (a)	2.95 (b)	1.83 (a)	9.96 (b)
A. Borges							
UV	4.02 (a)	2.78 (a)	31.12 (a)	4.28 (a)	5.54 (a)	1.24 (a)	9.91(a)
TOF	3.37 (b)	2.33 (a)	10.47 (b)	4.41 (a)	2.24 (b)	1.22 (a)	17.38 (b)
RF	3.37 (b)	2.72 (a)	12.26 (b)	4.84 (a)	2.52 (b)	1.57 (a)	19.77 (b)
H. Borges							
UV	10.25 (a)	7.41 (a)	56.67 (a)	3.28 (a)	5.98 (a)	1.13 (a)	33.29 (a)
TOF	9.76 (a)	6.56 (a)	33.00 (b)	3.47 (a)	2.26 (b)	0.99 (a)	38.23 (a,b)
RF	9.85 (a)	6.76 (a)	38.85 (b)	3.73 (a)	2.41 (b)	1.26 (a)	44.37 (b)
H. Hojiblanca							
UV	5.87 (a)	6.28 (a)	67.6 (a)	4.17 (a)	3.31(a)	2.27 (a)	26.78 (a)
TOF	5.21 (a)	5.35 (a)	43.47 (b)	4.15 (a)	0.68 (b)	2.51 (a)	32.20 (a,b)
RF	5.50 (a)	5.71 (a)	46.92 (b)	5.03 (a)	1.02 (b)	3.12 (b)	38.03 (b)
H. Carbonell							
UV	10.30 (a)	6.25 (a)	67.74 (a)	5.64 (a)	5.34 (a)	2.07 (a)	38.82 (a)
TOF	9.51 (a)	5.62 (a)	50.46 (b)	5.75 (a)	1.13 (b)	1.57 (a)	50.73 (b)
RF	8.83 (a)	5.44 (a)	52.18 (b)	6.29 (a)	1.56 (b)	1.95 (a)	53.22 (b)
P. Coosur							
UV	7.73 (a)	7.15 (a)	50.18 (a)	1.77 (a)	6.42 (a)	0.51 (a)	58.04 (a)
TOF	7.08 (a)	6.76 (a)	31.62 (b)	1.82 (a)	1.57 (b)	0.49 (a)	48.86 (a)
RF	6.95 (a)	6.63 (a)	35.79 (b)	2.12 (a)	2.03 (b)	0.64 (a)	53.48 (a)

P: Picual; A: Arbequina; H: Hojiblanca.
Means in the same table cell with different letters are significantly different ($p \leq 0.05$).

were incubated with a solution of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma, St. Louis, MO, USA) at a concentration of 5 mg/mL for 3 h at 37 °C. The supernatants were then carefully aspirated, 100 μL of DMSO was added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were read at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from the exposure of cells to each crude EVOO phenolic extract were analyzed as percentages of the control cell absorbances. For each treatment, cell viability was evaluated as a percentage using the following equation: (A_{570} of treated sample/A_{570} of untreated samples) × 100. Breast cancer cell sensitivity to crude EVOO phenolic extracts was expressed in terms of the concentration of extract (v/v) required to decrease by 50% cell viability (IC_{50} value). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{50} values were defined as the concentration of extracts that produced 50% reduction in control absorbance (by interpolation).

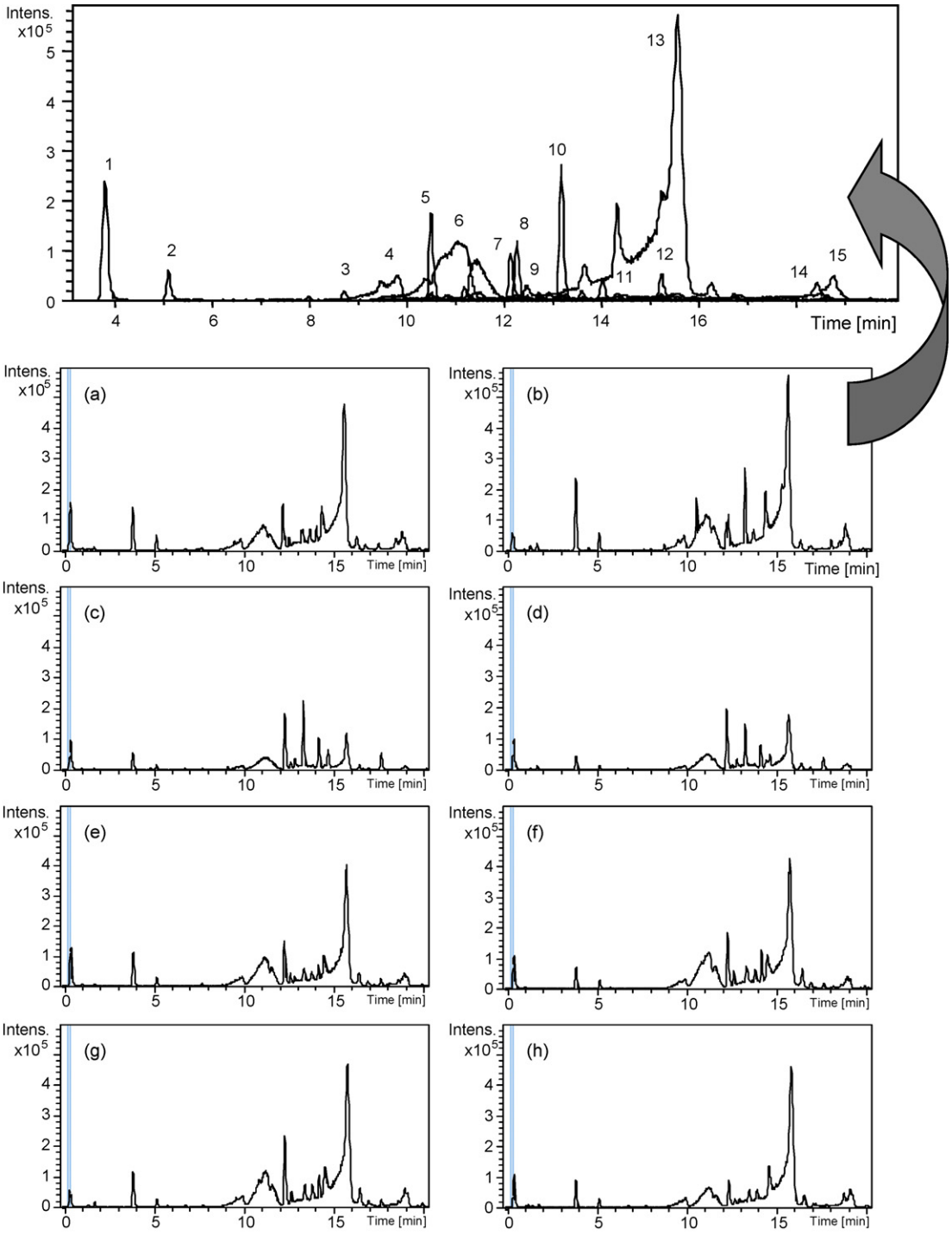

Fig. 3. (A) Extracted ion chromatograms (EICs) of the main phenolic compounds identified in the olive oil extract (Picual Borges) using the optimized RRLC-ESI-TOF method. Peak identification: 1, Hyty; 2, Ty; 3, H-EA; 4, EA; 5, H-DOA; 6, DOA; 7,Lut; 8, H-D-Lig Agl; 9, Pin; 10, H-Ol Agl; 11, Apig; 12, Methyl-DOA, 13, Ol Agl; 14, Methyl-Ol Agl; 15, Lig Agl. (B) Base peak chromatograms (BPCs) of the eight extra-virgin olive oils chosen for this study. (a) Picual Carbonell; (b) Picual Borges; (c) Arbequina Carbonell; (d) Arbequina Borges; (e) Hojiblanca Borges; (f) Hojiblanca Hojiblanca; (g) Hojiblanca Carbonell; (h) Picual Coosur.

Table 4
Response factors (RFs) of the seven phenols standards determined with ESI-TOF MS. RFs are expressed relative to Hyty, which is set to 1.00.

Compounds	Slope (a)	RFs
Hyty	39,934	1
Ty	12,596	3.17
EA	6,688	5.97
Pin	37,578	1.06
Lut	114,566	0.35
Apig	151,131	0.26
Lig Agl	9,019	4.43

For ESI-MS detection, a and b values are coefficients of the linear calibration dependence $y = ax + b$ and RFs are calculated as RF (phenolic compounds) = $a_{Hyty}/a_{phenolic\ compound}$.

2.8. Statistics

As far as phenolic compounds are concerned, the results reported in this study are the averages of at least three repetitions ($n = 3$), unless otherwise stated. Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK). Data were also analyzed by Multivariate Exploratory Techniques in particular factor analysis and principal components and classification analysis (using Statistica 6.0) to evidence the correlation between the determined analytes and the differences in the samples. The analytical data were arranged in a matrix with the rows corresponding to the samples (objects) and the columns corresponding to the analytical indices (variables).

Results from breast cancer cell viability assays are expressed as the mean of three independent experiments. For each independent experiment, three replicate determinations were performed and a mean value was calculated. A two-way analysis of variance model, including the effects for cell line, dose and interaction between cell line and dose, was used to evaluate the relationship between cell viability and those effects.

3. Results and discussion

3.1. Development of a RRLC method

Typically, the separation of complex EVOO samples has required longer run time, in some cases more than 60 min, using conventional HPLC methods (column packed with 5 μm particles) [12,19,20]. The introduction of a new kind of chromatography RRLC allows working with columns of very small particle size (1.8 μm) and high flow withstanding high pressures. In this way, reducing the particle size the efficiency of the column increases and the resolution becomes independent of analysis time. Working with the flow and temperature we can get shorter analysis time and a maximum resolution between peaks. Shorter run times mean less peak capacity; therefore users should choose a balance between peak capacity and run time [34–36].

Based on the chromatographic conditions of a previous HPLC method used so far in our research group (Gemini C18 column: 3 mm × 250 mm, 5 μm particle size) [12], the optimization of a new RRLC method was carried out (Column Zorbax C18: 4.6 mm × 150 mm and 1.8 μm particle size). Length was reduced roughly by half in order to obtain faster analyses, maintaining a diameter of 4.6 mm, and a small column particle size was chosen to increase the efficiency of the separation with an excellent time of life and resistant to high pressures. The gradient, injection volume, flow rate, column temperature and dilution of the sample were optimized, following the general rules for the conversion of a HPLC method to RRLC method.

As a starting point for optimization, maintaining the composition of the mobile phase (phase A: H_2O + 0.5% acetic acid, phase B: ACN) and other variables from the original HPLC method (0.5 mL/min at 25 °C) different gradients were tested, changing the gradient slope until no significant reduction in resolution was observed. The optimum gradient was: from 5 to 30% B in 10 min, from 30 to 33% B in 2 min, from 33 to 38% B in 5 min, from 38 to

Table 5
Total polyphenol content (TPC) expressed as caffeic acid equivalents (Folin Ciocalteau method) and concentrations (mg/kg) of 19 phenolic compounds identified in eight EVOO samples using the combination of the total polyphenol content, the area percentage of each phenol in the total area of the chromatogram and the response factors of each particular phenol.

	P. Carbonell	P. Borges	A. Carbonell	A. Borges	H. Borges	H. Hojiblanca	H. Carbonell	P. Coosur
TPC (mg/kg caffeic acid)	189.39	260.98	102.89	109.61	190.2	230.6	219.5	169.8
Compounds								
Hyty	9.25 (a)	17.90 (b)	3.80 (c)	3.37 (c)	9.85 (a)	5.50 (d)	8.83 (a)	6.95 (e)
Ty	7.89 (a)	10.31 (b)	3.22 (c)	2.72 (d)	6.76 (e)	5.71 (f)	5.44 (f)	6.63 (e)
H-EA[a]	0.92 (a,b)	5.09 (c)	0.33 (b,d)	0.28 (d)	1.21 (a)	1.10 (a)	1.05 (a)	0.61 (b)
Hyty-Acet[b]	0.30 (a,b)	0.14 (b)	0.72 (c)	0.35 (a)	0.30 (a,b)	0.37 (a)	0.48 (a)	0.44 (a)
EA	47.81 (a)	67.48 (b)	13.17 (c)	12.26 (c)	38.85 (d)	46.92 (a,e)	52.18 (e)	35.79 (d)
H-DOA[c]	9.30 (a)	41.51 (b)	3.79 (c)	3.56 (c)	13.66 (d)	19.22 (e)	13.65 (d)	5.64 (f)
DOA[c]	192.33 (a)	301.44 (b)	111.01 (c)	127.46 (d)	254.25 (e)	352.34 (f)	295.45 (b)	169.14 (g)
Lut	3.45 (a)	2.11 (b)	4.82 (c)	4.84 (c)	3.73 (d)	5.03 (c)	6.29 (e)	2.12 (b)
Syri[d]	0.78 (a)	0.29 (b)	1.82 (d)	1.31 (e)	0.63 (a,c)	0.68 (a,c)	0.61 (a,c)	0.47 (c,b)
H-D-Lig Agl[c]	11.83 (a)	31.14 (b)	3.95 (c)	3.57 (c)	9.00 (d)	9.45 (d)	8.60 (d,e)	7.53 (e)
Pin	1.80 (a)	1.22 (b,c)	2.95 (d)	2.52 (d)	2.41(d)	1.02 (c)	1.56 (a,b)	2.03 (a)
Ac Pin[d]	2.05 (a,b)	0.99 (c)	16.32 (d)	10.90 (e)	1.55 (a,c)	5.77 (f)	2.88 (b)	0.88 (c)
H-Ol Agl[c]	17.42 (a)	74.43 (b)	1.76 (c)	3.06 (c)	15.72 (a,d)	12.09 (e)	12.78 (d,e)	10.57 (e)
D-Lig Agl[c]	33.37 (a)	29.48 (b)	16.72 (c)	18.73 (c)	26.77 (b,d)	24.94 (d)	27.24 (b,d)	29.82 (b)
Methyl DOA[c]	5.69 (a)	6.91 (b)	1.40 (c)	2.32 (d)	4.54 (e)	5.19 (f)	5.62 (a)	4.45 (e)
Apig	1.28 (a)	0.74 (b)	1.83 (c)	1.57 (a)	1.26 (a)	3.12 (d)	1.95 (c)	0.64 (b)
Ol Agl[c]	310.00 (a)	366.43 (b)	64.45 (c)	107.93 (d)	261.36 (e)	315.05 (a)	306.45 (a)	305.71(a)
Methyl Ol Agl[c]	24.93 (a)	22.22 (a)	2.78 (b)	2.91 (b)	17.22 (c)	11.65 (d)	14.87 (c,d)	34.96 (e)
Lig Agl	47.67 (a)	65.83 (b)	9.96 (c)	19.77 (d)	44.37 (a)	38.03 (e)	53.22 (f)	53.48 (f)

P: Picual; A: Arbequina; H: Hojiblanca. Means in the same row with different letters are significantly different ($p \leq 0.05$).

[a] Quantified by using response factor of EA.

[b] Quantified by using response factor of Hyty.

[c] Quantified by using response factor of Lig Agl.

[d] Quantified by using response factor of Pin.

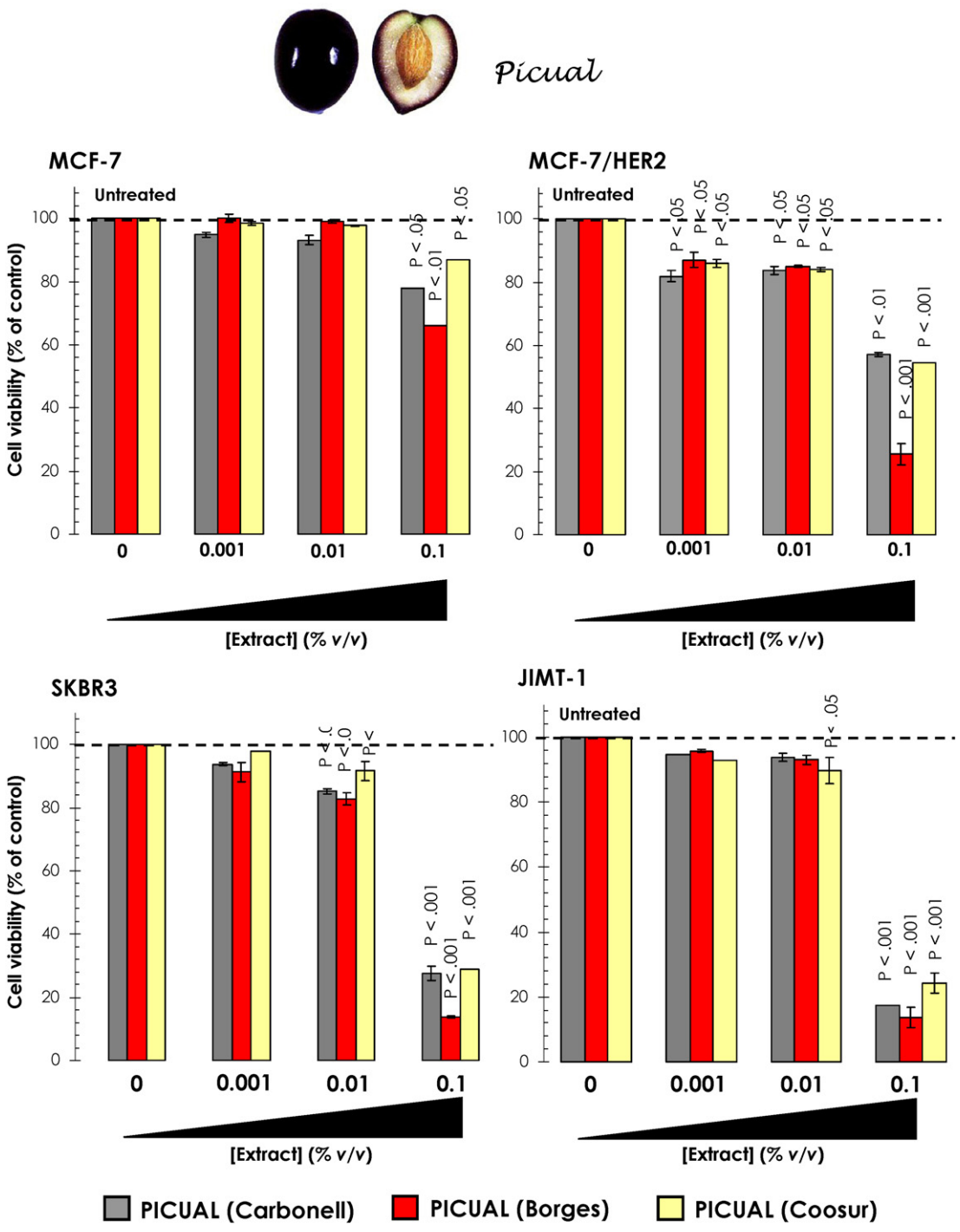

Fig. 4. Antiproliferative effects of crude EVOO phenolic extracts (PICUAL variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Picual variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

50% B in 3 min, and from 50 to 95% B in 3 min; then the percentage of B was reduced again to 5% B in 2 min and we kept the initial conditions for 10 min. With the optimized gradient the runtime could already be reduced by 40%. In order to increase the resolution between peaks the injected volume was reduced from 20 to 10 μL.

In the next step, the flow rate was increased from 0.5 to 2.0 mL/min (passing through the following steps: 0.5, 0.8, 1.0, 1.5, 2.0 mL/min). With increasing flow rates the back-pressure of the system rises until to reach the maximum value (80–90% of the pressure accepted by the chromatograph). However, if the temperature of the column also increases (25, 30, 35, 40 °C), the viscosity of the mobile phase decreases and the system back-pressure is reduced. Choosing a suitable temperature the flow could be further increased up to the maximum value. With acetonitrile as organic mobile phase the maximum flow rate until the maximum system back-pressure is reached is higher than for methanol because its viscosity is lower. In Fig. 1 we can observe that increasing the flow and the temperature shortened the run time without compromising too much resolution, but with temperatures above 40 °C there

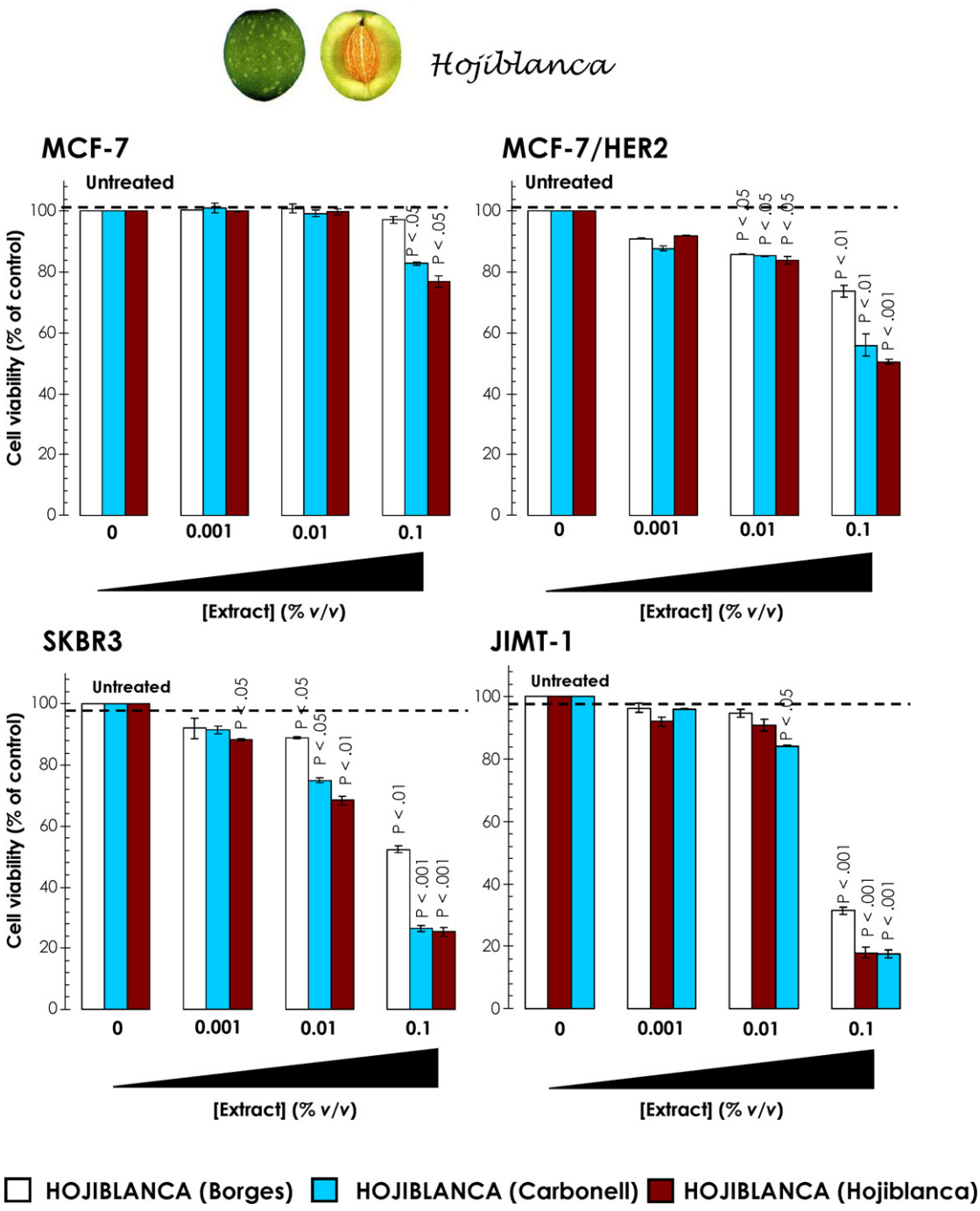

Fig. 5. Antiproliferative effects of crude EVOO phenolic extracts (HOJIBLANCA variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Hojiblanca variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

was overlapping of some peaks and loss of compounds. Finally, an optimum flow of 1.5 mL/min and temperature of 30 °C, maintained by a column thermostat, were selected since they ensure that the system pressure is not exceeded even later at the end of the run. The maximum pressure reached during this analysis was approximately 450 bar. As described in the experimental section this flow rate is too high for electrospray (ESI) so, it is necessary to use a splitter 1:6. The detection was carried out apart from mass spectrometry (TOF) with UV at two wavelength characteristics of the phenolic compounds of interest, 280 and 240 nm.

Fig. 2 shows the chromatograms of the same EVOO sample (Picual Borges) analyzed using the conventional HPLC and the RRLC method. With the optimum RRLC method the run time could be reduced from 60 min (Fig. 2a) to 20 min (Fig. 2b and c) and the analyst could achieve even better performance by using that method. This was achieved by using steeper gradients, increased temperature and higher flow rates. The peaks in Fig. 2b were narrower than those in Fig. 2a what means that the peak capacity increased. In general the results with the RRLC method were superior to those with the corresponding HPLC method providing better analysis time, separation and resolution.

3.2. Identification of EVOO phenolic compounds

Once analytical conditions for separation and detection were optimized, the RRLC-ESI-TOF method was used to identify and quantify the phenolic profile of eight commercial EVOOs. Peak identification was done by comparing both migration time and MS spectral data obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC); we also used the information previously reported [17,22,29] and the information provides with time of flight analyzer (TOF) by the mass spectrometer. TOF MS instrumentation with excellent mass resolution and mass accuracy in combination with true isotopic pattern is the perfect choice for molecular formula determination of small molecules using the editor SmartFormula^{TM}. For routine screening practice, a SigmaFit^{TM} tolerance of 0.05 and a mass tolerance of 5 ppm were chosen. Table 1 summarizes the main compounds identified in the Picual Borges variety including the information generated by TOF analyzer: retention time; product ions obtained spontaneously in

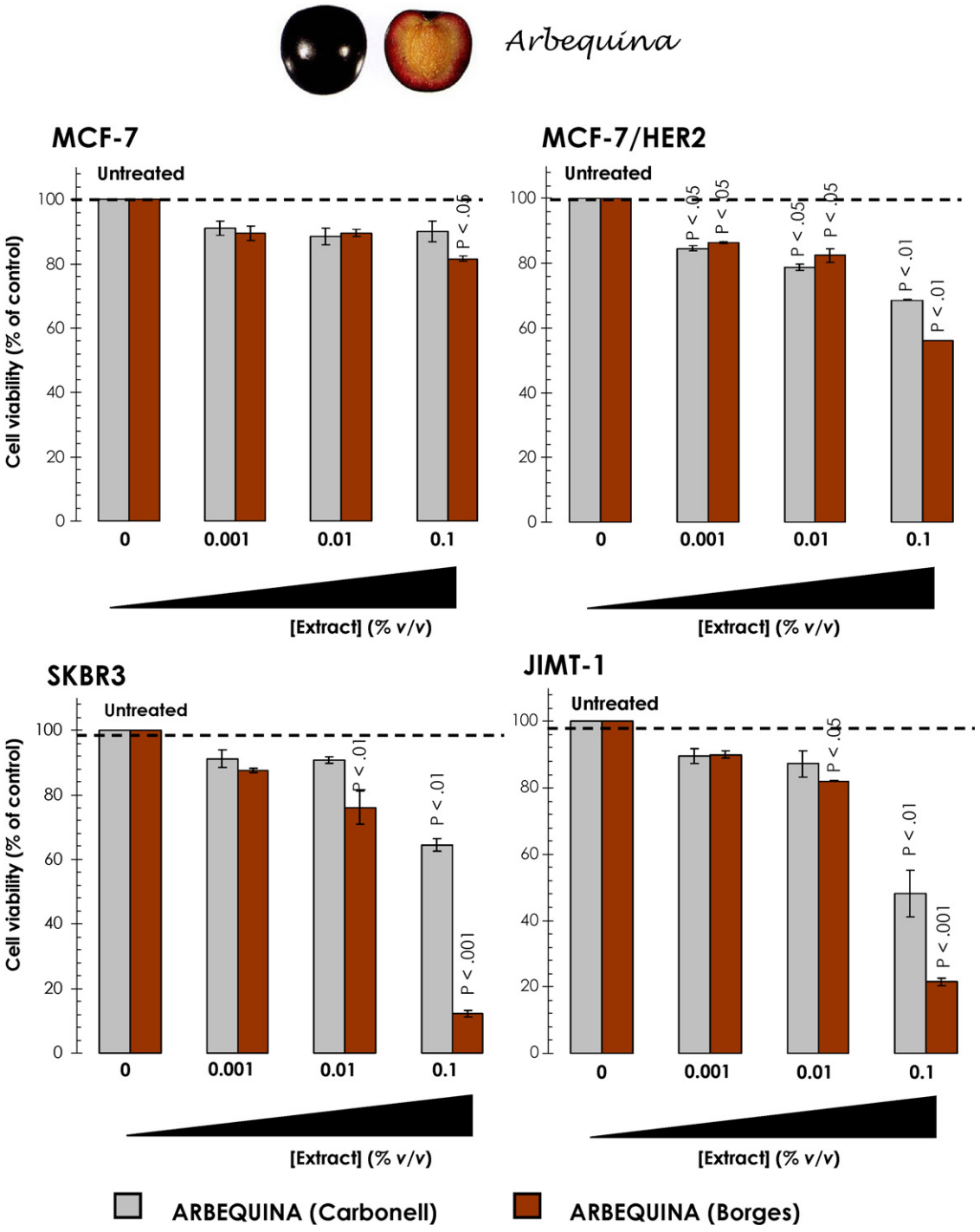

Fig. 6. Antiproliferative effects of crude EVOO phenolic extracts (ARBEQUINA variety) in human breast cancer cell lines. Cell viability (MTT assay) in MCF-7, MCF-7/HER2, SKBR3 and JIMT-1 cells after 5 days treatment with crude EVOO phenolic extracts obtained from the Arbequina variety. Cell viability is expressed as a ratio of the absorbance between treated cells and untreated control (=100% cell viability). Each point is a mean (*columns*) ± SE (*bars*) of three independent experiments performed in triplicate. Statistically significant differences are labeled.

the ionization source, m/z experimental and calculated, molecular formula, error and sigma value.

Finally, 19 phenolic compounds from different families (simple phenols, flavonoids, lignans and secoiridoids) were unequivocally identified. Fig. 3 represents the extracted ion chromatograms (EICs) of the main phenolic compounds identified in the Picual Borges variety and the base peak chromatograms (BPCs) obtained by RRLC-ESI-TOF for the eight EVOOs in the optimum conditions.

3.3. Quantification

3.3.1. Calibration curves

The quantification of seven phenols in the different EVOOs was carried out by both UV and ESI-TOF MS using commercially available standards and other pure standards previously isolated by semipreparative HPLC. Calibration curves were obtained using ten-point ($n = 3$) curves of each compound. Linear regression analysis using the least-square method was used to evaluate the MS and UV responses of each analyte as a function of its concentration. The responses fitted well to a straight line with r^2 values higher than 0.991 for both detectors. The limits of detection and quantification of the individual analytes in standard solutions were obtained by injecting diluted standard and were calculated according to the IUPAC method [37]. LODs were slightly better with MS detector in the range of 0.02–1.44 μg/ml. The linearity of the method was studied by injecting standard solutions in the range from 1 to 300 ppm obtaining less linearity responses in the mass spectrometer mainly because the degree of ionization in the ion source decreases when the amount of ions increases. Method precision based on within-day repeatability and expressed as relative standard deviation (RSD%), was estimated by measuring the peak areas of the different standards and, as it was expected, the repeatability was slightly better in UV. Table 2 shows the analytical parameters which enabled to evaluate the method performance: repeatability (% RSD), limits of detection (LOD) and quantification (LOQ), linearity, calibration curves and regression coefficient (r^2).

The described method was successfully applied to quantify these phenolic compounds in eight samples of different kinds of EVOOs. The phenolic compounds concentration was determined using the area of each individual compound (three replicates) and by interpolation in the corresponding calibration curve. Table 3 presents the content of the individual phenolic compounds found in commercial olive oils from RRLC-UV and RRLC-ESI-TOF MS. The results were statistically the same with both detectors for Hyty, Ty, Lut and Apig since no overlapping was detected in the zone of the chromatogram where these compounds were eluted. Opposite situation was found for Pin, EA and Lig Agl (in some cases), where some peaks could elute with the same retention time and create interferences in UV, making the quantification inaccurate by UV detector.

3.3.2. Folin Ciocalteu and response factor (RF)

In order to find a rapid and effective approach to quantify the largest number of olive oil phenolic compounds, a combination between the results obtained with the Folin Ciocalteau method and the percentage of each phenolic compound in the total area of the BPC in MS was developed by using the response factors (RFs) calculated for each compound.

Total polyphenol content (TPC) was analyzed according to the Folin Ciocalteau method and the results were given as caffeic acid equivalents. As a first approach, the content of each compound could be easily calculated without the necessity of calibration curves taking into account the percentage of each compound with respect to the total chromatogram area and the total polyphenol content calculated with the Folin Ciocalteau method. However, the main problem of this approach is that the mass spectrometry response for phenols changes significantly, mainly due to the different behaviour of compounds with variations in their chemical structure during the nebulization. Some parameters relating to the electrospray nebulizer (nebulizer pressure, gas flow rate, temperature, . . .) have a notable effect on RFs. This leads to systematic errors in the quantitation based on the relative peak areas. For this reason, a suitable approach for the quantitation of complex mixtures with a limited range of authentic standards is to use response factors (RFs).

We firstly had to look for an appropriate compound which could be our reference. To establish the mentioned reference compound, we compared the quantitation obtained with the calibration curves for the seven available standards and the one calculated directly with the total phenol content and the percentages of individual components. Finally, Hyty was chosen as reference because of its similar results with both ways of quantitation and its RF was set to 1. Other RFs were expressed in terms of this standard compound (Hyty) and linear concentration responses were calculated with the ratio of calibration slopes a(Hyty)/a(other phenolic compounds). RFs for the seven standards with respect to Hyty are shown in Table 4. As it can be observed some similarities can be found among the compounds which belong to the same family. So, EA and Lig Agl had very low responses, probably due to a poor electrospray nebulization. Better nebulization was shown for flavonoides with a very high response. The mass spectrometry response of pinoresinol was similar to that one of Hyty.

Finally, to calculate the real concentration of individual phenolic compounds in olive oil samples, the total polyphenol content is multiplied by the individual peak area percentage and then by the corresponding RF. The results obtained in this way for the seven standards are shown in Table 3 and, as it can be observed, are statistically the same as the previously found results achieved by using the calibration curves with RRLC-ESI-TOF MS. Due to the good results obtained with the new approach the quantitation of the other phenolic compounds identified in the eight EVOOs was carried out. In Table 5 we include the total polyphenol content as caffeic acid equivalents of the eight analyzed samples and the concentrations of the 19 phenols identified were calculated by using TPC, area percentages of each compound and response factors. The response factors of the other phenols which were not available as commercial standards were calculated assuming similar responses for compounds of the same family and with slight differences in their molecular structure.

Compared with other methods previously described in literature where external or internal standards are used, the proposed method using response factors together with total polyphenol content provides correct and faster quantitative results. The TPC and the calibration curve of the hyty are enough to quantify all the phenolic compounds using the response factors.

3.4. Inhibitory effects of crude EVOO phenolic extracts on proliferation in human breast cancer cells in vitro

Cell viability was evaluated by the MTT assay. First, we compared the anti-proliferative effects of crude EVOO phenolic extracts using two *in vitro* breast cancer cell models: MCF-7 breast cancer cells – which express physiological levels of HER2 (*i.e.* one single copy of HER2 gene) – and MCF-7/HER2 cells—an MCF-7 derived model engineered to overexpress HER2 gene (∼70-fold increase in HER2 oncoprotein expression when compared to MCF-7 parental cells). Cells were treated with a series of ethanolic dilutions that were made by diluting full strength (100%) EVOO phenolic extracts. At concentrations ranging from 0.001 to 0.1%, HER2-negative MCF-7 cells were mostly unresponsive to all the crude EVOO phenolic extracts tested (Figs. 4–6). However, a completely different picture emerged when Picual-, Hojiblanca- and Arbequina-derived crude EVOO phenolic extracts were tested on

Table 6
Effects of crude EVOO phenolic extracts on breast cancer cell viability.

	Picual (Carbonell)	Picual (Borges)	Picual (Coosur)	Hojiblanca (Carbonell)	Hojiblanca (Borges)	Hojiblanca (Hojiblanca)	Arbequina (Carbonell)	Arbequina (Borges)
MCF-7	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A	N.A
MCF-7/HER2	0.11	0.062	0.105	0.106	N.A.	0.102	N.A.	0.106
SKBR3	0.063	0.052	0.070	0.055	0.102	0.044	0.110	0.045
JIMT-1	0.061	0.059	0.064	0.055	0.069	0.060	0.073	0.056

The metabolic status of MCF-7, MCF-7/HER2, SKBR3, and JIMT-1 breast cancer cells cultured in the absence or presence of crude EVOO phenolic extracts was evaluated using a MTT-based cell viability assay as described in Section 2. Upon construction of dose–response curves, IC_{50} values (*i.e.* the concentration of each extract needed to reduce cell viability by 50% relative to untreated control cells) were calculated by interpolation. Values are means (in %, v/v) from three independent experiments made in triplicate. N.A.: not available (*i.e.* >0.150%, v/v).

their growth inhibitory activities against MCF-7/HER2 cells. Thus, MCF-7 cells stably overexpressing high levels of the human HER2 oncogene became significantly more sensitive to crude EVOO phenolic extracts in terms of decreased cell proliferation. Importantly, the inhibition of viability in MCF-7/HER2 cells was significantly more pronounced in the presence of Picual-derived crude phenolic extracts than in the presence of Arbequina-derived crude phenolic extracts.

To further evaluate whether crude EVOO phenolic extracts from Picual variety preferentially exhibited tumoricidal effects against HER2-overexpressing breast cancer cells, we explored the anti-proliferative effects of crude EVOO phenolic extracts in SKBR3 and JIMT-1, two human breast cancer-derived cell lines naturally exhibiting HER2 overexpression. SKBR3 cells represent a widely used breast cancer *in vitro* model characterized by naturally bearing HER2 gene amplification and HER2 protein overexpression. HER2-dependency for cell proliferation and survival is reflected by the fact that SKBR3 cells are highly sensitive to anti-HER2 therapies, including the anti-HER2 monoclonal antibody trastuzumab and small molecule HER2 tyrosine kinase inhibitors (TKIs). HER2-overexpressing JIMT-1 cells, however, provide a valuable experimental model for the studies of resistance to HER2 targeting therapies, as they are insensitive to trastuzumab and other HER2-inhibiting drugs including HER2 TKIs. When tested against SKBR3

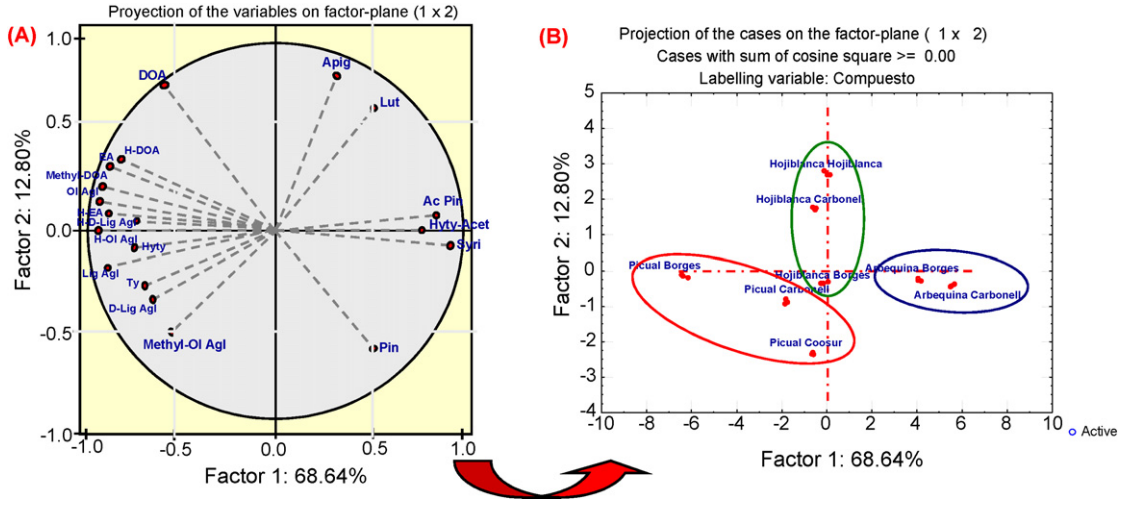

Fig. 7. (A) Projection of the variables on the factor-plane (PC1 × PC2) considering the 19 variables (phenolic compounds quantified in the current study). (B) Score plot for the two principal components showing the eight EVOO samples studied. The position of each sample depends on the variables shown in Section (A) of the current figure.

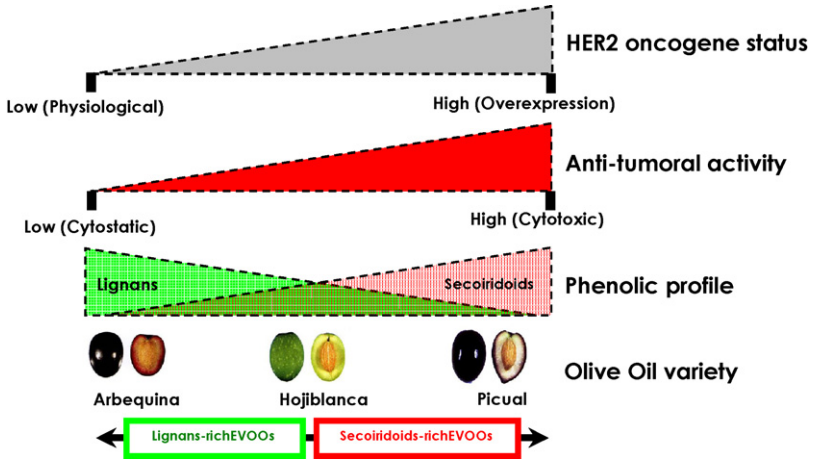

Fig. 8. Correlation between phenolic composition of crude EVOO extracts, cell viability effects, and HER2 oncogene status in human breast cancer cells.

cells, crude EVOO phenolic extracts exhibited the following anti-proliferative potencies: Picual > Hojiblanca > Arbequina (Figs. 4–6). Crude EVOO phenolic extracts failed to decrease JIMT-1 cell viability in a concentration-dependent manner. Intriguingly, exogenous supplementation with 0.1% (v/v) of Picual- and Hojiblanca-derived crude EVOO phenolic extracts drastically reduced JIMT-1 cell viability (>80% reduction; Figs. 4–6). This cytotoxic effect was less pronounced when JIMT-1 cells were cultured in the presence of crude EVOO phenolic extracts from the Arbequina variety.

3.5. Relationship between crude EVOO extracts' phenolic composition and anti-breast cancer activity

The above-mentioned findings strongly suggested that: (a) HER2-overexpressing cancer cells display exacerbated growth inhibitory responses when treated with crude EVOO phenolic extracts; (b) the anti-proliferative effects shown by crude EVOO extracts might relate to their different phenolic compositions (Table 6).

To evaluate the possibility of differentiating the samples taking into account the phenolic fraction, we applied a multivariate statistical analysis for the results of the RRLC-ESI-TOF MS analyses of the quantitative-phenolic profile. All the phenolic compounds quantified were considered to identify the two principal factors. Nineteen variables were selected for the PCA and the explained variance was higher than 80%. Fig. 7a represents the projection of the variables with regard to the single factor (PC1 or PC2) on the factor plane (PC1 × PC2). We can observe that each quadrant contains, at least, one of the variables. A map of samples (score plot) for the two principal components is shown in Fig. 7b. Extra-virgin olive oils made from Picual, Hojiblanca and Arbequina olives were shown to be quite different to the others, since the samples belonging to each family lied in different zones of the plot. The variables which were more decisive to discriminate among varieties were Ol Agl, Methyl-Ol Agl, DOA, Apig, Lut, Ac Pin and Syri; a finding which is in very good agreement with Carrasco-Pancorbo et al. [38]. The concentration of Ac Pin in the Arbequina extra-virgin olive oils under study was extremely high if we compare the oils from that variety with those from Picual and Hojiblanca. Some authors evidenced that fact previously; for example, Brenes et al. [39] observed the low Ac Pin content in Spanish EVOOs produced from Picual cultivar. Flavonoids, in general, were found at higher concentrations in Hojiblanca and Arbequina than in the Picual EVOOs analyzed. H. Hojiblanca was the richest variety concerning Apig and H. Carbonell, the richest in terms of Lut, a fact that could explain their position in the score plot. Picual EVOOs were found in a different quadrant of the plot, due to their high concentration in terms of secoiridoids (DOA, methyl-Ol Agl and Ol Agl).

However, we think it is important to stand out that our main aim was not only to discriminate among the analyzed samples, it was more to find out the phenolic compounds which could help to distinguish the samples. In that way, we could contribute to clarify what the phenols more responsible of the antiproliferative effect of the extracts from oil are.

Indeed, when we graphically represented EVOO varieties as a gradient of their major polyphenolic fractions, the anti-proliferative effects shown by crude EVOO phenolic extracts can be attributed to their enrichment in specific polyphenol classes (Fig. 8). Thus, crude EVOO phenolic extracts rich in lignans (*i.e.* Arbequina variety) appear to induce mostly cytostatic effects whereas secoiridoids-rich crude EVOO phenolic extracts (*i.e.* Picual variety) can be mostly related to strong cytotoxic effects. Moreover, our results further showed a close correlation between the ability of crude EVOO phenolic extracts to decrease cell viability and the expression status of HER2 in breast cancer cells (*i.e.* the effects on the cell viability were significantly more pronounced in the HER2-positive breast cancer cell lines than in HER2-negative cells). These findings, altogether, strongly suggest that the anti-proliferative effects shown by the phenolic compounds contained in three different varieties of EVOO may be attributed to the ability of specific phenolics to block HER2-dependent breast cancer cell viability. These findings support previous reports from our group showing a more pronounced anti-HER2 activity of individual secoiridoids (*e.g.* oleuropein aglycon, ligstroside aglycon) when compared to that of individual lignans (*e.g.* pinoresinol). They also suggest that the apparent HER2-related anti-breast cancer properties of crude EVOO phenolic extracts may result from antagonistic/synergistic properties of its individual phenolic compounds against HER2 activity and/or expression.

4. Conclusions

To the best of our knowledge, we report for the first time an easy, fast and effective RRLC-ESI-TOF MS method for the characterization/quantification of EVOOs of which we have demonstrated their potential anticancer value. Using columns of very small particle diameter (1.8 μm) and higher flows the analysis time was reduced to less than 20 min, without compromising chromatographic quality, with good resolution and reproducibility. Eight olive oils were analyzed and the quantification of the main phenolic compounds was developed in three ways, finding a new approach using the combination of the total polyphenol content, the area percentage of each phenol in the total area of the chromatogram and the response factors of each particular phenol. When coupled to the occurrence of correlations between the phenolic composition of EVOO-derived crude phenolic extracts and their anti-proliferative abilities toward human breast cancer-derived cell lines, this novel methodological approach may enable a rapid and objective identification of EVOO with potential anti-cancer value. When compared with lignans-rich EVOO varieties, secoiridoids-rich EVOOs had a significantly strong ability to alter cell viability in four different types of human breast carcinoma cells.

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CAPÍTULO 6:
NanoLC-ESI-TOF MS para el análisis de polifenoles del aceite de oliva. Comparación con un método convencional de HPLC-ESI-TOF MS

Como se puso de manifiesto en la introducción de esta sección, la fracción fenólica del aceite de oliva ha sido ampliamente estudiada por numerosos grupos de investigación, que han empleado principalmente HPLC y, en menor medida, GC y CE acopladas a diferentes detectores para su análisis.

En este capítulo y el siguiente, la idea que nos planteamos fue el desarrollo de nuevos métodos, donde se utilizasen plataformas analíticas que no hubieran sido empleadas hasta el momento en el análisis de polifenoles, con objeto de demostrar su potencialidad en este campo, lo que podría resultarnos muy útil en estudios futuros donde se persiguiera la determinación de este tipo de compuestos.

El objetivo concreto que perseguimos en este capítulo 6 fue demostrar el potencial de la nanocromatografía líquida acoplada a espectrometría de masas (nanoLC-ESI-TOF-MS) para la identificación y cuantificación de polifenoles. Para ello, lógicamente era necesario optimizar tanto el método cromatográfico, como las condiciones del espectrómetro de masas (especialmente aquellas de la interfase), identificar los compuestos (haciendo uso de los patrones comerciales y de los compuestos aislados en el capitulo 4), y establecer los parámetros analíticos del método.

Para llevar a cabo la identificación de los compuestos bajo estudio resulta de gran ayuda la información proporcionada por el TOF, que nos permite obtener la fórmula molecular del compuesto teniendo en cuenta la masa exacta y la distribución isotópica. Pero para que la información proporcionada por el TOF sea fiable, éste tiene que estar convenientemente calibrado. Existen dos tipos de calibración:

- *Calibración interna*: Se introduce el calibrante junto con la muestra y aparece durante todo el análisis. Proporciona mejor exactitud en los valores de masa obtenidos (errores por debajo de las 3 ppm), pero presenta el inconveniente de la contaminación de la muestra y la aparición de posibles efectos de supresión iónica en la interfase.
- *Calibración externa*: Se introduce el calibrante independientemente de la muestra, al principio o al final del análisis con una bomba externa, y proporciona errores que estarán por debajo de las 5 ppm. Es el tipo de calibración que se utiliza normalmente.

