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**TESIS DOCTORAL**

**DETERMINACIÓN DE COMPUESTOS FENÓLICOS EN ACEITE DE OLIVA MEDIANTE TÉCNICAS Y METODOLOGÍAS SEPARATIVAS AVANZADAS**

presentada por

**Alegría Carrasco Pancorbo**

para optar al grado de

**Doctor Europeo en Química**

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por

**Alegría Carrasco Pancorbo**

Visado en Granada a 21 de Septiembre de 2006

Fdo: Prof. Dr. D. Alberto Fernández Gutiérrez

Catedrático del Departamento de Química Analítica

Facultad de Ciencias. Universidad de Granada

Fdo: Dr. D. Antonio Segura Carretero

Profesor Titular del Departamento de Química Analítica

Facultad de Ciencias. Universidad de Granada

Trabajo presentado para optar al grado de Doctor Europeo en Química.

Fdo: Alegría Carrasco Pancorbo

El Prof. Dr. D. ALBERTO FERNÁNDEZ GUTIÉRREZ, Catedrático del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada, y director del grupo de investigación FQM-297 "Control analítico, ambiental, bioquímico y alimentario"

CERTIFICA:

Que el trabajo que se presenta en esta tesis doctoral bajo el título: **"DETERMINACIÓN DE COMPUESTOS FENÓLICOS EN ACEITE DE OLIVA MEDIANTE TÉCNICAS Y METODOLOGÍAS SEPARATIVAS AVANZADAS"**, que ha sido realizado bajo mi dirección y la del Dr. D. Antonio Segura Carretero en los laboratorios que el grupo FQM-297 tiene en el Departamento de Química Analítica y en los del Centro de Instrumentación Científica de la Universidad de Granada; también, parcialmente, en las instalaciones de la Universidad de Bologna (Italia) (Dipartimento di Scienze degli Alimenti, sede de Cesena), y en la sede de Bremen (Alemania) de la compañía Bruker Daltonik GMBH, reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dª. Alegría Carrasco Pancorbo pueda optar al grado de Doctor Europeo en Química.

Y para que conste, expido y firmo el presente certificado en Granada a 21 de Septiembre de 2006:

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**RESUMEN**

En esta memoria se reúnen los resultados obtenidos durante la realización de la tesis doctoral titulada "Determinación de compuestos fenólicos en aceite de oliva mediante técnicas y metodologías separativas avanzadas". Se ha dividido en una INTRODUCCIÓN, que incluye información acerca de la matriz estudiada (el aceite de oliva) y de las técnicas separativas y de detección empleadas (CE y HPLC con detección UV y MS).

Después, la sección de RESULTADOS Y DISCUSIÓN está dividida en once capítulos. En el primero de ellos se recogen y resumen los métodos analíticos para la determinación de compuestos fenólicos en aceite utilizando distintas técnicas publicados hasta la fecha de terminación del trabajo documental. La realización del review que compone este capítulo 1 nos dio una idea clara de qué había ya hecho y qué sería interesante hacer; también puso de manifiesto la escasez de datos obtenidos mediante CE hasta ese momento.

En el segundo capítulo se describe el desarrollo de un método para el análisis de ácidos fenólicos mediante CZE-UV. Estos compuestos han despertado gran interés por las funciones biológicas que pueden desempeñar como metabolitos secundarios y por el papel que juegan en la calidad de los alimentos y en sus propiedades organolépticas. El método desarrollado se aplicó al análisis de seis aceites de oliva virgen-extra, un aceite de oliva refinado y una mezcla de aceite refinado y virgen obteniendo interesantes resultados. Hasta el momento en el que se desarrolló este método, no existía ninguna alternativa a HPLC que ofreciese resultados fiables y reproducibles, con tiempos de análisis cortos, y que lograse detectar cantidades tan pequeñas de ácidos fenólicos en aceite.

El tercer capítulo centra su interés en la misma familia que se estudió en el capítulo 2: los ácidos fenólicos. Sin embargo, utiliza una metodología electroforética algo más innovadora en la que se invierte la polaridad de los electrodos en el equipo y con ello, a priori, se podrían reducir los tiempos de análisis. Además de la polaridad de los electrodos, también hay que invertir el FEO para hacer viable la puesta a punto de este método, y para ello, se modifica la superficie del capilar mediante varios recubrimientos dinámicos observando cuál da mejor resultado. El analizar la misma familia mediante otro método electroforético, nos dio la posibilidad de comparar los resultados y estudiar

la fiabilidad de los mismos. Los resultados obtenidos en el capítulo 2 y en el 3 están más que de acuerdo en orden de magnitud; las pequeñas diferencias obtenidas fueron debidas a que los aceites no eran exactamente del mismo período de la campaña oleícola.

El cuarto capítulo describe un nuevo procedimiento para la determinación de ácidos fenólicos mediante CE con polaridad invertida usando capilares recubiertos con EpyM-DMA. Se utilizó este novedoso recubrimiento interno sintetizado por un grupo de investigación del Instituto de Ciencia y Tecnología de Polímeros del CSIC (Madrid), que nos fue facilitado por el grupo de Dr. A. Cifuentes. El polímero mencionado da la posibilidad de obtener un recubrimiento físico simple, rápido, reproducible y compatible con el empleo de porcentajes notables de modificador orgánico en la disolución reguladora, sin que sea necesario añadir cantidades de esta sustancia en el tampón de separación.

Los resultados obtenidos fueron notablemente mejores (teniendo en cuenta la separación de los estándares comerciales) que los escritos en el capítulo 3, y muy similares a los expuestos en el capítulo 2; este capítulo fue más teórico que aplicado, con el objetivo de demostrar el potencial metodológico más que de llevar a cabo el análisis de diferentes extractos de aceite.

En el quinto capítulo se aborda el estudio de la capacidad antioxidante individual de ocho de los compuestos fenólicos más abundantes en la fracción más polar del aceite. El trabajo experimental incluido en este capítulo se desarrolló en la Universidad de Bolonia, Departamento de Ciencia de los Alimentos (Sede de Cesena) en el seno del grupo dirigido por el Prof. G. Lercker. Para poder evaluar de manera individual la capacidad antioxidante de cada compuesto, hubo que aislarlos (ya que no existen patrones comerciales); se analizaron aceites comerciales mediante HPLC-UV y HPLC-MS y, una vez que estaba caracterizada la fracción de interés, se procedió al coleccionamiento de varios de los compuestos individuales utilizando una columna semi-preparativa. Tras comprobar la pureza de los "estándares" aislados, se realizaron medidas de la capacidad antioxidante de los mismos mediante tres tests basados en técnicas totalmente diferentes, cosa que permitiría comparar los resultados y ver si todas las determinaciones estaban de acuerdo en cuanto a la capacidad de cada compuesto. Los tres tests fueron: un estudio de "radical scavenging" empleando el conocido DPPH,

una oxidación acelerada en sistema lipídico modelo (OSI, que es equiparable al sistema Rancimat), y un novedoso método electroquímico.

El capítulo 6versa sobre la puesta a punto de un método electroforético sencillo y rápido para caracterizar fenoles simples, lignanos, fenoles complejos, ácidos fenólicos y flavonoides en extractos de aceite de oliva virgen extra. Hasta el momento en que se desarrolló el método que se describe en este capítulo, habían sido descritos algunos otros métodos para CE, pero quedaban dos familias muy importantes dentro de la fracción fenólica del aceite de oliva aún por caracterizar: los secoiridoides y los flavonoides. El resultado de una concienzuda optimización de los parámetros electroforéticos, junto con el uso de toda la anterior información bibliográfica, los estándares coleccionados mediante HPLC (capítulo 5) y toda la información espectral, fue un potente y sencillo método que es capaz de determinar hasta 26 analitos pertenecientes a 5 familias diferentes en 10 minutos.