Con la instrumentación que utilizamos en este capítulo, nanoLC-ESI-TOF MS, no es posible realizar una calibración externa, ya que no se ha desarrollado un sistema adecuado para introducir el calibrante al principio o al final del análisis. Es por ello por lo que en este caso ha de llevarse a cabo una calibración interna. Pero no se realiza añadiendo ningún calibrante a la muestra, si no que se utiliza como calibrante un grupo de compuestos fenólicos muy conocidos presentes en el aceite, de los que tenemos patrones, con masas que abarcan todo el rango que queremos calibrar.

Con objeto de determinar las ventajas e inconvenientes que presenta nanoLC para el análisis de los polifenoles, tendríamos que comparar los parámetros analíticos y otros aspectos de la técnica, con aquellos relativos a una técnica de uso mucho más extendido, como es la cromatografía líquida convencional (HPLC). Para ello se van emplear los resultados obtenidos con el método optimizado en el capítulo 5.

Parte de este trabajo de investigación se llevó a cabo durante una estancia en el Departamento de Aplicaciones, en la sede que la empresa Bruker Daltonik tiene en Bremen (Alemania), donde tuvimos la oportunidad de profundizar en los aspectos más críticos que un analista halla a la hora de operar con esta técnica y aplicarla por primera vez al análisis de los polifenoles del aceite.

Nano-liquid chromatography versus rapid resolution LC, both coupled to electrospray ionization time of flight-mass spectrometry to identify and quantify polyphenols: the case of olive oil phenols

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Abstract

The applicability of nano-liquid chromatography coupled to electrospray ionization-time of flight-mass spectrometry (nanoLC-ESI-TOF MS) for the analysis of olive oil polyphenols was studied and compared with a HPLC method. After the injection, the compounds were focused on a short capillary trapping column (100 μm i.d., effective length 20 mm, 5 μm particle size) and then nanoLC analysis was carried out in a fused silica capillary column (75 μm i.d., effective length 10 cm, 3 μm particle size) packed with C18 stationary phase. The mobile phase was a mixture of water + 0.5% acetic acid and acetonitrile eluting at 300 nL/min in a gradient mode. Polyphenols from different families were identified and quantified. The quality parameters of the nanoLC method (linearity, limits of detection and quantification, repeatability) were evaluated and compare to those obtained with HPLC. The new methodology presents better sensitivity (reaching LOD values below 1 ppb) with less consumption of mobile phases, but worse repeatability, especially inter-day repeatability, doing more difficult to get highly accurate quantification. The results described in this paper open up the application fields of this technique to cover a larger variety of compounds and its advantages will make it especially useful for the analysis of samples containing low concentration of polyphenols, as for instance, in biological samples.

1 Introduction

Miniaturization of analytical techniques has recently become one of the most important areas of research and several groups have paid great attention to the study and development of new miniaturized separation methods. Among these, nano-liquid chromatography (nanoLC), firstly introduced by Karlsson and Novotny in 1988 [1], has emerged as a new powerful analytical tool, complementary and/or competitive to conventional HPLC, providing a wide number of important applications, especially in proteomics and related fields [2- 7], mainly due to the very low sample requirements. Other applications of nanoLC can be also found in fields such as pharmaceutical [8], environmental [9- 11], and enantiomeric analysis [12,13]. The use of nanoLC for food analysis has not been so widely extended so far, although in the last years some interesting works have demonstrated its potential in this field [14 - 19]. The analyses are carried out in capillaries of small internal diameter (10- 100 μm), in most of the cases either of fused silica or peek material and containing selected stationary phases usually used in HPLC with particle sizes of 3-5 μm. The use of smaller diameter allows achieving higher separation efficiency and lower volumes of sample and flow rate are requested. Besides, in theory by reducing the internal diameter of the capillary column, better sensitivity can be achieved because the lower flow rate causes a reduction of the chromatographic dilution. However, due to the low injected sample volumes required in column with smaller diameter, the sensitivity will not be high. The loss of sensitivity can be avoided using large volume injections with specific techniques that prevent column overloading: on-column and extra column focusing techniques. In the first one, solutes are dissolved in a solvent of lower eluting power compared to the mobile phase [20] and the second solution consists of using a pre-column combining with a switching system [14]. With both techniques large injection volumes could be used, increasing the sensitivity.

As far as detection system is concerned, UV and, in some cases, fluorescence detection are the most commonly used, although when sensitivity is of paramount importance, MS detection is gaining more interest due to its easy coupling to nanoLC instrumentation, where very low flow rates are currently used (in the order of nL/ min). Besides improving the sensitivity, the use of MS allows to achieve structural information, unequivocal identification of some compounds (depending on the MS analyzer used) and to solve problems of analyte co-migration. For coupling nanoLC to mass spectrometry,

several nanospray interfaces have been tested (sheatliquid and sheatless), some of them commercially available and, in many occasions, homemade [8,21].

Extra virgin olive oil (EVOO) is a valuable component of the traditional Mediterranean diet, unique among other vegetable oils because of its fatty acid composition (characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio) and its high concentration level of phenolic compounds. The phenolic fraction of EVOO consists of a heterogeneous and very complex mixture of compounds, mainly simple phenols, lignans, flavonoids and secoiridoids; every family of compounds varies in chemical properties and has a particular influence on the quality of EVOO [22,23]. There is evidence that phenolic compounds could play a major role in the healthy effects of EVOO, besides to be responsible of its antioxidant activity and organoleptic properties (flavour, astringency…) [24-26]. Therefore, the determination of this family of compounds in olive oil is of special relevance. Until now, different analytical methods (gas chromatography (GC) [27], HPLC [28,29], capillary electrophoresis (CE)) [30] coupled to different detectors (UV, fluorescence, mass spectrometry, etc) [31] have been developed to analyze olive oil polyphenols. However, although nanoLC have already been employed in food analysis, to the best of our knowledge it has not been applied to the analysis of polyphenols.

The aim of this work was to test and evaluate the potentiality of nanoLC coupled with mass spectrometry (ESI-TOF MS) for the analysis of olive oil polyphenols. When an analytical technique is applied by first time to face a particular problem, it is quite interesting to compare its potential and performance with those of other techniques more widely used. Therefore, a comparison between the performance of both nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS methodologies for the separation and quantitation of this type of compounds was made.

2 Materials and methods

2.1 Chemicals and samples

Methanol and *n*-hexane of HPLC grade used during sample extraction were purchased from Panreac (Barcelona, Spain). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac (Barcelona, Spain) were used in the mobile phase for the HPLC and nanoLC analysis. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA).

Standards of hydroxytyrosol, tyrosol, luteolin and apigenin were purchased by Sigma-Aldrich (St. Louis, MO, USA) and (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland). Other polyphenols used as pure standard samples, elenolic acid and ligstroside aglycon, were isolated from EVOOs by semipreparative HPLC. Stock solutions at concentration of 500 mg/L for each polyphenol were prepared in MeOH and then serially diluted to working concentrations. For the nanoLC analysis the standards were dissolved in mobile phase (water + 0.5% acetic acid) with 10% MeOH.

EVOO samples of three different olive fruit varieties so-called Picual, Hojiblanca and Arbequina used for the study were acquired from a supermarket (Granada, Spain).

2.2 Sample extraction

The extraction procedure was based on a specific solid phase extraction (SPE) method with Diol-cartridges which we use as routine extraction protocol in our research group [32]. Briefly, the extraction consisted of passing through a column, previously conditioned with 10 mL of methanol and 10 mL of hexane, 60 g of EVOO dissolved in 60 mL of hexane. After removing the non-polar fraction with 15 mL of hexane, the phenolic compounds were recovered with methanol (40 mL). The final volume was dried in a rotary evaporator under reduced pressure at 35°C and the residue was dissolved in 2 mL of methanol. After preparing the extracts, we made the proper dilutions depending on the technique used in each case (nanoLC or HPLC). For the HPLC analysis a 1:10 dilution in MeOH was used and for the injection into the nanoLC, the sample was diluted 1:500 in mobile phase (water + 0.5% acetic acid) with 10% MeOH.

2.3 Nano- liquid chromatography analyses

Experiments were performed in a commercial available instrumentation EASY-nLC^{TM} (Bruker Daltonik GmbH, Bremen, Germany), composed of one module and equipped with three pumps, three pressure sensors, four valves, two flowsensors, an autosampler and a touchscreen.

The chromatographic separation was performed in a capillary column BioSphere (75 μm i.d., packed length 10 cm and particle size 3 μm) packed with C18 particles. An on-line C18 trapping column (BioSphere (100 μm i.d., packed length 20 mm and particle size 5 μm)) was used before the nanoLC column in order to achieve both pre-concentration and clean up of samples.

Optima chromatographic conditions were achieved by using a mobile phase composed of water + 0.5% acetic acid (phase A) and acetonitrile (phase B) with the following gradient: 0 to 10 min, 20-33% B; 10 to 35 min, 33-40% B; 35 to 38 min, 40-95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. Before starting the following analysis the pre-column and column were re-equilibrated with phase A at 6 μL/min for 2 min and 0.6 μL/min for 8 min, respectively. A volume of 5 μL of the sample was injected into the loop and later loaded onto the pre-column using the phase A (water + 0.5% acetic acid) at a flow rate of 6 μL/min during 1 min, to trap de compounds of interest and to clean the sample. Afterwards, the valve changed position and switched the pre-column in-line with the analytical column eluting the compounds of interest at a flow-rate of 300 nL/min and 25ºC (the column was at room temperature because of the lack of thermostatization system). Figure 1 shows a schematic figure of the nanoLC system, showing the moment when the sample is loading onto the pre-column using pump A.

All nanoLC parts were controlled by Hystar (version 3.1) software. The compounds separated were analyzed with a mass spectrometry detector.

2.4 High performance liquid chromatography analyses

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. Polyphenolic compounds 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 30ºC and a flow rate of 1.5 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: 0 to 10 min, 5-30% B; 10 to 12 min, 30-33% B; 12 to 17 min, 33-38% B; 17 to 20 min, 38-50% B; 20 to 23 min, 50-95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 10 min. A volume of 10 μL of the 1:10 diluted methanolic extracts of olive oil was injected. The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass spectrometry detector.

2.5 Mass spectrometry

The nanoLC column and the RRLC system were coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using electrospray ionization (ESI).

For nanoLC-MS coupling the coaxial sheath flow interface is the most used system where the capillary column is inserted in a concentric stainless steel tube which carries a make-up liquid. Recently, in order to achieve better sensitivity, a sheathless interface has been developed [33,34] using capillary tips of small i.d. that transfer the sprayed droplets into the inlet of the MS without a make-up flow. Nanoflow sprayers are typically fabricated from tapered fused silica capillaries or alternatively from conductive materials, such as stainless steel [35,36]. In this study the nanoLC column was interfaced to the mass spectrometry using a commercial sheatless nano-spray interface with a tapered fused silica sprayer tip. The key parameters of the nano-ESI were adjusted for the flow rate used (300 nL/min) to achieve stable spray across the entire gradient range: pressure 0.4 bar, dry gas flow 4 L/min and dry gas temperature 150ºC.

The RRLC system was coupled to the mass spectrometer using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The flow rate used in the RRLC method 1.5 mL/min was too high for achieving an stable electrospray ionization (ESI) (maximum flow-rate is around 1 mL/min), therefore it was necessary to use a flow divisor 1:6, so the flow delivered into the mass spectrometer was reduced to 0.21 mL/min. According to this inflow the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190ºC.

The mass transfer parameters (radio frequencies and voltages in the different skimmers, hexapoles and lenses) were similar to those previously optimized in recent works where the same matrix (EVOO) was analyzed [37] acquiring spectra in the range of 50-800 m/z in the negative mode. So far, nanoflow ESI has become routine in the positive ion mode and just few applications have been developed in negative ion mode due to difficulties with spray instability. TOF analyzers provide greatly improved mass resolution (5,000–10,000 at 250 *m/z*) and significantly high sensitivity and accuracy when acquiring full-fragment spectra. In order to obtain high mass accuracy in TOF, mass calibration is required. After a good instrument calibration, the accurate mass data of the molecular ions can be processed by DataAnalysis 4.0 software (Bruker Daltonik GmbH) which provides information of elemental composition of compounds based on the accurate

mass measurement and the true isotopic pattern (TIP). The calibrant can either be measured within the sample itself (internal calibration) or, alternatively, can be introduced externally, for instance, with a pump at the beginning or at the end of the analysis (external calibration). In general, it is safest and more convenient to measure the calibrant externally to avoid signal suppression and contamination and to assure the calibrant signal is measured at an appropriate, controlled intensity level. However, the internal calibration provides better mass accuracy (less than 3 ppm error). With the instrumentation used in this work for the nanoLC analysis was not possible to do an external calibration because a system to introduce the calibrant at the beginning or the end of the chromatographic run has not been developed yet. Instead of this, an internal calibration was applied using a mixture of well-known phenols present in the olive oil extracts (Table 1), giving mass peaks throughout the desired range of 100-400 m/z. The seven phenolic compounds included in Table 1 (which were available as pure standards) were used to calibrate every analysis. First of all, we corroborated their presence in the analyzed samples (taking into account their retention time, MS spectra and also by analyzing spiked samples). After their unequivocal identification, we decided to use them to increase the accuracy of our results. So we made an average MS spectrum of the whole chromatogram and we re-calibrated every analysis by using the calibration list mentioned above. This procedure resulted in mass accuracies of less than 3 ppm.

2.6 Statistics

Results of phenolic compounds are the averages of at least three repetitions (n=3), unless otherwise stated. Tukey's honest significant difference multiple comparison (one-way ANOVA) and Pearson's linear correlations, both at $p < 0.05$, were evaluated using Statistica 6.0 (2001, StatSoft, Tulsa, OK).

3 Results and discussion

3.1 NanoLC-ESI-TOF MS method

3.1.1. Development of the method

In order to develop the nanoLC method for the separation of the olive oil phenolic compounds, a capillary column BioSphere C18 (75 μm i.d., packed length 10 cm and particle size 3 μm) coupled to a C18 trapping column BioSphere (100 μm i.d., packed length 20 mm and particle size 5 μm) was used.

Capillary columns of 75 μm i.d. usually have an ideal injection volume of few nanoliters (20-60 nL) but the use of trapping columns before the analytical column, as mentioned in the introduction, allows injecting relatively high sample volumes, improving the sensitivity. In the case under study, where the sample amount was not limited, 5 μL of the sample was initially chosen for the injection into the loop to get more sensitivity.

Preliminary studies were done analyzing the best way to load the sample from the loop to the pre-column in order to trap the analytes. With the instrumentation used in this work the solvent used to load the samples is always phase A (in this case water + 0.5% acetic acid), and it is not possible to change it. Using this solvent, an appropriate loading time and speed were chosen. With a flow rate of 6 μL/min, a very low loading time led to some analytes, especially the most hydrophobic, not to reach the pre-column and to keep sorbed onto the tubing connecting the loop and the pre-column. On the contrary, if the time is too large, the analytes, principally the most hydrophilic was displaced from the pre-column during the loading/washing phase of the analysis. Finally, the best loading conditions that allowed an optimum recovery of olive oil polyphenols into the separation columns were 6 μL/min for 1 min. This description results easier to understand observing Figure 1.

Once optimized the loading conditions other experimental variables affecting nanoLC analysis were studied in order to achieve optima separation conditions of the analytes under study.

Based on our previous studies with this type of compounds water + 0.5% acetic acid and acetonitrile were selected as mobile phases and different isocratic and gradient programs were tested. In general, because of the very different properties of the analyzed compounds, an isocratic elution at different percentages of organic solvents did not provide an appropriate separation of the selected compounds and thus, a gradient elution was required. Optimum separation was achieved by using the following gradient: 0 to 10 min, 20-33% B; 10 to 35 min, 33-40% B; 35 to 38 min, 40-95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. As it can be observed, the gradient is limited between 20 and 100% of organic solvent, as in most of the nanoLC-MS systems described in literature [17,38,39], in order to improve spray stability that can be an issue when predominantly aqueous solvents are used (due to the high surface tension of water). Solvents were degassed with ultrasonication prior to use.

Different flow rates were tested: 200, 300 and 400 nL/min (the maximum flow rate supported by the column is 600 nL/min). Worse efficiency and long retention times were obtained when lower flow rates were used, whilst for high flows we got shorter analysis time but a loss of sensitivity and resolution for some compounds. After the optimization, chromatographic separations were carried out at room temperature (25ºC) at a flow rate of 300 nL/min. Other lower injection volumes were also tested (500 nL, 1 μL, 2 μL) but the resolution did not improve and the sensitivity was lower.

As described in the experimental section the detection was carried out with mass spectrometry (TOF) using a sheathless nano-electrospray. MS was operated in the range between 50-800 m/z in negative polarity; however analyses of EVOO by nanoLC-ESI-TOF MS were performed in negative ion mode and in positive ion mode. In positive ion mode, in general, the profiles were worse and as far as the different families of phenolic compounds are concerned, flavonoids and lignans were ionized quite properly, whilst secoiridoids, simple phenols and phenolic acids (or very related compounds) were detected better in negative polarity. We decided to use negative polarity for the rest of the analyses. Figure 2 shows the chromatograms of the olive oil extract (Picual variety) obtained by using nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS. As it can be observed, the nanoLC separation was successfully carried out in a relatively short time (less than 30 min), comparable to some results reported in literature for olive oil polyphenols. The resolution and efficiency for some compounds were a bit worse than in HPLC, particularly at the beginning of the chromatogram where predominantly aqueous portion is present.

3.1.2 Identification of the compounds under study

The optimized nanoLC-ESI-TOF MS method was applied for the identification and quantification of phenolic compounds in different olive oil samples.

The identification of the compounds was easily performed by comparing both migration time and MS spectral data obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC), and by using the information about the polarity of the compounds and the wide information previously reported in literature [37,40]. Moreover, the information provided by the mass spectrometer with TOF analyzer relative to mass accuracy and true isotopic pattern is adequate to generate the molecular formula using the editor SmartFormula^{TM}. Although, as we have mentioned in section 2.5, it was not possible to do an external calibration because of the lack of a

suitable system to introduce the calibrant, a satisfactory internal calibration was developed using a combination of well-known phenols present in the analyzed samples (throughout the desired range of 100-400 m/z). Table 2 summarizes the main phenolic compounds identified in Picual EVOO by nanoLC-ESI-TOF MS including information about the retention time, product ions obtained spontaneously in the ionization source, m/z, molecular formula, error and sigma value. As shown in Table 2 calibration error for each mass were less than 3 ppm.

Figure 3 shows the base peak chromatogram (BPC) obtained by the developed nanoLC-ESI-TOF MS method operating at the optima conditions for the olive oil from Picual variety and the extracted ions chromatograms (EICs) of the main phenolic compounds identified.

A quite stable nanospray was obtained by using these optima conditions and, in general, we could observe minimal carryover in the samples. The most hydrophilic compounds (hydroxytyrosol and tyrosol) presented low efficiency and resolution, probably due to the higher aqueous content used at the beginning of the gradient. We tried to improve the first part of the chromatogram starting with higher organic solvent content (30 and 40% ACN), but with these conditions, the resolution in the rest of the chromatogram was quite worse. Compounds in the family of lignans (pinoresinol, acetoxipinoresinol and syringaresinol) and flavonoids (luteolin and apigenin) were detected with very high efficiency and good peak shape. Regarding secoiridoids, some of them (oleuropein aglycon, ligstroside aglycon, methyl oleuropein aglycon) showed several peaks corresponding to different isomeric forms some of them, previously described in literature [30,37]. The extra peaks that appear in the extracted ion chromatograms of tyrosol and elenolic acid correspond to the fragmentation of other compounds that elute later in the chromatogram.

Three different varieties of olive oil (Picual, Arbequina and Hojiblanca) were analyzed by using the optima conditions and the results are shown in Figure 4.

3.1.3 Analytical parameters and quantification of the compounds

The analytical method was then validated in terms of specificity, linearity and precision for the analysis of polyphenols in olive oil.

The specificity of the method was tested by screening analysis of blank (in terms of phenols) oil samples. There were no impurity peaks or contamination at the retention times corresponding to the analytes. In order to obtain the calibration curves, the analyte

peak area was plotted versus the analyte concentration. Ten points of different concentrations level (n=10) were chosen for the different phenols standards and for each point the appropriate standard solution was injected three times. In general, wide linearity ranges were observed for each analyte with reasonable linearity and correlation coefficients (r^2) from 0.9670 to 0.9974. To test the sensitivity of the method, the mixture of the seven compounds were diluted several times and injected into the nanoLC system. The limits of detection (LODs) were determined as three times the signal to noise ratio (S/N) and were ranged between very low values: 0.7 and 0.9 ppb for the flavonoids, luteolin and apigenin respectively, and 30 ppb for Hydroxytyrosol. These LODs values are much lower than those described in literature for the same compounds by HPLC-MS. Regarding the repeatability, it was assayed out by three consecutive injections (n = 3) of the standard mixture of polyphenols in the same day (intra-day repeatability) and in four different days (inter-day repeatability) obtaining values of relative standard deviation (RSD%) on the peak area ratio above 4.3 and 15.6%, respectively. These results show fairly good intra-day repeatability but, as expected, worse inter-day repeatability that could be explained by the instability of the nanospray.

Table 3 shows the main quality parameters of the nanoLC-ESI-TOF MS method: linear range, calibration curve, correlation coefficients (r^2), limits of detection (LODs), limits of quantification (LOQs) and compares them with those obtained for HPLC-ESI-TOF MS. Both methods were compared taking into account the best chromatographic and mass spectrometry conditions for each one.

As can be observed, better results were obtained employing the HPLC in terms of linearity and repeatability, especially inter-day repeatability. However, concerning the sensitivity, nanoLC-ESI-TOF method provided a much higher S/N ratio for the compounds, and therefore, a better sensitivity.

The described method was applied to quantify the polyphenols under study in 3 different varieties of EVOO samples. The analyses were performed in triplicate and the polyphenols concentration was determined using the area of each individual compound and making an interpolation in the corresponding calibration curve. Table 4 present the polyphenolic content found in commercial olive oils by nanoLC-ESI-TOF MS together with the results obtained with HPLC-ESI-TOF MS. The variability in the phenolic content among the studied varieties can be motivated by some environmental, genetic, geographical and agronomic factors; all those variables have been widely studied in literature.

Having a look at Table 4, we can say that, in general, the results obtained for the seven phenolic compounds quantified in terms of their standards in this study are in good agreement when we compare nanoLC-ESI-TOF MS and HPLC-ESI-TOF MS. Only for hydroxytyrosol and tyrosol - belonging to the family of simple phenols - the results achieved by both techniques were not statistically the same. This fact could be explained taking into account that simple phenols are the most hydrophilic compounds in the extracts from olive oil and they appear in the profile when aqueous proportion is more abundant in the mobile phase, resulting in poor spray stability. For the rest, nanoLC and HPLC quantitative results are statistically the same, except for the luteolin content in Arbequina olive oil.

From our point of view, it is pretty worth to highlight that as repeatability was higher in HPLC, the standard deviation of HPLC results was lower. Although nanoLC quantitative data were not excellent, we consider that it is very interesting the fact of evaluating that technique and checking its performance and its capability to quantify accurately this kind of compounds. So far, in part due to the low reproducibility of the nanospray technology, nanoLC has been mainly used for qualitative analysis.

3.2 Comparison between NanoLC and HPLC results

With regards to the analytical parameters, as mentioned before, nanoLC showed in general worse, although sufficient, resolution, efficiency and repeatability compared to HPLC. However, in terms of sensitivity, the LODs obtained with the nanoLC system used in this study are much lower that those reported previously by using HPLC methods. This high sensitivity could be explain because of the possibility to inject large volumes of samples using the on-line pre-column together with the reduction of the chromatographic dilution due to the use of small i.d. capillary columns [17]. Besides, the use of MS detector improves the sensitivity because the small solvent volume from nanoLC is almost completely evaporated in the nanospray interface where the tip is positioned at a very short distance from the orifice inlet of the mass spectrometer.

The use of nanoLC can also offer other attractive advantages over classical HPLC. The use of small amounts of stationary phases made the columns cheaper (ten-folder lower) than a conventional C18 column and allows the use of expensive packing materials. In nanoLC, the use of pre-columns is highly recommended, since capillaries can be easily blocked at the inlet when real samples have to be analyzed. Besides, it allows samples to be both pre-concentrated and partially cleaned up. It should also be indicated, that after

more than 500 injections of olive oil polyphenols, the column is still in perfect state for the analysis of this type of compounds.

Another important advantage of this miniaturized technique it is the use of relatively low flow rates (40-600 nL/min depending on the column). Mobile phases, especially acetonitrile, are quite expensive, and the small amount request for nanoLC makes this technique very attractive with lower cost and reduction of waste solvents. Similar results were obtained in both LC and nanoLC with 3000-fold reduction in reagent consumption. The reduction of flow rate also allows a good coupling with MS transferring the entire effluent from the column into the MS instrument, whereas with HPLC we normally need a splitter. Both systems can be easily coupled to the mass spectrometer although the nanoLC coupling is most delicate and present more technical problems, requiring significant expertise, mainly because of the delicate plumbing and the use of fragile fused-silica ESI emitters. Besides, the mass spectrometer gets dirty earlier with nanoLC because the higher flows of solvents used in HPLC clean the surfaces of the equipment.

Due to the low consumption of mobile and stationary phases, the nanoLC method seems to be cheaper than the traditional HPLC but, at the moment, because of the novelty of the technique, the instrumentation and packed column for some applications are still pretty expensive.

Other advantage of nanoLC over classical HPLC is the possibility to use low injected sample volumes (20–60 nL), fact which can be very useful for applications where sample availability is restricted, as for example in proteomic field. However, the injection of low sample volumes can reduce the sensitivity. The analyst has to reach always a compromise between nanoliter injection and sensitivity determination. In this study with olive oil polyphenols, where the sample availability is not a problem, better sensitivity was reached by using higher volumes injected.

Another advantage that we can stand out is the better baseline noise, due to a reduced background, which is necessary to detect minor components.

Tabla 5 summarizes the advantages and disadvantages of the nanoLC methodologies versus HPLC.

4 Concluding remarks

In this work, a nanoLC-ESI-TOF MS method has been developed to demonstrate, for the first time, its application on the determination of olive oil polyphenols. The use and analytical performance of nanoLC were compared with a HPLC method, and both of

them were applied for the identification and quantification of different polyphenols in olive oil. The most important analytical parameters of both methods (linearity, calibration range, detection and quantification limit, repeatability etc) were calculated to establish the comparison. The new nanoLC method provides comparable analysis time and offers better sensitivity with less consumption of mobile phases; however it presents worse inter-day repeatability and it can be a bit more difficult to operate by the analyst. NanoLC-ESI-TOF MS showed the potential to become a very promising alternative, in particular, for studies where the determination of extremely low concentrations of analytes is required (biological samples, for instance). Further studies are already ongoing in our laboratory using nanoLC-ESI-TOF for the analysis of polyphenols in biological samples.

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Figure captions

Figure 1. Schematic figure of the nano-LC system, showing the moment when the sample is loading onto the pre column using pump A. Valve S is switched to positon 1-6 directing the flow through the loop carrying the sample onto the pre column. Valve W is set to position 1-6, because of the higher pressure drop on the analytical column the flow is directed to waste.

Figure 2. BPC (Base Peak Chromatogram) of an olive oil extract (variety Picual) using nanoLC-ESI- TOF MS (A) and comparison with the HPLC-ESI-TOF MS method (B). Chromatographic conditions are described in Materials and Methods.

Figure 3. Base peak chromatogram (BPC) achieved by nanoLC-ESI-TOF MS at the optima conditions for the variety Picual and extracted ions chromatogram (EICs) of the main phenolic compounds identified: **1**, Hydroxytyrosol; **2**, Tyrosol; **3**, Hydroxy elenolic acid; **4**, Elenolic acid; **5**, Hydroxy decarboxymethyl oleuropein aglycon; **6**, Decarboxymethyl oleuropein aglycon and hydroxy decarboxymethyl ligstroside aglycon; **7**, Syringaresinol; **8**, Luteolin; **9** Pinoresinol; **10**, Acetoxypinoresinol; **11**, Hydroxy oleuropein aglycon; **12**, Decarboxymethyl ligstroside aglycon; **13**, Apigenin; **14**, Methyl decarboxymethyl oleuropein aglycon, **15**, Oleuropein aglycon; **16**, Methyl oleuropein aglycon; **17**, Ligstroside aglycon.

Figure 4. Base peak chromatograms obtained by nanoLC-ESI-TOF at optima conditions for olive oils of three different varieties: a) Picual, b) Arbequina, c) Hojiblanca.

Table 1. Mass calibration matrix for internal calibration.

	Name	Formula $[M-H]^-$	Theoretical Mass	Charge
1	*Hydroxytyrosol*	$C_8H_9O_3$	153.05572	-1
2	*Tyrosol*	$C_8H_9O_2$	137.06080	-1
3	*Elenolic acid*	$C_{11}H_{13}O_6$	241.07176	-1
4	*Apigenin*	$C_{15}H_9O_5$	269.04555	-1
5	*Luteolin*	$C_{15}H_9O_6$	285.04046	-1
6	*Pinoresinol*	$C_{20}H_{21}O_6$	357.13436	-1
7	*Ligstroside aglycon*	$C_{19}H_{21}O_7$	361.12928	-1

Table 2. Phenolic compounds identified in an olive oil extract (Picual variety) by nanoLC-ESI-TOF including: mass/charge ratio, retention time, ISCID (Internal source collision induced dissociation) fragments, molecular formula, error (ppm) and Sigma value. Internal calibration was used by using 7 well-known phenolic compounds.

m/z	Retention time (min)	Fragments	Formula $[M-H]^-$	Error	Sigma	Identified Compounds
153.0559	11.3	123	$C_8H_9O_3$	-1.3	0.005	Hydroxytyrosol
137.0610	12.6		$C_8H_9O_2$	-1.6	0.029	Tyrosol
257.0660	15.3	181,137	$C_{11}H_{13}O_7$	2.7	0.007	Hydroxy elenolic acid
241.0712	16.2	139	$C_{11}H_{13}O_6$	2.5	0.014	Elenolic acid
335.1141	16.8	199	$C_{17}H_{19}O_7$	-1.5	0.014	Hydroxy decarboxymethyl oleuropein aglycon
319.1180	17.7	183	$C_{17}H_{19}O_6$	2.2	0.022	Decarboxymethyl oleuropein aglycon
417.1606	18.1		$C_{22}H_{25}O_8$	1.7	0.086	Syringaresinol
319.1185	18.6	199	$C_{17}H_{19}O_6$	0.7	0.003	Hydroxy decarboxymethyl ligstroside aglycon
285.0399	18.8		$C_{15}H_9O_6$	2.1	0.018	Luteolin
357.1348	18.9		$C_{20}H_{21}O_6$	-1.1	0.033	Pinoresinol
415.1404	19.2		$C_{22}H_{23}O_8$	1.5	0.026	Acetoxypinoresinol
393.1203	19.3		$C_{19}H_{21}O_9$	3.0	0.005	10-Hydroxy oleuropein aglycon
303.1244	19.6	183	$C_{17}H_{19}O_5$	-2.0	0.015	Decarboxymethyl ligstroside aglycon
269.0459	20.8		$C_{15}H_9O_5$	-1.2	0.079	Apigenin
333.1339	21.1		$C_{18}H_{21}O_6$	1.2	0.010	Methyl decarboxymethyl oleuropein aglycon
377.1256	21.5	345,307,275	$C_{19}H_{21}O_8$	-1.2	0.002	Oleuropein aglycon
391.1406	23.9	345,275	$C_{20}H_{23}O_8$	-2.0	0.016	Methyl oleuropein aglycon
361.1295	24.4	291,241	$C_{19}H_{21}O_7$	-0.5	0.012	Ligstroside aglycon

Table 3. Analytical parameters for the nanoLC and HPLC-ESI-TOF MS methods: relative standard deviation (RSD%), limit of detection (LOD) and quantitation (LOQ), linearity, calibration curves and r^2.

Analytes		RSD% Intra-day	RSD% Inter-day	LOD (ppb)	LOQ (ppb)	Linearity (ppm)	Calibration curves	r^2
Hyty	nanoLC	6.8	20.4	30	90	LOQ-4	y = 887868x - 31503	0.994
	HPLC	4.6	6.8	90	300	LOQ-50	y = 39934x + 42004	0.993
Ty	nanoLC	8.0	15.6	10	30	LOQ-2	y = 757872x + 4292	0.997
	HPLC	2.1	5.3	310	1030	LOQ-50	y = 12596x + 26635	0.991
EA	nanoLC	7.3	26.3	8	16	LOQ-4	y = 1057886x + 28193	0.982
	HPLC	3.4	7.5	1440	4800	LOQ-300	y = 6687.8x + 76261	0.991
Pin	nanoLC	8.1	16.2	1.2	3.6	LOQ-0.5	y = 1545997x + 25558	0.988
	HPLC	3.3	4.6	60	200	LOQ-50	y = 37578x + 53556	0.991
Lut	nanoLC	4.3	16.1	0.9	2.7	LOQ-1	y = 3854172x + 83079	0.980
	HPLC	2.8	5.8	20	60	LOQ-25	y = 114566x + 59826	0.994
Apig	nanoLC	9.8	18.2	0.7	2.1	LOQ-0.2	y = 6301191x - 28625	0.969
	HPLC	2.0	4.6	20	60	LOQ-25	y = 150131x + 118916	0.991
Lig Agl	nanoLC	9.5	22.0	2	6	LOQ-6	y = 314076x + 405488	0.964
	HPLC	3.0	6.7	430	1430	LOQ-300	y = 9018.9x + 59184	0.993

a) Hyty: Hydroxytyrosol; Ty: Tyrosol; EA: Elenolic acid; Pin: Pinoresinol; Lut: Luteolin; Apig: Apigenin; Lig Agl: Ligstroside aglycon.

b) In mass spectrometry detection limit was calculated considering S/N=3

Table 4. Quantitative results (mg/kg) achieved by HPLC and nanoLC-ESI-TOF MS for the three varieties of olive oil (Picual, Arbequina and Hojiblanca) included in the study.

Compounds	*PICUAL*		*ARBEQUINA*		*HOJIBLANCA*	
	nanoLC	**HPLC**	**nanoLC**	**HPLC**	**nanoLC**	**HPLC**
Hyty	30.15 ± 2.34 (b)	20.20 ± 0.77 (a)	6.15 ± 0.28 (b)	3.37 ± 0.11 (a)	21.93 ± 1.39 (b)	9.76 ± 0.34 (a)
Ty	15.19 ± 0.75 (a)	11.84 ± 0.56 (a)	4.65 ± 0.21 (b)	2.33 ± 0.10 (a)	13.61 ± 0.97 (b)	6.56 ± 0.17 (a)
EA	54.76 ± 2.54 (a)	58.18 ± 1.46 (a)	14.93 ± 1.86 (a)	10.47 ± 0.84 (a)	37.85 ± 2.33 (a)	33.00 ± 0.98 (a)
Lut	1.64 ± 0.12 (a)	1.84 ± 0.06 (a)	3.72 ± 0.23 (b)	4.41 ± 0.12 (a)	2.95 ± 0.28 (a)	3.29 ± 0.10 (a)
Pin	0.76 ± 0.10 (a)	0.77 ± 0.03 (a)	1.90 ± 0.45 (a)	2.24 ± 0.21 (a)	2.13 ± 0.31 (a)	1.86 ± 0.09 (a)
Apig	0.32 ± 0.06 (a)	0.43 ± 0.04 (a)	0.96 ± 0.23 (a)	1.22 ± 0.08 (a)	0.74 ± 0.06 (a)	0.99 ± 0.07 (a)
Lig Agl	65.5± 3.17 (a)	64.78 ± 1.19 (a)	20.09 ± 1.37 (a)	17.38 ± 0.32 (a)	34.77 ± 3.20 (a)	38.23 ± 0.71 (a)

a) Hyty: Hydroxytyrosol; Ty: Tyrosol; EA: Elenolic acid; Pin: Pinoresinol; Lut: Luteolin; Apig: Apigenin; Lig Agl: Ligstroside aglycon.

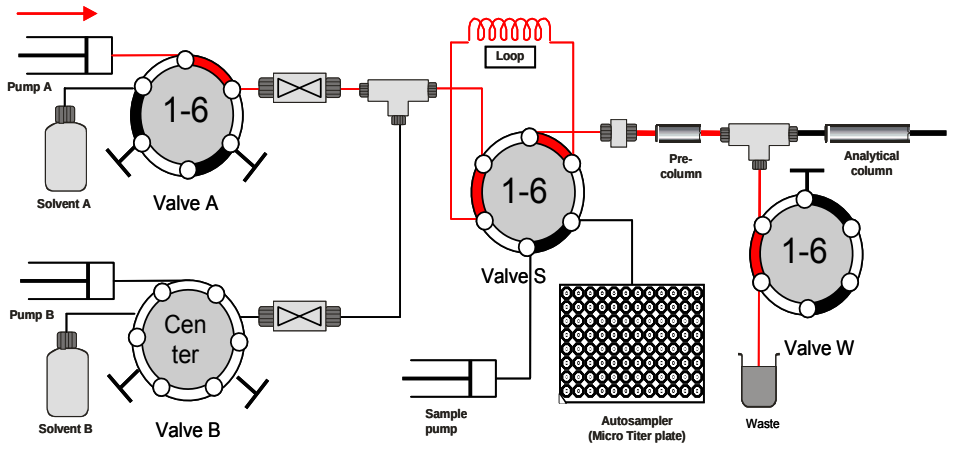
b) Values are given as Mean ± Standard deviation.

c) Means achieved by nanoLC-MS and HPLC-MS for the three varieties under study were compared. We indicated with different letters when means are significantly different ($p \leq 0.05$).

Table 5. Advantages and drawbacks of nanoLC (versus HPLC).

	nanoLC
Advantages	▪ Excellent sensitivity, very low LODs (ppb) when techniques to load large sample volumes are used ▪ Small amounts of stationary phase make, in most occasions, the columns cheaper ▪ Low flow rates (40-600 nL/min): lower cost and reduction of waste solvents ▪ Good coupling with MS ▪ Low sample consumption (20-60 nL) ▪ Better baseline noise due to the reduced background ▪ Very promising alternative for biological samples
Disadvantages	▪ Good intra-day repeatability but low inter-day repeatability ▪ Worse linearity ▪ Columns more easily blocked with real samples ▪ More technical problems because of the delicate plumbing and the use of fragile fused-silica ES emitters ▪ Mass spectrometry gets dirty early because of the use of lower flow rate.

Figure 1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Valve A
Loop
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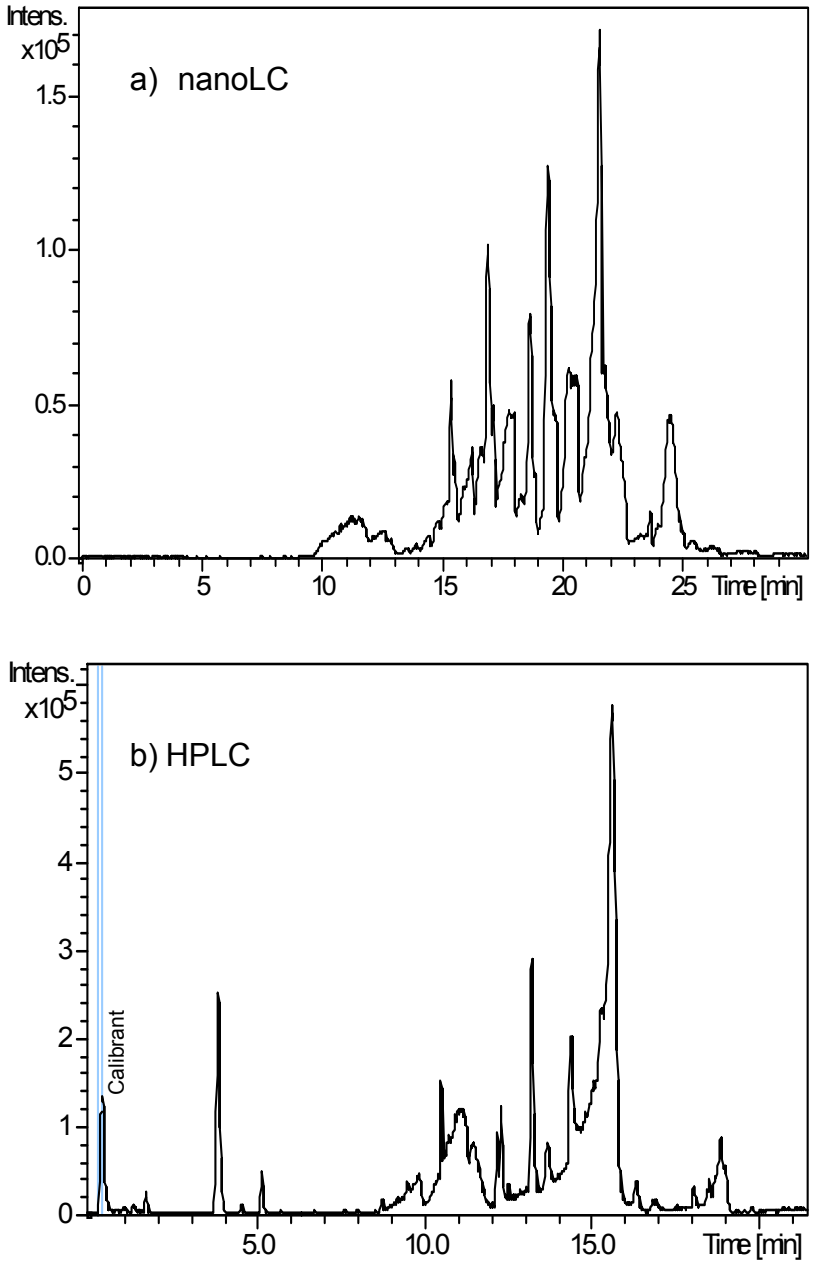
Valve S
Pre-
column
Analytical
column
1-6
Valve W
Pump B
Cen
ter
Solvent B
Valve B
Sample
pump
Autosampler
(Micro Titer plate)
Waste

Figure 2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0
5
10
15
20
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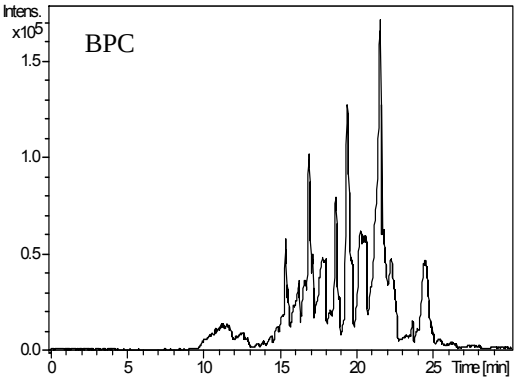
Time [min]
Intens.
x10^5
b) HPLC
Calibrant
5
4
3
2
1
0
5.0
10.0
15.0
Time [min]

Figure 3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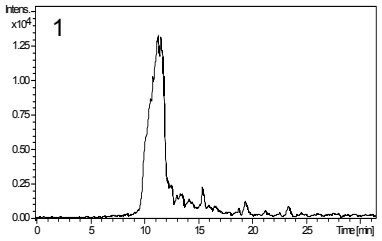
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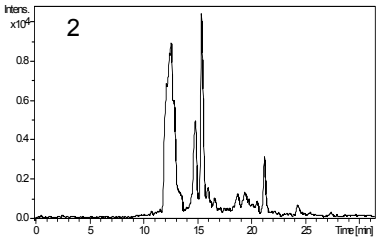
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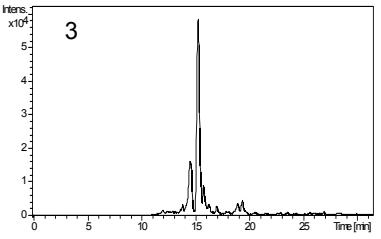
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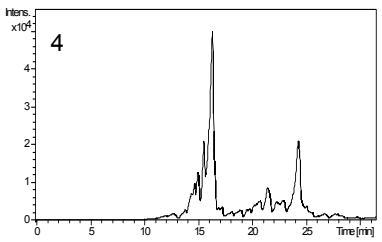
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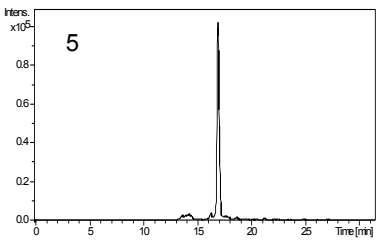
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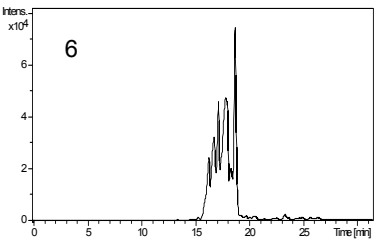
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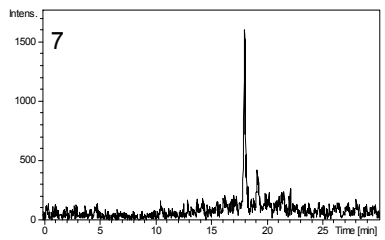
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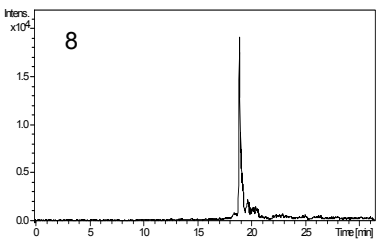
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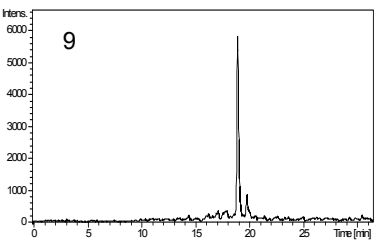
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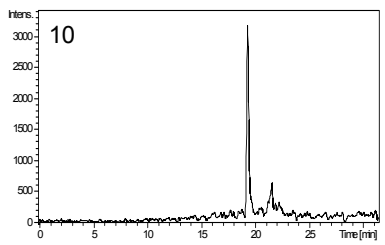
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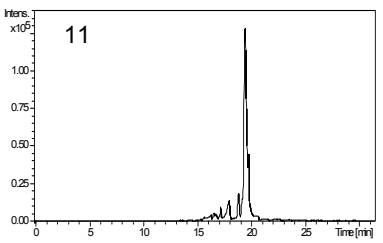
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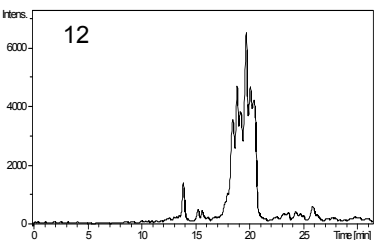
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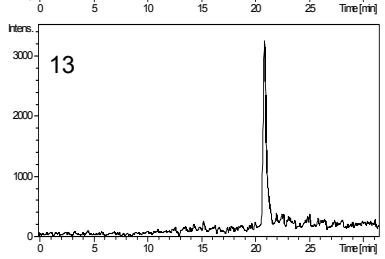
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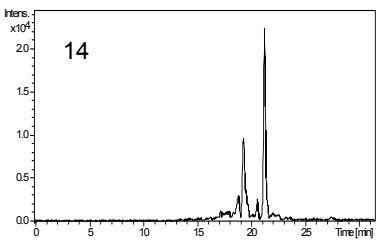
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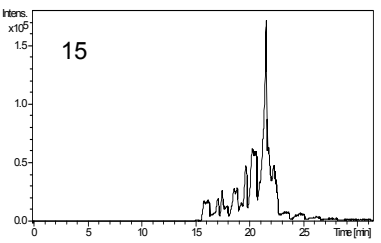
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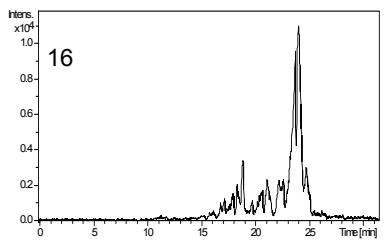
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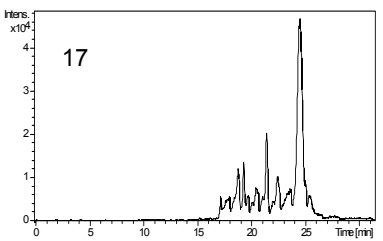
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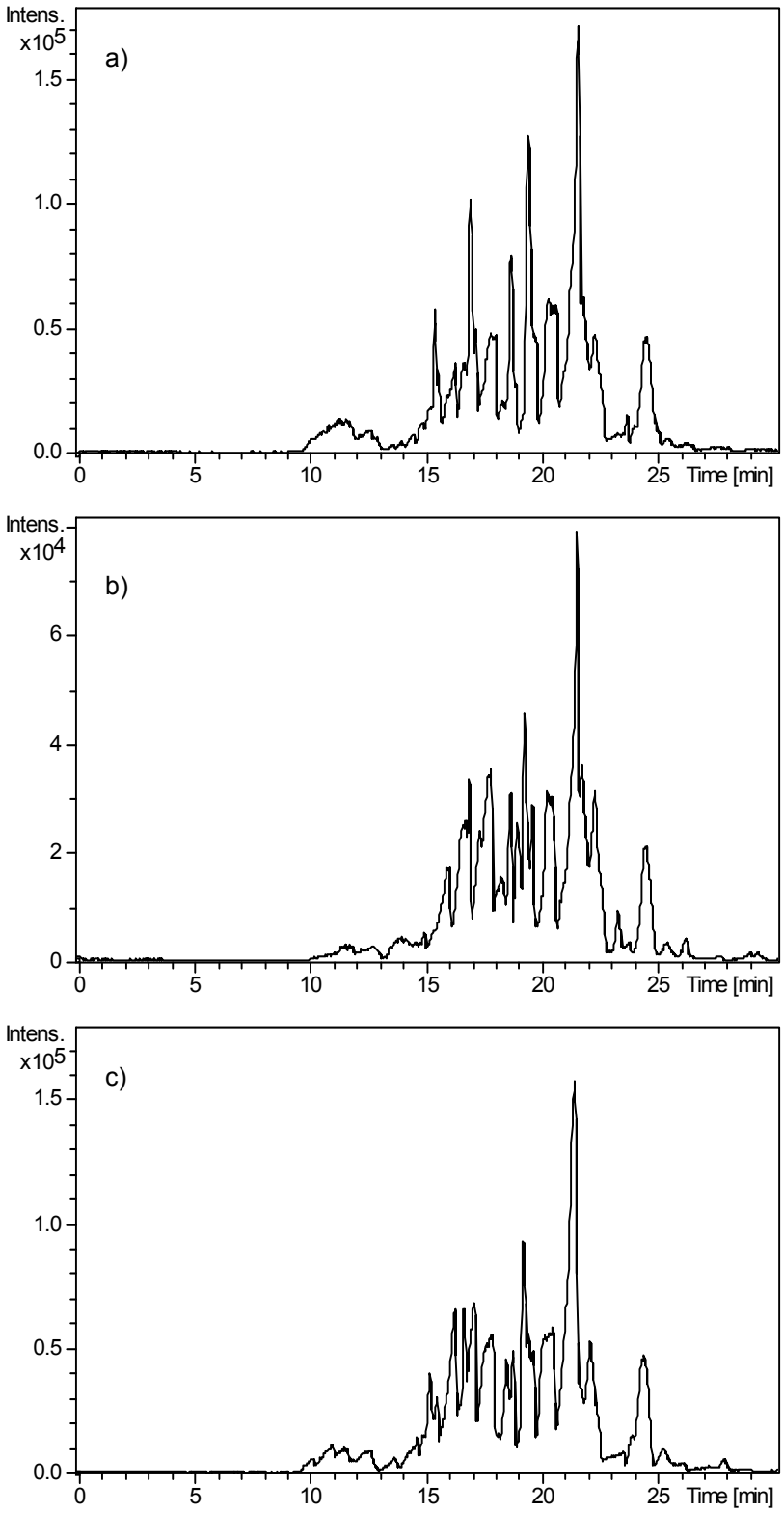
Time [min]

Figure 4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x10^5
a)
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0.0
0
5
10
15
20
25
Time [min]
Intens.
x10^4
b)
6
4
2
0
0
5
10
15
20
25
Time [min]
Intens.
x10^5
c)
1.5
1.0
0.5
0.0
0
5
10
15
20
25
Time [min]

CAPÍTULO 7:
Nueva plataforma analítica GC-APCI-MaXis MS para la determinación de polifenoles del aceite de oliva

La cromatografía de gases (GC) es una técnica que se ha empleado relativamente poco para el análisis de los polifenoles del aceite de oliva, principalmente debido a la necesidad de derivatización previa de estos compuestos antes de su análisis, como indicábamos en la introducción de este bloque. Además, las interfases utilizadas hasta el momento en el acoplamiento de GC con la espectrometría de masas -el impacto electrónico (EI) y la ionización química (CI)- presentan algunas limitaciones, como su alto poder de fragmentación, que dificulta la caracterización de los compuestos.

En este capítulo 7 se plantea el desarrollo de una metodología analítica en la que se emplee GC con un acoplamiento totalmente novedoso a través de una interfase APCI y que utilice un espectrómetro de masas de última generación (MaXis); una plataforma analítica (GC-APCI-MaXIs MS) nunca antes utilizada en el análisis de polifenoles. Se pretende demostrar el potencial analítico y la aplicabilidad de este método de ionización alternativo acoplado con un potente analizador de masas para el análisis de los compuestos fenólicos del aceite de oliva. Habría que optimizar la reacción de derivatización y evaluar los parámetros experimentales e instrumentales que influyen tanto en la sensibilidad, como en el poder resolutivo del método.

Una estancia llevada a cabo en la Unidad de Espectrometría de Masas Biomolecular del Centro Médico de la Universidad de Leiden (LUMC) en Holanda, nos dio la posibilidad de utilizar instrumentación y tecnologías que sólo están disponibles en varios laboratorios de todo el mundo.

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Role of GC-APCI-MaXis MS in food metabolomics: Determination of polyphenols from extra-virgin olive oil

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Abstract

We describe the first analytical application involving solid-phase extraction and gas chromatography coupled to atmospheric pressure chemical ionization-MaXis mass spectrometry (GC-APCI-MaXis MS) for achieving the chemical characterization of the phenolic fraction of extra-virgin olive oils. Both chromatographic and MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity of their determination. The BSTFA derivatized-phenols where injected in the instrument and separated in a HP-5-MS column (30 m, 0.25 mm ID, 0.25 μm film thickness). MS/MS analyses were carried out to achieve the unequivocal identification of the compounds under study. A complete validation of the method was carried out considering the specificity, linearity, sensitivity, precision and accuracy. The detection limits were found ranging between 0.50 and 4.20 ppm, for pinoresinol and homovanillic acid, respectively. Acceptable levels of precision were obtained for the developed method in terms of repeatability since in all cases RSDs calculated were lower than 6.07%. The accuracy ranged from 95.4% to 101.5%.

The method was used to carry out the quantitative characterization of different oils, offering to the analyst the chance to study important phenolic compounds such as phenolic alcohols, lignans, phenolic acids, flavonoids and complex phenols in extra-virgin olive oil.

Keywords: Gas chromatography / Mass spectrometry / Polyphenols / Metabolomics / APCI

1. Introduction

The beneficial effects of the Mediterranean diet on human health, reducing the risk of morbidity (particularly atherosclerosis, cardiovascular disease, and certain types of cancer) and mortality have been well-established [1,2]. These apparent health benefits have been partially attributed to the dietary consumption of virgin olive oil by Mediterranean populations; fat whose chemical composition consists of a glycerol fraction (that represents about 98% of the total weight) and a non-glycerol or unsaponificable fraction which contains minor components, such as phenolic compounds, tocopherols, sterols, pigment, etc [3]. Historically, the healthful properties of virgin olive oil have been attributed to the high proportion of monounsaturated fatty acids, however the hypothesis that minor components such as phenolic compounds could play an important role in the healthy effects of virgin olive oil has gradually become stronger [4,5,6]. Apart from that, the level of these substances is a very important parameter when the quality of virgin olive oil is evaluated, since phenols are strictly related to oil´s resistance to oxidation processes, due to their antioxidant properties [7,8], and to the organoleptic characteristic (flavour, astringency, pungency, bitterness…) [9,10,11].

VOO contains different classes of phenolic compounds, such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids and lignans [12]. Not all the mentioned families of phenols are present in every virgin olive oil and there is variation in the phenolic concentration between virgin olive oils; such variation is partially the result of diverse production factors (variety of the olive fruit, agricultural techniques used to cultivate the olive fruit, maturity of the olive fruit at harvest time, olive oil extraction, processing, and storage methods…) [1,1213,14].

The identification and quantification of the individual phenolic compounds present in VOO are of great interest. For example, in order to preserve phenolics in virgin olive oil, it is essential to identify the factors affecting the composition and concentration of olive oil phenols. Moreover, information on the absorption and disposition of olive oil phenolic compounds is pivotal in determining their potential healthy effects *in vivo*.

To achieve a deep knowledge about the composition of the phenolic fraction from VOO, the analyst need appropriate analytical methods. Analytical procedures for the characterization and quantification of the complete profile of phenolic components of olive oils usually entail extraction (LLE or SPE) of these compounds from the oil, followed by a separative technique (RP-HPLC, GC or CE coupled to different detection

systems) [12,15,16,17,18,19] or even a colorimetric assay (which obviously gives to the analyst only an estimation of total phenol content) [20]. GC has been mainly used with two different detectors, FID or MS; in HPLC, it is possible to find research works where UV (photodiode array), fluorescence, electrochemical, biosensors, NMR and MS detectors are used; whilst CE has been used with UV as detection system and, more recently, with MS detectors [12].

In literature, the 80% of the described applications about determination of polyphenols from olive oil were carried out by using HPLC (with different detectors); CE is opening up great expectations and becoming more popular; GC covers approx. the 15% of the papers published in the field. The results obtained by using GC are very reliable and interesting, but the use of this technique is less common because the derivatization step is essential and the use of high temperature which could damage the analytes.

The first work about the separation of phenolic compounds of olive oil by GC was made by Janer del Valle et al. [21] 25 years ago. This technique was used simultaneously by Solinas et al. [22,23] as well, who used GC for authentication purposes, identifying mixtures of virgin olive oils and refined oils. Forcadell et al. [24] developed a protocol for the preparation of trimethylsilyl (TMS) derivatives in 1987, and then, Solinas [25] published a paper where developed a GC method for the quali-quantitative evaluation of phenolic compounds in VOOs of different cultivars at diverse degrees of ripeness. Improvement in the identification of compounds was obtained with analytical sophisticated techniques such as GC-MS [26,27,28] and GC-MS/MS [29]. One of the newest applications was developed by Ríos et al. [15] when they optimized a SPE-GC-IT MS method for the qualitative evaluation of phenols in VOO and the structural confirmation of oleuropein and ligstroside aglycons and their oxidations products.

Atmospheric pressure ionization sources (API) are rarely used with GC instruments. Indeed, in most of the published papers about determination of phenols by GC-MS, the used systems carried out the ionization under vacuum conditions (electron ionization (EI) and, in some cases, chemical ionization (CI)); the analyzers were mainly quadrupoles and ion traps.

In the current manuscript our aim was to evaluate the role of a GC-APCI-MaXis MS platform in the field of food metabolomics, developing a method to analyze the polyphenols present in extra-VOO and carrying out a detailed examination of its analytical performance (repeatability, reproducibility, linearity, and detection limits). The excellent separation which can be achieved by using GC, together with a soft

ionization source (APCI) and a powerful MS analyzer (MaXis), able to carry out MS/MS experiments and provide excellent mass accuracy over a wide dynamic and measurements of the correct isotopic pattern, could become to one of the standard methods in metabolic profiling.

2. Materials and Methods

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Sinapinic acid, gentisic acid, 4-hydroxyphenylacetic acid, vanillin, syringic acid, vanillic acid, caffeic acid, gallic acid, *trans*-cinnamic acid, *m*-coumaric acid, protocatechuic acid, 4-hydroxybezoic acid, homovanillic acid, ferulic acid, *p*-coumaric acid (phenolic acids and related compounds), luteolin, apigenin, taxifolin (flavonoids and related compounds), tyrosol, hydroxytyrosol (simple phenols), and pinoresinol (lignans) were from Sigma-Aldrich, Fluka and Extrasynthèse, and they were used to evaluate the analytical characteristics of our method and to identify those compounds in the extract from EVOO. Dopac was acquired from Fluka and was used as internal standard (IS). Secoiridoids are not available as commercial standards, so we isolated them (see section 2.4).

The organic solvents, acetonitrile, methanol, and *n*-hexane, all from Sigma Aldrich (St. Louis, MO), were used to prepare the mobile phases in HPLC and to carry out the SPE extraction protocol. In HPLC, phase A contained acetic acid from Merck (Darmstadt, Germany). Deionized and organic-eliminated water was from the water purifier system (USF^{ELGA} from Purelab Plus, Ransbach-Baumbach, Germany).

N,*O*-bis(Trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) and N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA + 1% TMCS) from Pierce (Rockford, IL, USA) were used as derivatization reagents. These reagents were used from freshly opened 1 mL bottles. Methoxyamine hydrochloride was purchased from Supelco. Pyridine (>99%, ultra-pure GC grade) was acquired from Fluka.

2.2 Samples

Spanish EVOO samples used in the preliminary studies were obtained from a unique variety of olive fruit called Picual, Arbequina, Cornicabra, Frantoio and Hojiblanca (January 2009). For simplicity, to illustrate the results we got during the optimization,

we only show the GC profiles of extracts of a commercial mixture between Picual and Arbequina (50/50, v/v) extra-virgin olive oils, although we used the other varieties during the optimization of the separation conditions of our method. Using more than one variety, we could ensure the potential of the presented methodology for the analysis of these compounds in any kind of olive oil. In one of the last sections of the current paper, we will show the GC-APCI-MaXis MS profiles and quantitative data of three different commercial extra-VOOs (a mixture between Arbequina and Picual extra-VOOs, Hojiblanca extra-VOO Auchan, and Frantoio extra-VOO).

As mentioned before, some compounds belonging to the family of secoiridoids, which are very important in the phenolic fraction of this matrix, are not available as commercial standards. Thus, two varieties were chosen for being used along the study in order to isolate the different phenolic fractions; they were Picual and Arbequina. The reasons for choosing these two varieties were based on the high concentration of phenols and the number of peaks present in their profiles. We prepared a mixture EVOO Picual/EVOO Arbequina (1/1 v/v) to facilitate the isolation of the phenols.

2.3 SPE Procedure

Taking into account the previous results described elsewhere [30], we chose SPE with Diol-cartridges to isolate the phenolic fraction from EVOO. Briefly, the cartridge was placed in a vacuum elution apparatus and pre-conditioned passing 10 mL of methanol and then 10 mL of hexane. About 60 g of EVOO were thoroughly mixed with 60 mL of hexane and carefully loaded onto the pre-conditioned column, leaving the sample on the solid phase. After a wash with hexane (15 mL) to remove the non-polar fraction of the oil, the sample was eluted with methanol (40 mL). The methanolic extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 35ºC. The dried residue was then redissolved in 2mL of methanol and filtered through a 0.25 mm filter before the analysis.

2.4 HPLC isolation of polyphenolic compounds

Several strategies can be used to generate the chemical standards of the compounds under study in order to facilitate their identification and quantification in the sample; among them, to purchase them for the producer company is the most straightforward step. The limited availability of suitable commercially available standards is a problem

that can be overcome, in part, by synthesis of the relevant compounds (sometimes very tedious). Alternatively, the relevant compounds isolated by preparative scale chromatography can serve as reference standards. The last option was the one used in the current study (when the standards were not commercially available, as in the case of secoiridoids).

2.4.1 Isolation of polyphenols using semipreparative HPLC analysis

Once the mixture of olive oils rich in the phenolic compounds of interest was chosen, 9 phenolic fractions corresponding to different peaks in the UV chromatogram at 280 nm were selected for the isolation (**Fig. 1**).

In the semi-preparative HPLC analysis for the isolation of the reference compounds, the mobile phases were water with acetic acid (0.5%) (phase A) and ACN (phase B), and the solvent gradient changed according to the following conditions: from 0 to 30min, 95%(A):5%(B) to 80%(A):20%(B); from 30 to 40min, 80% (A):20% (B) to 70% (A):30% (B); from 40 to 50min, 70% (A):30% (B) to 65% (A):35% (B); from 50 to 60min, 65% (A):35% (B) to 50% (A):50% (B); from 60 to 70min, 50%(A):50%(B) to 5%(A):95%(B); from 70 to 75min, 5% (A):95% (B) to 95% (A):5% (B). This last value was maintained for 5 min, and the run was ended. The flow rate was set at 3 mL/min. The quantity of extract injected into the column was optimized in order to obtain concentrated profiles but with good resolution, and finally an injection of 200 μL was selected as optimum. The isolation of the compounds was carried out from Diol-SPE extracts of the mixture of EVOO and was done trying to obtain the fractions as pure as possible avoiding other potential interferences. We needed more than 100 injections to get quantities of the isolated fractions that could be weighed. These compounds were manually collected and kept at -20ºC. Once that enough quantity was collected, it was led to dryness in a rotary evaporator at 35ºC and the difference in weight between the empty flask and the flask after the evaporation of the solvent, gave us the amount of solute collected.

2.4.2 Analysis of the isolated compounds using analytical HPLC

After isolation, the analysis of these compounds was done with an analytical column (C18 Gemini column, 5 mm id, 25 cm x3.0 mm (Phenomenex, Torrance, CA, USA), equipped with a precolumn (Phenomenex) filter) to check the purity of the isolated compounds and confirm their identity, using the same gradient employed in the

semipreparative HPLC method. The injection volume was 10 μL. The wavelengths were set at 240, 280, and 330 nm. The detection was made using ESI-TOF MS as well, and the analyses were carried out using an electrospray interface operating in negative mode in the range of 50–800 m/z, using the following conditions: nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190ºC.

2.5 Derivatization reaction

A speed vacuum concentrator was used for drying both the standard mixture and the EVOO extracts to complete dryness. The dried samples were taken from store and warmed up to room temperature before starting derivatization. The derivatization reaction was carried out by adding 50 μL of BSTFA containing 1% TMCS (as the catalyst) to the dried sample containing the phenolic compounds under study. The solution was vortexed for 1 min and the trimethylsilylation reaction was performed at room temperature for 30 min. A minimum of 30 min equilibration time was necessary before sample injection.

The effect of including an intermediate step of methoxyamination was properly evaluated and we concluded that it was not necessary. The trimethylsilylation reaction was also tried by using MSTFA containing 1% TMCS; no significant changes were observed.

2.6 GC-APCI-MaXis MS optima conditions

An aliquot of the derivatized samples (1 μL) were applied by splitless injection with a programmable CTC PAL multipurpose-sampler (CTC Analytics AG, Zwingen, Switzerland) into an Agilent 7890A GC (Agilent, Palo Alto, USA) equipped with a HP-5-MS column (30 m, 0.25 mm ID, 0.25 μm film thickness). Injection programs included sequential washing steps of the 10 μL syringe before and after the injection, and a sample pumping step for removal of small air bubbles.

The injection temperature was set 250ºC. Helium was used as carrier gas at a constant flow rate of 0.5 mL/min through the column. For every analysis splitless injection time was 60 s and after this the injector was purged at 20 mL/min flow rate. The column temperature was initially kept at 170ºC for 5 min and then raised at 3º C/min to 255ºC, keeping that value for 1 min, finally the temperature was raised at 2º C/min to 310ºC

and held for 10 min. Using the described chromatographic conditions, the analysis time was about 70 min.

After identifying as many compounds as possible in the profile and knowing the areas of the chromatogram more crucial (because they contained some of the most important compounds of the phenolic fractions), we re-optimized the separation, looking for shortening the analysis time without loosing resolution in those areas. At the end, in the optimum method the column temperature was initially kept at 160ºC for 5 min and then raised at 3º C/min to 188ºC, keeping that value for 1 min. After that, the temperature raised at 15ºC/min till 241ºC (keeping that value for 1 min). From that T value to 280ºC the temperature changed according to the following rate: 1ºC. From 280ºC to 310ºC the temperature rate was 5ºC/min. When the T of 310ºC was achieved, it was held for 5 min. Helium was used as carrier gas at a constant flow rate of 1.0 mL/min through the column. With the described chromatographic conditions, the analysis time was about 50 min.

The GC transfer line to the mass spectrometer was kept at 300ºC. The APCI source and MS were operated in positive mode, temperature and flow rate of the dry gas (nitrogen) were 250°C and 5.00 L/min, respectively. The APCI vaporizer temperature was 450°C; the pressure of the nebulizer gas (nitrogen) was set to 2 bar, and the voltage of the corona discharge needle was 2000 nA. Capillary voltage was set at -1000 V and the end-plate offset at -1000 V.

As detector an orthogonal-accelerated MaXis mass spectrometer (oaMaXis-MS) (Bruker Daltonik, Bremen, Germany), which has the capability of determining the m/z with high accuracy considering the isotopic distribution and making MS/MS analyses, was used. The polarity of the APCI interface and all the parameters of TOF MS detector were optimized using the area of the MS signal for the polyphenols under study and the chromatographic resolution as analytical parameters. The position of the column in the transfer line, the transfer line temperature, the flow rate and pressure of nebulizer gas (nitrogen), the vaporizer temperature, voltages in the corona, source and ion transfer settings: all those parameters were optimized empirically. These are essential for optimal performance of an instrument but may vary from instrument to instrument.

Data were acquired for mass range from 50 to 1000 *m/z* with a repetition rate of 1 Hz. DataAnalysis 4.0 software (Bruker Daltonik) was used for data processing. The SmartFormula^{TM} tool within DataAnalysis was used for the calculation of elemental composition of compounds; it 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 theoretical with the measured isotope pattern (Sigma-Value) for increased confidence in the suggested molecular formula. 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 [31].

2.7 MS/MS analyses

Different MS/MS strategies were used to get as much information as possible about the extracts from extra-virgin olive oil under study. As a first step, we applied auto MS/MS mode to accumulate information about the energy which was necessary to use to achieve the proper fragmentation for each compound. After doing that, we proceeded to use multiple reaction monitoring (MRM) analyses. After checking the potential of MRM, we decided to create different segments in our MS method, changing some parameters (collision energy, isolation width, ISCID energy, amplitude) to assure the achievement of MS/MS spectra of high quality. The MS method was divided in the following segments: 0-9.1, 9.1-20, 20-40.8, 40.8-46.0, 46.0-52.0, 51.0-56.0, 56.0-62.2, and from 62.2 to the end of the run. Apart from the fragmentation conditions, we also changed the precursor ions that the analyzer was observing in each segment, keeping in mind the retention time of the analytes under study.

2.8 Validation experiments using EVOO quality control (QC) samples

The mentioned mixture of Picual and Arbequina EVOOs was used as QC sample.

2.8.1 Derivatized samples stability

The stability of BSTFA-derivatized secondary metabolites, kept at ambient temperature (20-25ºC), was determined periodically by injecting replicate preparations of the processed samples consecutively for up to 48 h. The peak areas of the analytes obtained at the initial cycle were used as the reference to determine the relative stability at subsequent sampling points.

2.8.2 Specificity

The specificity of the method was tested by screening analysis of blank oil samples (refined sunflower oil (phenolic-free)). There were no impurity peaks or contamination at the retention times corresponding to the analytes.

2.8.3 Linearity and sensitivity

Linearity of the detector response (MaXis-MS) was verified with standard solutions containing some of the analytes under study at ten different concentration levels over the range from the quantification limit to 250 ppm. We decided to make calibration curves for the following compounds: tyrosol, hydroxytyrosol, homovanillic acid, *p*-coumaric acid, ferulic acid, luteolin, apigenin, and pinoresinol. The reason was that after the preliminary studies, we checked that they were very likely present in the samples under study. Each point of the calibration graph corresponded to the mean value from three independent replicate injections. Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from GC-APCI-MaXis MS analyses. The sensitivity of the analytical procedure was calculated by defining the limits of detection (LOD) and quantification (LOQ) for the individual analytes in standard solutions according to the IUPAC method [32]. The smallest concentration that could be detected with a reasonable certainty for our analytical procedure (LOD) was considered S/N = 3, whilst LOQ was S/N = 10.

2.8.4 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, medium, high). Spiked quality control (QC) samples were tested in six replicates per concentration and calculated with calibration curves obtained daily. The precision of the analytical procedure was expressed as the relative standard deviation (RSD). The intra- and inter-day repeatability in the peak areas was determined as the RSD obtained for six consecutive injections of each phenol at each concentration value, carried out within the same day and on three different days.

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 four different concentration levels, i.e. close to LOQ, low, medium and high, by three determinations per concentration on different days. The analyte concentrations were calculated from daily calibration curves and the accuracy was

calculated by the ratio of this calculated concentration versus the theoretical spiked concentration.

3 Results and Discussion

3.1 Preliminary studies

BSTFA (+1% TMCS) and MSTFA (+1% TMCS) were used as derivatization reagents. They react with a range of polar compounds by replacing active hydrogen in alcohols, amines, carboxylic acids, etc. The derivatization reaction was carried out by adding 50 μL of the derivatization reagent containing 1% TMCS (as the catalyst) to the dried sample containing the phenolic compounds under study. The trimethylsilylation reaction was performed at room temperature for 30 min and minimum of 30 min equilibration time was necessary before sample injection. The effect of including an intermediate step of methoxyamination was properly evaluated and we concluded that it was not necessary. The trimethylsilylation reaction was also tried by using MSTFA containing 1% TMCS; no significant changes were observed.

Once we had some phenolic extracts from EVOO properly prepared, we started the optimization of the chromatographic and MS (APCI and MaXis) conditions, to get as many compounds as possible in our profiles with the best resolution and sensitivity. After the preliminary studies, the optima conditions resulted to be the following: Helium was used as carrier gas at a constant flow rate of 0.5 mL/min through the column. The column temperature was initially kept at 170ºC for 5 min and then raised at 3º C/min to 255ºC, keeping that value for 1 min, finally the temperature was raised at 2º C/min to 310ºC and held for 10 min. Using the described chromatographic conditions, the analysis time was about 70 min.

The GC transfer line to the mass spectrometer was kept at 300ºC. The APCI source and MS were operated in positive mode, temperature and flow rate of the dry gas (nitrogen) were 250°C and 5.00 L/min, respectively. The APCI vaporizer temperature was 450°C; the pressure of the nebulizer gas (nitrogen) was set to 2 bar, and the voltage of the corona discharge needle was 2000 nA. Capillary voltage was set at -1000 V and the end-plate offset at -1000 V.

3.2 GC-APCI-MaXis MS analysis of standard mixture

A standard mixture consisting of 22 commercially available phenolic compounds was analyzed by using the conditions we set as optimal after the preliminary studies. **Table**

1 shows all analytes detected, with their formula, retention time, measured and theoretical m/z, error (mDa) and mSigma value. All values were calculated from samples with concentrations close to the LOQ; nevertheless the mass position error remained within 1.5 mDa and high quality sigma fit values (< 20 mSigma) were obtained for all compounds. In each line in the table, we highlight in bold letter the prevalent ion which was observed in the MS spectrum. These results were pivotal to understand the signal that each phenolic compound included in the mixture produced in APCI-MaXis MS. (**Supporting information (fig 1)** shows the MS spectra for all the compounds included in the mix).

3.3 Analysis of the isolated phenolic fractions

As commented before, one of the problems that the analyst has to face when the phenolic fraction from extra-VOO is studied is the absence of commercially available standards of secoiridoids (complex phenols). These secoiridoids derive from olive secoiridoid glucosides, which are present only in plants belonging to the Oleaceae family, and they are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure [33]. The main olive secoiridoid glucosides are not present in extra-virgin olive oil because of their high solubility. However, during the crushing of olives oil-phase-soluble derivatives arise from the chemical and enzymatic hydrolysis of secoiridoid glucosides [34].This enzymatic hydrolysis explains the presence of so many isomeric or related forms in this family of compounds. The prevalent secoiridoid compounds in the olive oil are the dialdehydic form of elenolic acid linked to HYTY or TY and isomers of Ol Agl or Lig Agl [35,36,37].

We isolated with semi-preparative HPLC nine phenolic fractions (as we explained in section 2.4); then we made individual injections of every isolated fraction to study the GC-APCI-MaXis MS signal they produced. **Fig. 2** (on the top) shows the Base Peak Chromatogram (BPC) of the extra-VOO we used during the optimization together with the Extracted Ion Chromatograms (EICs) of the 9 isolated fractions (in colour). In the same figure, we can see the chromatograms which were observed when the 9 fractions were injected with the BPC of the oil under study in grey (to facilitate the identification of the peaks present in each fraction). **Table 2** completes the information showed in Fig 2, since it includes the number of the fraction (with a code which has to do with the most relevant compound which was observed in the HPLC analyses of each fraction),

retention time, m/z experimental, and molecular formula assigned (error and mSigma) when the compound was a well-known phenol.

So, in fraction 1 (Elenolic acid fraction) we observed 4 different peaks at retention times of 17.3, 17.9, 21.7 and 36.8. The most intense one was the peak which appeared at 17.3 which was identified as EA-H+1TMS+H. It was interesting to prove that in instances in which an HPLC method does not provide enough resolution, GC can be a very suitable alternative. For example, in this fraction 1, we just observed one peak in HPLC, whilst 4 different and well separated peaks can be seen in GC.

Some more peaks could be identified in the profile after injecting the other collected fractions. Particularly interesting was to study the analyses of fractions 6-9, since the lignan Ac Pin, and compounds related to Lig Agl, Ol Agl and their isomers were identified.

3.4 Identification of the compounds in the profile of an extra-VOO

Keeping in mind the results we got after doing the analysis of the standards (commercial standards and isolated standards) and the information previously published, we tried to identify as many compounds as possible in the optimum GC-MS profile.

Fig. 3 shows the optimum BPC of the Diol-SPE extract obtained from an extra-VOO obtained from a mixture between Arbequina and Picual oils under the optima GC-APCI-MaXis MS conditions. Elution windows of different families belonging to the phenolic fraction of EVOO are shown; simple phenols and phenolic acids appeared at the beginning of the chromatographic run, followed by secoiridoids, flavonoids and lignans. Individual peaks with the name in blue were identified with commercial standards; those with the name in red were identified by using the isolated phenolic fraction (with semi-preparative HPLC); and those in purple were identified keeping in mind our previous knowledge abut the fraction. In grey we can observe peaks with relevant intensity which were not fully identified.

3.5 MS/MS analyses

To assure the unequivocal identification of the compounds under study, we proceeded to carry out MS/MS analyses, taking advantage of the capabilities that the analyzer we were using had. Different MS/MS strategies were used; as a first step, we applied auto MS/MS mode to accumulate information about the energy which was necessary to use

to achieve the proper fragmentation for each compound. After doing that, we proceeded to use multiple reaction monitoring (MRM) analyses. After checking the potential of MRM, we decided to create different segments in our MS method, changing some parameters (collision energy, isolation width, ISCID energy, amplitude) to assure the achievement of MS/MS spectra of high quality. The MS method was divided in the following segments: 0-9.1, 9.1-20, 20-40.8, 40.8-46.0, 46.0-52.0, 51.0-56.0, 56.0-62.2, and from 62.2 to the end of the run. Apart from the fragmentation conditions, we also changed the precursor ions that the analyzer was observing in each segment, keeping in mind the retention time of the analytes under study.

Table 3 includes the m/z APCI-MaXis MS/MS signals detected for the most relevant phenolic compounds present in the extract of extra-virgin olive oil use during the optimization. In bold letter we highlight the prevalent ion in the MS spectrum for each compound. Every peak detected in the profile was isolated and further fragmented after applying the required energy to get a clean MS/MS spectrum. When a compound in particular did not only give one m/z signal in MS, we considered as precursor ions all the different m/z signals observed in the MS spectrum for making MS/MS analyses. The information included in the mentioned table, can be completed observing **supporting information (Fig. 2)**, where we included the MS/MS spectra of the most relevant compounds detected in the phenolic profiles of extra-VOOs.

In this way, we were able to identify unequivocally a great number of phenolic compounds in the GC-APCI-MaXis MS of an extra-VOO.

3.6 Re-optimization of the chromatographic conditions

When the phenolic profiles of the extracts of extra-VOO were completely characterized (after studying the signal and retention time of standards (commercial and isolated) and making MS/MS experiments), we were aware about which areas of the analysis were more crucial because they contained important phenols. At that moment, we re-optimized some GC parameters looking for a good compromise solution between the analysis time and resolution between the most relevant peaks. It is important to bear in mind that the analyzer was MaXis (new generation of TOF instrument, with powerful capabilities regarding accuracy, determination of isotopic distribution and MS/MS analysis), that means that in cases in which the resolution is not perfect, the analyzer still can determine properly the compounds under study. We studied the effect of changing the flow rate and the temperature gradient, and we finally decided to use as

optimum GC method the following one: temperature was initially kept at 160ºC for 5 min and then raised at 3º C/min to 188ºC, keeping that value for 1 min. After that, the temperature raised at 15ºC/min till 241ºC (keeping that value for 1 min). From that T value to 280ºC the temperature changed according to the following rate: 1ºC. From 280ºC to 310ºC the temperature rate was 5ºC/min. When the T of 310ºC was achieved, it was held for 5 min. Helium was used as carrier gas at a constant flow rate of 1.0 mL/min through the column. With the described chromatographic conditions, the analysis time was about 50 min.

The improvement of the analysis time achieving still good resolution can be seen in **Figure 4**, where we show a comparison between the profile obtained by using the method selected as optimum after the preliminary studies and the one that we finally selected. The changes we made in the method shortened the analysis time about 20 min.

3.7 Validation experiments. Analytical parameters of the method

The stability of derivatized samples is an important factor for large scale food metabolomics studies. To address this issue, we kept derivatized samples in 1.5 mL screw capped vials (with inserted microvials) at room temperature and performed analysis in equal time intervals between 0 and 48 h. Data proved to be rather consistent from 0 to 35 h. However, data collected on later time points demonstrated steadily increasing variability. Nevertheless, to avoid any possible risk of derivatization-depended variability material should preferably be processed within the first 24 hours.

The specificity of the method was tested by analysis of blank oil samples (refined sunflower oil) and there was no significant chromatographic interference around the retention times of the analytes (data not shown to contain the size of the paper).

Calibration curves were obtained for each standard by plotting the standard concentration as a function of the peak area obtained from GC-APCI-MaXis MS analyses. The parameters of the calibration functions, LOD, LOQ, linearity, calibration range, correlation coefficient, repeatability and accuracy are summarized in **Table 4**.

We decided to make calibration curves for the following compounds: tyrosol, hydroxytyrosol, homovanillic acid, *p*-coumaric acid, ferulic acid, luteolin, apigenin, and pinoresinol. The reason was that after the preliminary studies, we checked that they were very likely present in the samples under study. In order to calculate the calibration functions and LOD's, we took the EIC of the most intense or base peak in the mass spectrum for each compound in the standard mixture. If the compound was represented

by more than one silylated form, the one with higher linearity in the calibration range was used for calculation of analytical parameters. For example, in the case of tyrosol, we used for quantitation the m/z signal 193.1061; for homovanillic acid, we used m/z 209.1007; for *p*-coumaric, we took into account m/z 309.1333; for ferulic acid, m/z 249.0967; for luteolin, m/z 575.2142; for apigenin, we used m/z 487.1792; and for pinoresinol, we considered m/z 485.2189.

All calibration curves showed good linearity in the range of concentrations that we indicate in the table. LODs were found within the range between 0.5 and 4.20 ppm, for pinoresinol and homovanillic acid, respectively. The precision of the GC-APCI-MaXis MS method described was measured as repeatability (intra- and inter-day). The intra- and inter-day repeatability in the peak areas was determined as the RSD obtained for six consecutive injections of each phenol at an intermediate concentration value of the calibration curve, carried out within the same day and on three different days. **Table 4** 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 6.07%. The accuracy ranged from 95.4% to 101.5%.

3.8 Application of the method to the analysis of different extra-VOOs

In the final step of the work, extracts of three different commercial extra-VOOs (a mixture between Arbequina and Picual extra-VOOs, Hojiblanca extra-VOO Auchan, and Frantoio extra-VOO) were analyzed. All samples were injected in the GC-APCI-MaXis MS instrument three times (n =3). The polyphenolic profiles (BPCs) are shown in **Figure 5**, where a comparison of the three oils under study can be observed. The intensity scale was exactly the same in all the cases, to facilitate the visual comparison. The quantitative results are presented in **Table 5**, when we show the amount of phenolic compounds (mg/kg) or area (when pure standards were not available) found in the extra-virgin olive oils under study. Differences among the three oils studies were quite drastic. Frantoio extra-VOO was, in general, the oil which showed a lowest phenolic content; it was the poorest in terms of simple phenols (tyrosol and hydroxytyrosol) and secoiridoids (amounts of Ol Agl and some of its most important derivatives were not detected). However, it had a very similar content in terms of flavonoids, when it was compared with Hojiblanca extra-VOO. As far as Ac Pin content is concerned, Frantoio was the richest oil.

Levels of simple phenols found in Hojiblanca extra-VOO were quite high (if compared with the other oils under study), however, the phenolic profile obtained for the mix between Picual and Arbequina oils was the one which had higher secoiridoids concentration (Ol Agl, Lig Agl and their derivatives). From our point of view, it is quite interesting the fact that we can have an idea about the Ol Agl-derivatives and Lig Agl-derivatives which are present in an oil just by studying two EICs, 281.2481, and 193.1944, respectively. That can be observed in the red square on the top of Fig. 5, where in grey we show the BPC of the mixture between Arbequina and Picual extra-VOOs and in colour, we show the mentioned EICs. When Ol Agl-derivatives or related compounds are highlighted, we see in purple in the profile not only the area of the secoiridoids where they show up, but also the HYTY. That is easy to understand keeping in mind that HYTY is part of the structure of Ol Agl and related compounds. The same can be applied for TY and Lig Agl-derivatives.

4. Conclusions

The separation by GC with on-line detection by APCI-MaXis-MS was successfully applied to the analysis of the phenolic compounds present in extra-virgin olive oil samples for the first time. The fact is quite relevant since only EI and CI are the ionization techniques which have been used in GC-MS (both operating under vacuum condition) to carry out the analysis of these compounds so far.

The use of our previous knowledge, commercially available standards and isolated standards (by semi-preparative HPLC), together with the capabilities of the analyzer used (MaXis-new generation of Q-TOF instruments), gave us the opportunity to characterize in depth the phenolic profile of the extra-VOOs under study.

Moreover, a complete validation of the method was carried out considering the specificity, linearity, sensitivity, precision, and accuracy. The detection limits were found ranging between 0.50 and 4.20 ppm, for pinoresinol and homovanillic acid, respectively. Acceptable levels of precision were obtained for the developed method in terms of repeatability since in all cases RSDs calculated were lower than 6.07%. The accuracy ranged from 95.4% to 101.5%.

GC-APCI-MaXis MS is an analytical procedure, which combines the best of chromatography with one of the most robust MS interfaces and a powerful analyzer and we have demonstrated that it has a potential to become one of the standard methods in food metabolic profiling.

5. Abbreviations used

Ac Pin, (+)-1-acetoxypinoresinol; **Apig,** apigenin; **BPC,** Base Peak Chromatogram; **CI,** chemical ionization; **D-Lig Agl,** decarboxilated derivatives of Lig Agl; **DOA,** decarboxilated derivatives of Ol Agl; **EA**, elenolic acid; **EI,** electron ionization (electronic impact); **EIC,** Extracted Ion Chromatogram; **H-D-Ol Agl,** hydroxy-decarboxilated-oleuropein aglycon; **10-H-Ol Agl**, 10-hydroxy-oleuropein aglycon; **HYTY**, hydroxytyrosol; **Lig Agl**, ligstroside aglycon; **LLE,** liquid liquid extraction; **Lut,** luteolin; **Ol Agl**, oleuropein aglycon; **Pin**, (+)-pinoresinol; **SPE**, solid phase extraction; **TY**, tyrosol; **VOO,** virgin olive oil;

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Caption to figures

Fig 1. HPLC-DAD (280 mn) profile obtained for the phenolic fraction of the mixture of olive oils selected to isolate some standards which were not commercially available when a semi-preparative column was used (as described in section 2.4.1). The different collected fractions are indicated with a number (between 1-9).

Fig. 2. On the top shows the Base Peak Chromatogram (BPC) of the extra-VOO we used during the optimization together with the BPCs in colour produced when the 9 isolated fractions were analyzed. In the same figure, we can see the chromatograms which were observed when the 9 fractions were injected (with the BPC of the oil under study in grey).

Fig. 3. Base Peak Chromatogram (BPC) of the Diol-SPE extract obtained from an extra-VOO obtained from a mixture between Arbequina and Picual oils under the optima GC-APCI-MaXis MS conditions. Elution windows of different families belonging to the phenolic fraction of EVOO are shown. Individual peaks with name in blue were identified with commercial standards; those with name in red were identified by using the isolated phenolic fraction (with semi-preparative HPLC); and those in purple were identified keeping in mind our previous knowledge abut the fraction. In grey we can observe peaks with relevant intensity which were not fully identified.

Fig. 4. Comparison between the profile (BPC) obtained by using the method selected as optimum after the preliminary studies (A) and the one that we finally selected (B).

Fig. 5. Polyphenolic profiles (BPCs) of the three extra-VOOs under study. (A) Mixture between Arbequina and Picual extra-VOOs, (B) Frantoio extra-VOO, and (C) Hojiblanca extra-VOO Auchan.
Peak identification: 1, Ty-2H+2TMS; **2**, Hyty-3H+3TMS; **3**, Protocatechuic acid-3H+3TMS+H; **4**, Dopac-3H+3TMS+H; **5**, EA-1H+1TMS+H; **6**, D-Lig Agl; **7**, compound present in isolated fraction 6 (Lig Agl-related comp); **8**, compound present in isolated fraction 6 (Lig Agl-related comp); **9**, DOA-2H+2TMS+H; **10**, 501.3843 / 411.3312; **11**, Lig Agl-1H+1TMS+H; **12**, Methyl Ol Agl-2H+2TMS+H; **13**, H-D-Ol Agl-3H+3TMS+H; **14**, Lig Agl-2H+2TMS+H; **15**, Ol Agl-2H+2TMS+H; **16**, Ol Agl-3H+3TMS+H and related comp; **17**, Apigenin-3H+3TMS+H; **18**, Luteolin-4H+4TMS+H; **19**, Pinoresinol-2H+2TMS+H; **20**, 397.3825; **21**, Acetoxy-pinoresinol-2H+2TMS+H.
Square on the top: EICs of m/z 281.2480 and 193.1944, which facilitate the study of the Ol Agl-derivatives and Lig Agl-derivatives or related compounds.

Table 1. m/z APCI-MaXis MS signals detected for the phenolic compounds that we had as commercially available standards (potentially present in extra-virgin olive oil) previously separated by a GC method. In bold letter we highlight the prevalent ion in the MS spectrum for each compound. In red we indicate some silylated forms which can be observed for some compounds in particular and which were not expected considering the chemical structure of those analytes.

m/z experimental	**Retention time** (minutes)	**First possibility in Smartformula Editor**	**m/z theoretical**	**Error (mDa)**	**mSigma Value**	**Possible compounds**	**Other MS signals (silylated forms) or MS Fragments in-source**
225.0939	9.4	C11H17O3Si	225.0941	0.2	7.8	Vanillin-1H+1TMS+H	297.1337 / 197.1001 / 166.0457
221.0998	9.7	C12H17O2Si	221.0992	-0.6	4.1	*trans*-Cinnamic acid-1H+1TMS+H	293.1414 / 205.0698 / 149.0615 / **131.0507**
282.1470	10.0	C14H26O2Si2	282.1466	-0.4	5.1	Tyrosol-2H+2TMS	258.0970 / **193.1061**
283.1165	11.3	C13H23O3Si2	283.1180	1.5	5.7	4-Hydroxybenzoic acid-2H+2TMS+H	355.1632 / **267.0908** / 193.0713
178.9282	11.6	-	-	-	-	4-Hydroxyphenylacetic acid	369.1679 / 297.1016 / 283.0791 / 267.0379 / 178.9282
313.1287	14.9	C14H25O4Si2	313.1286	-0.1	3.1	Vanillic acid-2H+2TMS+H	385.1681 / 297.1022 / **223.0817** / 172.9583
370.1809	15.1	C17H34O3Si3	370.1810	0.1	10.6	Hydroxyyrosol-3H+3TMS	**281.1410** / 193.0691
327.1426	15.2	C15H27O4Si2	327.1442	0.1	7.1	Homovanillic acid-2H+2TMS+H	399.1829 / 281.1401 / **209.1007** / 137.0600
371.1523	15.4	C16H31O4Si3	371.1525	0.2	9.6	Gentisic acid-3H+3TMS+H	443.1956 / 355.1248 / **281.1060** / 209.0646
371.1535	16.6	C16H31O4Si3	371.1525	-1.0	15.1	Protocatechuic acid-3H+3TMS+H	443.1984 / 355.1273 / **281.1075**
385.1676	17.0	C17H33O4Si3	385.1681	0.5	9.4	Dopac-3H+3TMS+H	457.2091 **/ 267.0719** / 172.7891
309.1347	17.9	C15H25O3Si2	309.1337	-1.0	6.7	*m*-Coumaric-2H+2TMS+H	381.1736 **/** 293.1035 / 219.0852 / 172.9581
343.1400	18.8	C15H27O5Si2	343.1392	-0.8	7.1	Syringic acid-2H+2TMS+H	415.1813 / 327.1126 / 299.1528 / 253.0907 / 211.0791
309.1333	20.0	C15H25O3Si2	309.1337	-0.4	11.2	*p*-Coumaric acid-2H+2TMS+H	381.1740 / 293.1069 / 219.0868 / 195.0848
459.1860	20.8	C19H39O5Si4	459.1869	0.9	4.6	Gallic acid-4H+4TMS+H	415.2030 / 369.1424 / 327.1306 / 239.0597 / 172.9579
339.1447	24.4	C16H27O4Si2	339.1442	-0.5	10.2	Ferulic acid-2H+2TMS+H	411.1853 / 323.1124 / **249.0967**/ 177.0558
397.1680	25.8	C18H33O4Si3	397.1681	0.1	7.2	Caffeic acid-3H+3TMS+H	469.2071 / **307.1232** / 172.9582
369.1546	28.7	C17H29O5Si2	369.1548	0.2	9.2	Sinapinic acid-2H+2TMS+H	441.1983 / 353.1244 / **279.1084**
665.2635	49.9	C30H53O7Si5	665.2632	-0.3	18.3	Taxifolin-5H+5TMS	593.2320 / 297.1009 / **225.0608** / 172.9581
487.1792	54.5	C24H35O5Si3	487.1787	-0.5	9.2	Apigenin-3H+3TMS+H	415.1399 / 193.0695
575.2142	58.7	C27H43O6Si4	575.2131	-1.1	6.4	Luteolin-4H+4TMS+H	503.1744 / 281.1007
503.2283	60.0	C26H39O6Si2	503.2280	-0.3	15.2	Pinoresinol-2H+2TMS+H	**485.2189** / 414.1686 / 247.1154

Table 2. m/z APCI-MaXis MS signals produced by the isolated phenolic fractions when they were analyzed by using the optima GC and MS conditions. In bold letter we highlight the prevalent ion in the MS spectrum for each compound. *Most intense chromatographic peak in the isolated fraction.

Number of the isolated fraction and its code		Retention time (minutes)	m/z experimental	Molecular formula assigned to a known comp	Error (mDa); mSigma
1	***Elenolic acid fraction***	17.3*	315.1253 / 387.1644 / 283.0602 / **224.9861** / 138.7925	315.1253 = C14H23O6Si (EA-H+1TMS+H)	0.5; 5.1
		17.9	355.0620 / 283.0591 / **224.9847** / 138.7925		
		21.7	**355.1296** / 297.0831 / 222.9854		
		36.8	279.1205 / 166.8552 / **148.8043** / 112.8029		
2	***DOA fraction a***	16.6	429.2267 / **361.2942** / 169.1580		
		16.8	481.3377 / 363.2745 / 273.2070 / 149.1196		
		36.8*	279.2765 / 167.1187 / **149.1066** / 113.2015		
3	***DOA fraction b***	36.8	279.2765 / 167.1187 / **149.1066** / 113.2015		
		48.3	193.1953		
		41.8*	465.2122 / 411.3288 / 375.2898 / **281.2498** / 209.1942	465.2122 = C23H37O6Si2 (DOA-2H+2TMS+H)	0.1; 6.2
4	***DOA fraction c***	10.8	193.1952		
		36.4	249.2699		
		36.8	279.2765 / 167.1187 / **149.1066** / 113.2015		
		44.5*	**193.1950**		
		48.3	193.1949		
5	***DOA fraction d***	36.8*	279.2765 / 167.1187 / **149.1066** / 113.2015		
		44.7	193.1952		
		48.4	193.1947		
		68.0	545.3940 / 503.2283 / **337.2673**	503.2283 = C26H39O6Si2 (Syringaresinol-2H+2TMS+H)	-0.3; 8.3
6	***Ac Pin fraction***	36.8*	193.1950	D-Lig Agl	
		47.0	193.1935	Lig Agl-related comp	
		48.5	507.2232 / 475.3359 / **193.1944**	507.2232 = C25H39O7Si2 (Lig Agl-2H+2TMS+H)	0.4; 9.1
		61.1*	561.2330 / 501.3554 / **305.2331** / 275.2165	561.2330 = C28H41O8Si2 (Ac Pin-2H+2TMS)	0.4; 12.5
7	***Lignan fraction 2***	36.9*	193.1950	D-Lig Agl	
		48.5	507.2232 / 475.3359 / **193.1944**	507.2232 = C25H39O7Si2 (Lig Agl-2H+2TMS+H)	
		49.7	523.2185 / **281.2492** / 208.9914	523.2185= C25H39O8Si2 (Ol Agl-2H+2TMS+H)	-0.8; 11.1
		52.5*	611.2528 / 313.2290 / **281.2497** / 173.0419	611.2528 = C28H47O9Si3 (10-hydroxy-Ol Agl-3H+3TMS+H)	0.6; 15.6
8	***Ol Agl fraction***	36.8	279.2765 / 167.1187 / **149.1066** / 113.2015		
		47.8	523.2188 / 313.2261 / **193.1950**	523.2188 = C25H39O8Si2 (Ol Agl-2H+2TMS+H)	-1.1; 11.1
		49.7*	523.2188 / **281.2492** / 225.1723 /209.1937	523.2188 = C25H39O8Si2 (Ol Agl-2H+2TMS+H)	-1.1; 11.1
		51.8	521.3510 / **281.2480** / 209.1939	Ol Agl-related comp	
		53.1	**281.2483**	Ol Agl-related comp	
		53.8	281.2483 / 595.2556	595.2556 = C28H47O8Si3 (Ol Agl-3H+3TMS+H)	-0.8, 6.9
9	***Lig Agl fraction***	36.7*	279.2765 / 167.1187 / **149.1066** / 113.2015		
		44.7	435.1830 / **193.1966** / 281.9075	435.1830 = C22H31O7Si (Lig Agl-H+1TMS+H)	0.4; 13.1
		48.5	193.1965		

Table 3. m/z APCI-MaXis MS/MS signals detected for the most relevant phenolic compounds present in the extract of extra-virgin olive oil use during the optimization. In bold letter we highlight the prevalent ion in the MS spectrum for each compound.