Una vez puesto a punto el método descrito en el capítulo 6 quisimos demostrar su utilidad, en el capítulo 7 comparando varias muestras de aceites de oliva virgen-extra españoles llevando a cabo la cuantificación (tanto individual como en grupos teniendo en cuenta las distintas categorías de compuestos fenólicos presentes en el perfil electroforético) de catorce compuestos que pertenecían a las familias de fenoles simples, lignanos, fenoles complejos y flavonoides. Además, usando estadística multivariante, se intentó también lograr distinguir con claridad los aceites de oliva estudiados considerando su contenido fenólico, utilizando para lograr este fin la capacidad discriminatoria que ofrecen las variables electroforéticas determinadas en este estudio.

Siguiendo el enfoque aplicado del método descrito en el capítulo 6, en el capítulo 9 el objetivo planteado fue analizar los componentes fenólicos contenidos en la fracción insaponificable de aceites de oliva virgen extra, intentando diferenciar los aceites provenientes de una Denominación de Origen Protegida (DOP) española (Sierra de Segura) y una italiana (Chianti Classico) en función de este contenido polifenólico y utilizando estadística multivariante. A su vez, se persiguió establecer un paralelismo entre el análisis del aceite mediante CE y el proceso organoléptico de cata del mismo, intentando determinar qué compuestos eran aquellos que con su presencia, o que al estar

presentes en una determinada concentración en el aceite, pudieran otorgarle un atributo característico al aceite (picante, amargo, afrutado…).

En el capítulo 9, que recoge otra parte del trabajo experimental desarrollado en el Dpto. de Ciencia de los Alimentos de la Universidad de Bolonia, se aborda la evaluación de la influencia de la oxidación térmica en la composición fenólica y en la actividad antioxidante de aceites de oliva virgen extra. Nos pareció muy interesante comparar los resultados obtenidos mediante HPLC-UV, HPLC-MS y CE-UV para caracterizar el deterioro del aceite de oliva virgen extra durante un calentamiento que simulara la temperatura de fritura (180º C) durante diferentes intervalos de tiempo. Se determina en esta sección experimental de la presente tesis la concentración de compuestos fenólicos pertenecientes a cuatro familias (fenoles simples, lignanos, fenoles complejos y ácidos fenólicos) mediante ambas técnicas. Del mismo modo, se observó la formación de "nuevos" compuestos ("unkonwn") que se podían detectar en los perfiles de los aceites sometidos al tratamiento; estos compuestos se estudiaron mediante HPLC-MS y se cuantificaron. El índice de peróxidos y la capacidad antioxidante de la fracción fenólica (mediante el test OSI) también se determinaron tras cada intervalo del tratamiento térmico.

En el décimo capítulo se describe el desarrollo del primer método para el análisis de polifenoles en aceite de oliva mediante CE-ESI-IT MS. El objetivo era caracterizar el mayor número de compuestos fenólicos presentes en un extracto (SPE-Diol) de aceite de oliva virgen extra. Para demostrar, en este caso, el poder cuantitativo de la MS, se compararon también los perfiles polifenólicos de tres aceites obtenidos de variedades de aceituna diferentes. Se lograron determinar un total de once compuestos distintos, aunque en varios de los secoiridoides, una misma masa molecular producía dos picos (a causa de las formas isoméricas de algunos compuestos).

Aunque los resultados obtenidos por el primer método puesto a punto durante la realización de la presente memoria mediante detección MS (trampa de iones) fueron satisfactorios (capítulo 10), el afán por seguir caracterizando la fracción polifenólica del aceite de oliva, nos llevó a desarrollar dos métodos CE- y HPLC-ESI-TOF MS (en la sede de Bruker Daltonik de Bremen, Alemania) que se incluyen en el capítulo 11 de esta tesis doctoral. Se lograron determinar más de 45 compuestos mediante cada uno de los

dos métodos optimizados. De esos 45 analitos, un gran número de compuestos pertenecían a familias que han sido descritas por muchos autores (alcoholes fenólicos, ácidos fenólicos, lignanos, flavonoides y secoiridoides), pero también hubo un gran número de compuestos determinados que eran "nuevos".

# ENGLISH SUMMARY

This work is a summary of all the results obtained during the PhD: "Determination of phenolic compounds in olive oil using different methods based on separative techniques". The current work can be divided in several sections; the first one is the INTRODUCTION, which includes outstanding information about olive oil (its history, its economical importance, its composition, kinds of olive fruits…) and two separative techniques (CE and HPLC) and several detection systems (mainly, UV and MS).

Then, we can see the RESULTS AND DISCUSSION section, which has eleven chapters. The first one includes an overview of analytical methods for the measurement of polyphenols in olive oil. Many reports in the literature determine the total amount of phenolic compounds in olive oil by spectrophotometric analysis and characterize their phenolic patterns by GC and, mainly, by reverse-phase HPLC; however, CE has recently been applied to the analysis of the phenolic compounds of olive oil and has opened up great expectations, especially because of the higher resolution, reduced sample volume, and analysis duration.

The second chapter describes a sensitive, rapid, efficient and reliable CZE method for the separation and determination of phenolic acids from virgin olive oil. Phenolic acids are important compounds which have been associated with color, sensory qualities, and nutritional and antioxidant properties of foods. Recent interest in phenolic acids also stems from their potential protective role against oxidative damage diseases (coronary heart disease, stroke and cancers). This method was applied to six extra-virgin olive oil samples obtained from different olive fruit varieties; we also analyzed refined olive oils and commercial mixtures of refined and virgin olive oils to compare the amounts of phenolic acids present.

The chapter 3 explains the development of a method using co-electroosmotic capillary electrophoresis with a polycationic surfactant (HDB), which dynamically coats the inner surface of the capillary and causes a fast anodic electroosmotic flow, for the analysis of the same family studied in the latter chapter, the phenolic acids. The amounts of these substances were determined in several extracts obtained from different olive oil varieties (Arbequina, Lechín de Sevilla, Cornicabra, Hojiblanca, Lechín de Granada and Picual) and the method provided good repeatability and acceptable analysis times.

In chapter four, a family of 13 phenolic acids was separated by CE using a physically adsorbed polymer as capillary coating. The polymer used was DMA-EpyM and it provided a stable coating by only flushing the capillary with a DMA-EpyM aqueous solution for two minutes between runs. It is important to highlight that the experimental work included in this fourth chapter demonstrates that DMA-EpyM can be also used when the separation buffer contains organic modifiers, fact that makes broader the usefulness of this coating. The results were better than those obtained in chapter three and very similar to the data showed in chapter two; but we did not carried out applications in this work, we only wanted to demonstrate the potential of this methodology for the analysis of the extracts of a plenty of products (olive oil among them) which contain these phenolic compounds of great interest.

In chapter 5, we isolated several phenolic compounds of extra-virgin olive oil (simple phenols, lignans and complex phenols) by semipreparative HPLC and identified them using ultraviolet, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) detection. The purity of these extracts was confirmed by analytical HPLC using two different gradients. Finally, the antioxidant capacity of the isolated compounds was evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl, by accelerated oxidation in a lipid model system (OSI=Oxidative Stability Instrument) and by an electrochemical method.

It is interesting to stand out that this chapter was really important for the rest of the experimental work presented in this thesis, since there are not commercially available standards of the major part of the phenolic compounds of olive oil. In this chapter we got our "isolated-standards" and this fact helped a lot to continue the study of this fraction and identify these compounds in the electrophoretic profiles.