MS signal (when no defined before)	**Retention time** (minutes)	**Parent (precursor) ion isolated and further fragmented**	**MS fragments**	**Compounds**
	10.0	193.1061	144.7203 / 126.6961/ **108.6209**	Tyrosol-2H+2TMS
	13.4	299.2632	266.9440 / **192.9298**	
		281.2477	192.9426 / **118.7576** / 105.6919	
		193.1613	164.8823 / 144.7197 / **108.6199**	
	15.1	370.1754	267.0739 / **192.9301** / 178.8886	Hydroxyyrosol-3H+3TMS
		281.2480	**192.9304** / 165.8570 / 114.7144	
	16.6	281.2469	**192.9377** / 148.7980 / 118.7612 / 104.6898	Protocatechuic acid-3H+3TMS+H
	17.0	385.1642	**267.0723** / 178.8888	Dopac-3H+3TMS+H
		267.0716	**178.8913** / 148.7747 / 108.6205	
	17.3	315.1253	283.0594 / **224.9829** /183.1515 / 139.1157	Elenolic acid-H+1TMS+H
		224.9829	190.8610 / **164.8771** / 118.6764	
		283.0595	132.8196 / 118.6764 / **104.6874** / 90.6075	
	33.3	192.9661	177.9179 / 144.7184 / 127.7065 / **108.6197**	
	36.9	192.9667	164.8792 / 144.7203 / 126.6961/ **108.6209**	D-Lig Agl-related comp
	38.4	281.0971	192.9426 / 118.7612 / **104.6892**	Lig Agl-related comp
192.9717	40.0	192.9717	177.9152 / 144.7196 / 126.6981 / **108.6201**	Lig Agl-related comp
192.9717	40.9	192.9717	177.9152 / 144.7196 / 126.6981 / **108.6201**	Lig Agl-related comp
	42.0	411.3288	**128.7833** / 72.4970	DOA-2H+2TMS+H
		281.0977	**192.9331** / 168.8517 / 90.6110 / 72.4970	
		208.9862	190.9405 / **164.8767** / 135.7303 / 108.6181	
	42.2	411.3288	**128.7833** / 72.4970	DOA-2H+2TMS+H
		281.0977	**192.9331** / 168.8517 / 90.6110 / 72.4970	
		208.9862	190.9424 / **164.8771** / 135.7294 / 108.6234 / 90.6110	
501.3843 / **411.4616**	43.1	501.3843	**128.7831** / 102.6687 / 72.4960	
		411.4616	**128.7831** / 72.4960	
	44.8	281.0971	**192.9426** / 118.7612 / 104.6892	Lig Agl-H+1TMS+H
537.2533 / **281.0968**	45.8	281.0968	**192.9426** / 118.7612 / 104.6892	Methyl Ol Agl-2H+2TMS+H
553.2491 / **281.0975** / 192.9678	46.9	553.2491	281.0968 / **192.9296** / 122.7343	H-D-Ol Agl-3H+3TMS+H
		281.0975	192.9377 / **148.7980** / 118.7612 / 104.6898	
		192.9678	177.9152 / 144.7196 / 126.6981 / **108.6211**	
192.9717 / 462.2351	47.0	192.9717	177.9152 / 144.7196 / 126.6981 / **108.6211**	Lig Agl-2H+2TMS+H
		462.2351	**192.9622** / 177.9192 / 97.6840	
	47.9	192.9718	177.9152 / 144.7196 / 126.6981 / **108.6211**	Lig Agl-2H+2TMS+H
	48.6	475.1989	**192.9646** / 176.9046 / 148.8093 / 72.4060	Lig Agl-2H+2TMS+H
		297.0825	248.9216 / 208.9031 / 132.8241 / **118.7593** / 104.6874 / 90.6075 / 72.4970	
		192.9665	177.9152 / 144.7196 / 126.6981 / **108.6211**	

MS signal (when no defined before)	**Retention time** (minutes)	**Parent (precursor) ion isolated and further fragmented**	**MS fragments**	**Compounds**
	49.8	523.2188	**281.2492** / 225.1733 / 209.1937 / 193.1593	Ol Agl-2H+2TMS+H
		281.4481	**192.9304** / 165.8570 / 114.7144 / 90.6075 / 72.4970	
550.2678 / **281.0973**	51.8	550.2678	**281.0971** / 192.9302 / 177.9207 / 165.0849 / 97.6855	Ol Agl-related comp
		281.0973	192.9377 / 148.7980 / **118.7612** / 104.6898	
521.2068 / **281.0879** / 208.9912	51.9	521.2068	281.0971 / **192.9302**	Ol Agl-related comp
		281.0879	192.9377 / 148.7980 / **118.7612** / 104.6898	
		208.9912	190.9457 / 178.9249 / **164.8781** / 108.6224	
	52.5	313.0876	142.8366 / 132.8210 / **118.7623** / 104.6888 / 90.6088	10-hydroxy-Ol Agl-3H+3TMS+H
		281.0981	192.9377 / 148.7980 / **118.7612** / 104.6898 / 90.6075 / 72.4970	
	53.3	595.2540	281.0975 / **192.9300**	Ol Agl-3H+3TMS+H
		563.2299	281.0960 / **192.9300**	
		297.0929	266.9500 / 224.9536 / 192.9335 / **118.7611** / 104.6895 / 90.6055	
		281.0978	192.9377 / 148.7980 / **118.7612** / 104.6898	
	53.9	281.0975	192.9649 / 178.9214 / 154.8671 / **118.7611** / 104.6895 / 90.6055	Ol Agl-related comp
	54.0	281.0975	192.9649 / 178.9214 / 154.8671 / **118.7611** / 104.6895 / 90.6055	Ol Agl-related comp
	54.5	4857.1792	**471.1498** / 399.1083 / 415.1399 / 193.0695	Apigenin-3H+3TMS+H
	58.7	575.2142	**559.3394** / 487.2883 / 297.2119	Luteolin-4H+4TMS+H
		503.3205	431.2735 / 415.2367 / 225.1546 / 191.1419	
	59.9	485.3569	414.3040 / 384.1199 / 289.2369 / 259.0503 / **208.9846** / 178.8874 / 128.7817	Pinoresinol-2H+2TMS+H
		414.1698	146.8805 / **118.7618**	
		247.0525	201.9547 / 177.9131 / **164.8770** / 127.7674	
	60.2	397.3832	160.9312 / **146.8805** / 132.8342 / 118.7614 / 104.6907	
		160.9290	127.7686 / 114.7141 / 90.6062	
	61.1	501.2143	275.0644 / 247.0429 / **222.9837** / 208.9865	Ac Pin-2H+2TMS
		305.0929	228.9791 / **216.9663** / 202.9288 / 114.7153 / 72.4960	
		276.0701	**216.9666** / 202.9288 / 114.7153 / 72.4960	
		259.0576	**228.9806** / 202.9288 / 198.8934 / 104.6901	
	68.0	503.2283	**337.2673**	Syringaresinol-2H+2TMS+H

Table 4. Analytical parameters of the developed GC-APCI-MaXis MS method.

Analytes	***LOD (ppm)***	***LOQ(ppm)***	***Linearity (μg/ml)***	***Calibration curves*** [a]	r^2	**Repeat. Intra-day** [b]	**Repeat. Inter-day** [b]	**Accuracy** [c]
Tyrosol	1.58	4.74	LOQ-100	y = 29080x-135585	0.987	1.53	4.16	97.7
Hydroxytyrosol	0.80	2.40	LOQ-50	y = 5809x-6690	0.994	1.61	4.10	95.4
Homovanillic acid	4.00	12.00	LOQ-100	y = 4650x -17309	0.994	1.30	3.76	97.3
p-coumaric acid	0.75	2.25	LOQ-100	y = 6649x-13911	0.994	0.89	3.67	99.1
Ferulic acid	0.74	2.22	LOQ-100	y = 7313x-33576	0.985	0.73	6.01	98.6
Luteolin	4.20	12.60	LOQ-50	y = 22182x-14950	0.993	1.77	6.07	101.5
Apigenin	2.50	7.50	LOQ-100	y = 3223x -24031	0.998	1.45	4.74	100.5
Pinoresinol	0.50	1.50	LOQ-100	y = 2657x -18999	0.993	1.03	5.01	98.2

[a] A (peak area) = $a + b \times C$ (ppm) for five points (n=10).
[b] RSDs values (%) for peak areas corresponding to each compound; measured from three injections of each analyte within the same day (intra-) and on three different days (inter-).
[c] 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. (%Recovery, %RSD).

Table 5. Amount of phenolic compounds (mg/kg) or area (when pure standards were not available) found in the extra-virgin olive oils under study. (n=3)
(Value = mean value). RSD in all the cases ≤ 5%.

Analyte	***t_r (min)***	***Picual-Arbequina oil***	***Frantoio oil***	***Hojiblanca oil***
Tyrosol [a]	7.2	3.33	1.67	7.21
Hydroxytyrosol [b]	12.3	8.31	2.42	9.32
Protocatechuic acid [c]	13.6	0.25	n.d.	0.21
Dopac	14.1	*Internal standard (IS)*		
Elenolic acid [d]	14.2	356567	936997	228073
D-Lig Agl [d]	25.6	1460407	182971	821396
Lig Agl-related comp [d]	27.6	103272	68637	203280
Lig Agl-related comp [d]	28.3	49646	n.d.	148478
DOA [d]	29.2	1029476	276124	422605
501.3843 / 411.3312 [d]	30.0	167040	43682	92022
Lig Agl [d]	31.2	830890	172235	151760
Methyl Ol Agl [d]	32.0	56047	16797	n.d.
H-D-Ol Agl [d]	32.8	211872	219061	33328
Lig Agl [d]	34.2	3907685	3962249	1334142
Ol Agl [d]	35.3	3713714	n.d.	183453
Ol Agl-related comp [d]	37.0	540608	n.d.	25417
10-H-Ol Agl [d]	37.6	429370	n.d.	124260
Ol Agl [d]	38.3	2209565	n.d.	304794
Apigenin [e]	39.2	0.35	0.19	0.20
Luteolin [f]	42.9	1.65	n.d.	n.d.
Pinoresinol [g]	43.8	3.25	0.75	0.54
Ac Pin [g]	44.6	19.37	25.45	n.d.

[a]: quantified with the calibration curve of tyrosol.
[b]: quantified with the calibration curve of hydroxytyrosol.
[c]: quantified with the calibration curve of protocatechuic acid.
[d]: semi-quantitative information (mean value of area of the compound). Pure standards were not available.
[e]: quantified with the calibration curve of apigenin.
[f]: quantified with the calibration curve of luteolin.
[g]: quantified with the calibration curve of pinoresinol.
n.d.: non detected

Fig.

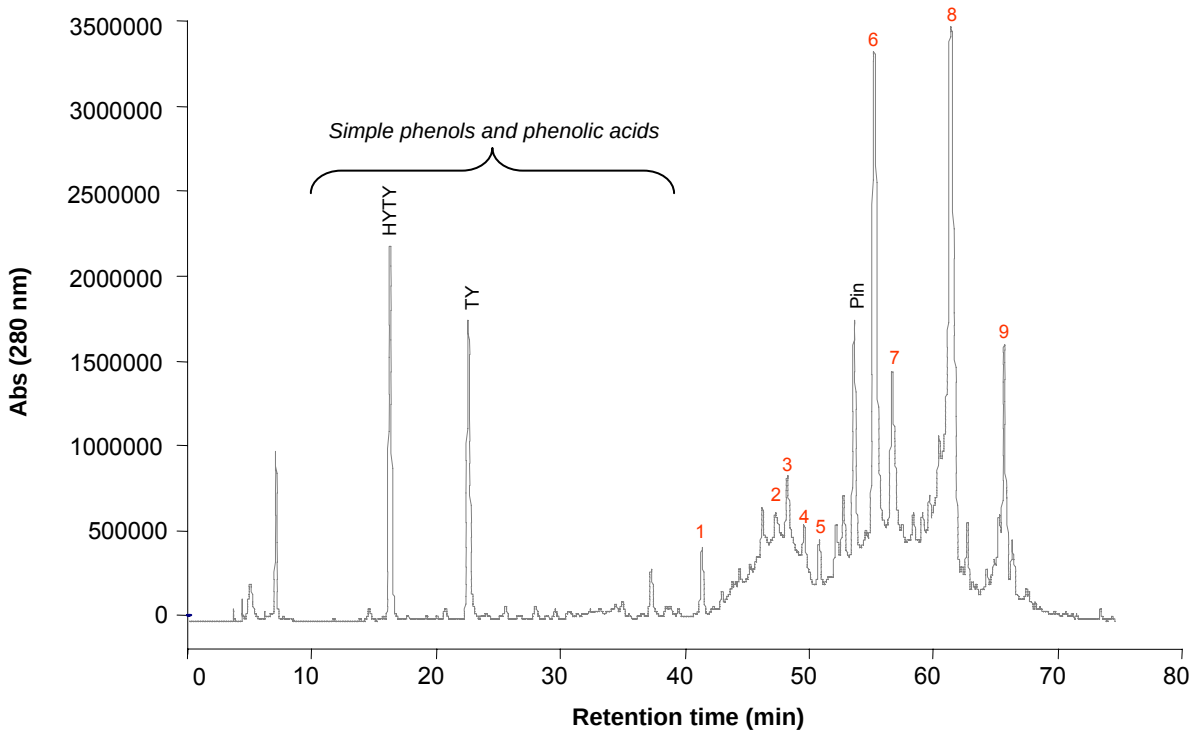

Simple phenols and phenolic acids
HYTY
TY
Pin
1
2
3
4
5
6
7
8
9
Abs (280 nm)
Retention time (min)
0
500000
1000000
1500000
2000000
2500000
3000000
3500000
0
10
20
30
40
50
60
70
80

Fig. 2

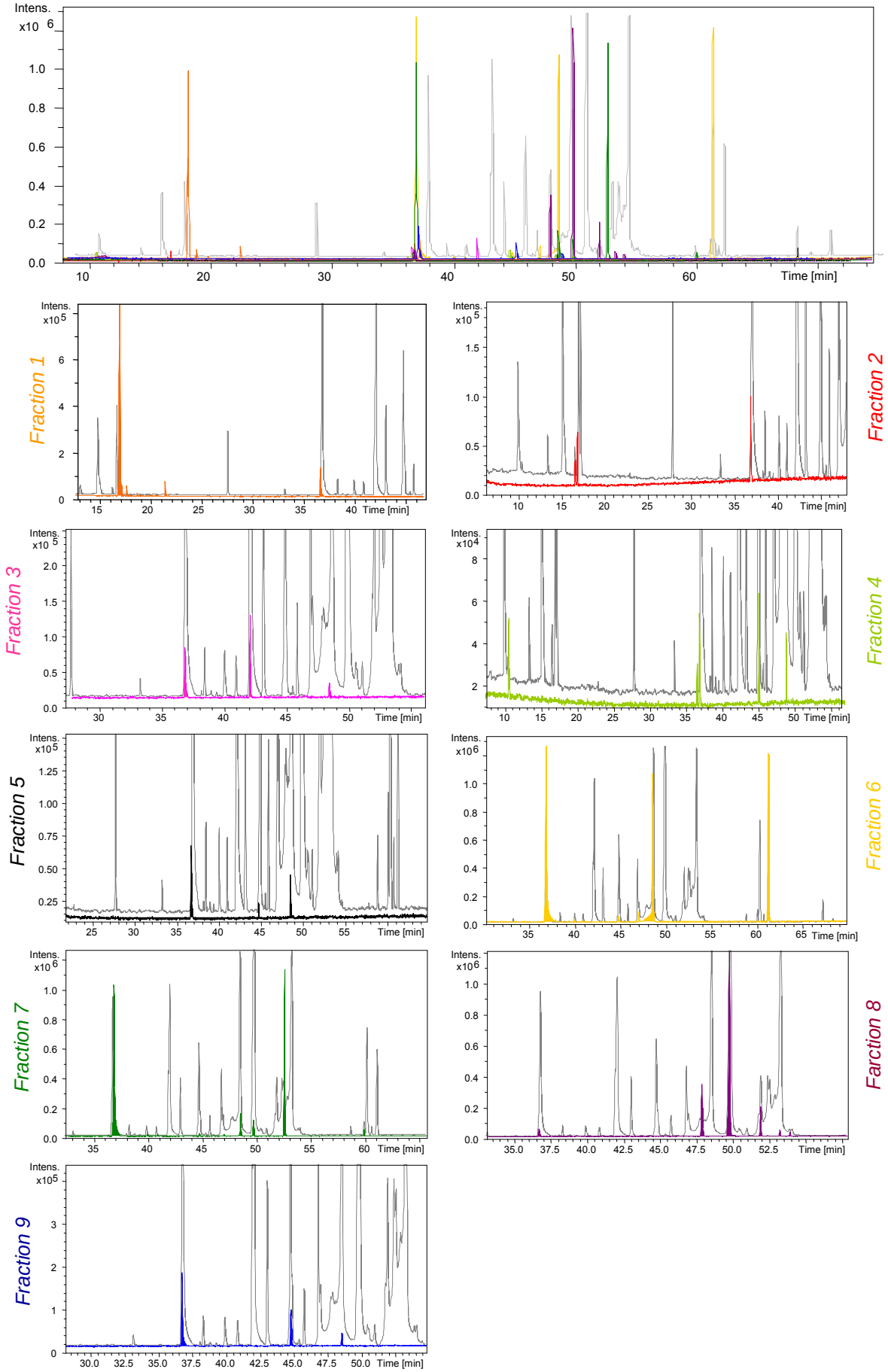

Intens.
x10 6
Time [min]
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Fraction 7
Farction 8
Fraction 9

Fig. 3

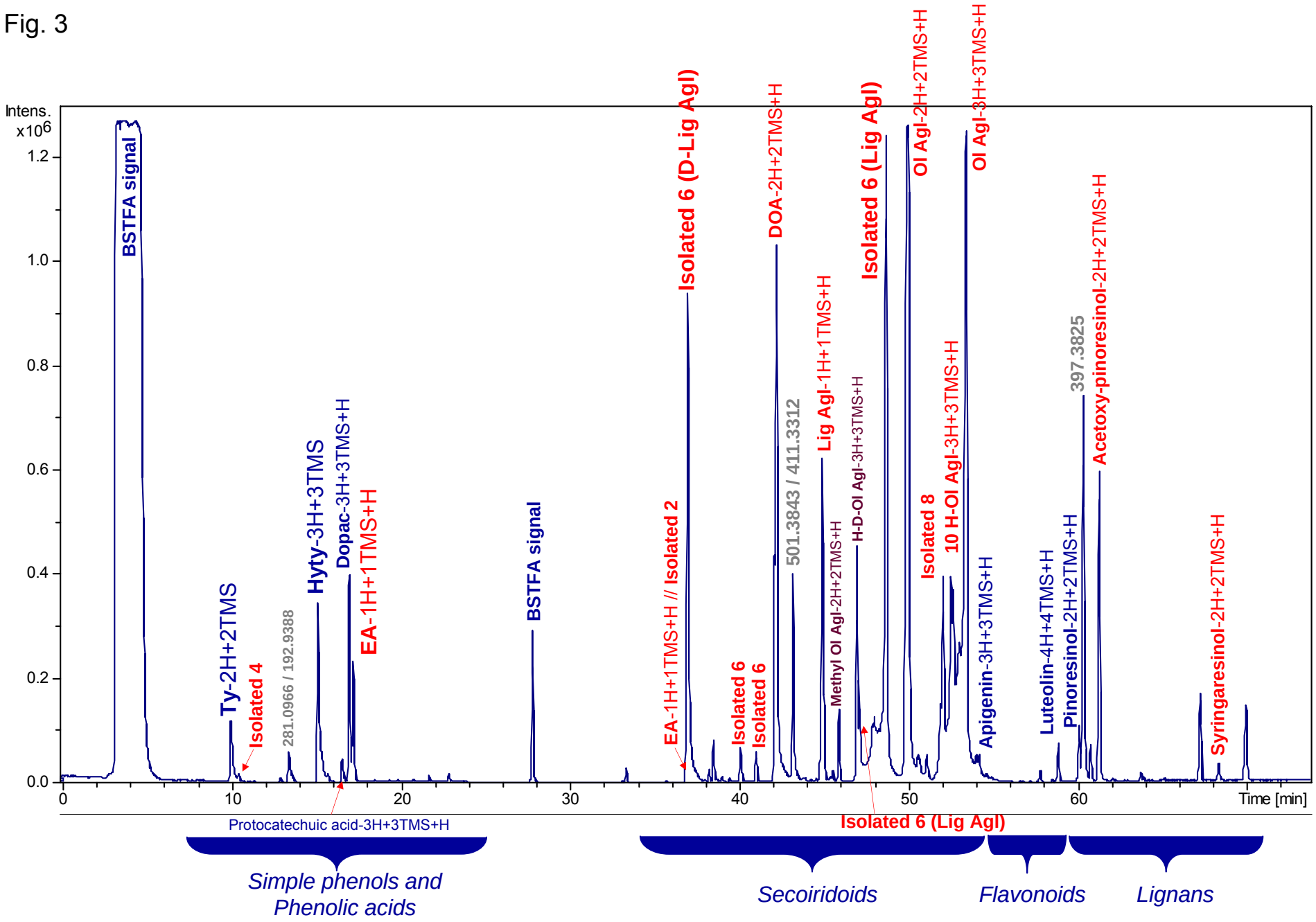

Intens.
x10^6
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time [min]
BSTFA signal
Ty-2H+2TMS
Isolated 4
281.0966 / 192.9388
Hyty-3H+3TMS
Dopac-3H+3TMS+H
EA-1H+1TMS+H
BSTFA signal
EA-1H+1TMS+H // Isolated 2
Isolated 6 (D-Lig Agl)
Isolated 6
Isolated 6
DOA-2H+2TMS+H
501.3843 / 411.3312
Lig Agl-1H+1TMS+H
Methyl Ol Agl-2H+2TMS+H
H-D-Ol Agl-3H+3TMS+H
Isolated 6 (Lig Agl)
Ol Agl-2H+2TMS+H
Isolated 8
10 H-Ol Agl-3H+3TMS+H
Ol Agl-3H+3TMS+H
Apigenin-3H+3TMS+H
Luteolin-4H+4TMS+H
Pinoresinol-2H+2TMS+H
397.3825
Acetoxy-pinoresinol-2H+2TMS+H
Syringaresinol-2H+2TMS+H
Protocatechuic acid-3H+3TMS+H
Isolated 6 (Lig Agl)
Simple phenols and Phenolic acids
Secoiridoids
Flavonoids
Lignans

Fig. 4

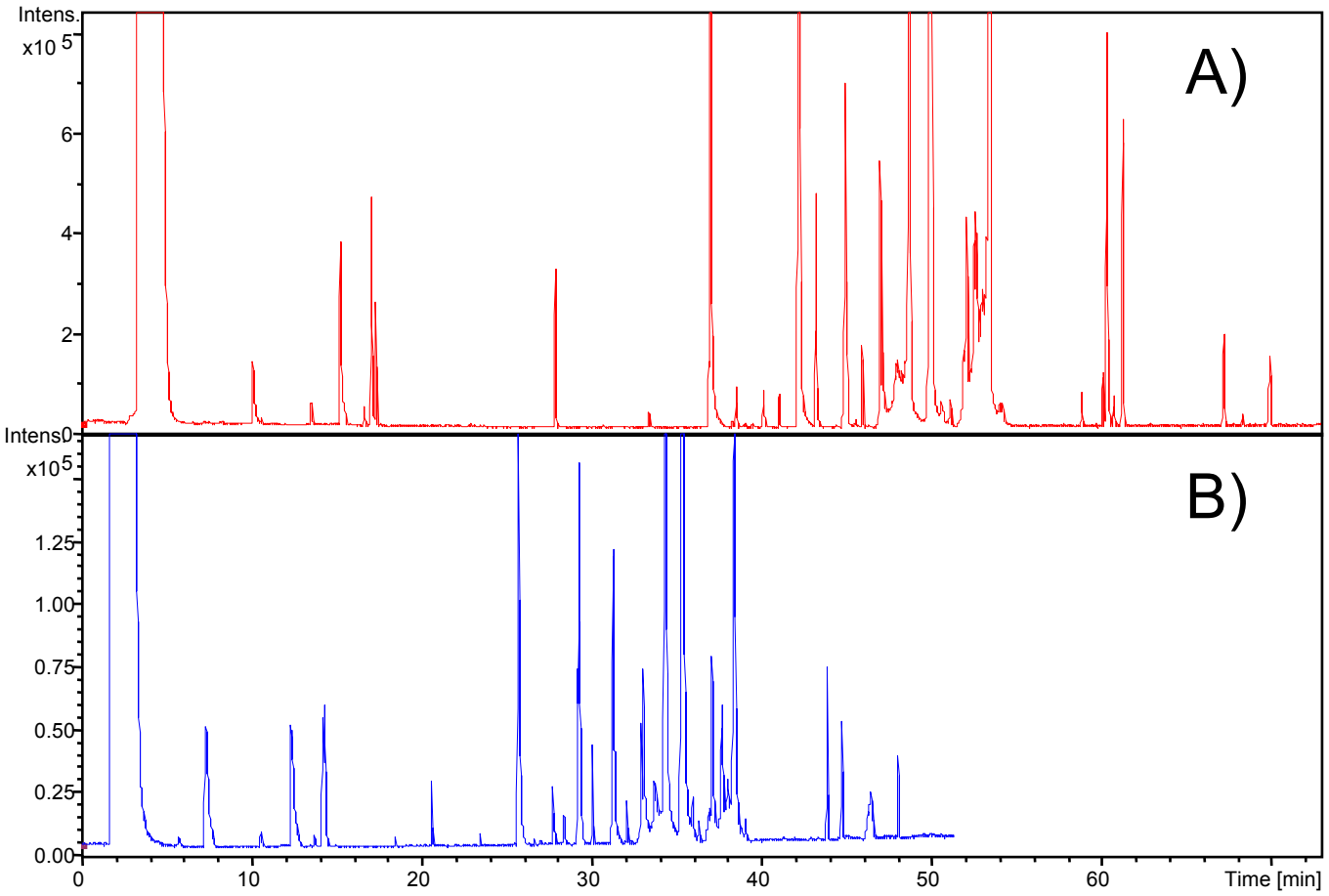
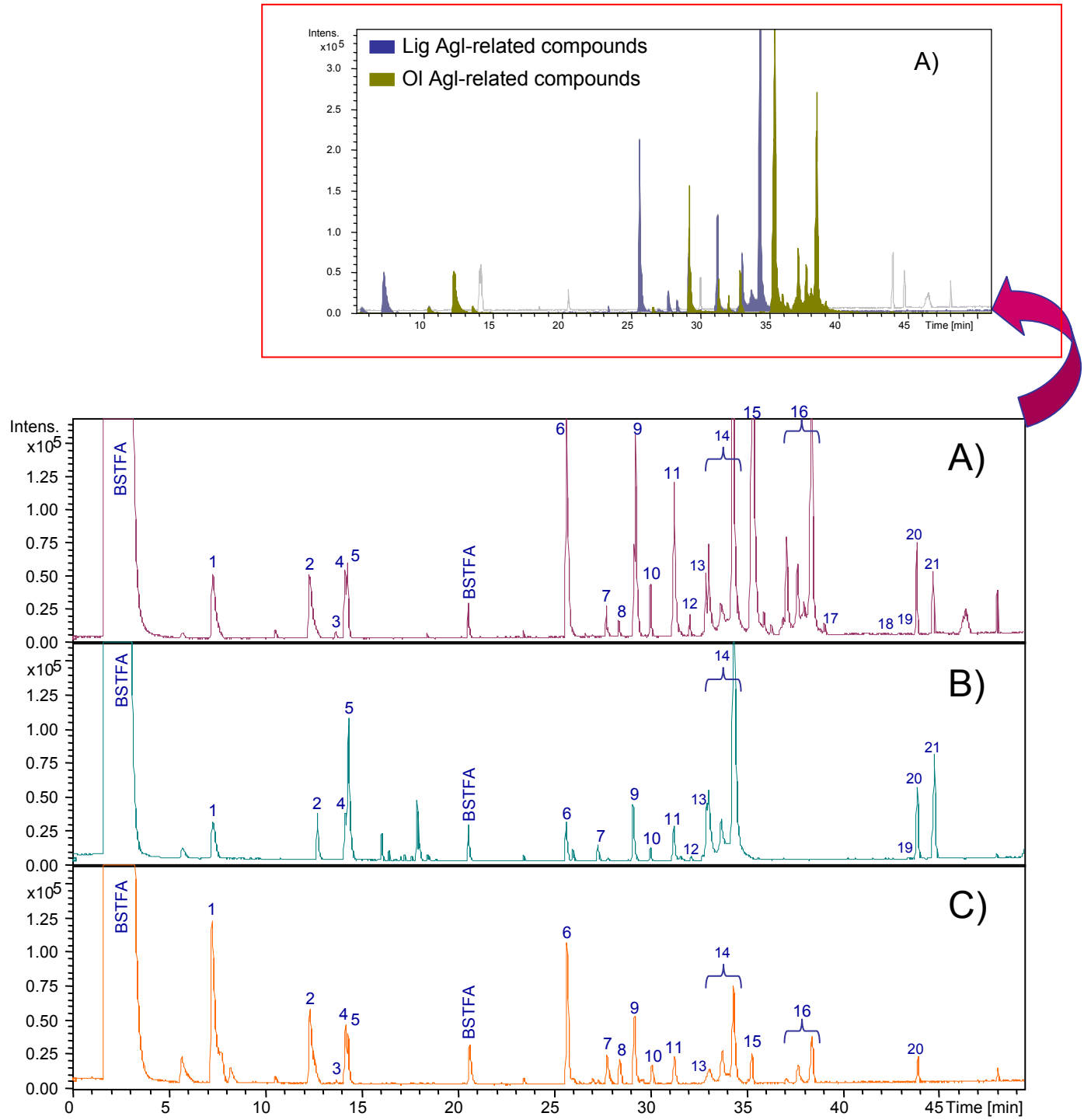
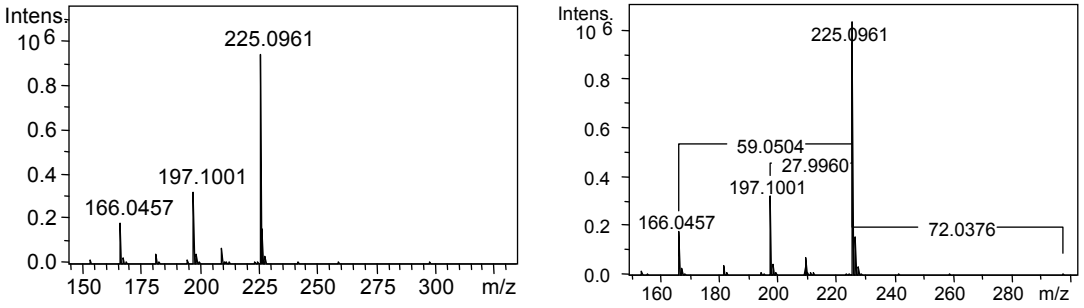

Intens.
x10 5
6
4
2
A)
Intens0
x10 5
1.25
1.00
0.75
0.50
0.25
0.00
B)
0
10
20
30
40
50
60
Time [min]

Fig. 5

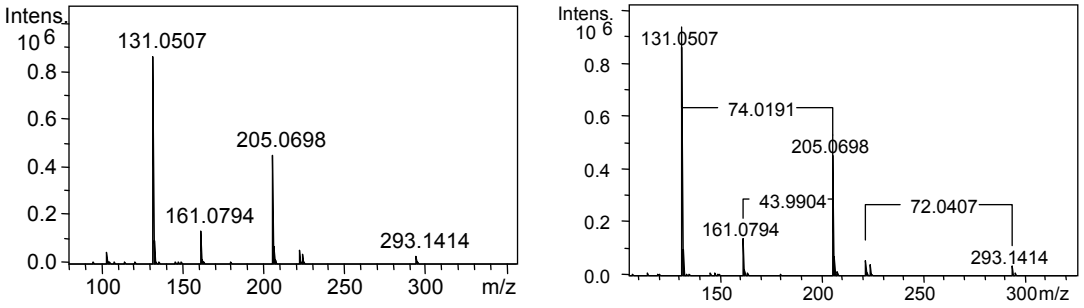

Intens.
x10 5
Lig AgI-related compounds
OI AgI-related compounds
A)
Time [min]
BSTFA
A)
B)
C)
Intens.
x10 5
Time [min]