The work summarized in chapter 6 reports a complete and rapid CZE method for the qualitative determination of extra-virgin olive oil phenolic compounds. The Diol-SPE extracts from extra-virgin olive oil were analyzed in less than 10 min and we were able to study several families of phenolic compounds present in this foodstuff, such as simple phenols, lignans, complex phenols, phenolic acids and flavonoids. The described method is not completely original regarding the electrophoretic conditions, but its novelty is clear in terms of identification of phenolic compounds in virgin olive oil,

since, to our knowledge, this is the first paper to show the determination of flavonoids and several isomeric forms of secoiridoid compounds in olive-oil samples using a CE method. Peak identification was done by comparing both migration time and spectral data obtained from olive oil samples and standards (commercial or isolated (by HPLC-MS) standards), with spiked methanol-water extracts of olive oil with HPLC-collected compounds and commercially available standards at several concentration levels, studying the information of the electropherograms obtained at several wavelengths and also using the information previously reported.

Furthermore, as the extraction protocol uses a considerable quantity of oil (60 g), it is possible to detect in the same run 26 compounds belonging to both majority and minority families which, to the date, have not been detected simultaneously in any CE method. In this study we have demonstrated the qualitative potential of the described method; its usefulness to carry out quantitative determinations is considered in following chapters.

The method optimized in the latter chapter, was used for carrying out an applicative or monitoring work in chapter 7. This was the first time in which an electrophoretic method demonstrates to have the ability to detect and quantify simultaneously members of five different families of phenolic compounds present in olive oil. Its usefulness was checked analyzing five monovarietal extra-virgin olive oils (January 2005), an Organic olive oil and two types of Picual olive oils with different commercial names related to their sensorial properties indicate and very interesting results were found. CE joined to statistical analysis permited the discrimination among different olive oils.

In chapter 8, we used again the method developed in chapter 6. Nowadays, there is a great interest in foods that belong to a Protected Designations of Origin (PDO) because the quality and other essential and exclusive characteristics due to a particular geographical environment are guaranteed. So, we thought it would be very interesting applying the mentioned method for the characterization and quantification of the polyphenolic fraction of extra-virgin olive oil from different PDOs, and we analyzed sixteen samples of a Spanish PDO and other nine samples belonging to an Italian PDO (n=5). In this way, it was possible to compare the phenolic profiles of the oils of different zones of the same PDO, as well as the phenolic profiles of Spanish and Italian extra-virgin olive oils. Multivariate statistics was used for differenciating the oils

produced in each PDO; furthermore, a statistical study considering three sensory atributes (fruity, bitter and pungent) of each oil and their relation with the concentration of the 18 phenolic compounds quantified was carried out as well.

A comparison between the results obtained by using HPLC-UV, HPLC-MS and CE-UV for characterizing the deterioration of extra-virgin olive oil during heating, simulating frying temperature, was investigated in chapter 9. The polar extracts of a commercial extra-virgin olive oils were analyzed by both techniques to determine changes in the phenolic compounds present in the oil as a result of heating at 180 ºC during six time intervals (30-180 min). The concentration of several compounds belonging to four families of phenols (simple phenols, lignans, complex phenols and phenolic acids) was determined in the samples after the thermal treatment by both techniques. Several "unknown" compounds were determined in the phenolic profiles of the oils after the thermal treatment and their presence was confirmed in refined olive oils. The antioxidant activity performed by the phenolic fraction of virgin olive oil was indirectly measured by the OSI test and, for each oil sample, we also determined the peroxide value.

In chapter 10, we describe the first analytical method involving solid-phase extraction (SPE) and capillary zone electrophoresis coupled to electrospray ionization-ion trap mass spectrometry (CZE-ESI-MS) used to identify and characterize phenolic compounds in olive oil samples. The SPE, CZE and ESI-MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity of their determination. To this end we devised a detailed method to find the best conditions for CE separation and the detection by MS of the phenolic compounds present in olive oil using a methanol-water extract of Picual extra-virgin olive oil. After demonstrating the potential of this CE-ESI-MS method to characterize the extracts obtained from an extra-VOO, it was used to compare the phenolic content in three monovarietal extra-VOOs: Picual, Lechín de Sevilla and Hojiblanca.

Although we achieved our purposes regarding the detection by Ion Trap MS in chapter 10, the characterization of the phenolic fraction of olive oil could be still improved. In chapter 11, we present an easy and rapid method for the analysis of phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis (CZE) coupled to

electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS), and we thus achieved the determination of several important families (phenyl alcohols, phenyl acids, lignans, flavonoids and secoiridoids) of the polar fraction of the olive oil. Furthermore, other "unknown" compounds were also identified.

In addition to the CZE method, HPLC analyses were made, separating compounds belonging to the main families present in this polyphenolic fraction, as well as other new compounds. We compared the results obtained with both techniques and found it was possible to determine more than 45 compounds with both methods.

The sensitivity, together with mass accuracy and true isotopic pattern of the TOF-MS, allowed identification of a broad series of known and so far not described phenolic compounds present in extra-virgin olive oil.

# INTRODUCCIÓN
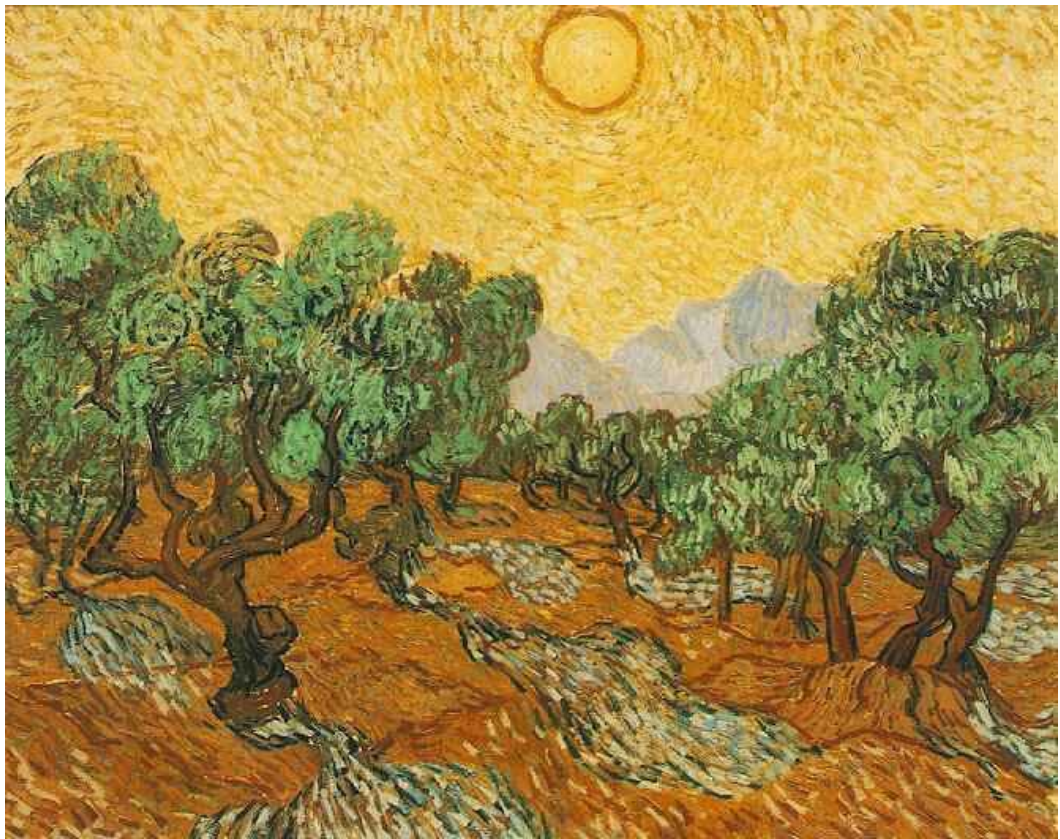
**El aceite de oliva**

# Un poco de historia

Actualmente el olivo (*Olea europea*) se cultiva en todos los países de la cuenca Mediterránea, especialmente en el centro y el sur de España e Italia, y en Grecia, Turquía, Túnez y Marruecos entre otros. Su situación actual es el resultado de un lento, pero continuo desarrollo de las distintas civilizaciones que habitaron las costas del Mediterráneo y zonas del interior de Oriente Medio.

El olivo tiene una gran importancia histórica. Ha jugado un papel preponderante en diversos aspectos de la vida humana como la dieta, la religión, y la decoración de cerámicas, muros y piezas de oro artísticas. También ha sido símbolo para representar la paz, la sabiduría y la victoria en distintas etapas de la historia de la humanidad [1].

El origen de *Olea europea* está aún en nuestros días sometido a discusión. Aunque los fósiles que datan del período terciario (hace un millón de años) demuestran que existió un antecesor del olivo en Italia [2], y los huesos de aceitunas hallados en los asentamientos humanos del período paleolítico (35000-8000 a. C.) confirman la presencia de una planta parecida al olivo, parece que el olivo, tal y como lo conocemos hoy, tuvo su origen en las regiones que se corresponden con las antiguas Persia y Mesopotamia [3,4]. Con el paso del tiempo el olivo se fue extendiendo desde estos países hasta territorios vecinos que hoy se corresponden con Siria y Palestina [5]. Según otras fuentes, los morteros y las prensas de piedra que se usaban para extraer aceite de las aceitunas datan del 5000 a. C..

La exportación posterior del olivo de estas originarias zonas a Egipto y el Mediterráneo occidental está bien documentada [6]. En la siguiente figura se pueden diferenciar tres

[1] A. Goor. "Place of olive in holy land and its history through ages". *Econ. Bot*. 20 (1966) 223-&.

[2] E. Palamarev. "Paleobotanical evidences of the Terciary history and origin of the Mediterranean clerophyll dendroflora". *Plant Systematics and Evolution* 162 (1989) 93-107.

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[4] D. Boskou. "History and characteristic of olive tree" en "Olive oil: Chemistry and Technology". Champaign, IL. AOCS Press. (1996) Pp 1-11.

[5] M. Heltzer. "Olive growing and olive oil in Ugarit" en "Olive oil and Antiquity". M. Heltzer, D. Eitam (Ed.). University of Haifa Press, Haifa. (1987) Pp 106-120.

[6] L. E. Stager. "First fruits of civilization" en "Palestine in the Bronze and Iron Age: papers in honour of Olga Tufnell". J. N. Tubb (Ed.). Instutute of Archaeology, London. (1985) Pp. 172-187.

tipos de centros: primarios, secundarios y terciarios, atendiendo a desde dónde partía y hasta dónde se fue propagando el cultivo del olivo.
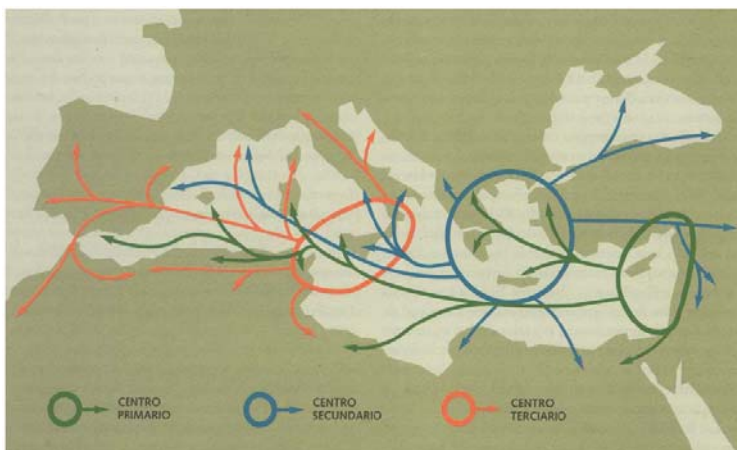


*Figura*. Difusión del cultivo del olivo en la cuenca del Mediterráneo

La civilización cretense o minoica, que experimentó un gran crecimiento entre el 2000 y el 1450 a. C. fomentó especialmente la producción y el comercio de la aceituna, con lo que se estimuló la exportación de aceite a otros países mediterráneos, especialmente a Egipto [7]. Aquí, el olivo se cultivaba para utilizar su aceite en ceremonias religiosas como ungüento [8] (ver figura). El Antiguo Testamento y otros libros sagrados atestiguan, con numerosas citas, el cultivo del olivo.



*Figura*: Interior de un laboratorio de un preparador de ungüentos: Tres aprendices machacan aceitunas e hierbas en el mortero de una tumba en Tebas (Egipto) alrededor del 1500 a.C..

Los fenicios a través del comercio marítimo que desarrollaron en el Mediterráneo, contribuyeron a la expansión del cultivo del olivar en los países mediterráneos más occidentales.

En las sucesivas civilizaciones que se desarrollaron en los territorios del litoral mediterráneo, tanto el olivo como su aceite ocuparon un importante papel tanto en la

[7] R. Standish. "The first of trees. The story of the olive". Phoenix House Ltd, London. (1960).
[8] P. Ghaliongui. "La medicina en el Egipto faraónico" en "Historia universal de la Medicina". P. Laín Entralgo (Ed.). Salvat. Barcelona. (1992) Pp 95-124.

economía agrícola del país productor como en el comercio con poblaciones vecinas. El olivo estaba ampliamente extendido, sobre todo en la antigua Grecia [9], como se puede verificar en la mitología griega y los numerosos testimonios históricos y literarios, especialmente en los poemas de Homero. El pueblo griego introdujo el cultivo de la aceituna en las colonias del sur de Italia (Magna Grecia) [ 10 ]. Los romanos incrementaron su cultivo y desarrollaron su comercio con los países conquistados que componían el Imperio Romano [11].

A pesar de que los romanos, al igual que los griegos, utilizaban este aceite principalmente como ungüento y como producto de uso farmacéutico y de iluminación (aceite lampante), fueron pioneros en usarlo como alimento. También contribuyeron al desarrollo tecnológico del tratamiento de la aceituna, facilitando su molienda con una piedra de molino llamada Trapetum (trapiche en castellano) [12] y mejorando el sistema de separación de las fases sólida y líquida con la introducción de prensas (ver figura).
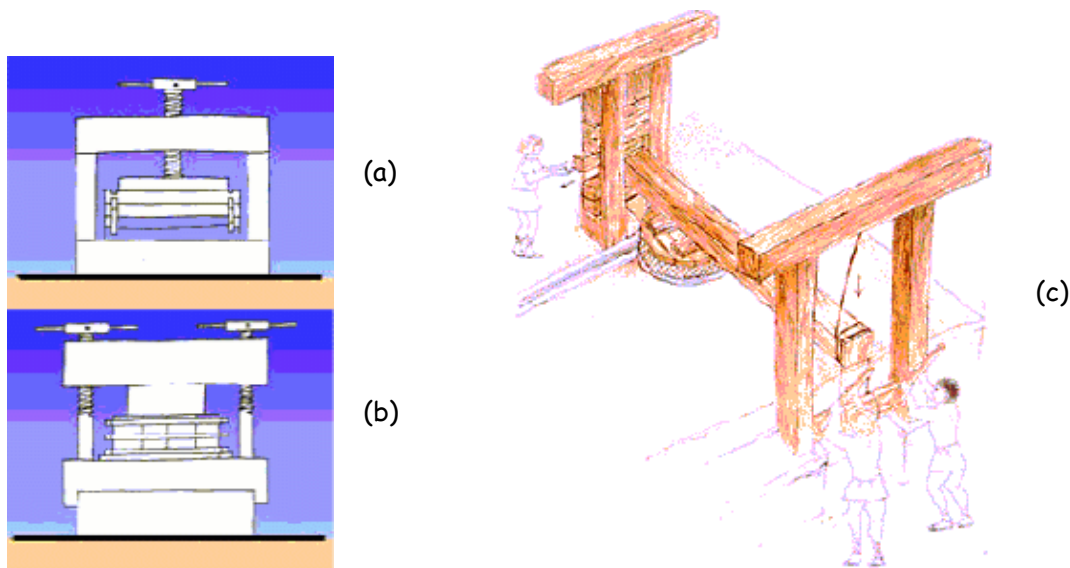


*Figura*: Prensa con un único torno (a), prensa con dos tornos (b), y torno a palanca y cabestrante (c).