Supporting info-Fig. 1

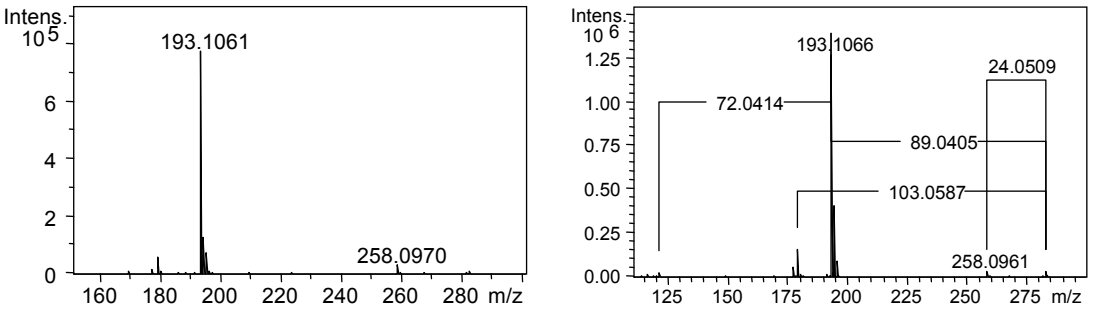
Vanillin



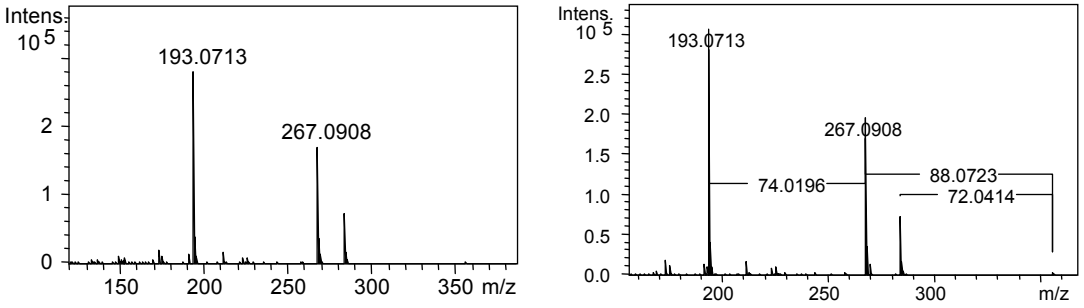
Trans-cinnamic acid



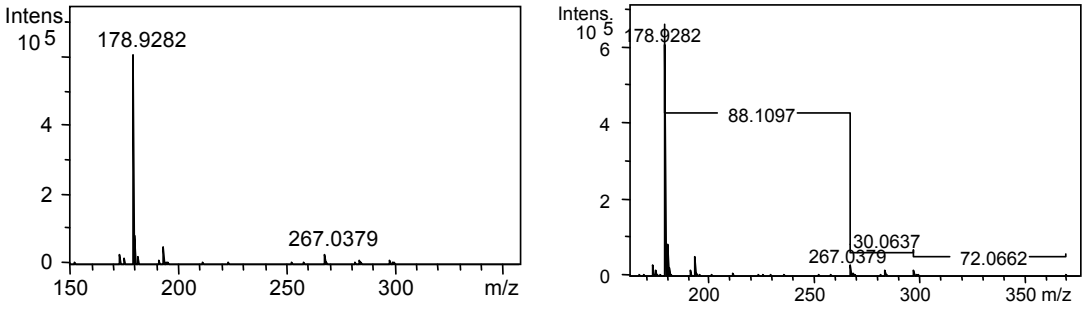
Tyrosol



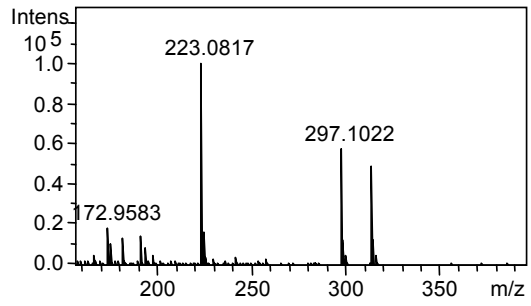
4-hydroxybenzoic acid

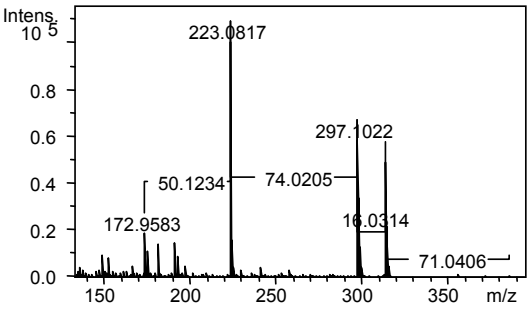


4-hydroxyphenylacetic acid

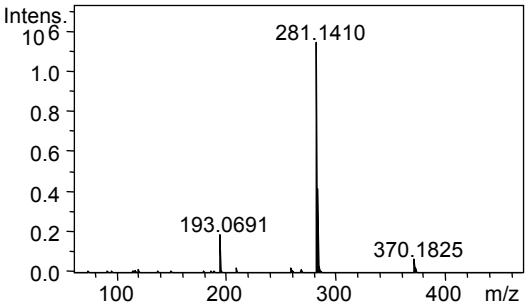


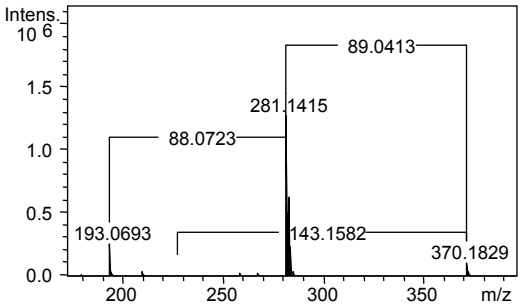
Vanillic acid



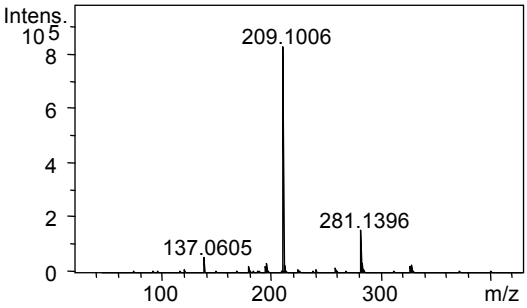


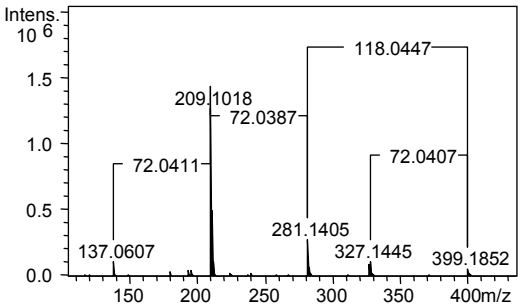
Hydroxytyrosol



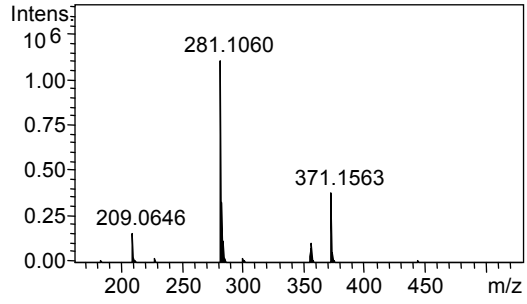


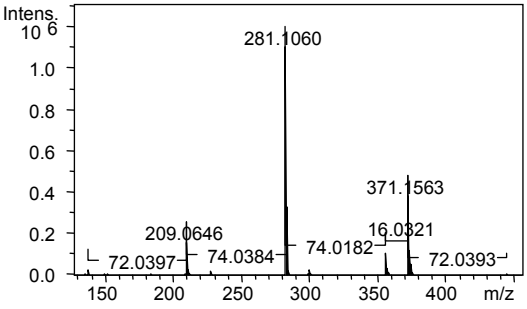
Homovanillic acid



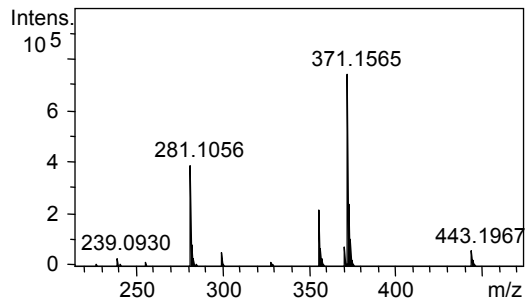


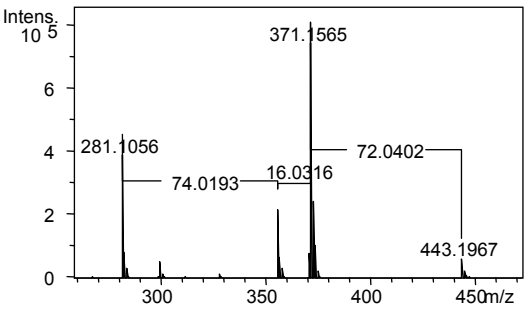
Gentisic acid



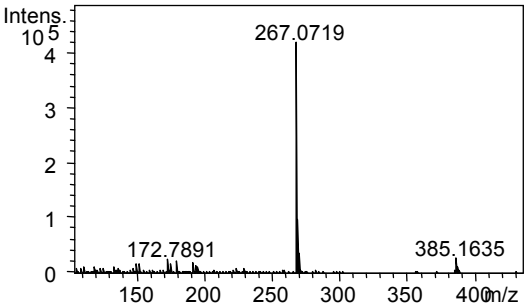


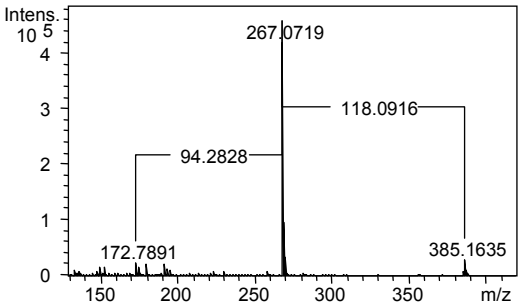
Protocatechuic acid



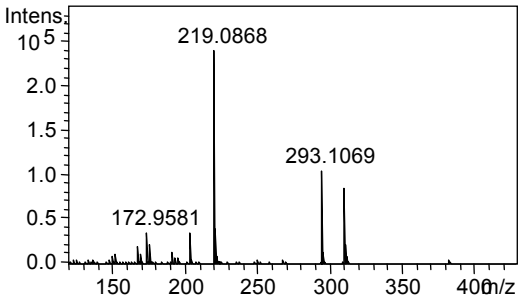


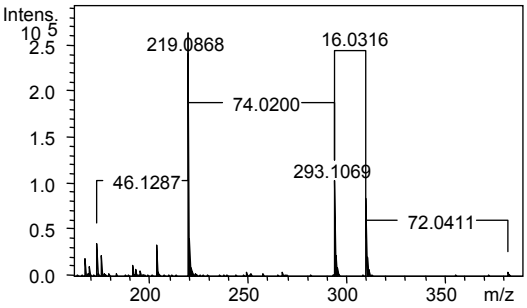
Dopac



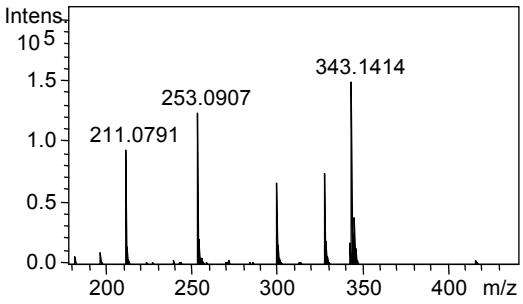


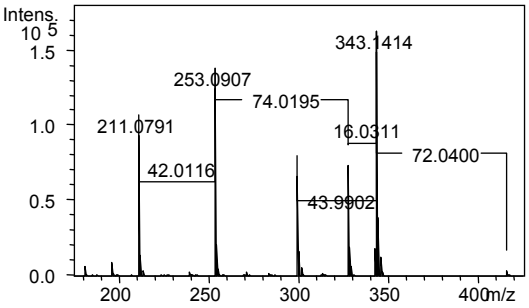
m-coumaric acid



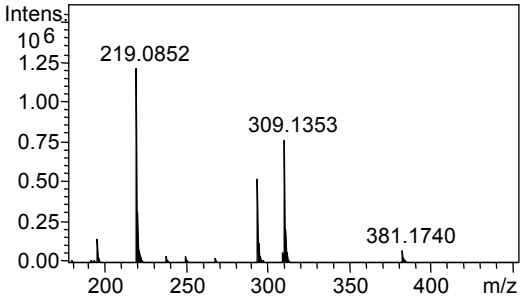


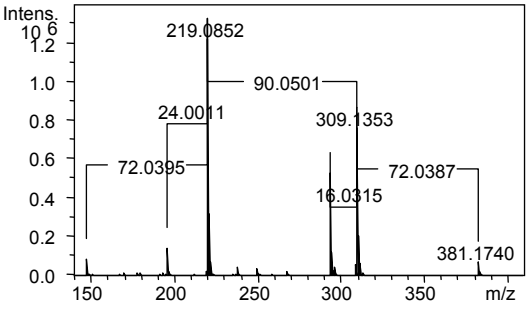
Syringic acid



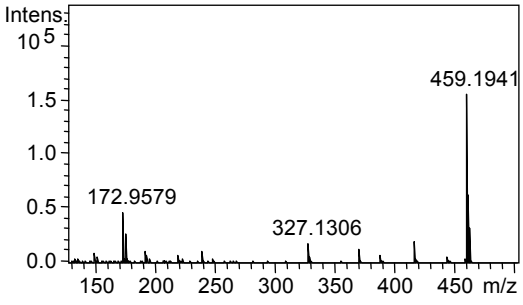


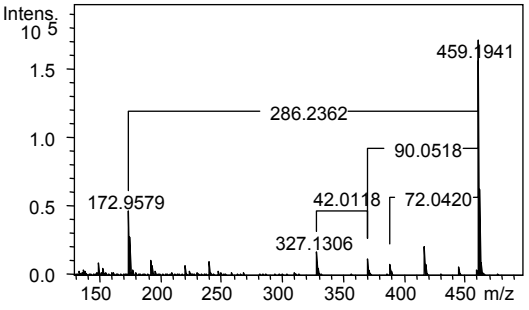
p-coumaric acid



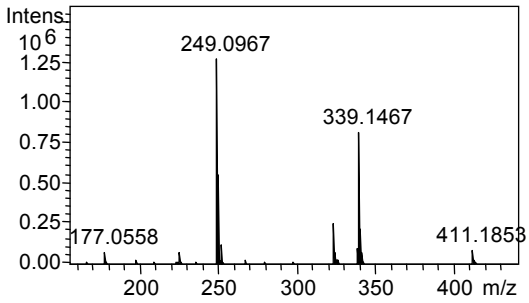


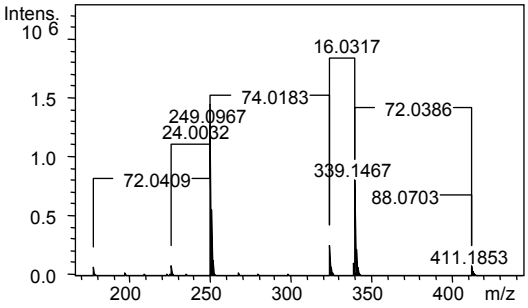
Gallic acid



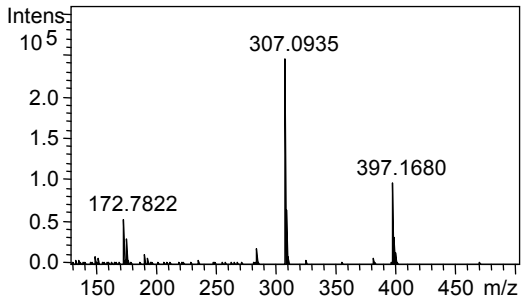


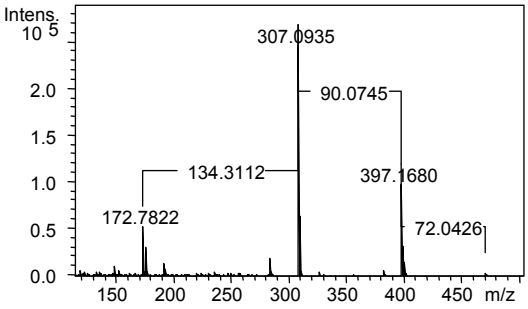
Ferulic acid



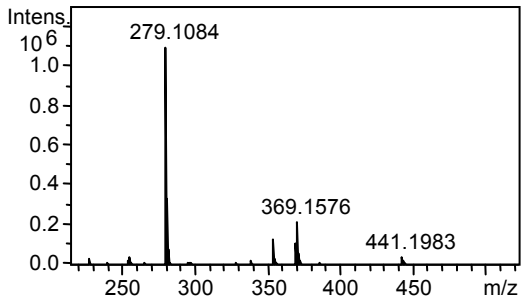


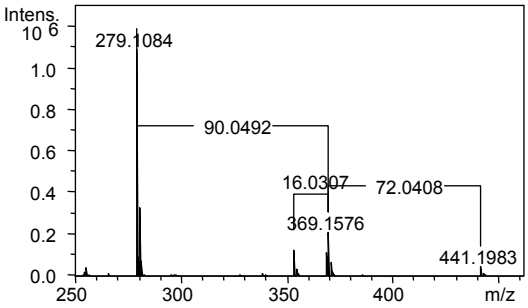
Caffeic acid



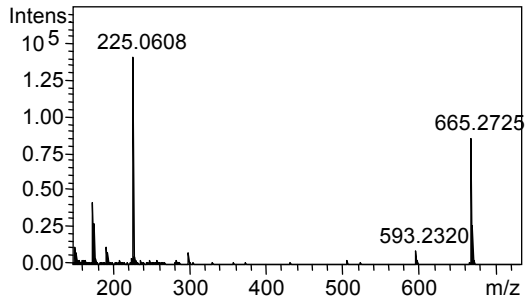


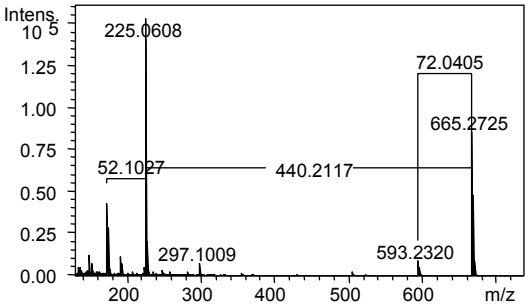
Sinapinic acid



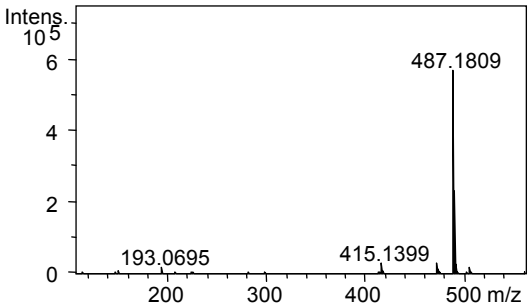


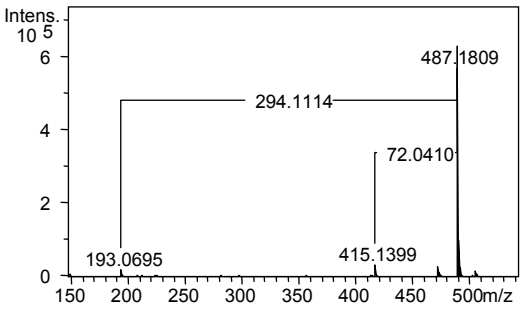
Taxifolin



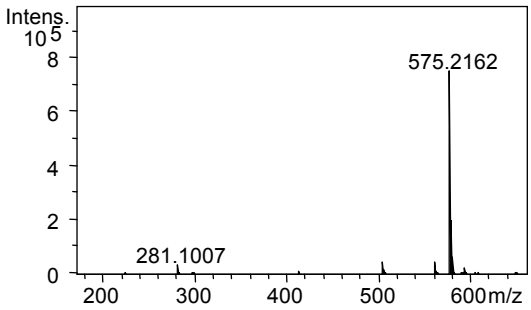


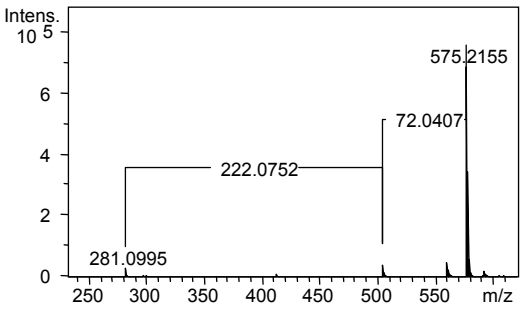
Apigenin



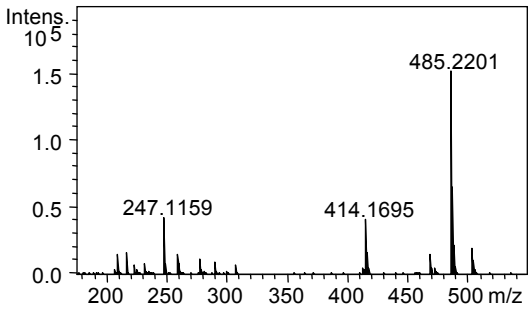


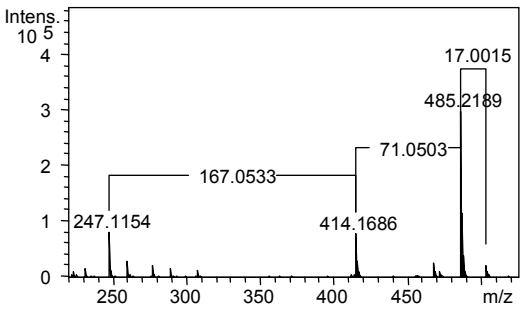
Luteolin



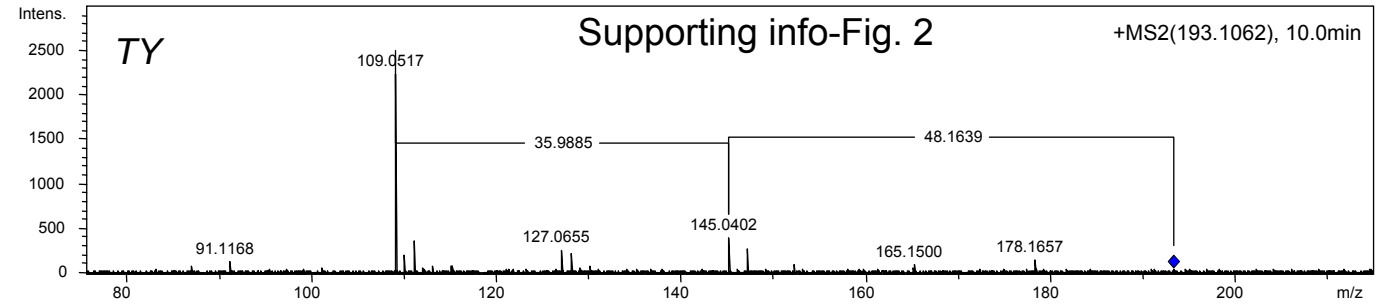


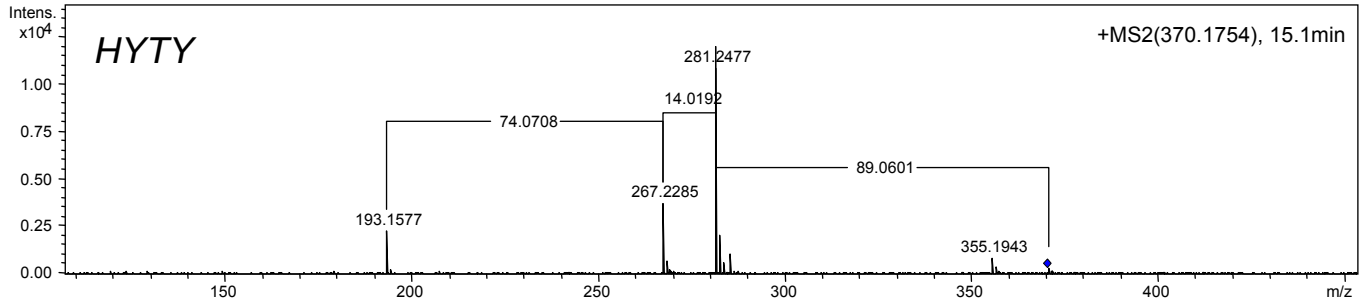
Pinoresinol

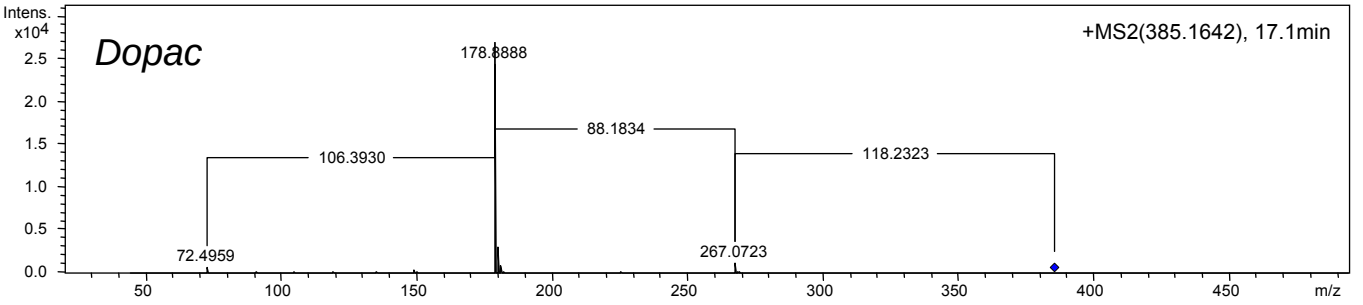


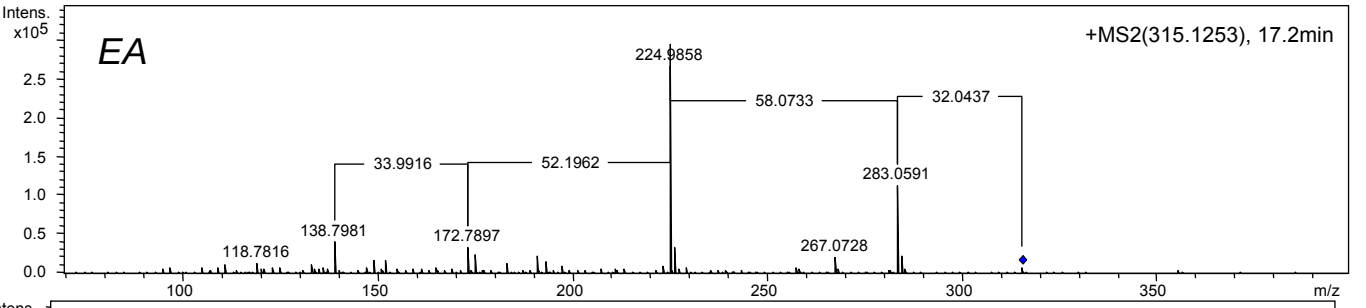


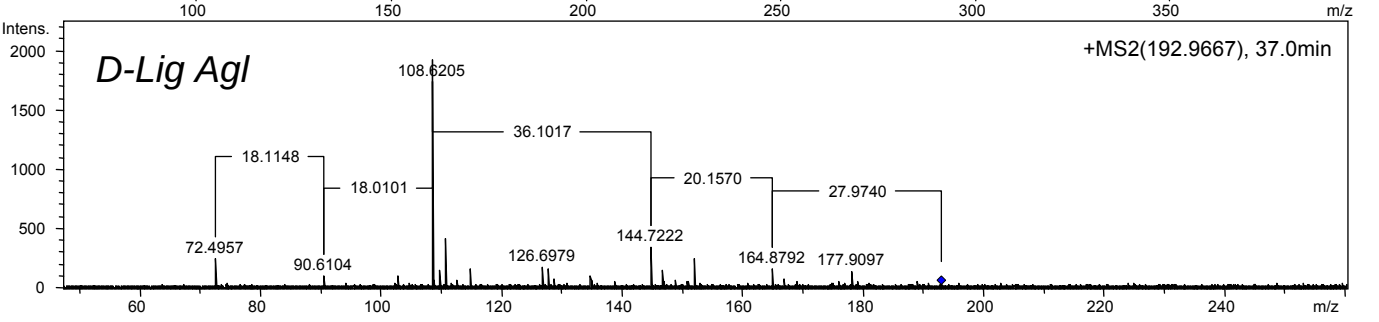
Supporting info-Fig. 2

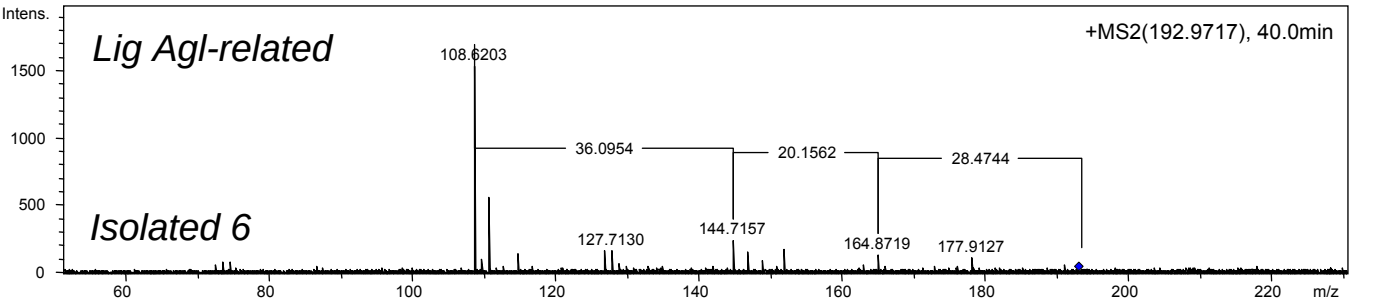


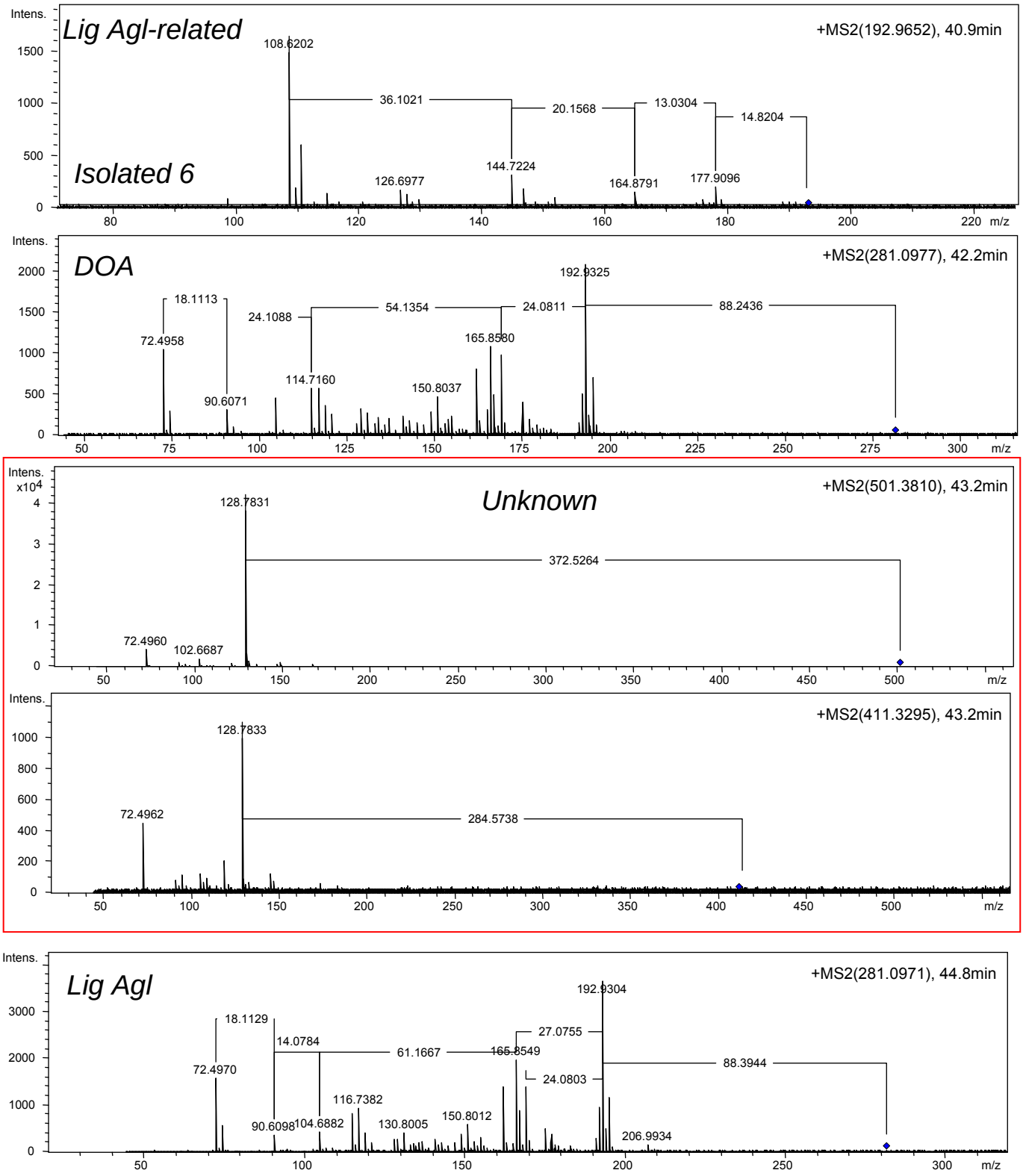


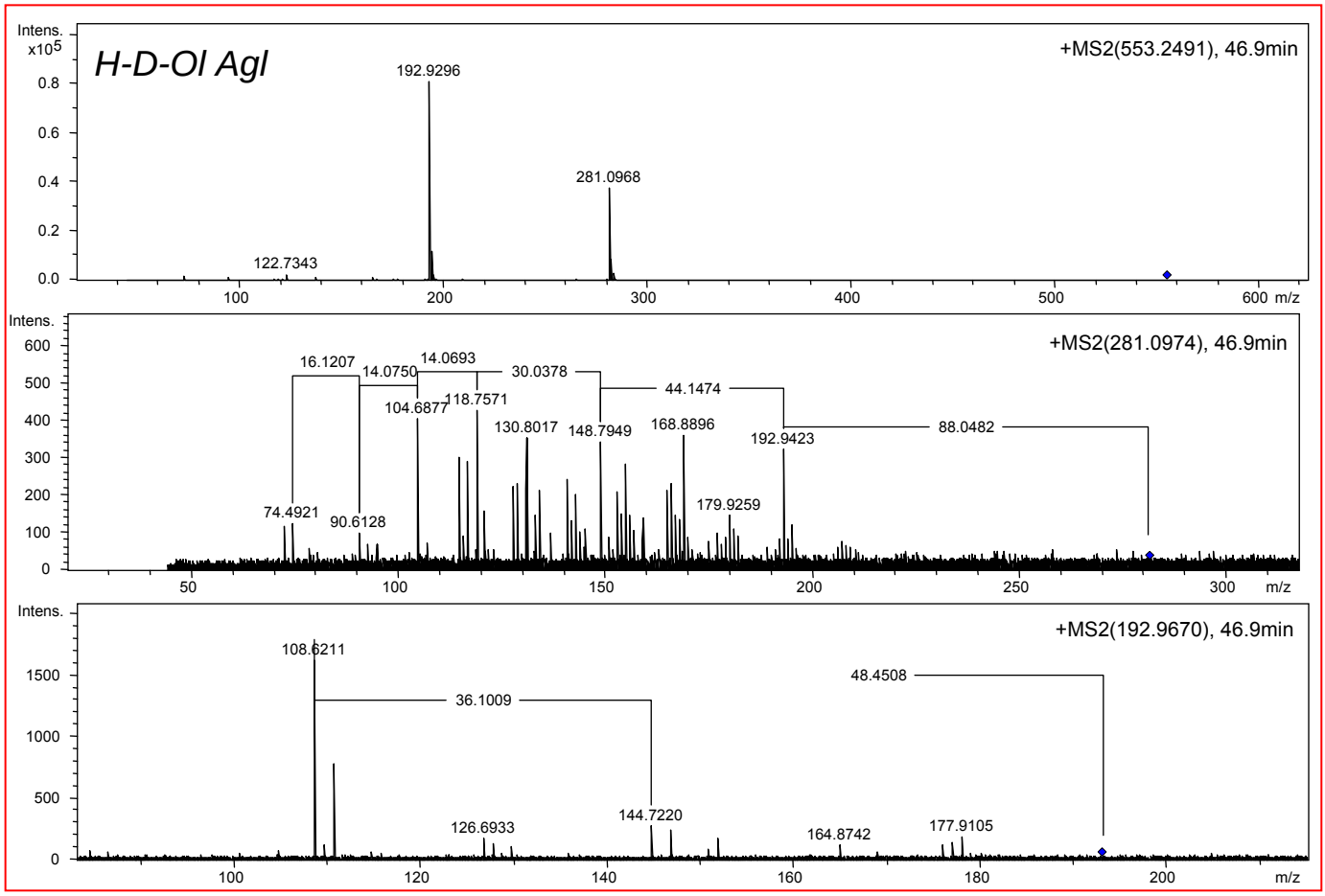


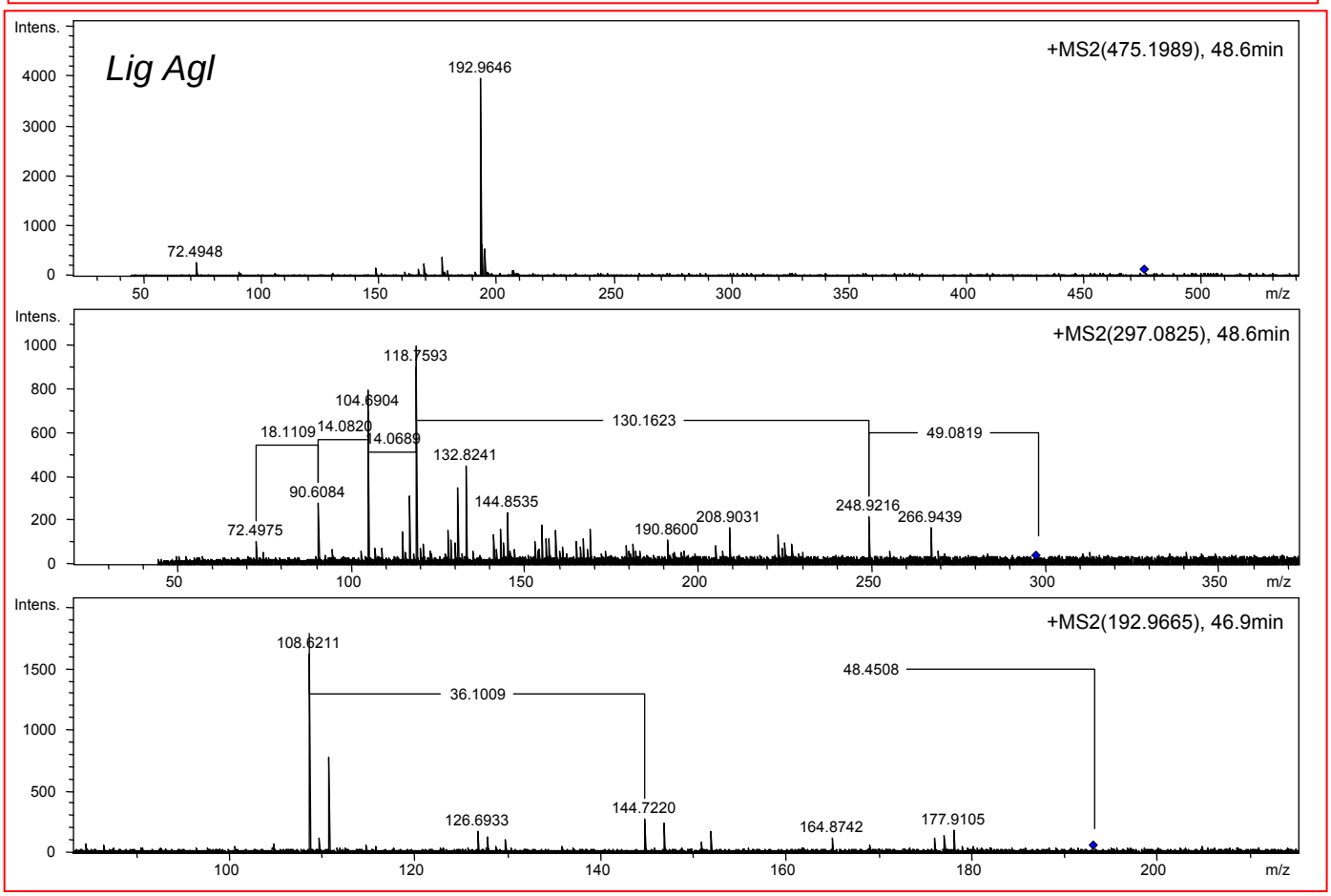


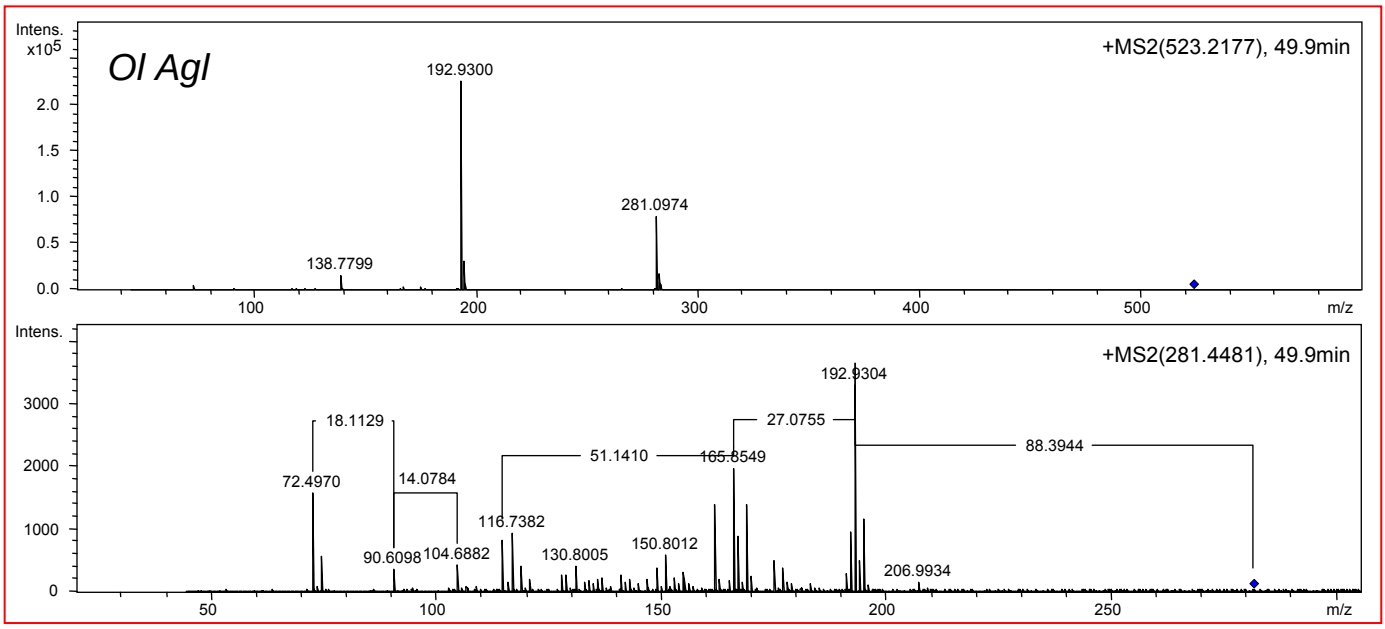


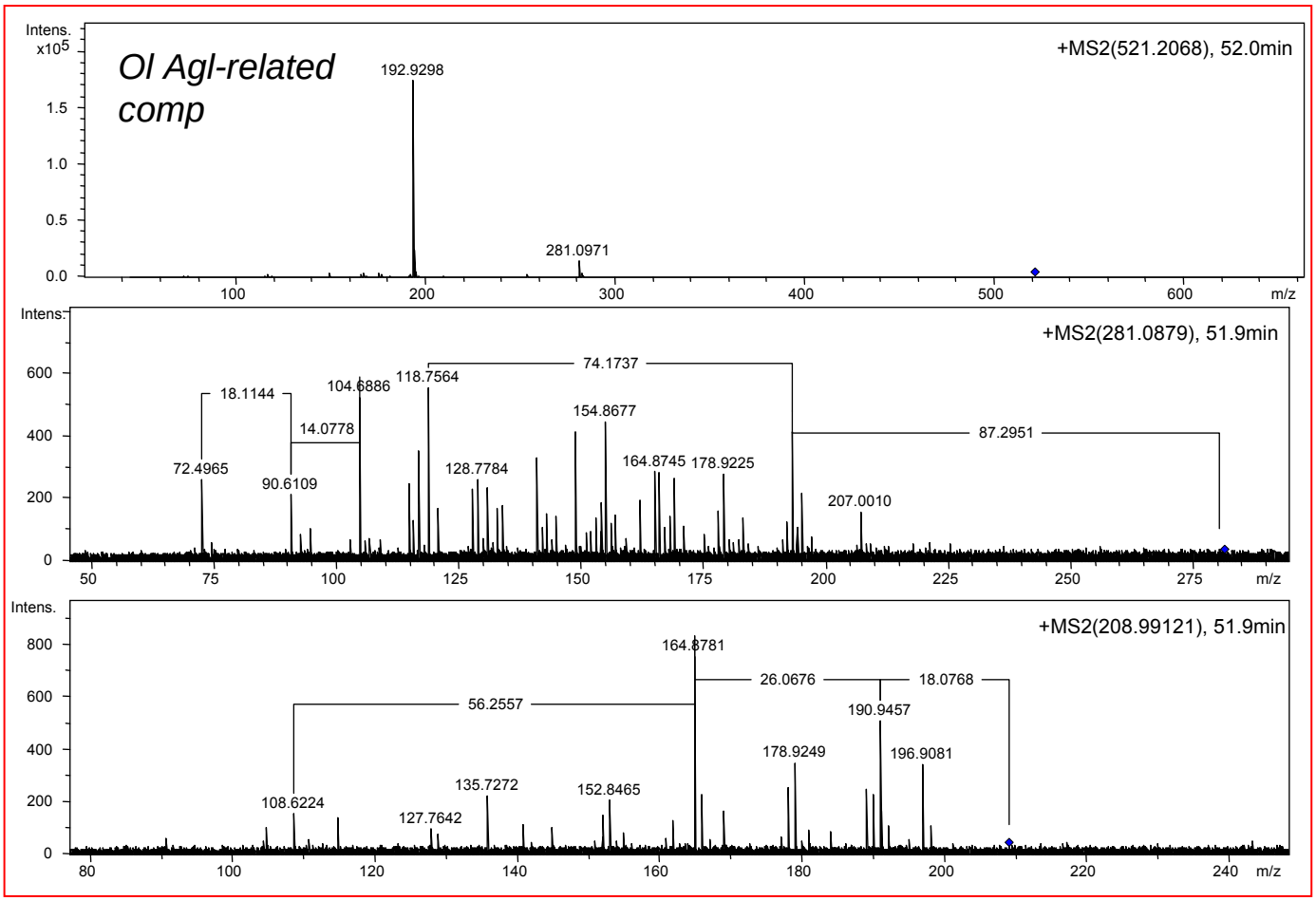


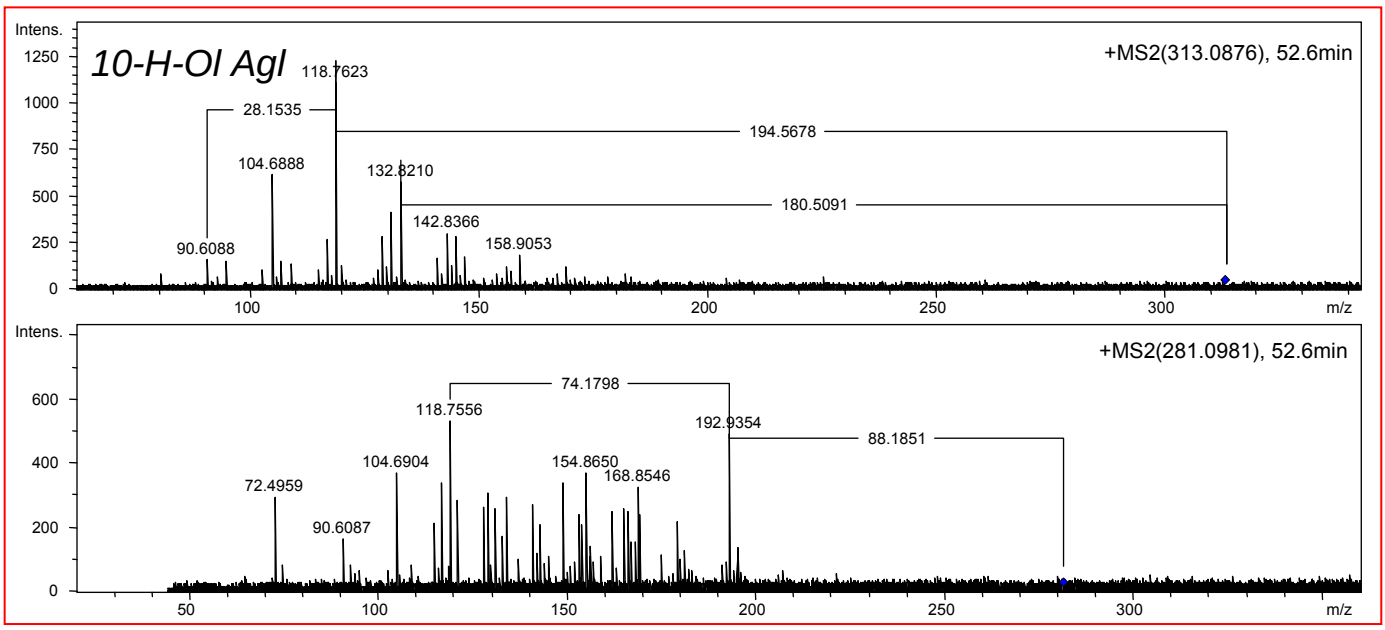

Lig Agl-related
+MS2(192.9652), 40.9min
Isolated 6
DOA
+MS2(281.0977), 42.2min
Unknown
+MS2(501.3810), 43.2min
+MS2(411.3295), 43.2min
Lig Agl
+MS2(281.0971), 44.8min
Intens.
m/z

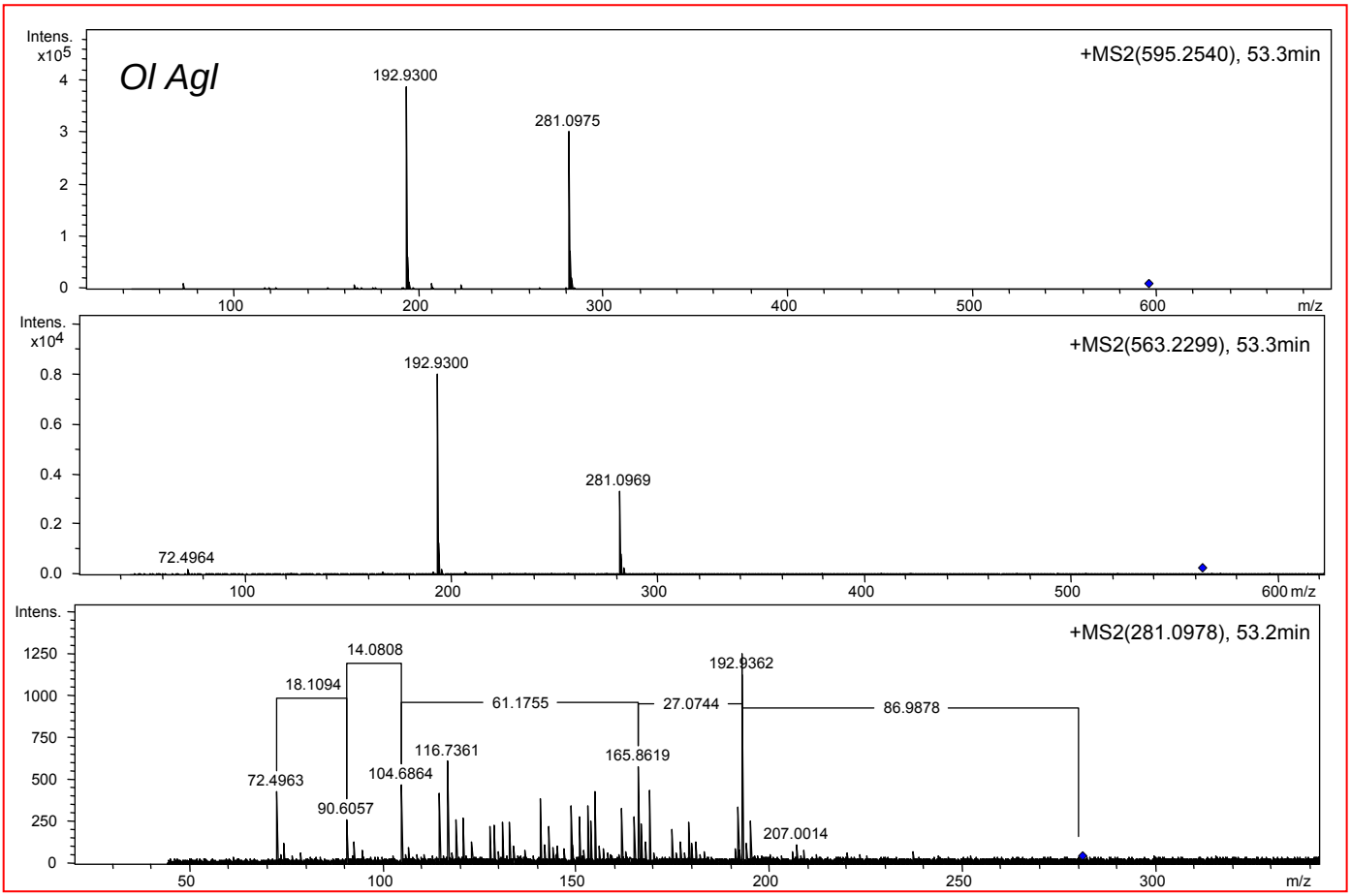

H-D-OI Agl
+MS2(553.2491), 46.9min
192.9296
281.0968
122.7343
+MS2(281.0974), 46.9min
16.1207
14.0750
14.0693
30.0378
44.1474
104.6877
118.7571
130.8017
148.7949
168.8896
192.9423
88.0482
74.4921
90.6128
179.9259
+MS2(192.9670), 46.9min
108.6211
36.1009
48.4508
126.6933
144.7220
164.8742
177.9105
Intens.
m/z


Lig Agl
+MS2(475.1989), 48.6min
192.9646
72.4948
+MS2(297.0825), 48.6min
118.7593
104.6904
130.1623
49.0819
18.1109
14.0820
14.0689
132.8241
90.6084
144.8535
72.4975
190.8600
208.9031
248.9216
266.9439
+MS2(192.9665), 46.9min
108.6211
36.1009
48.4508
126.6933
144.7220
164.8742
177.9105
Intens.
m/z


Ol Agl
+MS2(523.2177), 49.9min
Intens.
x10^5
192.9300
281.0974
138.7799
m/z
+MS2(281.4481), 49.9min
192.9304
18.1129
27.0755
88.3944
51.1410
165.8549
72.4970
14.0784
116.7382
150.8012
90.6098
104.6882
130.8005
206.9934


Ol Agl-related comp
+MS2(521.2068), 52.0min
192.9298
281.0971
+MS2(281.0879), 51.9min
74.1737
18.1144
104.6886
118.7564
154.8677
14.0778
87.2951
72.4965
90.6109
128.7784
164.8745
178.9225
207.0010
+MS2(208.99121), 51.9min
164.8781
26.0676
18.0768
56.2557
190.9457
178.9249
196.9081
135.7272
152.8465
108.6224
127.7642


10-H-Ol Agl
+MS2(313.0876), 52.6min
Intens.
118.7623
28.1535
194.5678
104.6888
132.8210
180.5091
142.8366
158.9053
90.6088
m/z
+MS2(281.0981), 52.6min
74.1798
118.7556
192.9354
88.1851
104.6904
154.8650
168.8546
72.4959
90.6087


Ol Agl
+MS2(595.2540), 53.3min
Intens.
x10^5
192.9300
281.0975
m/z
+MS2(563.2299), 53.3min
x10^4
192.9300
281.0969
72.4964
+MS2(281.0978), 53.2min
14.0808
192.9362
18.1094
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27.0744
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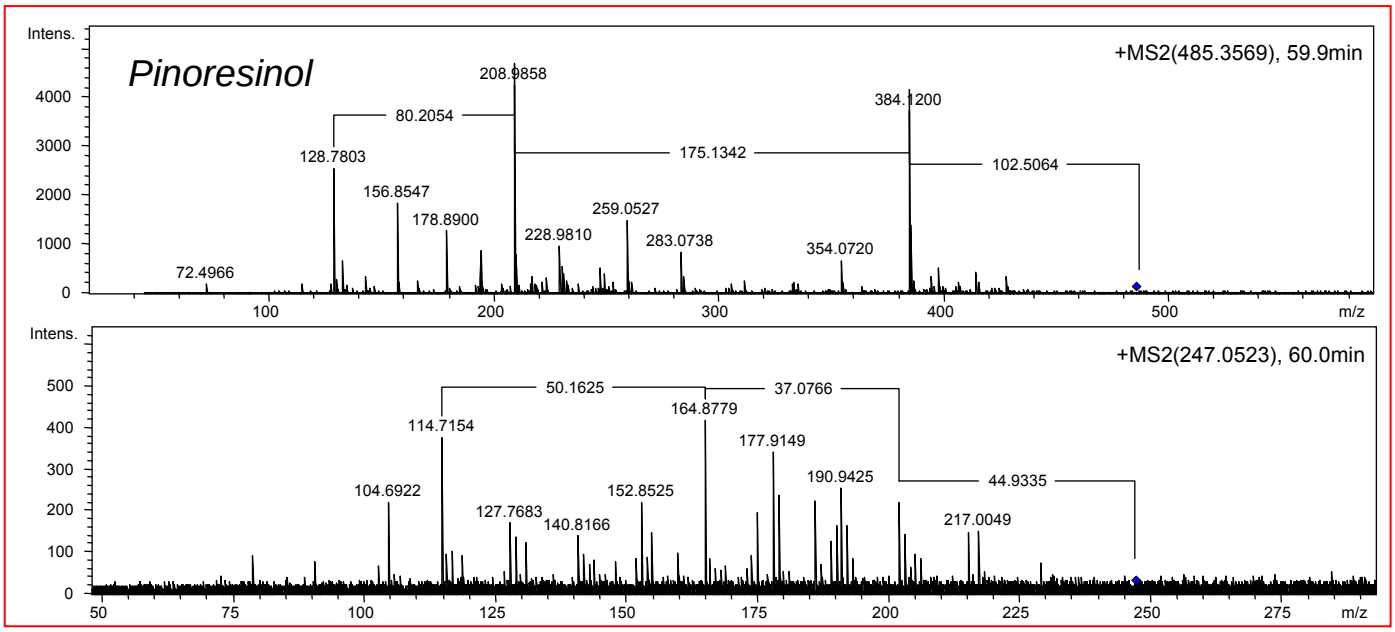
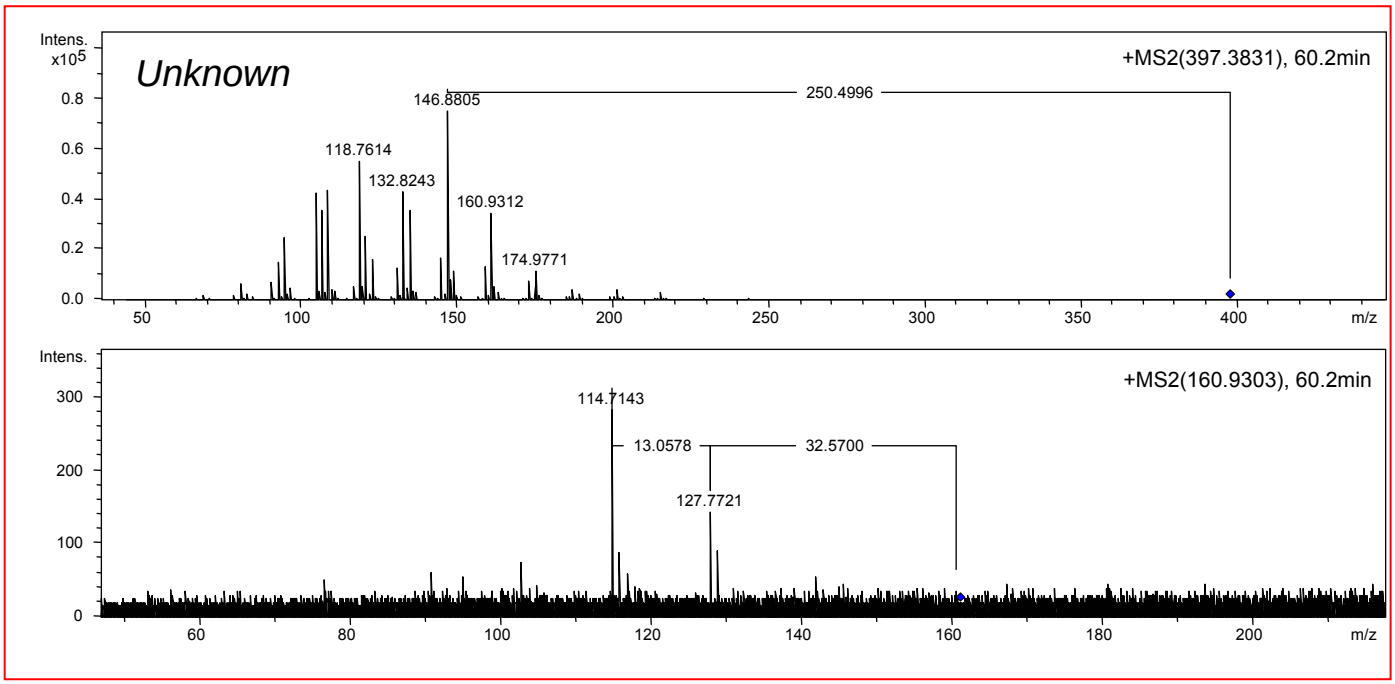
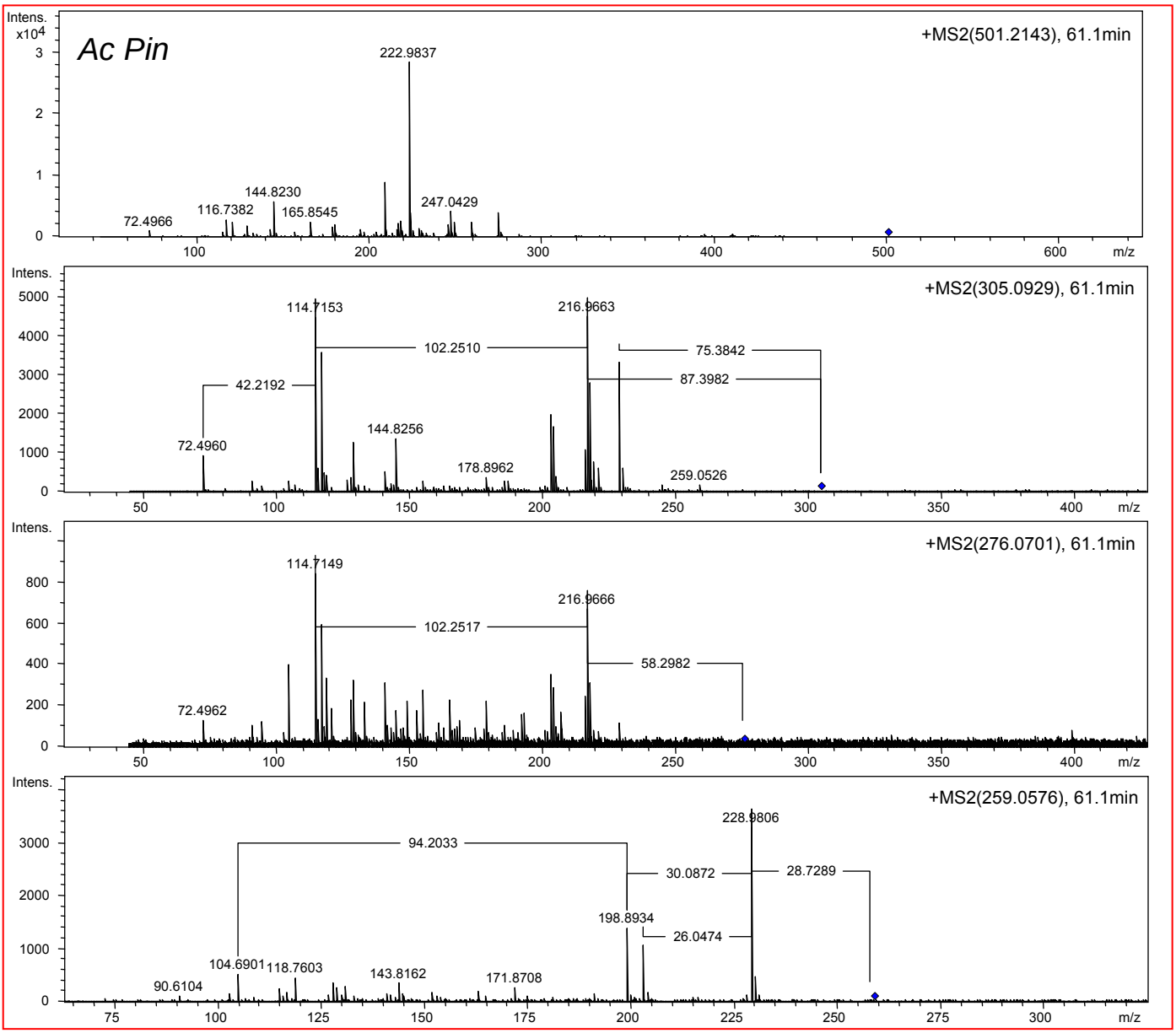
116.7361
165.8619
72.4963
104.6864
90.6057
207.0014


Pinoresinol
+MS2(485.3569), 59.9min
Intens.
208.9858
384.1200
80.2054
175.1342
102.5064
128.7803
156.8547
178.8900
259.0527
228.9810
283.0738
354.0720
72.4966
m/z
+MS2(247.0523), 60.0min
50.1625
37.0766
164.8779
114.7154
177.9149
190.9425
44.9335
104.6922
152.8525
127.7683
140.8166
217.0049


Unknown
Intens.
x10^5
+MS2(397.3831), 60.2min
250.4996
146.8805
118.7614
132.8243
160.9312
174.9771
m/z
+MS2(160.9303), 60.2min
114.7143
13.0578
32.5700
127.7721


Ac Pin
+MS2(501.2143), 61.1min
Intens.
x10^4
222.9837
144.8230
116.7382
165.8545
247.0429
72.4966
m/z
+MS2(305.0929), 61.1min
114.7153
216.9663
102.2510
75.3842
87.3982
42.2192
144.8256
72.4960
178.8962
259.0526
+MS2(276.0701), 61.1min
114.7149
216.9666
102.2517
58.2982
72.4962
+MS2(259.0576), 61.1min
228.9806
94.2033
30.0872
28.7289
198.8934
26.0474
104.6901
118.7603
143.8162
171.8708
90.6104

CAPÍTULO 8:
Estudio de la absorción y metabolismo de los polifenoles del aceite de oliva en células de cáncer de mama mediante nanoLC-ESI-TOF MS

En los capítulos 4 y 5, estudios *in vitro* llevados a cabo en diferentes líneas de cáncer de mama incubadas con polifenoles del aceite de oliva -tanto individualmente como en extractos complejos- demostraron los efectos anti-tumorales de la mayoría de los compuestos fenólicos, siendo el grupo de los lignanos y, especialmente de los secoiridoides, aquellos que resultaron los más efectivos.

En base a los resultados obtenidos, creímos interesante profundizar un poco más en esta línea de investigación, estudiando la absorción y metabolismo de estos compuestos fenólicos a nivel celular. Para ello, siguiendo la colaboración con el Insituto Catalán de Oncología de Gerona, medios de cultivo -tanto en ausencia de células, como en presencia de células de cáncer de mama de la línea JIMT-1- se pusieron en contacto con extractos polifenólicos del aceite de oliva (teniendo así en cuenta los posibles efectos sinérgicos) y se tomaron muestras a diferentes intervalos de tiempo.

Para estudiar los procesos de absorción y metabolización en las células cancerígenas disponemos de muestras tanto del medio de cultivo, como de las células lisadas y partes sólidas (núcleos, paredes celulares, etc.). Los dos principales inconvenientes que nos podemos encontrar a la hora de afrontar este estudio son: las bajas concentraciones en las diferentes muestras de los compuestos fenólicos añadidos y, sobre todo de los metabolitos formados, y el desconocimiento acerca de las reacciones metabólicas que tienen lugar en este tipo de células. Hay que tener en cuenta que en las células tienen lugar millares de reacciones químicas catalizadas por enzimas y que además de las rutas metabólicas centrales, comunes para la mayoría de las células, los diversos órganos muestran metabolismos propios.

Para llevar a cabo estos estudios se va a emplear una metodología nanoLC-ESI-TOF MS aprovechando las ventajas que presenta en cuanto a sensibilidad y exactitud a la hora de determinar la m/z de los compuestos estudiados. El potencial de esta plataforma analítica para la determinación de polifenoles del aceite de oliva ya fue demostrado en el capítulo 6, donde se establecieron sus ventajas e incovenientes comparándola con HPLC. En dicho capítulo se resaltó la ventaja de su gran sensibilidad, que la convierte en una técnica muy útil para el análisis de muestras donde los analitos se encuentran en bajas concentraciones, como es el caso del estudio que se incluye en este capitulo 8.

Uptake and metabolism of olive oil polyphenols in human breast cancer cells using nano-liquid chromatography coupled to electrospray ionization-time of flight-mass spectrometry

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Metabolomics (under review)

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Fax: +34 958 249510

Abreviations used

Ac Pin, (+)-1-acetoxypinoresinol; **Apig**, apigenin; **D-Lig Agl,** deacetoxy ligstroside aglycon; **DOA,** deacetoxy oleuropein aglycon; **EA**, elenolic acid; **EVOO,** Extra virgin olive oil; **Hyty**, hydroxytyrosol; **Hyty-Acet**, hydroxytyrosol acetate; **Lig Agl**, ligstroside aglycon; **Lut**, luteolin; **Ol Agl**, oleuropein aglycon; **Pin**, (+)-pinoresinol; **RSD%**, relative standard deviation; **Ty**, tyrosol

Abstract

Polyphenols from Extra Virgin Olive Oil (EVOO), a main component of the Mediterranean diet, have demonstrated repeatedly anti-tumoral activity in several *in vitro* and *in vivo* studies. However, little is known about the efficiency of the absorption process and metabolic conversion of these compounds at the cellular level. In this study, a nano liquid chromatography-electrospray ionization-time of flight mass spectrometry (nanoLC-ESI-TOF MS) method was developed to study the cellular uptake and metabolism of olive oil phenols in JIMT-1 human breast cancer cells. After incubation for different time periods with EVOO-derived phenolic extracts, culture media, cytoplasm and solid parts of JIMT-1 breast cancer cells were separated, subjected to different extraction procedures and analyzed. A short capillary trapping column (100 μm ID, effective length 20 mm, 5 μm particle size) packed with C18 stationary phase, was used. The mobile phase was a mixture of water + 0.5% acetic acid and acetonitrile eluting at 300 nL/min and 25ºC in a gradient mode. Most of the free phenols disappeared in the culture media in different extent and at different times according to the type of compound. The appearance of metabolites, especially from secoiridoids as well as from the flavonoid luteolin, could indicate absorption and intracellular metabolism followed by rapid cellular export. Low intracellular accumulation was observed with only traces of some compounds detected in the cytoplasm and solid parts. Methylated conjugates were the major metabolites detected, strongly suggesting a catalytic action of catechol-O-methyl transferase (COMT) in cancer cells.

Keywords nano liquid chromatography / Electrospray-time of flight-mass spectrometry/ Olive oil / phenolic compounds/ cancer cells/ metabolites

1 Introduction

Several studies have reported that the consumption of olive oil have a potential protective effect towards several malignancies, especially in relation to colon and breast cancer (Escrich et al. 2006; Owen et al. 2004; Di Fronzo et al. 2007; López et al. 2004). The beneficial effects of olive oil are related not only to its high content in the monounsaturated fatty acid (MUFA) oleic acid (Gerber 1997; Escrich et al. 2007; Menéndez and Lupu 2006) but also to the presence of nonsaponificable fraction, including biologically active phenolic compounds (Sotiroudis et al. 2003; Owen et al. 2000; Cicerale et al. 2009). The main families of phenolic compounds in olive oil are: simple phenols, lignans, flavonoids and secoiridoids (García-Villalba et al. 2010). A large body of evidence indicates that polyphenols can exert chemopreventive effects toward different cancers and our and other groups have explored the anti-tumoral activity of olive oil polyphenols. These *in vitro* studies mainly involved incubations of different types of human cancer cell lines (e.g., colon, prostate, leukaemia, breast) with individual polyphenols or whole olive oil phenolic extracts (Fabiani et al. 2008; Gill et al. 2005; Han et al. 2009; Fabiani et al. 2006; Hashim et al. 2008). These studies have suggested consistently that olive oil polyphenols are capable of significantly affecting the overall process of carcinogenesis due to their abilities to inhibit cell cycle, cell proliferation or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system. We have recently assessed the effects of olive oil polyphenols, supplemented individually or in the oil matrix, in different human breast cancer lines naturally exhibiting clinically relevant molecular markers. We have revealed that olive oil phenolics, especially those fractions enriched in lignans and secoiridoids, had a significantly stronger ability to decrease breast cancer cell viability by promoting an efficient blockade in the activity and expression of the tyrosine kinase receptor HER2 (*erb*B-2), one of the most commonly analyzed proto-oncogenes in human cancer studies (Menéndez et al. 2009; Menéndez et al. 2008; Menéndez et al. 2008).

Despite the strong experimental evidence suggesting that olive oil-derived polyphenols exhibit antitumoral activity, yet remains to be definitely elucidated the molecular mechanism(s) underlying both the uptake and the metabolism of these compounds in human cancer cells. This prompted us to investigate the efficiency of absorption and metabolic conversion of different families of phenolic compounds in human breast

cancer cells. To elucidate for the first time the uptake and metabolism of the main families of olive oil polyphenols in human breast cancer cells, we took advantage of the *HER2* gene-amplified JIMT-1 cell line originally established from a ductal carcinoma pleural metastasis of a 62-year-old patient, who did not respond to HER2-targeted therapies (Tanner et al. 2004; Rennstam et al. 2007). JIMT-1 cells were cultured in the absence or presence of crude extra virgin olive oil-derived phenolic extracts and nano liquid chromatography (nanoLC) coupled to electrospray ionization time of flight mass spectrometry (nanoLC-ESI-TOF MS) was used in culture media, cytoplasm and solid parts of JIMT-1 breast cancer cells. This technique has emerged as a new powerful analytical tool, providing a wide number of important applications, especially in proteomics and also in fields such as pharmaceutical, environmental and enantiomeric analysis (Hernández-Borges et al. 2007). The application of nanoLC in the field of food analysis is very limited (Fanali et al. 2004; Hernández Borges et al. 2007) but recently we have demonstrated the potential of this technique in the determination and quantification of polyphenols from olive oil samples (under review). This technique has demonstrated to offer several advantages over classical analytical methods, especially better sensibility with less consumption of mobile phases what become it in a very promising alternative for the analysis of samples containing low concentration of analytes, as for example biological fluids. However, its potential in the metabolomic field has not been fully explored and to the best of our knowledge it is the first time that this technique is used for the analysis of small bio-active molecules (i.e., polyphenols) in biological samples (i.e., human breast cancer cells).

2 Materials and Methods

2.1 Materials

All chemicals were of analytical reagent grade. For the optimization of the extraction procedure methanol and hexane was purchased from Panreac (Barcelona, Spain), hydrochloric acid from Scharlau (Barcelona, Spain) and ethyl acetate from Lab-Scan (Dublin Ireland). Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Fluka (Buchs, Switzerland) were used for preparing mobile phase and also for the extraction procedures. Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). For the trypsin digestion sequencing grade modified trypsin was purchased from Promega Corporation (Madison, WI, USA) and ammonium bicarbonate

from Panreac (Barcelona, Spain). Solid-phase extraction cartridges ENV+ were purchased from International Sorbent Technology (Cambridge, UK) and ziptip from Millipore (Bedford, MA, USA). Culture medium and fetal bovine sure from Sigma-Aldrich (St. Louis, MO, USA) were used in the optimization of the extraction protocol to simulate the real samples. EVOO sample of the variety Cornezuelo used for the incubation with cancer cells was obtained from CTAEX.

2.2 Extraction of phenolic compounds from virgin olive oil

The extraction procedure was based on a specific solid phase extraction (SPE) method with Diol-cartridges, previously described in literature (Gómez-Caravaca et al. 2005). Briefly, the extraction consisted of passing through a column, previously conditioned with 10 mL of methanol and 10 mL of hexane, 60 g of EVOO dissolved in 60 mL of hexane. After removing the non-polar fraction with 15 mL of hexane, the phenolic compounds were recovered with methanol (40 mL). The final volume was dried in a rotary evaporator under reduced pressure at 35°C and the residue was dissolved in 2 mL of methanol. For the supplementation to the cells 1 mL of the methanolic extract was evaporated and reconstituted in 125 µL of ethanol.

2.3 Human breast cancer cell line and culture conditions

JIMT-1 human breast cancer cell line was established at Tampere University and is available from the German Collection of Microorganisms and Cell Cultures (http://www.dsmz.de/). JIMT-1 cells were grown in F-12/DMEM (1:1) supplemented with 10% FBS and 2 mM L-glutamine. Cells were maintained at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

JIMT-1 cells were plated in 100-mm tissue culture dishes and cultured in regular media containing 10% FBS until they reached 75%-80% confluence. The cells were then washed twice with serum-free medium and incubated overnight upon serum-free conditions. Cells were then stimulated to grow in low-serum (0.1% FBS)-containing medium in the presence or absence of 0.01% of olive oil extracts dissolved in ethanol for 0, 15 min, 30 min, 1h, 2h, 6h and 24h. At each of such time points, the supernatants were collected, centrifuged at 1,000 x g, aliquoted, and immediately stored at -80^{o}C until utilization. Cells were rinsed with cold phosphate-buffered saline (PBS) and immediately solubilized in NP-40 lysis buffer [1% NP-40, 20 mmol/L Tris-HCl (pH

8.0), 137 mmol/L NaCl, 10% glycerol, 2 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonylfluoride, and complete protease inhibitor cocktail (Sigma-Chemicals)] by rocking the lysates gently at 4°C for 30 min. Following microcentrifugation at 14,000 x *g* for 5 min, supernatants (i.e. cytosolic fraction) and membrane + nuclear fractions (i.e. solid particles fraction) were obtained. Cytosolic fractions were transferred into a clean test tube and sample protein concentrations were determined using the Pierce Protein Assay Kit (Rockford, IL, USA) and stored immediately at -80°C until utilization. Blank controls containing solely olive oil extract in the absence of cells and cells cultured in the presence of *v/v* concentrations of olive oil extract solvent (i.e. 0.01% ethanol) were prepared strictly in parallel.

2.4 Sample preparation

Different extraction procedures were assayed for the different samples in order to extract the major number of compounds and with the highest recoveries. The optima extraction procedure was basically a liquid-liquid extraction with ethyl acetate including some slight differences according to the type of sample. For the culture medium the best extraction procedure was as follow: 1mL of the culture medium was mixed with 1 mL of ethyl acetate, extracted for 10 min using a vortex, centrifuged at 13000 r.p.m for 10 min and the supernatant was evaporated to dryness. The dried sample was reconstituted in 200 μL of mobile phase (water + 0.5% acetic acid) with 20% MeOH.

For the cytoplasm samples the extraction procedure consisted in stirring 100 μL of cytoplasm with 100 μL of 0.5M HCl/MeOH and 200 μL of ethyl acetate for 10 min using a vortex. The mixture was maintained in the freezer for 1 h at -20ºC. After samples reached the room temperature were centrifuged at 13000 r.p.m for 10 min and the supernatant was evaporated to dryness. The dried sample was reconstituted in 150 μL in mobile phase (water + 0.5% acetic acid) with 20% MeOH, because lower volumes were prone to erratic recoveries.

The solid parts were firstly washed with 100 μL of hexane to remove lipids. The samples were vortexing for 10 min and then centrifugated for 10 min at 13.000 r.p.m. The supernatant was removed and the solid parts were extracted as described above for the cytoplasm samples.

2.5 Instrumentation

2.5.1. HPLC

The optimization of the extraction procedure was carried out operating on an Agilent 1100-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV-Vis detector. The analytical column used for the separation of the compounds was a C_{18} Gemini, 3 μm i.d., 15 cm x 2.0 mm (Phenomenex, Torrance, CA, USA), protected by a guard cartridge of the same packing, operating at room temperature and a flow rate of 0.2 mL/min. The mobile phases consisted of water plus 0.5% acetic acid (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: 0 to 10 min, 5-33% B; 10 to 25 min, 33-40% B; 25 to 32 min 40-95% B. This value was maintained for 2 min and then the B content was decreased to the initial conditions, with which the column was re-equilibrated for 10 min. A volume of 10 μL samples was injected.

2.5.2. Nano LC

A commercial available instrumentation EASY-nLC^{TM} (Bruker Daltonik GmbH, Bremen, Germany), composed of one module and equipped with three pumps, three pressure sensors, four valves, two flowsensors, an autosampler and a touchscreen was used for the study.

A short capillary trapping column (100 μm ID, effective length 20 mm, 5 μm particle size) and a fused silica capillary column (75 μm ID, effective length 10 cm, 3 μm particle size)-both packed with C18 stationary phase-were used for the chromatographic separation. The mobile phases were composed of water + 0.5% acetic acid (phase A) and acetonitrile (phase B) with the following gradient: 0 to 20 min, 20-33% B; 20 to 45 min, 33-40% B; 45 to 48 min, 40-95% B. Finally, the B content was decreased to the initial conditions (20%) within 2 min and the column rinsed with these conditions for 5 min. Before starting the following analysis the pre-column and column were re-equilibrated with phase A at 6 μL/min for 2 min and 0.6 μL/min for 8 min, respectively. The flow rate used to elute the compounds in the analytical column was 300 nL/min at 25ºC and 5 μL of the samples was injected into the loop.

2.5.3. Mass spectrometry

The nanoLC column and the HPLC system were coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using electrospray ionization (ESI).

The HPLC system was coupled to the mass spectrometer using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The ESI parameters were chosen according to the flow delivered into the mass spectrometer (0.2 mL/min):nebulizer pressure was set at 2 bar, dry gas flow 9 L/min and dry gas temperature 190ºC.

The nanoLC column was interfaced to the mass spectrometry using a commercial sheatless nano-spray interface with a tapered fused silica sprayer tip. The key parameters of the nano-ESI were adjusted for the flow rate used (300 nL/min) to achieve stable spray across the entire gradient range: pressure 0.4 bar, dry gas flow 4 L/min and dry gas temperature 150 ºC.

The mass transfer parameters (radio frequencies and voltages in the different skimmers, hexapoles and lenses) were optimized in recent works, where the same families of polyphenols from olive oil samples were analyzed (García-Villalba et al. 2009). The mass spectrometer was run in the negative mode and was operated to acquire spectra in the range of 50-800 m/z. The accurate mass data of the molecular ions, provided by the TOF analyzers, was processed by DataAnalysis 4.0 software (Bruker Daltonik GmbH) which lists possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). In order to obtain this high mass accuracy mass calibration is required.

With the HPLC instrumentation the calibration of the MS was performed using sodium formate clusters by introducing externally a solution containing sodium hydroxide in the sheat liquid of some formic acid in water:isopropanol 1:1 v/v with a pump at the beginning of the analysis (external calibration). For the nanoLC analysis was not possible to do an external calibration because a system to introduce the calibrant at the beginning or the end of the chromatographic run has not been developed yet. Instead of this, an internal calibration was applied to quality control samples (culture medium spiked with olive oil extracts), introduced every three analysis, where a mixture of well-known phenols present in the olive oil extracts were used as calibrant, giving mass peaks throughout the desired range of 100-450 m/z . For the rest of the samples where the parent phenolic compounds were present in very low concentrations, the calibration

was made using the calibration parameters of the nearest control sample. This procedure resulted in mass accuracies of less than 5 ppm.

3. Results and discussion

3.1 Optimization of metabolites extraction from cancer cells

To extract the major number of phenolic compounds and metabolites from the samples with good recoveries in a reproducible way different extraction protocols were tested for the different samples. HPLC-ESI-TOF MS was initially used to monitorize the results of the extractions and the most representative components of the phenolic fraction of virgin olive oil were selected to calculate the recoveries: simple phenols (Hyty and Ty), flavonoids (Lut, Apig), lignans (Pin, Ac Pin and Syri) and secoiridoids (Ol Agl, Lig Agl, DOA, D-Lig Agl).

Regarding the culture media, commercially available culture media cell-free was used to optimize the extraction procedure. To simulate the real samples 5 μL of fetal bovine serum (0.1%) was added to 5mL of culture media and then was spiked with 5 μL of an olive oil extract in ethanol (0.1%). The whole olive oil extract was chosen for the study to see the behavior of the compounds in a complex mixture of phenols from real food and to consider the possible synergistic/antagonist interactions among them. At the beginning very conservative extraction procedures were testing, solid phase and liquid-liquid extraction followed with trypsin digestion, to ensure the elimination of possible proteins that could interference in the analysis. Our first attempt was to use solid phase extractions with ISOLUTE ENV+ cartridges (200 mg) very useful for the extraction of polar compounds from aqueous matrices. Different amounts of sample (2 mL (protocol 3) and 5 mL (protocols 1 and 2) were loaded onto the column and two different solvents, methanol (protocol 1 and 3) and acetonitrile (protocol 2) both with 1% acetic acid, were tested for the equilibration and the elution step. The elute was dried and the last step was to do a digestion with trypsin to ensure the elimination of proteins. The recoveries were very low for the flavonoids (1-15%) and practically zero for all the secoiridoids. Recovery percentages higher than 100% were obtained for the simple phenols, probably due to the degradation of the secoiridoids, and for the lignans perhaps because of the presence of additive interactions. The results were better with 2 mL loading sample for the flavonoids and slightly better with methanol for the recovery of simple phenols. A liquid-liquid extraction with ethyl acetate was also assayed (protocol

5) and , in some cases, a previous acidification with 0.5M HCl/MeOH was attempted (protocol 4) for disrupting the hydrogen bond formed between the proteins and phenolic compounds. At the last step a digestion with trypsin was carried out. The recoveries of flavonoids were higher than with the SPE extraction (46-74%) whereas the behaviour of lignans and simple phenols was similar with recoveries higher than 100%. Again the low recoveries of secoiridoids could be attributed to some decomposition during the extraction and the transformation in simple phenols. Because this decomposition could be produced by the use of trypsin, we tested the liquid-liquid extractions with ethyl acetate (with (protocol 6) and without (protocol 7) the previous step of acidification) but removing the last step of the trypsin digestion, obtaining in both cases good recoveries of practically all the compounds. Finally, the most simple extraction procedure only with ethyl acetate was chosen as optima because without the previous acidification, at least two more compounds (D-Lig Agl and DOA) were extracted and also the interferences produced by the use of HCL/ MeOH were eliminated. Besides, we could check that the protein content in the culture medium was too low to interfere in the analysis. Table 1 shows the recoveries of the main phenolic compounds identified in olive oil for the different extraction procedures assayed using HPLC-ESI-TOF MS.

To simulate the cytoplasm samples 100 μL of cytoplasm was spiked with an olive oil extract in ethanol assuming that 10% of the polyphenols added to the culture medium passed into the cytoplasm. With the cytoplasm samples the main problem was how to isolate the polyphenols with good recovery and minimal interference, especially from proteins. In this case we avoided the use of trypsin because of the problems of decomposition of secoiridoids, described above. A solid phase extraction with ziptip was tested eluting the compounds with acetonitrile or methanol and with a previous step of precipitation of proteins with 0.5 M HCl/MeOH or TCA, respectivey. The recoveries obtained with TCA and elution with MeOH was practically zero (data not show) whereas with 0.5 M HCl/MeOH and elution with acetonitrile (protocol 1) most of the compounds (with the exception of the secoiridoids DOA and D-Lig Agl) were recovered, although in general with low percentages. A liquid-liquid extraction with ethyl acetate and a previous acidification with 0.5 M HCL/MeOH was also assayed (protocol 2). In the cytoplasm case, the sample acidification should not be dispensed in order to disrupt protein binding and enhance recovery. The liquid-liquid extraction found to be advantageous in terms of maximum recoveries especially for the family of secoiridoids, some of which could not be recovered with the ziptip protocol.

The solid parts (membranes, nucleous, etc..) could not be simulated, so the extraction procedure was the same used for the cytoplasm with a previous step of washing with hexane to remove the lipids.

3.2 NanoLC analysis of cancer cells treated with olive oil extracts

Once optimize the extraction protocol, the culture medium and the cytoplasm spiked with the olive oil extract were analyzed by nanoLC-ESI-TOF MS and chromatographic and mass spectrometry conditions were optimized based on previous study with olive oil samples (paper under review). Recoveries were again calculated for the culture medium at two level of concentration, 0.1 and also 0.01% to make a closer approach to the real samples, and the results were in agreement with those obtained with HPLC (data not shown). The precision of the assay was evaluated by three consecutive injections ($n = 3$) of the quality control sample (culture medium spiked with the olive oil extract) in the same day (intra-day repeatability) and in three different days (inter-day repetibility) obtaining values of relative standard deviation (RSD%) on the peak area ratio above 3.8 and 18.6% respectively. These results showed good intra-day repeatability although poor inter-day repeatability, one of the disadvantages observed with the nanoLC technique.

The optima nanoLC-ESI-TOF MS method was applied to analyze all the real samples (culture medium, cytoplasm and solid parts) before and at different times after the administration of the olive oil extract. Each three analysis, a quality control sample was injected for correcting the variability in terms of signal sensitivity

Figure 1 shows the base peak chromatograms (BPC) of culture medium at time 0 h in the absence of olive oil (blank sample) (a) and just after the addition of the olive oil extract (b). In the same figure the extracted ion chromatograms (EIC´s) of the most representative olive oil phenols recovered from the culture medium can be observed (c). The method proved specificity for these compounds, since no interfering endogenous compounds could be seen at the elution positions of the polyphenols in the extracted ion chromatograms, when blank samples were analyzed.

To identify the phenolic compounds in free form and the possible metabolites formed after incubation, we used the information available in literature, the polarity of the compounds in reversed-phase and the valuable information provided by TOF analyzer. This gives us a list of possible molecular formula using the information about mass accuracy and the isotopic pattern of the compounds. Although, as we have mentioned in

Material and Methods, it was not possible to do an external calibration because of the lack of a suitable system to introduce the calibrant, a satisfactory internal calibration was developed using a combination of well-known phenols present in the quality control samples and the parameters of calibration of these samples were used for the subsequent analysis.

3.3 Cellular uptake

JIMT-1 human breast cancer cells were incubated in culture medium exogenously supplemented with olive oil extracts. The most representative compounds belonging to the different phenolic families described in olive oil were monitored in the medium at different periods of incubation: 0, 15 min, 30 min, 1h, 2h, 6h and 24h. All the compounds were identified taking into account all the available information described in the previous section.

The time of course of cellular uptake of the parent compounds expressed as % of the quantity initially incorporated in the culture medium can be seen on the Fig. 2. Considering that the disappearance of some compounds could be due to their rapid degradation in the medium two experiments were carried out in parallel: one of them with the culture medium in the presence of cells to study how the compounds from the extract are absorbed and biologically metabolized by the cells and the other, in the absence of cells, to represent the spontaneous or basal degradation of the compounds without any biological metabolization.

Different uptake was observed for each compound in relation to their different chemical structure. In the case of flavonoids, luteolin decreased significantly in the culture medium from 2 to 24 h: 38% of the quantity incorporated was present after 6h of incubation and only traces of this compound were observed after 24h. Apigenin appeared to be more slowly and poorly taken up with 39% of the nonmetabolized molecules detected in the culture medium after 24h. This could be due to the limited diffusion into the cell of compounds with a lower degree of hydroxylation, as in the case of apigenin with one hydroxyl group less than luteolin.

Regarding lignans, pinoresinol and syringaresinol remained practically intact in the culture medium for 2 hours. The compounds were absorbed, although poorly, between 2 and 6 hours, detecting approximately 50% of the quantity initially added in the medium after 6h of incubation. Syringaresinol was a bit more absorbed, reaching 35% after 24 h. In the case of acetoxypinoresinol the same quantity (50%) was found after 6 h of

incubation but the absorption started a few hours before (around 1 h) what indicates a faster uptake of this compound. The rapid increase between 6 and 24 h of incubation could be due to the appearance of the acetoxylated metabolite of pinoresinol.

For the family of secoiridoids it is important to take into account that these compounds are susceptible to the spontaneous degradation in free cells culture media, particularly since 2 h of incubation. The cellular uptake appeared to slightly differ between the secoiridoids containing in their structure a molecule of hydroxytyrosol (Ol Agl and DOA) and those with a molecule of ty (Lig Agl and D-lig Agl). Ol Agl and DOA were absorbed very rapidly after the administration and a gradual uptake, starting from 15 min, was observed along the time. In Fig. 2 both compounds were observed to be taken up at all exposure time points although the pattern of association differed in a time-dependent manner, probably due to the differences in the structure. During the first minutes the absorption was a bit faster for the DOA whereas the levels of Ol Agl fell more slowly within 30 min and reached the maximum of absorption between 30 min and 2 h. Limited uptake of the secoiridoids derived from ty (Lig Agl and D-Lig Agl) was observed at short incubation time: 15 min-1h, reaching the maximum absorption a bit later between 1 and 2 h for Lig Agl and after 6 h of incubation for D-Lig Agl. This slower uptake could be due to the lower degree of hydroxylation, as mentioned before. The remaining quantity of compounds that disappeared from 2 to 24 h was probably due to the spontaneous degradation of the compounds as can be observed in the experiments in the absence of cells.

The results obtained from the simple phenols (hyty and ty) were more difficult to interpret because part of them could come from the decomposition of their respective secoiridoids in the culture medium. Hyty, as in the case of its secoiridoids derivatives, was incorporated in the first minutes reaching 50% after 1 h of incubation. The increase observed after 1 h could be due to the decomposition of Ol Agl and the rapid decrease from 2 h to the absorption and in part to its decomposition in the culture medium. For hyty-acet the absorption was significant from 2 h and only 4% of the parent compound was detected after 6 h of incubation.

No significant changes were observed in the concentration of tyrosol throughout the incubation period what it is in agreement with the results obtained in literature about the limited diffusion of this compound in HepG2 cells (Mateos et al. 2005). Besides, a high increase in this compound was observed after 2 h probably due to the decomposition of the secoiridoids containing this molecule in their structure.

Removal from the culture medium could suggest accumulation of these compounds in the cells but also in the cell membranes. However, we failed to distinguish these compounds when analyzing cytoplasm and solid parts of JIMT-1 cells, maybe due to the low intracellular accumulation (Mateos et al. 2005). Therefore, the decrease of the amount of some compounds in the culture medium along the time may indicate the uptake of intact compounds by the cells.

3.4 Metabolism of olive oil polyphenols in breast cancer cell

Several compounds formed as a consequence of cell metabolism were observed in the extracellular culture medium at different incubation times. Table 2 shows all the metabolites tentatively identified in the culture medium grouped by families, including m/z experimental, retention time, error and sigma value and molecular formula. The time range where these compounds appear and the time at which the highest intensity of each compound is reached are also indicated in the table. The extracted ion chromatograms of all the metabolites tentatively identified at the time at which they present the highest intensity can be seen in Fig. 3. Control samples cell-free and control samples with no added phenolics were also analyzed because some compounds could also be formed spontaneously in the culture medium.

Simple phenols. One metabolite with very low intensity and m/z 167.707 was tentatively identified as the methyl conjugate of Hyty. This compound started to appear in the culture medium after 2 h of incubation, when part of the parent compound had already been absorbed, but reached the maximum concentration after 24 h. As it was mentioned before Ty was poorly absorbed and subsequently none metabolites of this compound were found in the culture medium.

Secoiridoids. Compounds belonging to the secoiridoids group were the most extensively metabolized, especially those derived from Hyty (Ol Agl and DOA). Methylation was the preferential pathways for both compounds followed or preceded, in some cases, by a hydrogenation reaction. All the methylated forms increased in amount with time, in parallel with the decrease of the parent compounds in the culture medium, reaching the maximum value after 2 h of incubation. No methyl conjugates of secoiridoids derived from Ty (Lig Agl and D-Lig Agl) were observed, as could be expected since methylation by COMT requires and ortho-diphenolic structure. Two new

peaks with m/z 379.1390 and 393.1560 were tentatively identified as the dihydrogenated metabolite of Ol Agl and Lig Agl, respectively. This metabolite of the Ol Agl appeared in the medium with high intensity at earlier times (30 min) indicating a faster metabolism and excretion compared with the methylation. Regarding the Lig Agl, this was the only metabolite detected, confirming the poor metabolism of the secoiridoids containing in their structure a molecule of ty. Besides, this compound appeared with high intensity a bit later (2h) compared with the same metabolite of the Ol Agl, probably because of the slower uptake of the parent compound, as indicated in the previous section. After 24 h of incubation these compounds were almost disappeared.

Flavonoids. As could be observed in the above section the cellular uptake of flavonoids was slow, with most of the compounds found as the original molecules in the first hours of incubation. Only traces of three oxidation products of luteolin (hydroxylated, dihydroxylated and hydrated) were observed after 2 h, when the absorption of the compound is still very limited. At longer incubation time (24 h), an intensive peak with m/z 299.0559 and later retention time was tentatively identified as the methyl-luteolin. The other flavonoid, apigenin, appeared to be poorly metabolized, and presumably this compound cannot take the same metabolic route as luteolin because of the lower degree of hydroxylation. Only a peak observed after 6 hours of incubation with m/z 447.0939 could be identified as dehydrogenated apigenin glucuronide and the same metabolite of luteolin (dehydrogenated luteolin glucuronide) could be attributed to another smaller peak observed at tr 20.789 with m/z 463.0865. However, some doubts about this identification appeared because both metabolites were detected in the culture medium at too early times (6 and 2 h) when the parent compounds were poorly absorbed. Besides, according with the literature the gluronoconjugates have great difficulty to cross the membrane because of their polarity and it would be expected to appear at longer times or even not to appear. It would be necessary further experimentation to confirm the notion that human breast cancer cell contain UDp-glucuronosyl- transferase activity capable of originate glucuronidated flavonoid forms.

Lignans. The metabolites formed from the lignan precursors were practically absent, in part because of their low absorption into the cells. Two metabolites of pinoresinol appeared in small traces, one of them could correspond to the loss of a methoxy group

and the other the possible sulfoconjugate metabolite. Therefore a limited sulfation activity could be attributed to this cancer cell line although no other sulfated metabolites were found and further attempts should be done to confirm the identity. At longer incubation time (24h) a small peak with m/z 475.1605 was identified as acetoxi-syringaresinol, the same metabolite that was described for pinoresinol in the previous section (acetoxipinoresinol).

Other peaks with high intensity were detected in the culture medium at different incubation time but it was not possible to identify them with the instrumentation used in this study and it was only possible to show their molecular formula. Some molecular formulas seemed to be related.

The content of the metabolites identified was also measured in cell lysates but only small traces were observed after different hours of incubation. This could be because the concentration of the added compounds is too low to detect them in the cytoplasm or could suggest no intracellular accumulation of the metabolites and a rapid excretion into the culture medium. No metabolites were detected in the solid parts either.

Therefore, the appearance of the metabolites in the medium with time indicates that the compounds are absorbed, intracellularly metabolized to different forms and excreted to culture medium. Alternatively, they can be produced directly in the medium by secreted cellular enzymes. Since the methyl conjugates prevailed in the culture medium, these results might suggest that JIMT-1 human breast cancer cells possess the enzymatic activity of catechol-O-methyltransferasa (COMT), as in the case of cells obtained from the gastrointestinal tract (Mateos et al. 2005; Manna et al. 2000). However further studies would be required to confirm that JIMT-1 human breast cancer cells possesses enzymatic activity for metabolizing flavonoids and lignans to glucuronide and sulphate conjugates.

We have studied in what extent the main compounds of olive oil are taken up and modified by human breast cancer cells. However, this study has two limitations: the concentrations used probably are higher than those physiologically achievable; indeed phenolic compounds actually reaching target tissues probably are metabolites of the original compounds. It is necessary a better understanding of the mechanism of action of olive oil polyphenols to definitely establish both the nature and the plasma concentration of phenolic. Also, future studies should elucidate whether retention of olive oil polyphenols and/or their metabolites differentially occurs among specific target

tissues. Because only limited information on the tissue distribution is available at present, we have used the original compounds. Further studies are required to clarify the concentrations and metabolic forms that tissues and cells are exposed following dietary ingestion of olive oil polyphenols.

4. Concluding remarks

The extent of uptake and the level of metabolism of olive oil polyphenols in cancer cells will be central to understand their mechanism of action. In this work, for the firs time the cellular uptake and metabolism of olive oil polyphenols in human breast cancer cells has been evaluated at different incubation time, taking advantage in sensitivity of the nanoLC-ESI-TOF MS. It is the first time that this analytical platform is used in a metabolomic studied and it has demonstrated its potential in the analysis of small molecules in biological fluids. The analysis of culture medium, cytoplasm and solid parts at different incubation time, suggested that some compounds, mainly hydroxytyrosol, secoiridoids, and the flavonoid luteolin are absorbed, metabolized in the cytoplasmic compartment of cancer cells and excreted to the extra cellular medium. The secoiridoids, especially those derivatives from Hyty (DOA and D-Lig Agl) appeared to be the most rapidly and extensively taken up and metabolized, mainly as methylconjugates. The methylated derivatives were also predominant for the hyty and luteolin, this last one together with trace amounts of other oxidation metabolites. The presence of the methylconjugates suggests that this breast cancer cell line possesses catechol-O-methyltransferase activity for metabolizing polyphenols. Limited metabolism was observed for the lignans, apigenin, ty and their related secoiridoids (Lig Agl and D-Lig Agl), a phenomenon likely related to a less efficient transport through human breast cancer cell membranes.

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Caption figures

Fig. 1 Base peak chromatograms (BPC) obtained by nanoLC-ESI TOF MS at the optima conditions for culture medium at time 0 h in the absence of olive oil (blank sample) (a) and just after the addition of the olive oil extract (b). Extracted ion chromatograms (EIC´s) of the most representative olive oil phenols obtained from Fig 1b (c).

Fig. 2 Time course of cellular uptake of the parent olive oil phenolic compounds in the culture medium at different incubation time: 0, 15 min, 30 min, 1h, 2h, 6h and 24h. The values show the amounts of compounds remaining in the culture medium expressed as % of the quantity initially incorporated. ♦ culture medium in the absence of cells ■ culture medium in the presence of cells.

Fig. 3 Extracted ion chromatograms (EIC´s) of the main metabolites tentatively identified in culture medium of human breast cancer cells incubated with olive oil extracts. They are grouped according with the time at which they presented the highest intensity.

Table 1 Recovery percentages (%) of the main phenolic compounds identified in olive oil from culture medium and cytoplasm spiked with ethanolic extracts of olive oil, using different extraction protocols. Samples were analyzed by HPLC-ESI-TOF MS. Each determination was in triplicate.

	CULTURE MEDIUM							CYTOPLAMS	
Compounds	**Prot.1**	**Prot. 2**	**Prot. 3**	**Prot. 4**	**Prot. 5**	**Prot. 6**	**Prot.7**	**Prot.1**	**Prot.2**
Hyty	228	62	387	443	428	78	82	49	70
Ty	105	90	216	216	140	69	73	34	63
Lut	1	4	13	46	53	56	60	20	57
Apig	3	6	15	69	74	60	64	31	64
Pin	126	127	213	153	197	162	148	119	151
Ac Pin	186	162	336	189	377	190	157	134	172
Siri	141	139	301	125	341	120	138	123	158
Lig Agl	0	0	0	3	3	52	55	24	68
Ol Agl	0	0	1	1	0	67	62	19	48
D- Lig Agl	0	0	0	0	1	1	30	0	4
DOA	0	0	0	0	0	2	21	0	3

Table 2 Metabolites tentatively identify in the culture medium of cancer cells exogenously supplemented with olive oil extracts at different incubation times.

	m/z experimental	Retention time	Error ppm	Sigma	Molecular Formula	Proposed metabolite	Maximum concentration (range)
Simple phenols	167.707*	18.097	4.2	56.5	$C_9H_{11}O_3$	Methyl Hyty	24 h (2-24 h)
Secoiridoids	335.1499	25.605	0.3	17.8	$C_{18}H_{23}O_6$	Dihydrogenated methyl DOA	2 h (1-24 h)
	333.1347	26.190	-0.9	63.1	$C_{18}H_{21}O_6$	Methyl DOA	2 h (30'-6 h)
	379.1390	26.625	2.3	17.1	$C_{19}H_{23}O_8$	Dihydrogenated Ol Agl	30' (15'-2 h)
	363.1445	28.933	1.3	4.9	$C_{19}H_{23}O_7$	Dihydrogenated Lig Agl	2 h (30'- 24 h)
	393.1560	29.602	-1.3	5.3	$C_{20}H_{25}O_8$	Dihydrogenated methyl Ol Agl	2 h (15'-24 h)
	391.1397	33.599	0.4	28.2	$C_{20}H_{23}O_8$	Methyl Ol Agl	2 h (15'-2 h)
Flavonoids	317.0305*	19.501	-0.7	95.1	$C_{15}H_9O_8$	Dihydroxy luteolin	2 h
	303.0495*	21.692	5.2	94.9	$C_{15}H_{11}O_7$	Luteolin hydrated	2 h
	301.033	26.709	4.1	58.6	$C_{15}H_9O_7$	Hydroxy luteolin	2 h
	299.0559	29.117	0.8	3.6	$C_{16}H_{11}O_6$	Methyl luteolin	24 h
Lignans	327.1218*	24.250	6.0	135.6	$C_{19}H_{19}O_5$	Demethoxy pino	2 h (1 -6 h)
	437.0923*	24.920	2.5	120.6	$C_{20}H_{21}O_9S$	Pino sulfate	24 h
	475.1605*	29.284	0.9	28.5	$C_{24}H_{27}O_{10}$	Acetoxi -siri	6 h (2-24 h)
Unknown	463.0865 *	20.789	3.6	37.4	$C_{21}H_{19}O_{12}$		2 h (30'-2 h)
	349.0581	20.872	-4.6	32.5	$C_{16}H_{13}O_9$		2 h
	447.0939	22.026	-1.5	6.2	$C_{21}H_{19}O_{11}$		6 h
	433.1502	22.880	0.5	57.0	$C_{22}H_{25}O_9$		15'
	431.100	23.548	-3.8	4.8	$C_{21}H_{19}O_{10}$		6 h
	609.1249*	24.501	9.7	66.5	$C_{23}H_{29}O_{19}$		2 h
	273.1692	25.037	5.8	74.9	$C_{14}H_{25}O_5$		24 h
	281.1035	25.672	-1.7	31.4	$C_{14}H_{17}O_6$		2 h
	265.1072	27.010	3.5	110.7	$C_{14}H_{17}O_5$		2 h
	249.1120	29.267	5.1	2.6	$C_{14}H_{17}O_4$		2 h
	437.1130	29.736	-9.4	32.8	$C_{20}H_{21}O_{11}$		6 h
	403.1398*	36.609	0.2	306.2	$C_{21}H_{23}O_8$		1 h (15'-2 h)

* means compounds present in small traces.