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[9] L. Foxal. "Oil extraction and processing equipment in Classical Greece" en "La production du vin et de l´huile en Mediterranée". M. C. Amouretti, J. P. Brun (Ed.). École Française d´Athènes, Athens. (1993) Pp 183-200.

[10] H. T. Hartmann, P. C. Bougas. "Olive production in Greece". *Econ. Bot*. 24(4) (1970) 443&.

[11] H. Camps-Fabrer. "L'olivier et son importance économique dans l'Afrique du Nord Antique" *Olivae* 2 (1984) 9-22.

[12] F. Angerosa. "L´estrazione dell´olio dalle olive. Breve storia della tecnologia estrattiva" en "Olio d´oliva. All´origine della qualità". Ed. Organizzazione Nazionale Assaggiatori Olio di Oliva. (2005) Pp 8-13.

Con la caída del Imperio Romano y la invasión de los pueblos bárbaros, también cae el cultivo de la aceituna que no se recupera hasta la Edad Media gracias, en su mayor parte, al el esfuerzo de las comunidades religiosas. De todos modos, durante la Edad Media, el aceite de oliva escaseó de tal manera que, en determinados casos, llegó a ser considerado como dinero en efectivo. La producción aceitera medieval no alcanzó la cantidad y la difusión de aquella de la antigüedad romana, pero siguió siendo utilizado en el ámbito alimenticio, la iluminación y sobre todo para usos religiosos.

La crisis europea a mediados del siglo XIV, causada por oleadas de la peste que diezmaron la población, y por un empeoramiento de las condiciones atmosféricas, comportaron algunos cambios en la gestión agrícola de extensas regiones. Se difunde así el cultivo del olivo en amplias áreas Mediterráneas más templadas, ya que su gran fuerza vegetativa es a largo plazo una garantía de inversión. Aparece una intensa economía de cambio entre el Mediterráneo Occidental y el área del Mar del Norte y Báltico; el aceite de oliva es protagonista, por cuanto es utilizado para la conservación de gran variedad de productos alimenticios, para la iluminación y, con sus derivados, para otros diversos empleos. En la Edad Moderna, se realizan también interesantes innovaciones tecnológicas en el campo de la elaboración y en el sistema de lavado de los orujos (principio del siglo XVIII).

Durante este largo período de tiempo, salvo lo anteriormente dicho, no se producen innovaciones en la tecnología de obtención del aceite de oliva, que continúa basándose en la presión manual ejercida por prensas de tornillo de metal o de madera. El sistema se perfecciona cuando se introduce la primera prensa hidráulica a finales del siglo XIX. Durante el siglo XX se presentan otras posibilidades de extracción mecánica de aceite como resultado de los estudios realizados sobre los sistemas de extracción parcial y centrifugación. Estos innovadores sistemas se materializaron en 1951 con un sistema de extracción parcial, y hacia finales de 1960 con la introducción del equipo Centriolive, el primer decánter (o centrífuga horizontal) industrial basado en la centrifugación continua de la pasta de aceitunas.

Hoy el aceite de oliva se ha extendido más allá de las zonas mediterráneas, hasta difundirse por todos los continentes exceptuando la Antártida.

# Producción e importancia económica del aceite de oliva

El tener una visión lo más general y certera posible acerca de la producción, comercio y especialmente consumo del aceite de oliva en la actualidad nos hace conscientes de la importancia que esta grasa comestible puede tener en las economías de ciertas partes del mundo.

Respecto al consumo medio de grasa y aceite en el contexto mundial, el aceite de soja (32%) y el de palma (19%) son las grasas dominantes en la nutrición, mientras que el aceite de oliva, con una cuota del 4,6% ocupa el séptimo puesto en el ranking de las grasas y aceite más usados [13,14]. Sin embargo, en términos de valor del producto, los aceites de oliva representan un notable porcentaje del comercio mundial (15%) debido a que su valor por unidad de producto es significativamente mayor que el de otros aceites alternativos.

La importancia económica de los aceites de oliva varía de un país a otro, pero ésta es relevante cuando se analizan las economías de ciertas regiones del mundo. Sin lugar a dudas, su relevancia puede llegar a ser crucial en los países mediterráneos. Además de sus profundas raíces en la historia y civilización, el sector del aceite de oliva contribuye de manera notable a la actividad económica de varios de estos países.

En España más de medio millón de agricultores trabajan en el cultivo del olivar, cuyo sector demanda anualmente 46 millones de jornadas agrícolas. En Túnez, más de una décima parte de la población percibe todo o parte de sus ingresos del cultivo de olivo. Además de los empleos fijos, el sector proporciona aproximadamente un 30% del empleo temporal en agricultura [15].

La importancia económica y la resultante social del sector del aceite de oliva son, por tanto, 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.

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[13] Todos los datos estadísticos que se presentan en esta sección pertenecen a las bases de datos del COI (Consejo Oleícola Internacional), los cuales son el resultado de recopilar las cifras suministradas regularmente por los estados miembros. También se ha recurrido para complementar estos datos a los resultados estadísticos publicados por organizaciones internacionales como la FAO (Food and Agriculture Organization) de Naciones Unidas, a las Estadísticas Agrícolas de la Comisión Europea (http://europa.eu.int/comm/agriculture) y a aquellos datos publicados por revistas especializadas como *Oil World, GeoJournal, Agriculture and Human value* entre otras.

[14] TDC.Olive. "Enciclopedia del Olivo". Capítulo: "Estudios de consumo", elaborado por Unilever.

[15] F. Luchetti. "Introducción al estudio del aceite de oliva" en R. Aparicio, J. Hardwood. "Manual del aceite de oliva". Ed. Mundi-Prensa. Madrid. (2003) Pp 13-19.

En todo el mundo hay unos 805 millones de olivos, que ocupan aproximadamente unos 10 millones de hectáreas. Hoy en día se producen aceitunas en más de 40 países de todos los continentes, incluidos lugares exóticos como Hawai, aunque el 98% de la producción mundial de aceitunas (≈20 millones de toneladas) aún se sigue recogiendo en la región del Mediterráneo. En lo que a producción de aceite de oliva se refiere, ésta se concentra en los países mediterráneos como España, Italia, Grecia, Siria, Turquía, Marruecos y Argelia; entre ellos suman el 90% de la producción mundial.
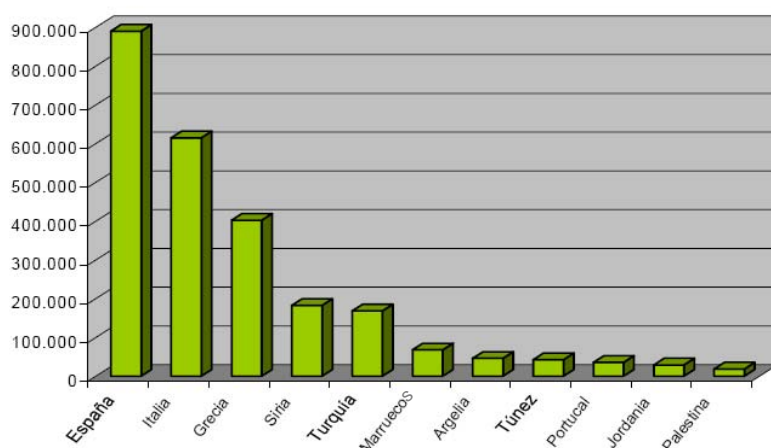


*Figura.* Producción mundial de aceite de oliva en 2004-2005.