Fig. 1

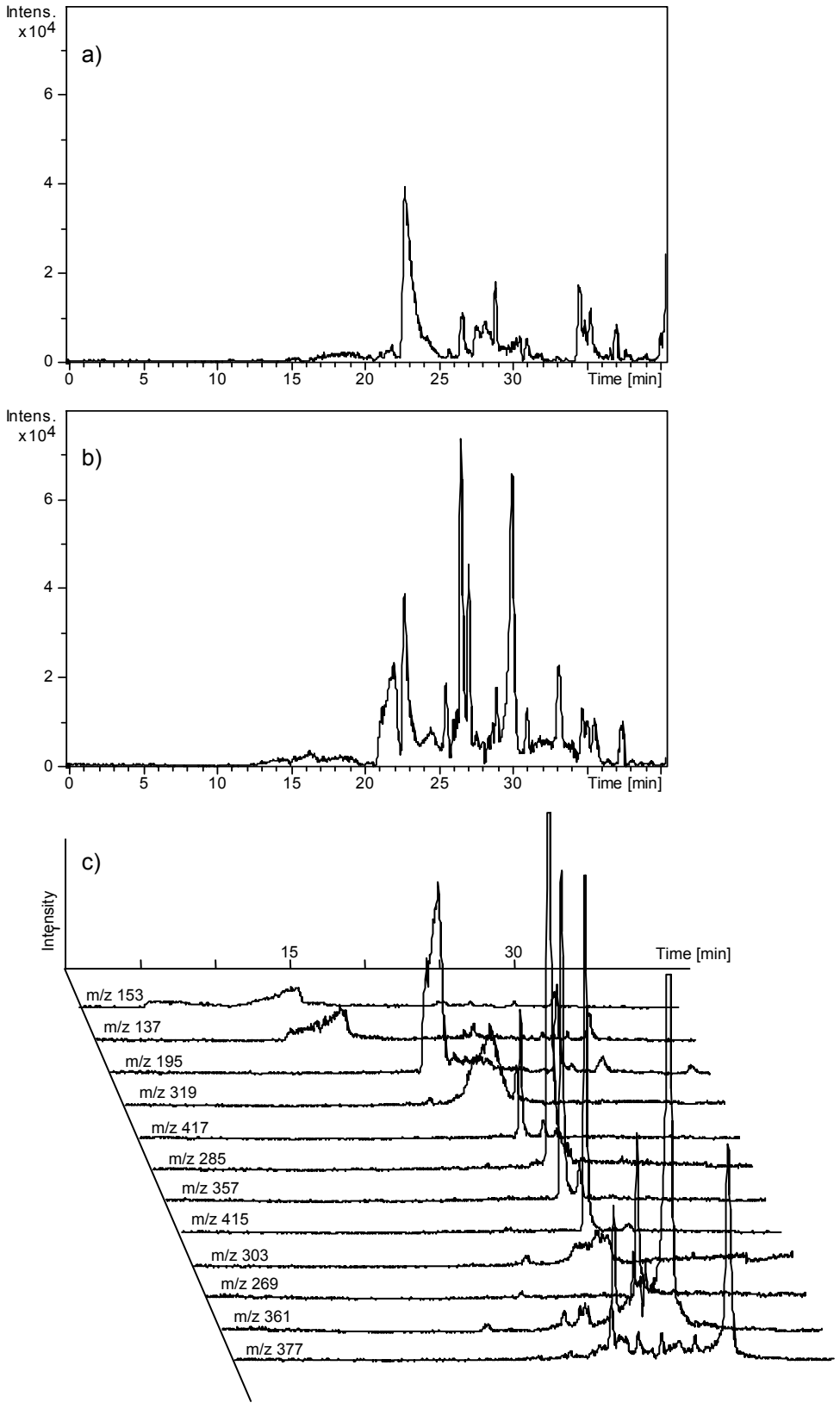
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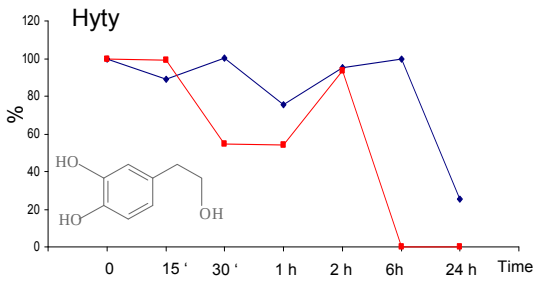
Fig. 2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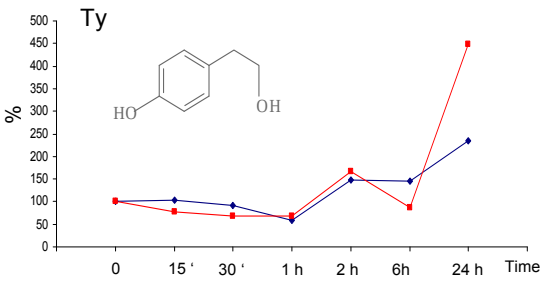
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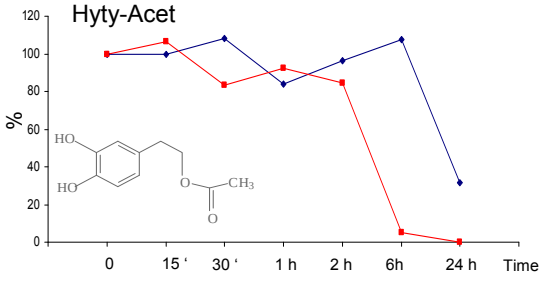
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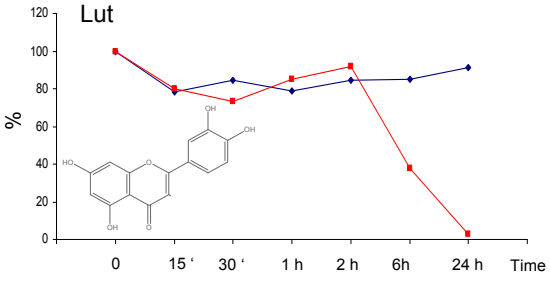
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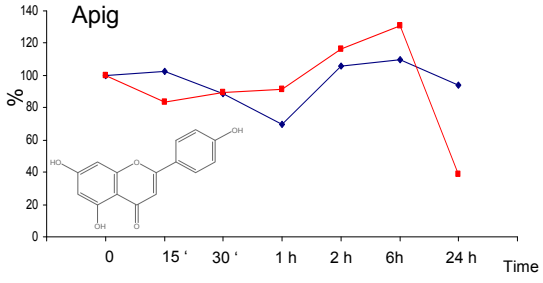
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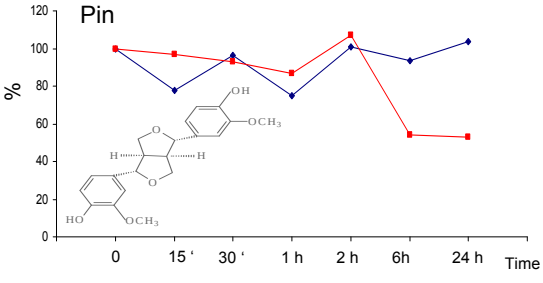
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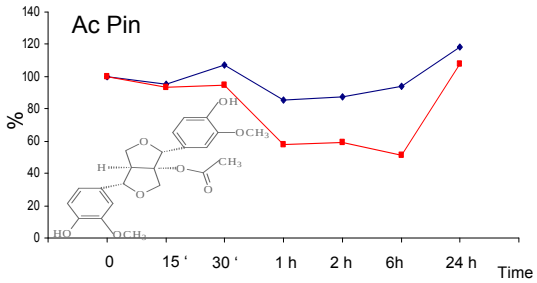
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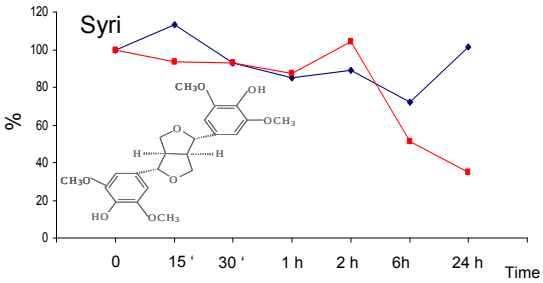
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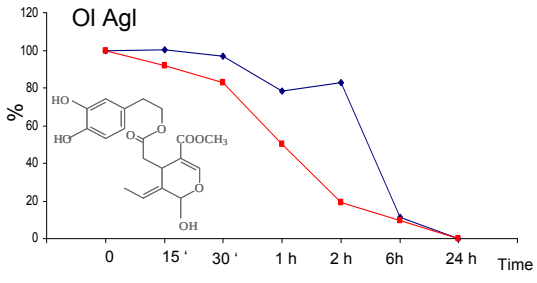
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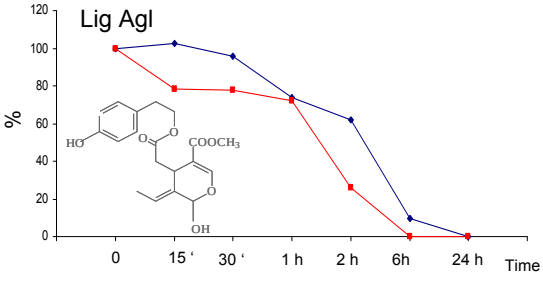
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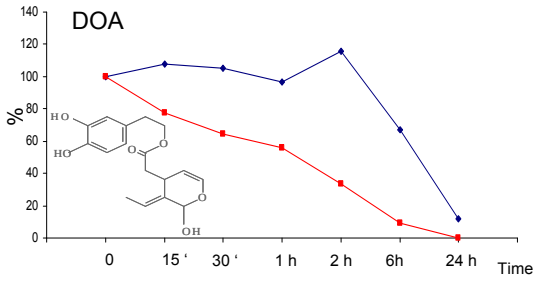
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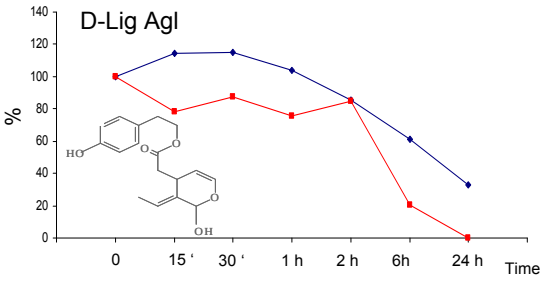
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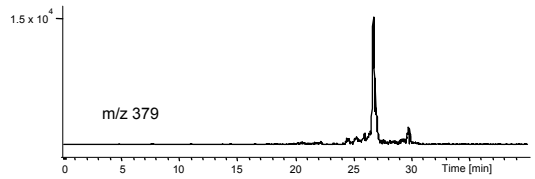
Fig. 3

a) 30 min



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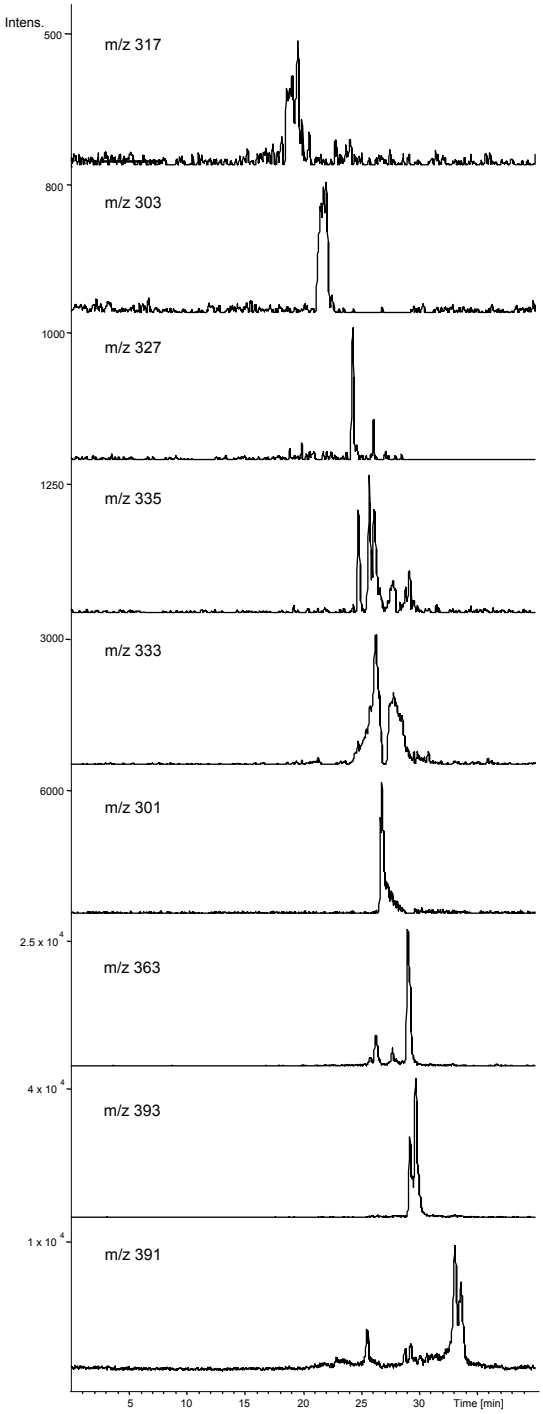
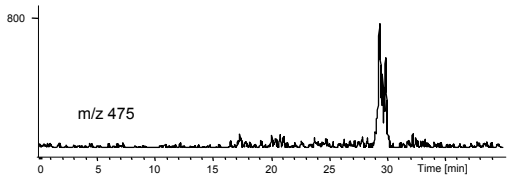
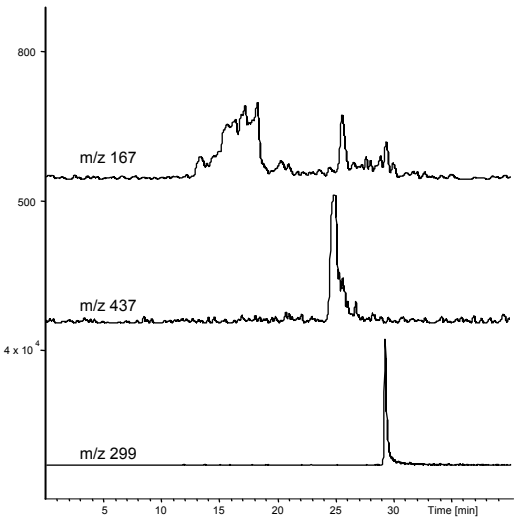


c) 6 horas



d) 24 horas



CAPÍTULO 9:

Análisis exploratorio de muestras de orina humana tras la ingesta de aceite de oliva empleando RRLC-ESI-TOF MS. Absorción y metabolismo de los polifenoles

Para poner de manifiesto la acción antioxidante de los polifenoles y su papel en la prevención de enfermedades, es crucial conocer en qué extensión son absorbidos y metabolizados, su destino en el organismo (interacción con los tejidos diana) y la forma en que son excretados, es decir su biodisponibilidad.

Los estudios *in vitro* con polifenoles del aceite de oliva que se llevaron a cabo en los capítulos 4 y 5, demostraron sus efectos anticancerígenos en diferentes líneas celulares. Tras ello, en el capítulo 8 se estudiaron los procesos de absorción y metabolización de estos compuestos fenólicos en las células. Sin embargo, estos estudios y otros recogidos en bibliografía con células *in vitro* presentan dos limitaciones fundamentales:

1) Se determina la acción de los compuestos fenólicos *in vitro* a concentraciones probablemente superiores a las podrían encontrarse en plasma y, por supuesto, a las que llegarían a los tejidos diana.

2) Se está trabajando con los compuestos "intactos" sin saber con seguridad si son ellos o sus metabolitos los que alcanzan el tejido diana, ya que es posible que el compuesto original sea excretado o metabolizado y no llegue a su destino. Además, las actividades biológicas de los metabolitos originados pueden mantener las propias de los compuestos de partida o modificarlas sustancialmente.

Por lo tanto, para poder extrapolar los resultados obtenidos *in vitro* a lo que ocurre en un organismo *in vivo* se debe tener en cuenta si los compuestos activos se encuentran a disposición de las células, a qué concentraciones y la aparición de derivados metabólicos después de su ingestión. Es, por tanto, esencial conocer la absorción y el metabolismo de estos compuestos en el organismo humano mediante estudios de su biodisponibilidad *in vivo*.

Investigaciones llevadas a cabo con animales y humanos han demostrado que algunos compuestos fenólicos del aceite de oliva como Hyty y Ty son biodisponibles, pero todavía se conoce poco sobre los mecanismos de absorción y rutas de biotransformación de otros polifenoles presentes en la matriz, como lignanos y secoiridoides, que además han demostrado elevados efectos anticancerígenos *in vitro*. Será necesario conocer si dichos compuestos pueden ser absorbidos por el organismo

para ejercer su efecto protector después de la ingestión y cuáles son los derivados metabólicos producidos a partir de las moléculas parentales, que podrían presentar efectos biológicos diferentes a los de sus precursores.

Por lo tanto, el objetivo de este capítulo 9 de la tesis fue estudiar la biodisponibilidad (absorción, metabolismo y excreción) de compuestos fenólicos del aceite de oliva pertenecientes a diferentes familias, analizando muestras de orina humana tras la ingesta forzada de aceite de oliva virgen. Para llevar a cabo este trabajo se van a seguir 4 pasos, comunes en cualquier estudio metabólico:

1) *Diseño del estudio.* Habrá que elegir los voluntarios para el estudio, establecer la dieta baja en polifenoles que se va seguir durante el mismo, las condiciones de la ingesta, asi como la recolección y el almacenamiento de muestras.

2) *Procedimiento analítico*. Este apartado incluye el procedimiento de extracción de los polifenoles de las muestras de orina y el método analítico desarrollado mediante RRLC-ESI-TOF MS para el análisis de los extractos obtenidos.

3) *Estudio estadístico*. Una vez obtenidos los cromatogramas y dada la complejidad de interpretación de los datos procedentes de muestras biológicas, el uso de técnicas quimiométricas (PCA, PLS) puede resultar muy interesante para discriminar entre las muestras de orina antes y después de la ingesta y mostrar los compuestos responsables de esta discriminación (posibles biomarcadores).

4) *Identificación de biomarcadores y otros metabolitos*. Usando toda la información disponible (polaridad de los compuestos, información del TOF, bases de datos) se procederá a la identificación de los posibles biomarcadores y otros metabolitos presentes en las muestras de orina.

Exploratory analysis of human urine by LC-ESI-TOF MS after high intake of olive oil: understanding metabolism of polyphenols

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Analytical and Bioanalytical Chemistry (under review)

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Abstract

Olive oil polyphenols have demonstrated to exhibit important biological properties which depend on their bioavailability: absorption, metabolism and elimination of the body. An analytical methodology based on rapid resolution liquid chromatography (RRLC) coupled to mass spectrometry detection with a time of flight analyzer (RRLC-ESI-TOF MS) was developed for the determination of the main olive oil phenolic compounds and their metabolites in human urine. Urine samples from ten healthy volunteers were collected before and 2, 4 and 6 h after the intake of 50 mL of extra-virgin olive oil. Samples were prepared by liquid-liquid extraction using ethyl acetate with recoveries between 35 and 75% of the phenolic compounds studied. The chromatographic separation was performed in a C18 column (150 mm × 4.6 mm, 1.8 μm) using water with 0.5% acetic acid and acetonitrile as mobile phases. Good repeatability was obtained, since the relative standard deviations (RSDs) of peak areas in the intra- and inter-day studies were 4.3 and 6.5%, respectively.

Statistical studies allowed us to discriminate between the urine samples before and after the intake, and facilitated to find out the m/z values responsible of this discrimination. Based on the very accurate mass information and the isotopic pattern provided by TOF-MS analyzer, together with other available information, ten of these biomarkers and more than 50 metabolites, obtained through phase I and phase II biotransformation reactions, were tentatively identified. Additionally, kinetic studies of the metabolites identified as possible biomarkers were developed, obtaining maximal values in the first two hours for most compounds.

Keywords RRLC-ESI-TOF MS/ Olive oil / Phenolic compounds/ Urine sample / Metabolites

Introduction

Nowadays, a growing number of studies point to the important role that extra virgin olive oil (EVOO) plays as a crucial ingredient of the Mediterranean diet due to its beneficial effects on health, associated with a lower incidence of atherosclerosis, cardiovascular disease, and certain types of cancer [1-3]. These beneficial effects of olive oil have been attributed to a high content of monounsaturated fatty acids (MUFAs) [4-6], mainly oleic acid and more recently to the presence of antioxidants in the nonsaponificable fraction, specially its high level of phenolic compounds [7, 8]. The phenolic content of olive oil depends on several agronomic and technological factors [9], but regardless the amount, the main families of phenols that have been described in olive oil are: simple phenols, lignans, flavonoids and secoiridoids [10,11]

Numerous experiments *in vitro* have shown antioxidant properties of these phenolic compounds: strong radical-scavenging activity and the ability to inhibit prooxidation processes on human low density lipoproteins (LDL) [12-14]. Moreover, these compounds have demonstrated other biological properties including activity against platelet aggregation and anti-proliferative effects in different cancer cell models [15,16].

One of the requirements to extent their protective effect and to evaluate their biological activity *in vivo* is the knowledge of their bioavailability and metabolism in humans. The recovery of polyphenols in plasma or urine after intake of olive oil phenols, and the amount and form in which they are present, may explain their absorption and metabolism in human body [17].

Different analytical methods have been proposed to investigate the *in vivo* effect of the olive oil phenols in biological fluids so far, especially the effect of hydroxytyrosol, (Hyty) tyrosol (Ty) and oleuropein [18, 19]. Several papers have described the absorption of Hyty and oleuropein in rat plasma after their administration as pure compounds by GC-MS [20] and HPLC with UV [21], fluorescence [22], mass spectrometry detection [23] and radioanalysis [24]. The oral or intravenous administration of these compounds to experimental animals and their detection in plasma has provided evidence of its absorption. However, studies in which phenolic ingestion is closer to typical dietary patterns may be more appropriate for estimating bioavailability that those after the administration of pure phenolic compounds. Besides, some studies suggest that the rat is not the appropriate model for the study of Hyty metabolism [25]. Miró-Casas et al proposed a GC-MS method to quantify Hyty and 3-

O-methyl-Hyty in human plasma and urine after real-life doses of virgin olive oil [26]. Both compounds appear rapidly in plasma (32 and 53 min respectively) as conjugated forms, mainly glucuronoconjugates. Due to data on plasma phenol concentrations are scarce, an alternative is to look at olive oil phenols excreted in urine, which may provide information about the form in which phenols are present in plasma. Several human and animal studies have shown that the main olive oil phenols Hyty and Ty are bioavailable. The urinary level of Ty and especially Hyty were determined mainly by GC-MS after olive oil consumption or olive oil enriched with a phenolic extracts [27-31] and HPLC- radiometric detection after consumption of labeled Hyty. [32]. In these studies it was initially postulated that Hyty and Ty were absorbed, metabolized and eliminated in urine in either free form or conjugated, mainly as glucuronides. Different metabolites of Hyty (sulfoconjugate, 3-O glucuronide conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenyl acetaldehyde (DOPAL), 3,4-dihydroxyphenylacetic acid (DOPAC)) were identified in rat urine by radyoanalysis after orally or intravenously dosing of the labelled Hyty [24,33]. Caruso et al. described novel pathways of Hyty metabolism identifying for the first time homovanillic alcohol and homovanillic acid in human urine after ingestion of virgin olive oil [34].

As can be seen, literature data on the metabolism of olive oil phenolic compounds in humans are very limited, and the majority of studies conducted in this area have been focused on Hyty, Ty and oleuropein. There are no previous reports about the bioavailability of some other phenolic compounds described in olive oil, despite recent studies have demonstrated that individual phenolic fractions rich in lignans and especially in secoiridoids have a significantly stronger ability to decrease cell viability and the expression status of HER2 in different types of human breast carcinoma cells in comparison with simple phenols [11,35,36]. This lack of information is due to the absence of commercially available pure standards and the difficulty of developing sensitive method to detect the presumptively low concentrations of these compounds in biological systems.

Thus, the aim of the present study was to develop an analytical methodology based on RRLC-ESI-TOF MS to identify by first time, the urinary excretion of different families of phenolic compounds as free compounds or in conjugated form after ingestion of commercially available virgin olive oils, allowing to further elucidate the *in vivo* kinetics of olive oil phenolics in habitual consumption quantities.

Experimental

Chemicals and samples

Acetonitrile from Lab-Scan (Dublin, Ireland) and acetic acid from Fluka (Buchs, Switzerland) were used for preparing the mobile phases. For the extraction procedure, sodium acetate and methanol were purchased from Panreac (Barcelona, Spain), hydrochloric acid from Scharlau (Barcelona, Spain) and ethyl acetate from Lab-Scan (Dublin Ireland). Water was deionized by using a Milli-Q-system (Millipore, Bedford, MA, USA). β-glucuronidase (type H-2 crude solution from Helix pomatia; 111000 U/mL of β-glucuronidase and 1079 U/mL of sulfatase) and taxifolin, used as internal standard (I.S.), were supplied by Sigma-Aldrich (St. Louis, MO, USA). A stock solution of taxifolin was prepared in methanol with a final concentration 100 µg/mL and stored at -20ºC until use. Synthetic urine, free of the tested compounds was purchased from Alltech Associates (Deerfield, IL, USA) and was prepared according to the manufacturer's instructions, dissolved in water.

Aiming to chose a very high-phenol olive oil for the intake, several olive oils were selected and analyzed and, finally, a mixture of two extra virgin olive oils bought in a supermarket: 50% Arbequina, 50% Picual was used.

All chemical were analytical grade and used without further purification

Human experiments

Ten healthy volunteers (5 male and 5 female) with an age ranging between 24 and 35 years old participated in the intervention study. Subjects had an average weight of 71.8±11.5 kg (men, 81.2±7.3 kg, women 62.4±4.6 kg) and a body mass index of 24.4±2.9 (men, 26.2±2.7 women, 22.5±1.8). The study was approved by the ethical committee at the University of Granada. The subjects volunteered for the study and gave their written consent after receiving carefully information about the study. All volunteers followed a phenolic-free diet for 2 days (wash-out period), avoiding several foods and beverages from their diet (fruits, vegetables, cereals, chocolate, coffee, tea, wine, beer etc…) and specifically excluding olive oil. On day 3 after the overnight fast, all subjects consumed 50 mL of olive oil with 30 g of bread (intake of olive oil in the Mediterranean countries is estimated to be 30-50 mL/day [18]) between 8.00 and 8.30 h at the laboratory. Subjects stayed at the laboratory the whole day and were allowed to drink only water for 6 h in order to assure that olive oil was the only source of phenolic

compounds. Urine samples were collected in special urine-collecting plastic bottles during the washout period (first void spot urine in the morning) and in three different time collection periods (at 0-2, 2-4, 4-6 h) after the ingestion. After the measurement of the urinary volume, the samples were immediately stored at -20ºC. A human urine sample obtained from fasted healthy individuals was prepared and used as a blank.

Extraction procedure

Urine is one of the easiest biological fluids to collect, but contains a high salt concentration that can easily interfere with the electrospray ionization process, so it is important to remove the electrolytes that are present prior to analysis. This is usually achieved in part by extraction into solvents, particularly those that are inmiscible with water (ether or ethyl acetate).

2 mL of urine (pH~7) was acidified at pH 2.5 with 0.5 M HCl/MeOH (50/50, v/v) and a liquid-liquid extraction was carried out by addition to each sample of 3 mL ethyl acetate. After 10 min stirring in a vortex and 10 min centrifugation at 3500 r.p.m, the supernatant was evaporated to dryness. The dried sample was reconstituted in 100 µL of methanol. To assay the performance of the mass spectrometer and to correct for variability, 10 µL of taxifolin (100 µg/mL), a flavanonol with similar structure to the compounds under study and that was not present in urine, was chosen as internal standard and added to the extract of the sample. Finally the extracts were stored at -20 ºC until analysis.

Enzymatic hydrolysis

To verify the presence of the conjugated forms of polyphenols and their metabolites urine samples were subjected to enzymatic hydrolysis with deconjugating enzymes that hydrolyze glucuronides and sulphate ester.

2 mL of urine sample was adjusted to pH 5 by the addition of 800 µL 1M sodium acetate buffer. An aliquot of 45 µL of the crude enzymatic preparation (β-glucuronidase from Helix Pomatia), containing 5000 units of β-glucuronidase and 49 units of sulfatase, was added to the sample and incubated for 4 h at 37 ºC in a heating bath. After samples cooled to room temperature the pH was adjusted to pH 5 by the addition of 0.5 M HCl/MeOH (50/50, v/v) and the free aglycones were extracted with 3 mL of ethyl acetate, as described above.

The most suitable type of β-glucuronidase and the optimal incubation time for the enzymatic hydrolysis were investigated previously in literature [26].
Urines from the wash-out period were subjected to the same extraction procedures (with and without enzymatic hydrolysis) to establish basal background and to preclude possible interferences.

RRLC-ESI-TOF MS

Analyses were carried out operating on an Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV-Vis detector. The analytical column used for the separation of the compounds was a Zorbax C18 (4.6 x 150 mm, 1.8 μm particle size) protected by a guard cartridge of the same packing, operating at 30 ºC and a flow rate of 0.8 mL/min. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the separation was performed using the gradient elution program: 0 min, 95% A and 5% B; 10 min, 70% A and 30% B; 12 min, 67% A and 33% B; 17 min 62% A and 38% B; 20 min, 50% A and 50% B; 23 min, 5% A, 95% B; 25 min, 95% A and 5% B. Finally, the column was re-equilibrated for 10 min. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA) and degassed in an ultrasonic bath prior to HPLC analysis. A volume of 10 μL urine extracts was injected.
The RRLC system was coupled to a Bruker Daltonik microTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). TOF analyzers provide greatly improved mass resolution (5,000–10,000 at 250 *m/z*) and significantly higher sensitivity and accuracy when acquiring full-fragment spectra compared with traditional instruments. Due to the high flows of mobile phase used (0.8 mL/min) and in order to achieve a stable electrospray, a flow divisor 1:3 was used to reduce the flow delivered into the mass spectrometry to 0.2 mL/min. According to this inflow, the ESI parameters were chosen: nebulizer pressure was set at 2 bar, dry gas flow, 9 L/min, and dry gas temperature, 190 ºC.
The transfer parameters of the mass spectrometer were similar to those previously optimized in recent works with the same families of phenolic compounds [11] acquiring spectra in the range of 50-800 *m/z* in negative mode. The negative ionization mode was

found to be more sensitive than positive ionization mode as has been similarly described for other classes of phenolic compounds [37, 38]

SmartFormula^{TM} tool within DataAnalysis was used for the calculation of elemental composition of compounds; it lists and rates possible molecular formulas consistent with the accurate mass measurement and the true isotopic pattern (TIP). If the given mass accuracy leads to multiple possible formulas, the TIP adds a second dimension to the analysis doing a sophisticated comparison of the theoretical with the measured isotope pattern (SigmaValue^{TM}). For routine screening, an error of 5 ppm and a threshold sigma value of 0.05 are generally considered appropriate.

The calibration of the MS was performed using sodium formate clusters by introducing externally a solution containing sodium hydroxide in the sheat liquid of some formic acid in water:isopropanol 1:1 v/v, for instance, with a pump at the beginning of the analysis (external calibration).

Statistic

All the datafiles were exported into mzXML format using Bruker Daltonics's DataAnalysis software. Only the chromatographic region from 1 to 29 min was used for further analysis.The alignment of chromatograms was performed using XCMS software (The Scripps Research Institute, La Jolla, USA) with default parameters [39]. The resulting tables included the detected ion features and their peak areas. As the primary interest was in metabolites of polyphenols, only those peaks were selected which were not present at 0 time point, but were present at later ones. These peaks were imported into Simca-P+ software package, version 12.0 (Umetrics, Umeå, Sweden) for further multivariate analysis. No normalization of the peak areas was performed firstly due to low systemic variance in the data (RSD of the total areas was within 30%) and secondly to avoid constrain effects of this type of normalization on data covariance structure [40].

Results and discussion

RRLC-ESI-TOF MS analysis

Based on the chromatographic and mass spectrometry conditions of previous studies where the same families of polyphenols from olive oil samples were the analytes of interest [11], the optimization of the RRLC-ESI-TOF MS method for the determination of metabolites of olive oil polyphenols in human urine was developed. To achieve this

purpose, two different control samples were used and compared: synthetic urine and blank urine spiked with diluted olive oil extracts containing the different families of polyphenols at low concentrations. Synthetic urine was used to ensure the absence of any endogenous compound that could interfere with the assay, and blank urines, obtained from volunteers after 2 days with free polyphenols diet and 12 h fasting, to make a closer approach to the real samples.

Figure 1 shows the base peak chromatogram (BPC) of (a) synthetic urine and (b) blank urine spiked with diluted olive oil extracts under the optimum RRLC-ESI-TOF MS conditions. The most representative components of the phenolic fraction of virgin olive oil (two analytes belonging to each family of phenolic compounds) were selected to calculate the recovery and to carry out the optimization: simple phenols (Ty and Hyty), flavonoids (luteolin (Lut) and apigenin (Apig)), lignans (pinoresinol (Pin) and acetoxipinoresinol (Ac Pin)) and secoiridoids (Oleuropein aglycon (Ol Agl) and Ligstroside aglycon (Lig Agl)).

As can be seen, optimum separation with good response and peak shape were obtained for the eight compounds studied with both control samples. Even with the blank urine, the RRLC-ESI-TOF MS method provided a separation good enough among the eight polyphenols from interfering compounds present in the sample over a short run time of 25 min. Moreover, it is important to keep in mind that the application of mass spectrometry for the analysis of these analytes confers a high specificity and selectivity to the assay.

Recoveries of the main compounds were calculated by comparison between peak areas (corrected by using the I.S.) of every compound in the olive oil extract analyzed by the normal procedure and those obtained after adding the same amounts of the olive oil extract to synthetic urine and blank urine and extracting the compounds with ethyl acetate as explained above. Extraction efficiency ranged from 46.9 to 74.9% for the families of simple phenols, lignans and flavonoids, and were a bit lower (around 35%) for the family of secoiridoids (Table 1). The results were similar for both control samples (synthetic and blank urine) used, fact which indicates the specificity of the method with regard to the interference of endogenous compounds.

The precision of the assay was evaluated by three consecutive injections (n = 3) of the control samples in the same day (intra-day repeatability) and in three different days

(inter-day repeatability) obtaining values of relative standard deviation (RSD%) on the peak area ratio above 4.3 and 6.5% respectively (data not shown to contain the size of the paper). These RSD indicate that the proposed methodology is reproducible and suitable for carrying out bioavailability studies.

To evaluate the applicability of the method, all urine samples collected before (wash-out period) and after the olive oil consumption were analyzed. After each series of analysis, a quality control sample (synthetic urine spiked with an olive oil extract) was injected for assessing the performance of the chromatographic procedure in terms of retention time stability and signal sensitivity. Figure 2 shows the base peak chromatogram (BPC) of a human urine sample collected 2 hours after the oral administration with the optima RRLC-ESI-TOF MS conditions (a). It can be easily observed the complexity of this type of samples. In the same figure, it is possible to observe the extracted ion chromatograms (EIC´s) of some possible metabolites found in the human urine samples (b).

Discrimination of urine samples using statistical analysis

As shown in Fig. 2 biological samples are often very complex and as because of that, full interpretation of the acquired data can be extremely difficult, making manual analysis not feasible due to a large number of samples in biological and medical research. For this purpose, chemometrics offers a variety of methods which allow investigation of the structure of all the data at once (e.g. Principal Component Analysis(PCA)) as well as discrimination and classification of the samples (e.g. Partial Least Squares (PLS), Artifitial Neural Networks (ANN) based methods).

There can be a number of processes that occur in the organism after the large intake of olive oil, which might be reflected in the composition of body fluids, but we have deliberately focused on metabolites of polyphenols. These metabolites should not be present at 0 time point, due to the low polyphenol diet that the subjects had before the experiment, and should appear at later time points. Thus among all the peaks picked by XCMS we selected for the analysis those with no or low intensity at time point 0.

PCA was used to evaluate the quality of the data and observe its structure. A lot of the variation present in the data is explained by the first two principal components (more than 65%) and as can be seen on the Fig. 3. The samples before the intake are tightly

clustered together which was expected with selection of peaks absent at this time point. Other samples are more scattered with 6 h time point tending to be closer to 0 time point, which suggests that after 6 h the excretion of polyphenol metabolites declines and the organisms starts to return to its normal state. To justify this and to find the peaks that have most influence on the difference between samples at different time points batch PLS was used. The peaks, most influential for the model, can be selected on the basis of variable importance in the projection (VIP) scores, the list of these peaks with VIP>1 is given in Table 2.

Identification of the metabolites

In order to identify the compounds which resulted to be more responsible of the discrimination of the samples (biomarkers) after the statistical analysis and other possible metabolites, the urine profiles were studied in depth taking into account the polarity of the compounds (they leave the column in decreasing order of polarity) and the information about the possible metabolism reaction that can occur in the organism. Furthermore, ESI-TOF MS analyzer provides information about accurate mass and isotopic pattern that allow obtaining a reduced number of possible molecular formula that then can be matched against available databases.

The chemical reaction of metabolism can be classified in *Phase I reactions* and *Phase II reactions*. Phase I reactions usually precede Phase II, though not necessarily, and the most important ones are oxidation, hydrogenation, hydration, decarboxilation, hydroxylation and methylation. Phase II reactions are usually known as conjugation reactions and include glucuronidation, sulfoconjugation, acetilation, glutamination.

To confirm the presence of the conjugated forms of polyphenols and their metabolites, analyses of the urine samples were conducted before and after enzymatic hydrolysis with deconjugating enzymes. It was possible to identify glucuronidic form of the analytes, as well as other conjugated forms, like sulfatase derivatives, because β-glucuronidase exhibits limited sulfatase activity.

Using all the mentioned information, 60 metabolites could be tentatively identified in the urine profile. All metabolites are summarized in Tables 3 (phase I metabolites) and 4 (phase II metabolites) including retention time, *m/z* experimental, the error and sigma value (comparison of the theoretical with the measured isotope pattern), molecular formula and a list of possible compounds. The compounds identified as biomarkers in

the statistical analysis are indicated in bold. As can be seen all the compounds are phase I metabolites, specially those derivates from deacetoxy oleuropein aglycon (DOA) and only one of them, is a glucuronoconjugate.

Metabolites obtained through phase I reaction are shown in Table 3 and classified by the type of metabolism reaction. Hydrogenation, hydroxylation, hydration and methylation were the reactions more frequently conducted, although the bioavailability (absorption, metabolism and elimination) appears to differ greatly between the various polyphenols. In Fig. 4, we can observe the extracted ion chromatograms (EIC´s) of some metabolites identified in table 3 during the wash-out period (I) and 2 hours after the intake of olive oil (II). Peaks corresponding to metabolites appeared after 2 h of the olive oil intake, whereas none of these compounds were detected during the wash-out period.

In general, the most abundant metabolites found in human urine come from compounds of the family of secoiridoids (Ol Agl, DOA, Lig agl and deacetoxy ligstroside aglycon (D-Lig Agl)) that were the most likely to be absorbed and metabolized. There are no available information in literature about the absorption and metabolism of these compounds and, so far, it was thought that Ol Agl and Lig Agl may be hydrolyzed in the gastrointestinal tract and the resulting polar phenols, Hyty and Ty, absorbed and metabolized [30]. However, the results obtained in the present trial suggest that, at least, part of these compounds is absorbed and metabolized and as well they represent the most abundant metabolites.

One of the most probable pathways for the metabolism of these compounds is the hydrogenation. The elenolic acid (EA), compound which is part of the molecular structure of the secoiridods, was also subjected to this hydrogenation reaction which could explain the presence of the hydrogenated metabolites of the secoiridoids. Some previous authors failed to detected EA metabolites in rat urine after olive oil ingestion [31]. In the present study the hydrogenated metabolite of EA appeared with high intensity and was identified in the statistical analysis as one of the possible biomarkers. Its bioavailability results very interesting and it could be rather useful as a biomarker because it is not present in other food sources as, for instance, Hyty or Ty.

Hydroxylation and hydration were also common pathways of the secoiridoids, especially for Ol Agl and DOA (compounds containing in their structures a molecule of Hyty). Some doubts appeared about Lig Agl and D-Lig Agl (compounds containing in their structure a molecule of Ty) because the *m/z* values of their possible hydroxylated

and hydrated metabolites matched up with those of other metabolites of DOA and Ol Agl. Taking into account the information available in literature about the limited diffusion into the cell of compounds with a lower degree of hydroxylation -such as ty and their derivatives [41]- the most adequate way of interpretation it would be that these masses correspond to Hyty derivates, however with the techniques used in this study it was not possible to confirm this information. Anyway, we have indicated in the table all the available possibilities. Small amounts of hydroxylated and hydrated metabolites of hydroxytyrosol acetate (Hyty-Acet) were detected as well.

Methylation was the preferential pathway for Hyty and we could also find the methyl conjugate of DOA with high intensity. The results indicate that these compounds underwent the action of catechol-O-methyl transferase (COMT), enzyme involved in the catecholamine metabolism. Other phase I reactions, mainly hydroxylation and hydration, could precede or follow the action of COMT on compounds as Hyty, DOA and Ol Agl. In this way, metabolites as methyl Hyty + OH, methyl Ol Agl + OH, methyl DOA + OH, methyl Ol Agl + H_2O and methyl DOA + H_2O were found in the urine. Some even suffered a double hydroxylation before or after the methylation (see table 3). The presence, although in low quantities, of MOPAL (Methyl Hyty – H_2) and homovanillic alcohol (HVA) (methyl Hyty- H_2 + OH) previously identified in literature [24, 32, 34] implies a sequential oxidation of methyl-Hyty ethanol side chain catalyzed by alcohol and aldehyde dehydrogenase, respectively. In the same pathway of HVA, a compound with *m/z* 347.1158 was tentatively identified as (Methyl DOA – H_2 + OH).

It is the first time that the presence of methylated derivatives, different from alcohol homovanillic, are studied and found in urine samples. No methyl conjugates of Ty and derivatives compounds (Lig agl and D-Lig Agl) were observed as could be expected, since methylation by COMT requires an ortho-diphenolic structure, absent in these compounds [41].

It is worth noting that the secoiridoid DOA participated in all the phase I reactions described in our study and all its metabolites showed up with high intensity. Besides, most of them (DOA + H_2, DOA- H_2, methyl DOA+ OH, DOA + H_2O, methyl DOA+ H_2O and DOA + CH_3) were identified as biomarkers of the olive oil intake.

The compounds in Table 4 are phase II metabolites, most of them identified as glucuronoconjugates. The identity of these compounds was confirmed with enzymatic hydrolysis, showing the disappearance of the peaks suspected of being

glucuronoconjugates and the parallel increase of the corresponding unconjugated compounds (frees polyphenols or phase I metabolites). Figure 5a shows, as an example, the extracted ion chromatograms (EIC´s) of three compounds identified as glucuronides and sulfoconjugates (on the left) and the corresponding unconjugated compounds obtained after the enzymatic hydrolysis (on the right).

As an example, the chromatographic peak of *m/z* 329.0868 disappeared after treatment of the urine samples with β-glucuronidase with the peak *m/z* 153.0562 increasing in size, assuming that this peak posses a glucuronide group. Both peaks showed the same $[M-H]^-$ ion at *m/z* 329.0868 characteristic of a monoglucuronidated metabolite of Hyty. These data together with that obtained from the enzymatic hydrolysis experiments confirmed these metabolites corresponded to the monoglucuronides of Hyty, although we can not distinguish between structural isomers [41]. Many of the glucuronoconjugated compounds showed more than one peak with exactly the same *m/z* (isomers). Similarly, peak wit *m/z* 313.0929 and 555.1719 disappeared after hydrolysis with β-glucuronidase and suggesting that these metabolites correspond to the glucuronides of Ty and Ol Agl + H_2 respectively.

Figure 5b shows the extracted ion chromatograms (EIC´s) of other important metabolites identified as glucuronoconjugates. However, the position of the substituents on the aromatic ring was not possible to confirm with the technique used in this study and it would be necessary to use nuclear magnetic resonance spectroscopy (NMR) to get more structural information.

It is worth noting the presence of glucuronoconjugates of compounds belonging to the most representative phenolic families described in olive oil: simple phenols (Hyty, Ty and Hyty-Acet), secoiridoids (DOA, Ol Agl, D-Lig Agl and Lig Agl), and lignans (Pin, and Ac pin). To the best of our knowledge, the metabolism of lignans has not been reported in any detail and one of the few references has been developed recently [42]. In that study, pinoresinol-glucuronide was identified after incubation of pinoresinol using differentiated Caco-2/Tc7 cells monolayers as a model of the human intestinal epithelium.

As far as flavonoids are concerned, the product of both methylation and glucuronidation were observed and peaks with *m/z* 459.0975 and 475.0868 were identified as methyl-monoglucuronides of apigenin and luteolin, respectively. The latter mentioned metabolite, luteolin-methyl glucuronide was also identified by Soler et al [42]. Although new metabolites were identified in the intestinal epithelial cells, they showed limited

metabolism of olive oil phenolics, and it is most likely that glucuronides are products of hepatic metabolism [41, 42].
Besides, almost all the phase I metabolites identified in table 3 were subsequently subjected to glucuronoconjugation reactions and new glucuronoconjugates appeared especially those related with the Ol Agl (Ol Agl – H_2 + glucuronide, Ol Agl + CH_3 + glucuronide, Mehtyl Ol Agl - H_2 + glucuronide).

The absence of the excretion of sulfated metabolites in human urine (with the exception of Hyty- sulfoconjugate) is in agreement with data from previous studies in humans [27, 28, 29, 30] and *in vitro* studies with HepG2 cells in which methylated and glucuronidated metabolites were the main conjugated observed [41].

Not all the compounds that were ingested were subsequently found in the urines in free forms or as phase I or phase II metabolites. This could be because these compounds were not absorbed, excreted with the faeces, destroyed in the gut, accumulated in organs, or excreted through another metabolic pathway [17]

Metabolic kinetic of polyphenols in human urine

The metabolic kinetic of the compounds identified as biomarkers in human urine after the olive oil intake was followed along six hours. Figure 6 clearly illustrates the metabolic pattern of the compounds under study, showing the average results from the ten volunteers as μg excreted of taxifolin (I.S). Due to the absence of pure standards, the metabolites were quantified on the basis of the response factors of I.S., which was also used to correct any possible deviations. The results indicated that the majority of the metabolites studied followed the same kinetic, reaching maximum concentrations in the human urine at 2 hours after the administration, except for the compound with *m/z* 349.1283 (methyl DOA + OH) which appeared with higher intensity at 4 h. In the samples taken 6 h after the large intake of oil, a fast decrease of the intensity of the compounds was observed with a trend toward basal conditions. These results are in good agreement with previous studies about the kinetic of Hyty and Ty, where the maximum concentrations of the metabolites were reached in the first 2-4 h after the intake [28, 29].

Furthermore, during this period, the concentration of the compounds fluctuated widely with the individual, reflected as high SD value; but no significant difference in urinary recovery could be observed between men and women for any of the polyphenols analyzed. The basal levels of the studied metabolites found in the blank urine samples and in the wash-out period were practically zero.

Conclusions

A direct and reproducible RRLC-ESI-TOF MS method was developed to carry out the exploratory analysis of human urine after high intake of olive oil. Simultaneous identification of more than 60 metabolites of olive oil polyphenols was achieved by using the information obtained from the statistical analysis, the different metabolism reaction, polarity of the compounds, enzymatic hydrolysis and the valuable information from the time-of flight mass analyzer.

In other works previously developed specific information about the type of conjugates was not provided, and the authors were mainly focused on the determination of metabolites of Hyty, Ty and oleuropein. This is the first report in which metabolites of practically all the compound described in olive oil have been found in human urine samples, suggesting that all the compounds are absorbed to a greater or lesser extent. However, bioavailability appeared to differ greatly between the various polyphenols, and the most abundant metabolites came from those phenolic compounds containing a catechol group, such as Hyty and the secoiridoids Ol Agl and DOA. Phenolic compounds were subjected to different Phase I and Phase II reactions, but the most common metabolic reactions were methylation and glucuronidation.

Statistical analysis allowed us to discriminate between the urine samples and to identify at least ten possible metabolites (mainly derived from DOA) that could be used as biomarkers of olive oil intake. The developed method was also successfully applied to monitor the levels of these biomarkers in human urine after the intake of olive oil, and the results indicated that the highest level of most compounds was detected at 2h after administration.

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Figure Captions

Fig. 1 Chromatographic separation of the phenolic compounds recovered from synthetic urine (a) and blank urine (b) spiked with a diluted olive oil extract. The well-known compounds described in olive oil are represented in grey: 1: hydroxytyrosol, 2: tyrosol, 3: luteolin, 4: pinoresinol, 5: acetoxipinoresinol, 6: apigenin, 7: Oleuropein Aglycone, 8: Ligstroside Aglycone

Fig. 2 (a) Base peak chromatogram (BPC) of a human urine sample (2 h after the intake of olive oil) analyzed using the optima RRLC-ESI-TOF MS method. (b) A more detailed view of this BPC highlighting in grey all possible metabolites found in the human urine 2 after the olive oil intake

Fig. 3 Results of the multivariate statistical analysis of urine samples after the intake of olive oil. PCA scores plot, first two principal components cover 63.9% and 11.5% of the variation respectively

Fig. 4 Extracted ion chromatograms (EIC´s) of the main Phase I metabolites identified in human urine samples during the wash-out period (I) and 2 h after oral administration of 50 ml of olive oil (II). The numbering of the peaks in this figure refers to the numbering of the compounds in table 3

Fig. 5 (a) Extracted ion chromatograms (EIC´s) of three compounds identified as glucuronides (on the left) and the corresponding unconjugated compounds obtained after the enzymatic hydrolysis (on the right). (b) More examples of compounds identified as glucuronides and sulfoconjugated. (I) samples in the was-out period (II) 2 h after oral administration (III) 2 h after oral administration and with the enzymatic hydrolysis (IV) samples in the washout period subjected to the same enzymatic hydrolysis. The numbering of the peaks in this figure refers to the numbering of the compounds in table 4

Fig. 6 Bioavailability of the main biomarkers identified in the human urine after the olive oil intake. Bars represent the average of data obtained from ten individuals expressed as μg excreted of I.S during each time period and errors (SD) are expressed

as line on the top of each bar. Concentrations at wash-out period (urine collected just before oil administration) were practically zero. To facilitate the interpretation of the figure compounds were put in pairs according to the intensity

Table 1 Extraction recoveries of the main phenolic compounds identified in olive oil from synthetic and blank urine samples spiked with diluted extracts of olive oil. Samples were analyzed by RRLC-ESI-TOF MS and recoveries calculated as described in the experimental section. Values are means from three independent experiments.