Dentro de estos países las zonas olivareras están bastante localizadas. Por ejemplo, un 60% del olivar español está localizado en Andalucía, donde se produce el 75% del aceite de oliva español. Italia se sitúa al mismo nivel, con un 63% tanto de la superficie como de la producción localizado en las regiones del sur (por ejemplo, Apulia o Calabria). El 60% del olivar tunecino está situado en la zona centro del país y contribuye con un 30% a la producción nacional, mientras que el sur que tiene sólo la cuarta parte del olivar produce más de la mitad del aceite de oliva.

La gran concentración de olivos en ciertas regiones se explica por las condiciones climáticas que necesita el olivo para dar frutos, a la vez que por el hecho de que estos árboles ofrecen una oportunidad única para rentabilizar al máximo los recursos de agua y suelo que, normalmente, son limitados en las zonas áridas del mundo.

La producción mundial de aceite de oliva en la campaña 2004-2005 ascendió a 2,8 millones de toneladas. Los países de la Comunidad Europea producen anualmente casi el 78% del total mundial. Los principales productores de la Comunidad son España

(43% del total para Europa), Italia (35%) y Grecia (19%). Siria, Turquía y Túnez también son grandes productores de aceite de oliva, aunque con menor porcentaje [16,17]. En la campaña 2005-2006 se estima una producción ligeramente superior a los 2,58 millones de toneladas, con porcentajes de producción repartidos de modo muy similar a los de la campaña 2004-2005.

Sin embargo, aunque son pocos los productores, el aceite de oliva se consume en todo el mundo, de ahí la importancia que juega el comercio internacional en el sector olivarero [18]. Estados Unidos es uno de los principales países importadores, y casi ha doblado sus importaciones en los últimos años. En el Reino Unido, Alemania y Japón, los índices de crecimiento de las importaciones registrados son incluso mayores. Por otra parte, España no es sólo el mayor productor mundial, sino también el mayor país exportador del mundo.

Al igual que la producción mundial, el consumo de aceites de oliva está prácticamente concentrado en las regiones productoras; el 71% está centrado en la CEE, del cual un 92% corresponde a los tres principales países productores, España, Italia y Grecia.
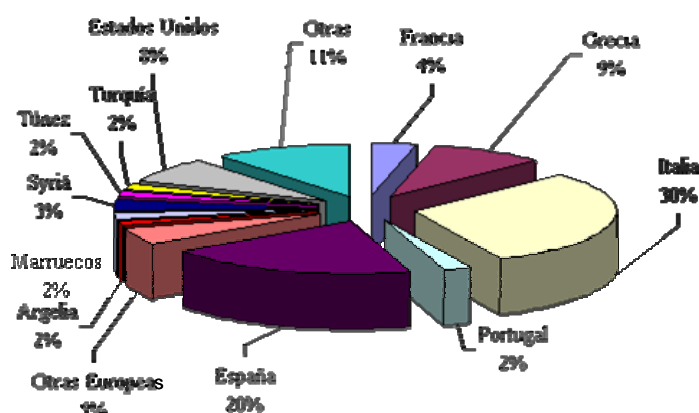


*Figura*. Reparto del consumo de aceite en todo el mundo [19].

El aceite de oliva es un alimento muy importante en la dieta mediterránea. Los griegos son los que mayor consumo *per cápita* tienen con 25 kilos por habitante y año. Le

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[16] Comunicado de prensa (texto no oficial destinado a la información) de la UNCTAD (Conferencia de las Naciones Unidas sobre Comercio y Desarrollo). UNCTAD/PRESS/PR/2005/014 con fecha de 22/04/05.
[17] G. Cladini, L. Cerretani, A. Bendini, T. Gallina Toschi. "Produzione e consumo di oli di oliva in Italia e Spagna". *L´informatore agrario* 30 (2005) 33-39.
[18] Market Researches (Investigaciones de Mercado): www.marketresearch.com
[19] Sitio web de las Naciones Unidas sobre Comercio y Desarrollo:
http://r0.unctad.org/infocomm/espagnol/olivo/mercado.htm

siguen los españoles con 15 kilos y los italianos con 13 kilos por habitante y año. En Estados Unidos, el consumo de aceite de oliva ha aumentado de forma significativa durante estos últimos años, encontrándose en la actualidad en 5.14 kilos.

# Elaboración del aceite de oliva. Aspectos tecnológicos.

El aceite de oliva se extrae de la aceituna, que es el fruto del olivo. La aceituna es una drupa en la que se distinguen las siguientes partes: pedúnculo o rabillo, epicarpio o piel, mesocarpio o carne, endocarpio o hueso y embrión o semilla.
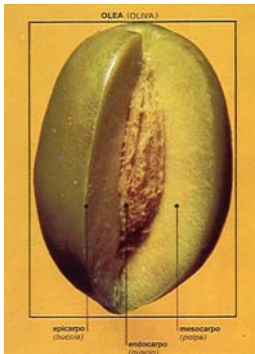


*Figura*. Esquema que muestra el epicarpio, endocarpio y mesocarpio de una aceituna.

La aceituna va experimentando cambios en su coloración al tiempo que engorda. Desde un verde intenso al comienzo de su cuajado, a un verde amarillento según va desarrollándose, aparecen manchas púrpuras al iniciar el envero, sigue una tonalidad púrpura azulada, para terminar, cuando alcanza su madurez plena en una tonalidad negro azulada.

La composición de este fruto en el momento de la recolección es muy variable, dependiendo de la variedad de aceitunas, del suelo, del clima y del cultivo. Por término medio, las aceitunas llevan en su composición:

- aceite: 18-32%
- agua de vegetación: 40-55%
- hueso y tejidos vegetales: 23-35%.

La siguiente tabla muestra la composición de la pulpa, el hueso y la semilla de una aceituna atendiendo al contenido de agua, aceite, azúcares, celulosa, materia nitrogenada, cenizas, polifenoles y otros compuestos:

*Tabla*. Composición de la pulpa, hueso y semilla de una aceituna.

|                     | Pulpa   | Hueso | Semilla |
|---------------------|---------|-------|---------|
| **Agua**            | 50-60   | 9.3   | 30      |
| Aceite              | 15-30   | 0.7   | 27.3    |
| Azúcares            | 3-7.5   | 41    | 26.6    |
| Celulosa            | 3-6     | 38    | 1.9     |
| Materia nitrogenada | 2-5     | 3.4   | 10.2    |
| Cenizas             | 1-2     | 4.1   | 1.5     |
| Polifenoles         | 2-2.5   | 0.1   | 1-1.5   |
| Indeterminados      |         | 3.4   | 2.4     |

El aceite constituye como decimos un porcentaje no muy elevado de la aceituna y se encuentra en las vacuolas dentro de las células de las aceitunas. La producción de aceite de oliva consiste en la separación del aceite contenido en el fruto de los componentes sólidos y el agua de vegetación de las aceitunas. La molienda rompe estas células, desprendiendo el aceite que contienen las aceitunas.

La planta dónde se realiza este proceso se llama "Almazara" en España, "Moulin" en Francia y "Olificio" en Italia.

La recolección; el lavado, triturado y molturación; el prensado y decantación, son pasos a seguir en la elaboración del aceite de oliva. Veámoslos a continuación a grandes rasgos.

### La recolección

Para obtener un aceite de calidad se debe de partir de unas aceitunas enteras, sanas y maduras. Es fundamental recolectar la aceituna en el momento óptimo de maduración, cuando la mayoría está cambiando de color (envero), apenas quedan aceitunas verdes y algunas están completamente maduras.

El método ideal de recolección es el ordeño o vareo, a mano o con rasquetas. Puesto que la recogida manual es muy cara, a veces es necesario recoger las aceitunas de forma mecánica para obtener beneficios. Existen distintos tipos de máquinas, pero las más utilizadas por los agricultores son las máquinas vibradoras manejadas desde tractores (excéntrica con cable, vibradores de inercia y, fundamentalmente, vibradores multidireccionales) o por los operarios.

Lo más importante es no dañar la aceituna y transportarla lo antes posible a la almazara, para que el fruto no se deteriore. Ahora bien, el trato que la aceituna reciba en la industria va a determinar también la calidad, pues si no se actúa correctamente en todas la fases de la elaboración, la buena calidad potencial encerrada en las aceitunas se puede perjudicar [20].

**Transporte y almacenamiento**

El mejor medio de transporte de las aceitunas recogidas es la caja de plástico con orificios especiales que permitan la ventilación y ayuden a dispersar el calor producido por la actividad catabólica del fruto [21]. Generalmente se usan cajas de 25 a 250-300 kg de capacidad que limitan la profundidad de las capas de las aceitunas reduciendo así el riesgo de aplastamiento. Estas cajas son también el mejor método para almacenar las aceitunas en espera de ser molidas.

Las aceitunas deben ser procesadas en la almazara tan pronto como les sea posible, sobre todo si están maduras. Si no fuera así, y éstas tuvieran que ser almacenadas, el almacenamiento debería ser lo más corto posible y llevarse a cabo de tal modo que se proteja siempre la calidad del producto.

**Limpieza y lavado**

La separación de hojas y el lavado son procesos necesarios para eliminar de las aceitunas materiales extraños, tanto vegetales como no vegetales, que puedan ser dañinos para la maquinaria o contaminar el producto. Este proceso se lleva a cabo con maquinarias equipadas con un potente aspirador, que elimina hojas y pequeñas ramas, y un tubo con circulación forzada de agua que permite lavar las aceitunas.

[20] A. Guerrero García. "Nueva Olivicultura". Ed. Mundi-Prensa. (2003).
[21] L. Ibar Albiñana. "Guía completa del cultivo del olivo". Ed. Vecchi. (2002).

*Figura*. Instalación para el lavado y pesado de la aceituna.

### Procesamiento de la aceituna

El objetivo de procesar las aceitunas es obtener un aceite de oliva virgen tal y como lo define el Reglamento CE Nº 1989/2003 de la Comisión de 6 de Noviembre de 2003 que modifica el Reglamento (CEE) Nº 2568/1991. La extracción mecánica del aceite de las aceitunas implica liberarlo de los tejidos del fruto de tal forma que las gotitas se unan formando gotas mayores hasta constituir una fase líquida continua. La mayor parte del aceite de oliva se localiza dentro de las vacuolas de las células del mesocarpio, aunque también existe algo de aceite disperso dentro del sistema coloidal del citoplasma y, un pequeño tanto por ciento dentro del epicarpio y del endosperma [22].

El aceite incluido en las vacuolas puede ser liberado por medios mecánicos, aunque el aceite disperso dentro del citoplasma es bastante difícil de extraer y normalmente se pierde con el orujo o el alpechín. A veces cuando la pasta de aceitunas está siendo sometida al proceso de batido se forma una emulsión que dificulta la separación del aceite. Por esta razón, la preparación de la pasta de aceitunas para la obtención de aceite con medios mecánicos, debe llevarse a cabo para alcanzar dos objetivos básicos: 1) la ruptura completa de las células del mesocarpio que contienen el aceite; y 2) la eliminación o restricción de la emulsión de la pasta.

Hoy día para la extracción del aceite de oliva se emplean dos procesos, uno de ellos se basa en una separación en una centrífuga horizontal ("decánter") de las tres fases presentes en la aceitunas (aceite, agua de vegetación y sólido); en el otro (de dos fases) se separa el aceite del resto de componentes presentes en los frutos (sólido y agua de

[22] L. Di Giovacchino. "Aspectos tecnológicos" en R. Aparicio, J. Hardwood. "Manual del aceite de oliva". Ed. Mundi-Prensa. Madrid. (2003) 33-67.

vegetación). Ello, influye significativamente en la cantidad y composición de los distintos subproductos que se generan. Además, todavía en algunos lugares se emplea el llamado "proceso tradicional" en el que el aceite se extrae mediante prensas [23]. En la figura se muestra un esquema de las diferentes operaciones que se realizan para la extracción del aceite de oliva para cada uno de los tres de sistemas:
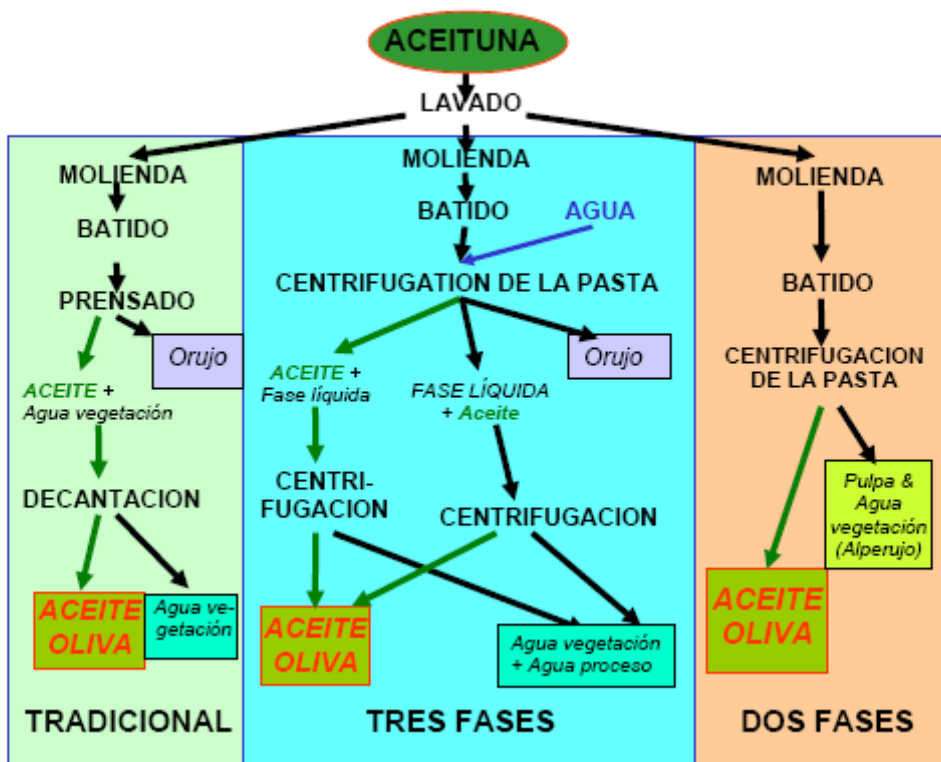


Se podría mencionar también el método "Sinolea" que se basa en el principio de filtración selectiva. Es un viejo sistema tecnológico (que se utilizó por primera vez en España en 1911) que, aunque escasamente, aún hoy en día se utiliza para algunas producciones típicas. Consiste en un sistema de láminas metálicas donde queda adherido el aceite que se extrae posteriormente de la pasta. El rendimiento es más bajo comparado con los sistemas anteriores, por lo que es aconsejable acoplarle un sistema de centrifugación [ 24 ]. Por dichas razones, este sistema no lo describiremos posteriormente.

[23] L. Cerretani, S. Cerni. "Tecniche estrattive ed influenza sulle caractteristiche chimico-fisiche ed organolettiche Delhi oil" en "Dalle olive all'olio: Un viaggio alla scoperta del più nobile dei condimenti". Ed. Regione Emilia-Romagna y Alma Mater Studiorum (Universidad de Bologna) Pp. 32-47 (2005).
[24] V. Sciancalepore, G. De Stefano, P. Piacquadio. "Influence of Sinolea cold extraction system on virgin olive oil quality". *Industrie Alimentari* 39(394) (2000) 824-828.