Compounds	m/z	Recovery %	
		Blank urine	Synthetic urine
1. Hydroxytyrosol	153	51.2	46.9
2. Tyrosol	137	60.4	56.1
3. Luteolin	285	74.9	66.5
4. Pinoresinol	357	58.5	64.1
5. Acetoxipinoresinol	415	69.3	69.7
6. Apigenin	269	73.4	63.7
7. Oleuropein aglycon	377	35.1	37.3
8. Ligstroside aglycon	361	40.4	46.5

Table 2 m/z values and retention times of the possible biomarkers of the olive oil intake obtained by statistical analysis.

m/z	Retention time, s
393.119	1002.32
349.128	898.141
321.134	847.395
243.086	682.154
434.223	860.882
337.128	752.647
229.076	513.481
391.205	1159.82
363.145	1190.31
431.203	837.384
555.170	810.844
351.144	865.707
445.255	1372.96
181.055	1019.49
457.212	935.351
317.102	1018.47
322.144	851.324
459.109	992.087
333.134	1169.11
414.231	1367.6

Table 3 Phase I metabolites, classified by the metabolism reaction, identified in human urine 2 h after the intake of olive oil

Phase I reactions		**Retention time (min)**	**m/z experimental**	**error**	**sigma**	**Molecular Formula**	**Posible Compounds**
Hydrogenation (+ H_2)	**1**	**11.308**	**243.0868**	**2.3**	**0.004**	**$C_{11}H_{15}O_6$**	**EA + H_2**
	2	**14.084**	**321.1344**	**-2.3**	**0.014**	**$C_{17}H_{21}O_6$**	**DOA + H_2**
	3	15.355	305.1397	-0.9	0.107	$C_{17}H_{21}O_5$	D- Lig Agl + H_2 *
	4	14.686	379.1410	-3.1	0.029	$C_{19}H_{23}O_8$	Ol Agl + H_2 (isomer)
	5	**19.720**	**363.1455**	**-1.5**	**0.021**	**$C_{19}H_{23}O_7$**	**Lig Agl + H_2**
Dehydrogenation (-H_2)	**6**	**16.860**	**317.1027**	**1.0**	**0.019**	**$C_{17}H_{17}O_6$**	**DOA-H_2**
Hydroxylation (+ OH)	7	10.650	211.0615	-1.2	0.063	$C_{10}H_{11}O_5$	Hyty-Acet + OH *
	8	13.710	335.1132	1.2	0.052	$C_{17}H_{19}O_7$	DOA + OH
	9	16.493	319.1188	-0.3	0.008	$C_{17}H_{19}O_6$	D-Lig Agl + OH = DOA
	10	**16,660**	**393.1191**	**0.1**	**0.004**	**$C_{19}H_{21}O_9$**	**Ol Agl + OH**
	11	19.336	377.1248	-1.7	0.037	$C_{19}H_{21}O_8$	Lig Agl + OH = Ol Agl
Hydration (+ H_2O)	12	7.562	213.0768	4.8	0.063	$C_{10}H_{13}O_5$	Hyty- Acet + H_2O *
	13	**12.495**	**337.1282**	**3.3**	**0.007**	**$C_{17}H_{21}O_7$**	**DOA + H_2O**
	14	**14.084**	**321.1344**	**-2.3**	**0.014**	**$C_{17}H_{21}O_6$**	**D-Lig Agl + H_2O = DOA + H_2**
	15	14.686	379.1410	-3.1	0.029	$C_{19}H_{23}O_8$	Lig Agl + H_2O = Ol Agl + H_2
	16	16.626	395.1361	-3.4	0.080	$C_{19}H_{23}O_9$	Ol Agl + H_2O
Methylation (+ CH_3)	17	10.639	167.0716	-1.3	0.164	$C_9H_{11}O_3$	Hyty + CH_3 *
	18	**19.326**	**333.1345**	**-0.3**	**0.046**	**$C_{18}H_{21}O_6$**	**DOA + CH_3**
Methylation + others reactions	19	8.532	185.0817	1.5	0.057	$C_9H1_3O_4$	Methyl Hyty + OH
	20	10.121	165.0543	8.6	0.325	$C_9H_9O_3$	Methyl Hyty - H_2 (mopal) *
	21	11.342	181.0516	-5.4	0.228	$C_9H_9O_4$	Methyl Hyty- H_2+OH (HVA) *
	22	13.164	365.1242	0.1	0.013	$C_{18}H_{21}O_8$	Methyl DOA + 2 OH *
	23	**14.319**	**351.1444**	**1.5**	**0.015**	**$C_{18}H_{23}O_7$**	**Methyl DOA + H_2O**
	24	14.890	423.1315	-4.3	0.112	$C_{20}H_{23}O_{10}$	Methyl Ol Agl + 2 OH *
	25	14.904	409.1520	-3.8	0.022	$C_{20}H_{25}O_9$	Methyl Ol Agl + H_2O
	26	**14.921**	**349.1283**	**2.8**	**0.018**	**$C_{18}H_{21}O_7$**	**Methyl DOA + OH**
	27	14.971	347.1158	-6.2	0.149	$C_{18}H_{19}O_7$	Methyl DOA- H_2 + OH
	28	19.720	407.1355	-1.9	0.019	$C_{20}H_{23}O_9$	Methyl Ol Agl + OH

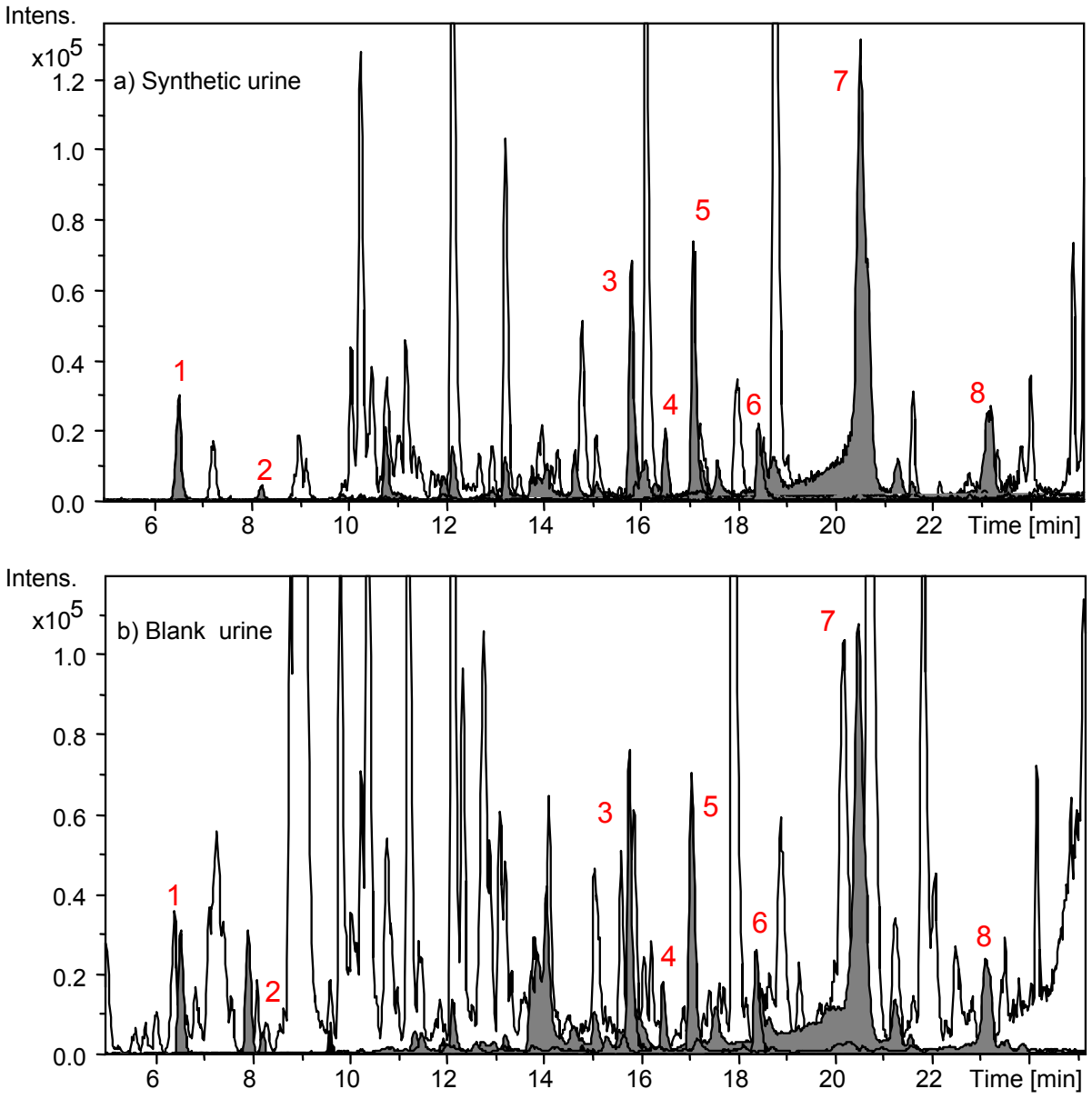
* means compounds present in small traces.
We represent in bold compounds found out in the statistical analysis responsible of the discrimination between urine samples before and after the intake of olive oil (biomarkers)

Table 4 Phase II metabolites, classified by the conjugation reaction, identified in human urine 2 h after the intake of olive oil.

Phase II reactions		**Retention time (min)**	**m/z experimental**	**error**	**sigma**	**Molecular Formula**	**Posible Compounds**
Glucuronidation	1	5.672-6.107	329.0868	3.1	0.030	$C_{14}H_{17}O_9$	Hyty- glucuronide
	2	5.756	313.0912	5.3	0.012	$C_{14}H_{17}O_8$	Ty- glucuronide
	3	10.071,10.355	371.1071	-1.4	0.128	$C_{16}H_{19}O_{10}$	Hyty- Acet- glucuronide
	4	13.198	495.1497	2.2	0.130	$C_{23}H_{26}O_{12}$	DOA- glucuronide *
	5	11.709	533.1639	4.7	0.053	$C_{26}H_{30}O_{12}$	Pin- glucuronide
	6	12.04,12.362	591.1705	2.4	0.160	$C_{28}H_{31}O_{14}$	Ac Pin- glucuronide *
	7	12.914	479.1687	-14.2	0.093	$C_{23}H_{26}O_{11}$	D- Lig Agl- glucuronide
	8	13.014	553.1550	2.2	0.061	$C_{25}H_{29}O_{14}$	Ol Agl- glucuronide
	9	13.399	537.1626	-2.4	0.070	$C_{25}H_{29}O_{13}$	Lig Agl- glucuronide
Hydrogenation + Glucuronidation	10	10.907,11.592	497.1651	2.7	0.011	$C_{23}H_{29}O_{12}$	DOA + H_2- glucuronide
	11	12.830	481.1715	1.5	0.017	$C_{23}H_{29}O_{11}$	D-Lig Agl + H_2- glucuronide
	12	**13.064, 13.449**	**555.1709**	**1.8**	**0.006**	**$C_{25}H_{31}O_{14}$**	**Ol Agl+ H_2- glucuronide**
	13	13.551,14.723	539.1766	0.7	0.013	$C_{25}H_{31}O_{13}$	Lig Agl + H_2- glucuronide
Dehydrogenation + Glucuronidation	14	10.572,10.288	327.0713	2.5	0.019	$C_{14}H_{15}O_9$	Hyty- H_2- glucuronide
	15	14.904	551.1386	3.7	0.084	$C_{25}H_{27}O_{14}$	Ol Agl – H_2- glucuronide
Hydroxylation + glucuronidation	16	7.512	387.0918	3.8	0.041	$C_{16}H_{19}O_{11}$	Hyty-Acet + OH- glucuronide *
	17	13.750	569.1538	-4.6	0.077	$C_{25}H_{29}O_{15}$	Ol Agl + OH- glucuronide
Hydration + glucuronidation	18	10.723	513.1597	3.3	0.016	$C_{23}H_{29}O_{13}$	DOA + H_2O- glucuronide
	19	11.158	571.1649	3.4	0.037	$C_{25}H_{31}O_{15}$	Ol Agl + H_2O- glucuronide *
Methylation + glucuronidation	20	6.425-7.328	343.1022	3.6	0.023	$C_{15}H_{19}O_9$	Hyty + CH_3- glucuronide
	21	12.763	475.0868	3.0	0.124	$C_{22}H_{19}O_{12}$	Luteolin + CH_3- glucuronide *
	22	17.262	459.0975	3.6	0.044	$C_{22}H_{19}O_{11}$	Apigenin + CH_3- glucuronide
	23	13.315	567.1719	1.2	0.115	$C_{26}H_{31}O_{14}$	Ol Agl + CH_3- glucuronide *
Methylation + other reactions+ glucuronidation	24	6.040,7.261	357.0809	5.0	0.098	$C_{15}H_{17}O_{10}$	Methyl Hyty-H_2+OH-glucuronide*
	25	7.913, 8.465	341.0878	-1.6	0.095	$C_{15}H_{17}O_9$	Methyl Hyty- H_2- glucuronide*
	26	11.308, 11.776	527.1757	2.4	0.072	$C_{24}H_{31}O_{13}$	Methyl DOA + H_2O- glucuronide
	27	11.408	541.1553	1.9	0.070	$C_{14}H_{29}O_{14}$	Methyl DOA +2 OH- glucuronide
	28	11.709	525.1638	-4.6	0.173	$C_{24}H_{29}O_{13}$	Methyl DOA + OH- glucuronide*
	29	13.466, 14.250	569.1905	-5.1	0.007	$C_{26}H_{33}O_{14}$	Methyl Ol Agl + H_2- glucuronide
	30	14.268, 14.954	583.1642	4.6	0.037	$C_{26}H_{31}O_{15}$	Methyl Ol Agl + OH- glucuronide
	31	15.288	565.1563	7.3	0.147	$C_{26}H_{29}O_{14}$	Methyl Ol Agl- H_2- glucuronide *
Sulfoconjugation	32	6.559	233.0125	0.1	0.006	$C_8H_9O_6S$	Hyty -sulfate

* means compounds present in small traces.
We represent in bold compounds found out in the statistical analysis responsible of the discrimination between urine samples before and after the intake of olive oil (biomarkers)

Fig.1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x10^5
a) Synthetic urine
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1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
5
6
7
8
6
8
10
12
14
16
18
20
22
Time [min]
Intens.
x10^5
b) Blank urine
1.0
0.8
0.6
0.4
0.2
0.0
1
2
3
4
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7
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10
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14
16
18
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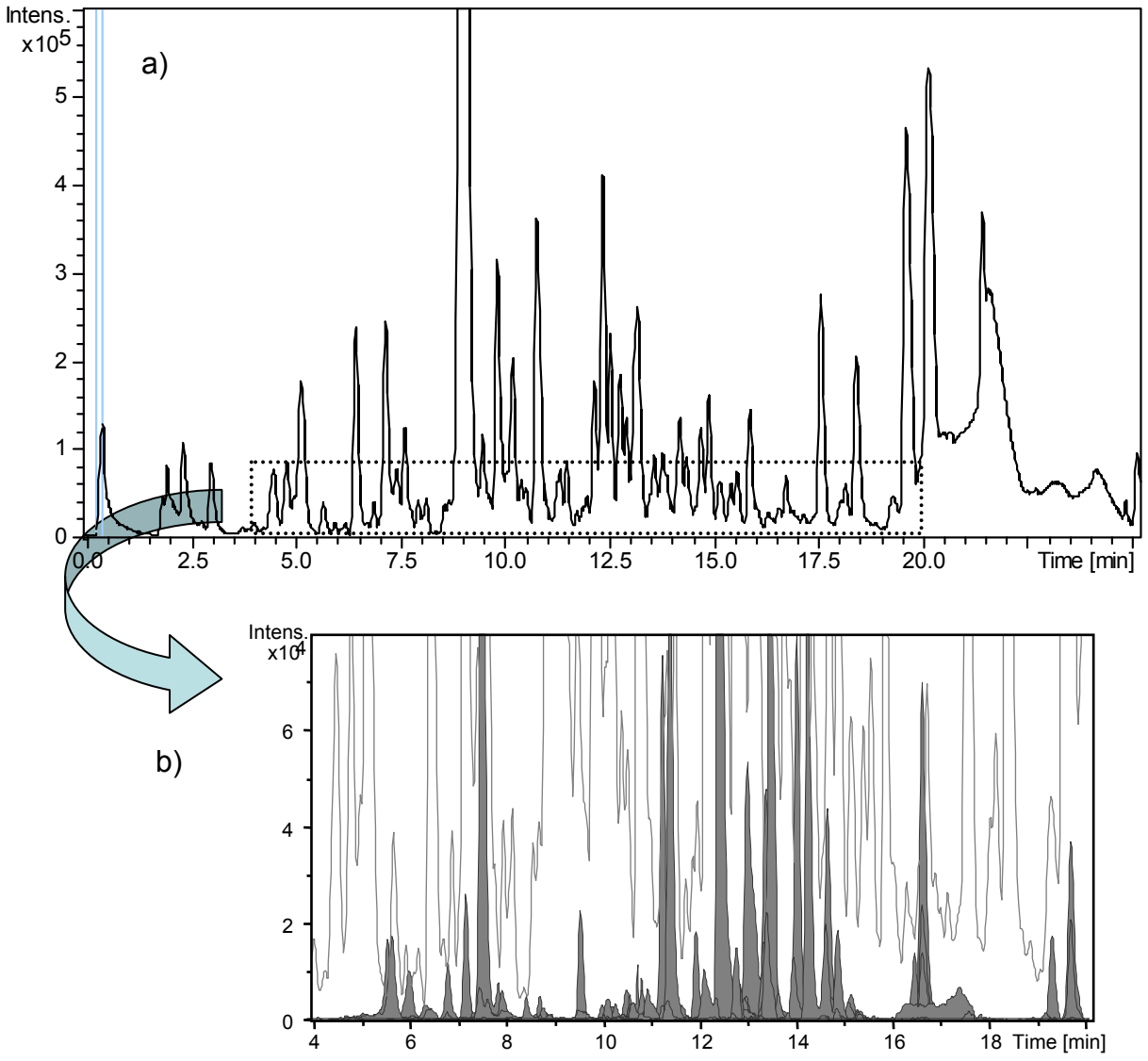
22
Time [min]

Fig. 2


Intens.
x10^5
a)
5
4
3
2
1
0
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2.5
5.0
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10.0
12.5
15.0
17.5
20.0
Time [min]
b)
Intens.
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4
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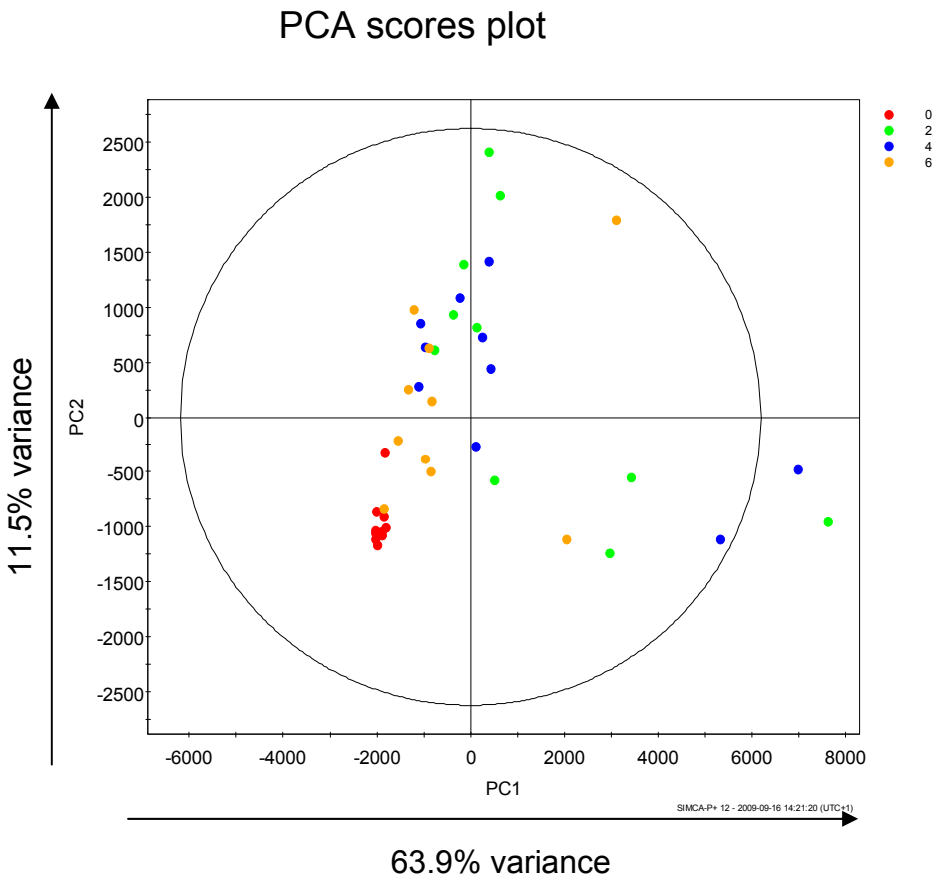
0
4
6
8
10
12
14
16
18
Time [min]

Fig. 3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2
4
6
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2000
1500
1000
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-1000
-1500
-2000
-2500
PC2
11.5% variance
-6000
-4000
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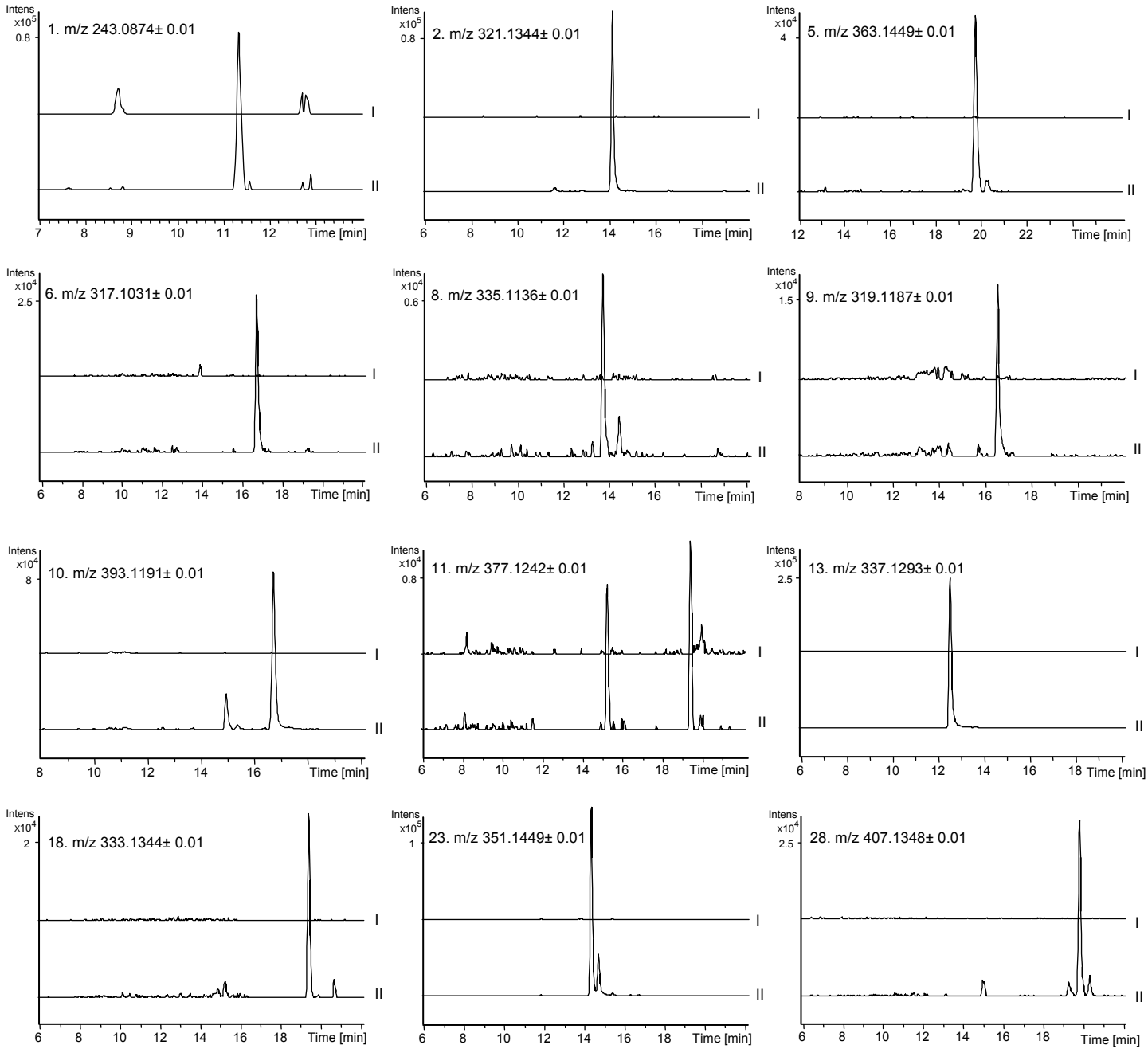
63.9% variance

Fig. 4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x10^5
0.8
1. m/z 243.0874± 0.01
I
II
7 8 9 10 11 12 Time [min]
Intens
x10^5
0.8
2. m/z 321.1344± 0.01
I
II
6 8 10 12 14 16 Time [min]
Intens
x10^4
4
5. m/z 363.1449± 0.01
I
II
12 14 16 18 20 22 Time [min]
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x10^4
2.5
6. m/z 317.1031± 0.01
I
II
6 8 10 12 14 16 18 Time [min]
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x10^4
0.6
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I
II
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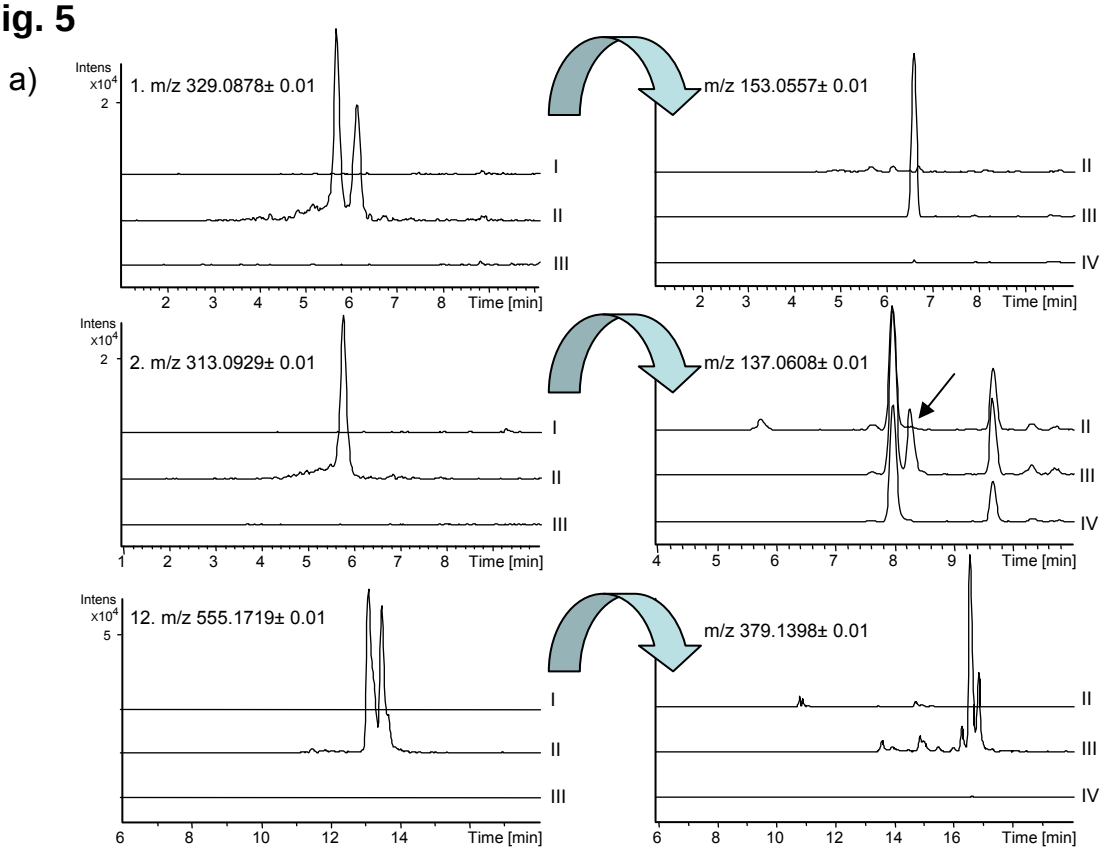
1.5
9. m/z 319.1187± 0.01
I
II
8 10 12 14 16 18 Time [min]
Intens
x10^4
8
10. m/z 393.1191± 0.01
I
II
8 10 12 14 16 Time [min]
Intens
x10^4
0.8
11. m/z 377.1242± 0.01
I
II
6 8 10 12 14 16 18 Time [min]
Intens
x10^5
2.5
13. m/z 337.1293± 0.01
I
II
6 8 10 12 14 16 18 Time [min]
Intens
x10^4
2
18. m/z 333.1344± 0.01
I
II
6 8 10 12 14 16 18 Time [min]
Intens
x10^5
1
23. m/z 351.1449± 0.01
I
II
6 8 10 12 14 16 18 Time [min]
Intens
x10^4
2.5
28. m/z 407.1348± 0.01
I
II
6 8 10 12 14 16 18 Time [min]

Fig. 5


a)
Intens
x10^4
1. m/z 329.0878± 0.01
m/z 153.0557± 0.01
2. m/z 313.0929± 0.01
m/z 137.0608± 0.01
12. m/z 555.1719± 0.01
m/z 379.1398± 0.01
I
II
III
IV
Time [min]

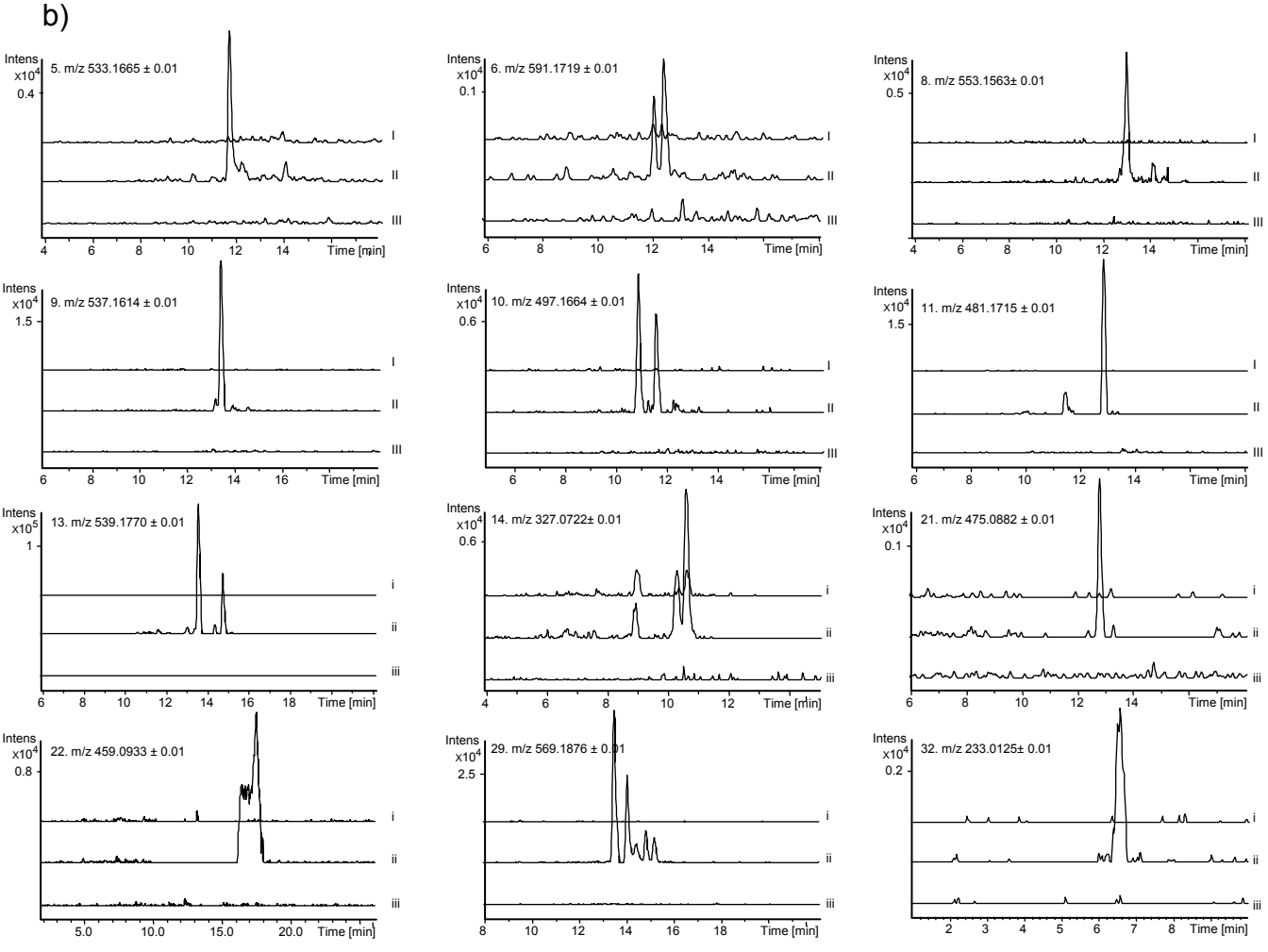
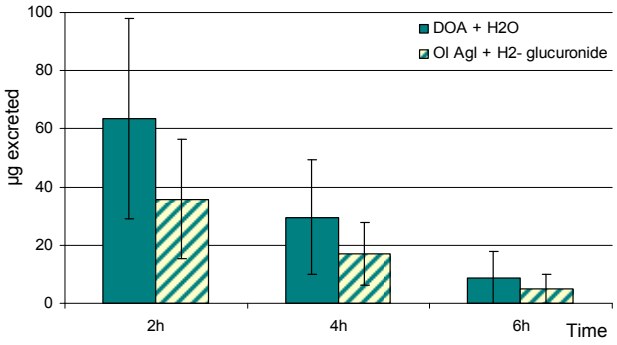
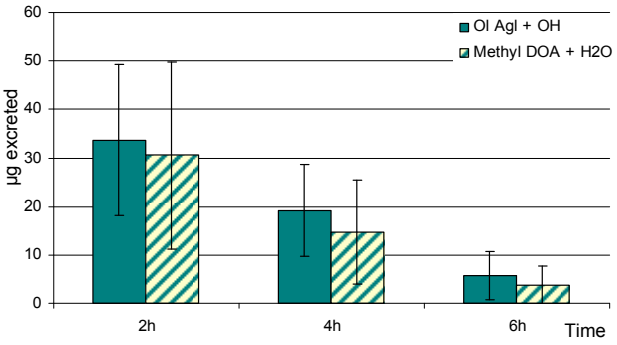
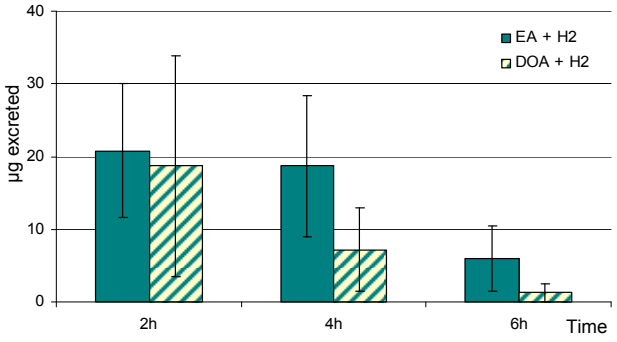
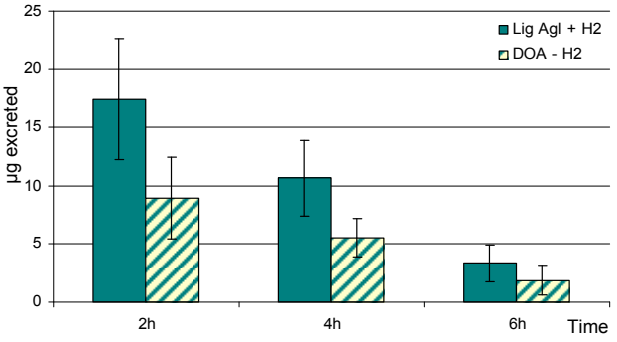

b)
Intens
5. m/z 533.1665 ± 0.01
6. m/z 591.1719 ± 0.01
8. m/z 553.1563± 0.01
9. m/z 537.1614 ± 0.01
10. m/z 497.1664 ± 0.01
11. m/z 481.1715 ± 0.01
13. m/z 539.1770 ± 0.01
14. m/z 327.0722± 0.01
21. m/z 475.0882 ± 0.01
22. m/z 459.0933 ± 0.01
29. m/z 569.1876 ± 0.01
32. m/z 233.0125± 0.01
I
II
III
i
ii
iii
Time [min]

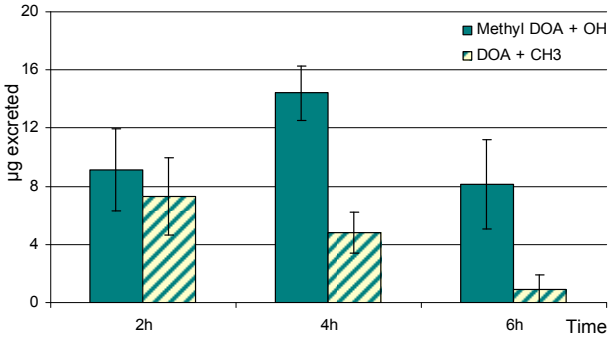
Fig. 6


DOA + H2O
OI AgI + H2- glucuronide
µg excreted
100
80
60
40
20
0
2h
4h
6h
Time


OI AgI + OH
Methyl DOA + H2O
µg excreted
60
50
40
30
20
10
0
2h
4h
6h
Time


EA + H2
DOA + H2
µg excreted
40
30
20
10
0
2h
4h
6h
Time


Lig AgI + H2
DOA - H2
µg excreted
25
20
15
10
5
0
2h
4h
6h
Time


Methyl DOA + OH
DOA + CH3
µg excreted
20
16
12
8
4
0
2h
4h
6h
Time

CONCLUSIONES

CONCLUSIONES

1) Se ha utilizado un método de CZE con detección UV para el análisis del perfil proteico (hordeínas) de muestras de cebada tomadas en diferentes etapas del proceso de malteado. Las proteínas fueron separadas obteniendo tres grupos de picos identificados como: albúminas y globulinas, B-hordeínas y C-hordeínas y se observaron cambios en cada una de las tres zonas, siendo la zona de las C-hordeínas la más afectada. Los resultados obtenidos proporcionan información sobre los cambios en la composición proteica de la cebada que pueden resultar útiles para la evaluación de su calidad durante el proceso de malteado y para monitorizar este proceso.

2) Se ha desarrollado un método que conlleva el empleo de CE-ESI-IT MS y permite la identificación de los α- y β- ácidos del lúpulo y sus productos de oxidación obtenidos bajo distintas condiciones de almacenamiento. Se estudiaron cuatro variedades de lúpulo: Nugget, Saaz, Magnum y Columbus, que mostraron diferentes tendencias a la oxidación. La electroforesis capilar acoplada a espectrometría de masas ha demostrado ser una herramienta analítica adecuada para el análisis de este tipo de compuestos y para el control de su estado de oxidación con objeto de obtener una cerveza de calidad. Además, esta misma metodología se ha aplicado con éxito para el estudio cualitativo y cuantitativo de los iso-α-ácidos de la cerveza.

3) Se ha puesto a punto un método de CE-ESI-TOF MS para estudiar comparativamente soja convencional y soja modificada genéticamente. Esta plataforma analítica ha demostrado que posee un gran potencial en este ámbito, pues se han identificado tentativamente 45 compuestos pertenecientes a diferentes familias: aminoácidos, péptidos, ácidos carboxílicos, isoflavonas y otros flavonoides, y se ha podido diferenciar entre soja convencional y transgénica observando el perfil metabolómico. Aunque la mayoría de los metabolitos identificados permanecen inalterados, se han observado diferencias significativas en las intensidades de algunos de ellos e incluso la desaparición de un posible aminoácido en la soja transgénica.

4) Se ha estudiado en profundidad el perfil polifenólico de muestras de aceite de oliva mediante un método bidimensional HPLC-CE-ESI-TOF MS. Diferentes fracciones fenólicas fueron aisladas a partir de extractos de una mezcla de aceites de oliva empleando HPLC semipreparativa y posteriormente fueron caracterizadas mediante CE-ESI-TOF MS. El empleo de dos técnicas complementarias que sen basan en principios completamente distintos (HPLC y CE) junto con el potencial del TOF y Q-TOF ha permitido la identificación de una gran cantidad de compuestos, algunos de ellos no descritos antes en bibliografía.

   Además las fracciones fenólicas aisladas, tan importantes debido a la ausencia de estándares comerciales para la mayoría de los compuestos, se han utilizado para mejorar la cuantificación de estos compuestos y para llevar a cabo estudio *in vitro*. Los compuestos fenólicos aislados como puros se usaron para cuantificar y se comparó dicha cuantificación con la que se venía haciendo tradicionalmente empleando estándares externos, encontrando grandes diferencias y corroborando la idea inicial de una cuantificación más veraz con el empleo de los estándares aislados. Algunas de las fracciones aisladas se han empleado en estudios *in vitro* con células de cáncer de mama con objeto de demostrar sus propiedades anticancerígenas. Se evidenció que tanto las fracciones ricas en lignanos (en particular, Ac Pin) como en secoiridoides (sobre todo Ol Agl y Lig Agl) son especialmente efectivas en la disminución de la proliferación celular, la inducción de muerte celular y en la reducción de los niveles de expresión y actividad del oncogén HER2.

   5) Se ha puesto a punto un método rápido, fácil y efectivo empleando RRLC-ESI-TOF MS para caracterizar y cuantificar los extractos polifenólicos de 8 aceites de oliva que en estudios previos habían demostrado actividad anticancerígena *in vitro*. 19 compuestos fenólicos fueron identificados en el perfil y cuantificados usando una nueva aproximación que tiene en cuenta el contenido total de polifenoles, el área relativa de cada compuesto con respecto al área total del cromatograma y su factor de respuesta en el MS. Finalmente, empleando herramientas estadísticas se pudo establecer una correlación entre la composición fenólica de los extractos de aceite de oliva y su capacidad antiproliferativa. Las variedades ricas en secoiridoides, como

por ejemplo la variedad Picual, mostraron mayor capacidad para alterar la viabilidad celular.

6) Se ha evaluado y demostrado el potencial de la nanocromatografía líquida acoplada a espectrometría de masas (nanoLC-ESI-TOF MS) para la identificación y cuantificación de los polifenoles del aceite de oliva, comparándola con la cromatografía líquida convencional, HPLC. El nuevo método de nanoLC ofreció mejor sensibilidad, con menos consumo de fase móvil y tiempos de análisis semejantes, pero presentó peor repetibilidad y mayor dificultad de operación por parte del analista. La plataforma nanoLC-ESI-TOF MS muestra el potencial suficiente para convertirse en una alternativa prometedora para el análisis de muestras donde los analitos se encuentren en bajas concentraciones, como por ejemplo en el análisis de fluidos biológicos.

7) Se ha demostrado el potencial del acoplamiento GC-APCI-MaXis MS para el análisis de polifenoles del aceite de oliva. El estudio de los estándares puros disponibles comercialmente nos permitió observar el comportamiento de cada familia de compuestos en el sistema empleado (especialmente en la fuente APCI) y conocer el tipo de señal que cada analito origina. La reacción de derivatización que se realizaba durante la preparación de las muestras, provocaba que todos los hidrógenos activos de los fenoles fueran sustituidos por grupos TMS; sin embargo, no todas las familias de fenoles demostraban el mismo comportamiento en APCI. En la mayor parte de los casos, la señal que se detectaba con más probabilidad era $[M\text{-}xH+xTMS+H]^+$ (en función de los hidrógenos sustituidos por TMS), aunque en el caso de los fenoles simples, se observó la presencia de radicales en la fuente de ionización.

Los cromatogramas obtenidos al hacer el análisis de los extractos fenólicos del aceite (tanto en MaXis como en FID) proporcionaron una gran cantidad de información identificando una gran cantidad de compuestos en el perfil.

8) Se han evaluado los procesos de absorción y metabolización de los principales compuestos fenólicos del aceite de oliva en células de cáncer de mama de la línea JIMT-1, utilizando nanoLC-ESI-TOF MS. Es la primera vez que esta plataforma

analítica se ha usado en estudios metabolómicos y ha demostrado su potencial en este campo. Se analizaron los medios de cultivo, citoplasmas y partes sólidas a diferentes tiempos de incubación y los resultados obtenidos sugieren que algunos compuestos, especialmente el Hyty, sus derivados secoiridoides y el flavonoide luteolina son absorbidos, metabolizados en las células y excretados rápidamente al medio de cultivo. La acumulación intracelular parece ser baja pues sólo se detectaron trazas de algunos compuestos en el citoplasma y las partes sólidas. Los derivados metilados fueron los metabolitos predominantes para la mayoría de los compuestos, lo que sugiere que esta línea celular de cáncer de mama posee actividad catecol-O-metiltransferasa.

9) Se ha llevado a cabo un estudio de la biodisponibilidad de los polifenoles del aceite de oliva analizando muestras de orina de 10 voluntarios tras la ingesta de 50 ml de una mezcla de aceites de oliva virgen extra. Las muestras se analizaron mediante RRLC-ESI-TOF MS y los resultados obtenidos se estudiaron mediante técnicas estadísticas de análisis multivariante lo que permitió discriminar entre las muestras antes y después de la ingesta y establecer los compuestos responsables de esta discriminación (biomarcadores). Se han identificado 10 biomarcadores y otros 50 metabolitos de polifenoles del aceite de oliva pertenecientes a distintas familias, lo que demuestra que todos los compuestos son absorbidos en mayor o menor medida. Sin embargo, la biodisponibilidad difiere mucho dependiendo de la estructura del compuesto; así compuestos con estrucura orto-difenólica como el Hyty y sus derivados secoiridoides parecen ser los más absorbidos y metabolizados. Los compuestos fenólicos fueron sometidos a diferentes tipos de reacciones metabólicas (Fase I y Fase II), pero las más frecuentes fueron la metilación y glucuronidación. El método desarrollado se ha aplicado para el estudio cinético de los biomarcadores tras la ingesta, encontrando el nivel más alto de la mayoría de los compuestos 2 horas después de la administración.

FINAL CONCLUSIONS

FINAL CONCLUSIONS

1) A CZE method with UV detection has been developed and used for the analysis of hordein patterns of barley samples taken at different stages of the malting process. The proteins were resolved into three groups of peaks identified as: albumins and globulins, B-hordeins and C-hordeins. Some changes were observed in the three zones during malting, although the most significant ones were shown in the zone of the C-hordeins. The results provided information about changes in the protein composition of barley that could be useful for evaluating its quality during malting process and for monitoring the stages of this process.

2) The development of a CE-ESI-IT MS method has allowed the identification of hop acids (α- and β-acids) and their oxidation products obtained under different storage conditions. Four varieties of hops were studied: Nugget, Saaz, Magnum and Columbus, and each one showed different tendency to oxidation. CE coupled to MS has proved to be a suitable analytical tool for the analysis of this type of compounds and for the control of their oxidation state in order to obtain a high quality beer. Besides, the same methodology has been successfully applied to the qualitative and quantitative study of the iso-α-acids in beer.

3) A complete analytical method (including an extraction protocol, CE-ESI-TOF MS analysis and data evaluation) has been developed to comparatively study the metabolic profile of conventional and transgenic soybean. This method allowed the tentative identification of forty five compounds belonging to different families: amino acids, peptides, carboxylic acids, isoflavones and other flavonoids and it was possible to differentiate between conventional and transgenic soybeans based on their metabolic profile. Although most of the identified metabolites remained unchanged, significant differences were detected in the intensities of some of them and even one compound identified as an amino acid seemed to disappear in the transgenic soybean.

4) The phenolic profile of olive oil extracts was studied in depth by a bidimensional study using HPLC-ESI-TOF MS. Different phenolic fractions were isolated from olive oil extracts using semipreparative HPLC as a first dimension of separation and

then they were characterized by CE-ESI-TOF MS as a second dimension. The use of two complementary techniques (HPLC and CE), together with the potential of TOF and Q-TOF allowed the identification of a large number of compounds, some of them not previously described in literature. Furthermore, the importance of the isolated fractions is remarkable, since most of the compounds can not be purchased as commercial standards.

Using the isolated phenolic fractions identified as pure, some phenolic compounds were quantified and this quantification was compared with those traditionally done using external standards. Some differences were found between both quantifications, corroborating the idea that a better quantification can be achieved, closer to reality, using the isolated compounds. Besides, some of the isolated fractions have been used in *in vitro* studies with breast cancer cell lines demonstrating their anticancer properties. The studies showed that fractions rich in lignans (especially Ac Pin) and secoiridoids (especially Ol Agl and Lig Agl) are effective decreasing cell proliferation, causing cell death and reducing the expression levels and activity of HER2 oncogens.

5) A rapid, easy and effective method has been developed using RRLC-ESI-TOF MS for the characterization and quantification of phenolic extracts of eight olive oils, which had shown anticancer activity toward human breast cancer cell lines in previous studies. 19 phenolic compounds were identified and quantified by using three different strategies, finding a new approach using the combination of the total polyphenol content, the area percentage of each phenol in the total area of the chromatogram and the response factor of each particular phenol. Finally, using statistical tools, a correlation between the phenolic composition of olive oil extracts and their antiproliferative capacity was established. Those varieties rich in secoiridoids, such as the variety Picual, shown the major ability to alter cell viability in four different types of human breast carcinoma cells.

6) The potential of nanoliquid chromatography coupled to mass spectrometry (nanoLC-ESI-TOF MS) for the identification and quantification of olive oil polyphenols was demonstrated and compared with other technique widely used in the analysis of these compounds, HPLC. The new nanoLC method provided comparable analysis time and offered better sensitivity with less consumption of mobile phases; however

it presented worse inter-day repeatability and it was a bit more difficult to operate by the analyst. NanoLC-ESI-TOF MS showed the potential to become a very promising alternative, in particular, for studies where the determination of extremely low concentrations of analytes is required (biological samples, for instance).

7) The separation by GC coupled with APCI-MaXis MS has been applied to the identification and quantification of olive oil phenolic compounds. First of all, the study with standards allowed us to observe the behaviour of each family of compounds in the system (especially in the APCI source) and the signal originated by each analyte. A derivatization reaction was carried out to assure that every compound could be properly ionized; in all the cases the active hydrogen present in the phenolic structures were replaced by TMS groups. In the major part of cases, the signal which was more likely observed was $[M\text{-}xH+xTMS+H]^+$ (depending on the number of active hydrogen in the molecule), except for simple phenols, where radicals were observed in the source.

   The chromatograms obtained with the olive oil extracts (both with FID and MaXis) provided a lot of information, identifying a large number of compounds in the profile. GC-APCI-MaXis MS is an analytical procedure, which combines the best of chromatography with one of the most robust MS interfaces and a powerful analyzer and we have demonstrated that it has a potential to become one of the standard methods in food metabolic profiling.

8) The absorption and metabolism of the main olive oil phenolic compounds in the human breast cancer cell line JIMT-1 were evaluated at different incubation times using nanoLC-ESI-TOF MS. It is the first time that this analytical platform is used in a metabolomic studied and its potential in this field has been demonstrated. The analysis of culture medium, cytoplasm and solid parts at different incubation time, suggested that some compounds, mainly Hyty, secoiridoids, and the flavonoid luteolin are absorbed, metabolized in the cytoplasmic compartment of cancer cells and excreted to the extra cellular medium. Low intracellular accumulation was observed with only traces of some compounds detected in the cytoplasm and solid parts. The methylated derivatives were the predominat metabolites for most of the

compounds, suggesting that this breast cancer cell line possesses catechol-O-methyltransferase activity.

9) The bioavailability of olive oils polyphenols was studied by analyzing urine samples from 10 volunteers after the intake of 50 ml of extra virgin olive oils. The samples were analyzed by RRLC-ESI-TOF MS and the results were studied by multivariate analysis techniques which discriminated the urine samples before and after the intake of olive oil and established the compounds responsible of this discrimination. 10 biomarkers and other 50 metabolites of practically all the compound described in olive oil were identified, suggesting that all the compounds are absorbed to a greater or lesser extent. However, bioavailability appeared to differ greatly depending on the structure of the compounds. So, compounds with orto-diphenolic structure such as Hyty and its secoiridoids derivatives seem to be the most absorbed and metabolized. Phenolic compounds were subjected to different Phase I and Phase II reactions, but the most common metabolic reactions were methylation and glucuronidation. The developed method was also successfully applied to monitor the levels of these biomarkers in human urine after the intake of olive oil, and the results indicated that the highest level of most compounds was detected at 2h after administration.