**<u>Proceso tradicional</u>**

Este proceso de extracción, que ha sido el único método para obtener aceite de oliva virgen durante muchos siglos, está basado en distintos procesos mecánicos que dependen del tipo de prensado llevado a cabo. En él la fase líquida (aceite de oliva + agua de vegetación) se separa de la fase sólida de las aceitunas por presión. Seguidamente, se realiza la separación del aceite de oliva contenido en el líquido obtenido en el prensado por decantación.

Las etapas más interesantes que hemos de considerar en este tipo de proceso son: la molienda, el batido, el prensado y la decantación.

Las aceitunas se trituran en molinos, que generalmente son de piedra. Una vez molidas las aceitunas, la pasta obtenida tiene que ser agitada lentamente (batido). El objeto de esta etapa es aumentar el tamaño de las gotas de aceite para que puedan separarse más fácilmente [25,26]; además, se rompen las emulsiones aceite/agua.

La etapa del prensado se basa en el principio de que cuando se aplastan las aceitunas se libera la fase líquida (el agua de vegetación y el aceite), separándose de la fase sólida. En la prensa, la extracción consiste en la aplicación de presión a una pila de discos filtrantes ("capacho" en España), entre cada dos de ellos se dispone una capa de pasta de aceituna [27].

Al prensar la pasta de aceitunas se obtiene un líquido que contiene aceite de oliva, agua de vegetación y una cantidad pequeña de sólidos que se escapan en la prensa. Si se deja reposar (decantación) la mezcla líquida que se obtiene en el prensado, el aceite se queda en la superficie debido a que es menos denso (alrededor de 0.91 kg/l) que el agua de vegetación (aproximadamente 1.01). El procedimiento consiste en disponer una serie de depósitos conectados mutuamente. El aceite pasa de depósito en depósito por la parte superior, teniendo lugar la sedimentación del agua. El agua de vegetación circula en el

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[25] J. M. Martínez Moreno, C. Gómez Herrera, C. Janer del Valle. "Estudios físico-químicos sobre las pastas de aceitunas molidas. Las gotas de aceite". *Grasas Aceites* 8 (1957) 112-120.

[26] J. M. Martínez Moreno, C. Gómez Herrera, C. Janer del Valle. "Estudios físico-químicos sobre las pastas de aceitunas molidas. V". *Grasas Aceites* 8 (1957) 155-161.

[27] J. M. Martínez Moreno, C. Gómez Herrera, C. Janer del Valle, J. Pereda. "Estudios físico-químicos sobre las pastas de aceitunas molidas. La prensada de la pasta como un proceso de filtración". *Grasas Aceites* 15 (1964) 299-307.

sentido opuesto, por medio de sifones en la parte inferior de los depósitos; con ello, se retiene en la superficie la mayor cantidad posible de aceite.



Esquema de los pasos seguidos durante el proceso tradicional: Molino de piedras troncocónicas → Batidoras cilíndricas → Carro con capacho y prensa → Conjunto de decantadores.
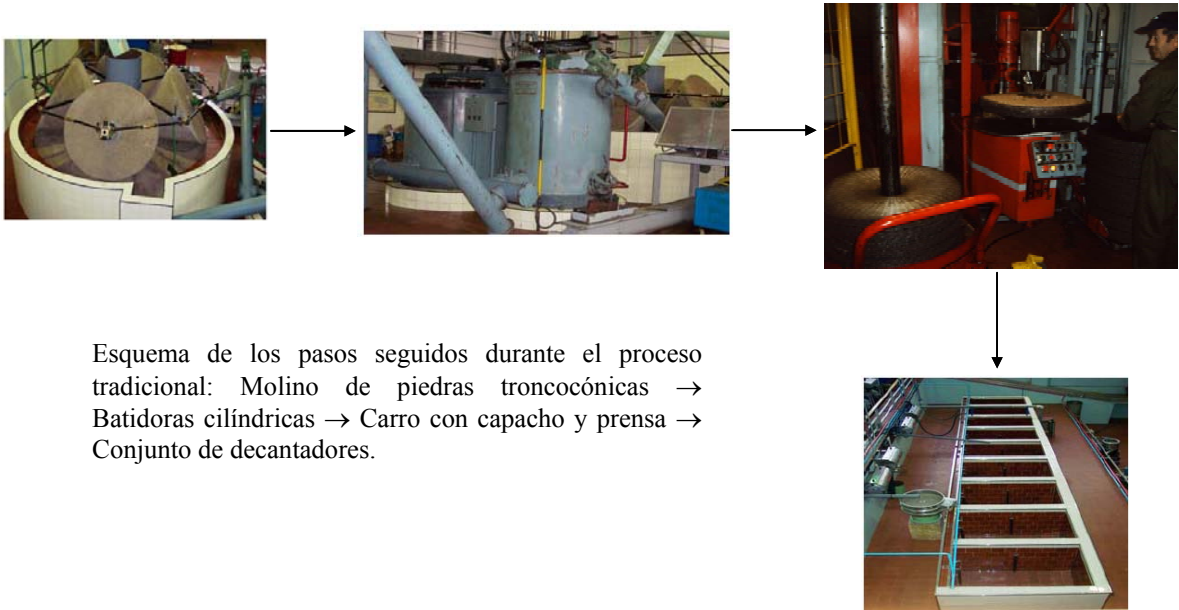
### Proceso de tres fases

La extracción de aceite de oliva por centrifugación de la pasta de aceituna y la obtención separada de aceite, agua de vegetación y el residuo sólido (orujo) se efectúa en plantas modulares que trabajan en continuo, cuyo esquema general se muestra a continuación:
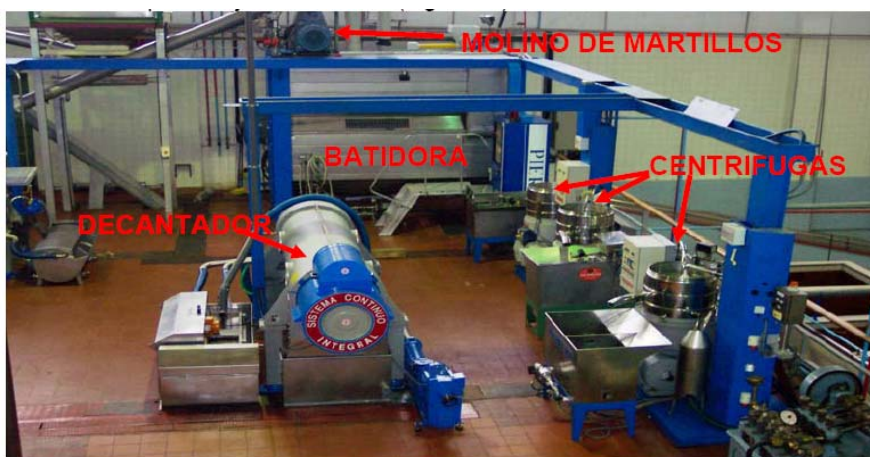
Las fases de molienda y batido son comunes al proceso tradicional, pero en lugar del prensado y la decantación, en este caso se incorporan las fases de centrifugación [28]. Habitualmente, en los sistemas continuos se emplean molinos metálicos (martillo, dientes, discos, cilíndricos o de rodillos) para triturar la aceituna. El más usado es el molino de martillo, aunque puede dar lugar a la formación de emulsiones entre el aceite y el agua. Por ello, es necesario realizar el batido de la pasta para aumentar el tamaño de las gotas de aceite.



*Figura*. Molino de martillos

Centrifugación de la pasta

En este proceso las fases líquidas se separan de la fase sólida por medio de la aplicación de fuerzas centrífugas que aumentan las diferencias entre las densidades específicas del aceite, agua de vegetación [29] y la materia sólida. Esta operación se realiza en una centrífuga horizontal (decantador).
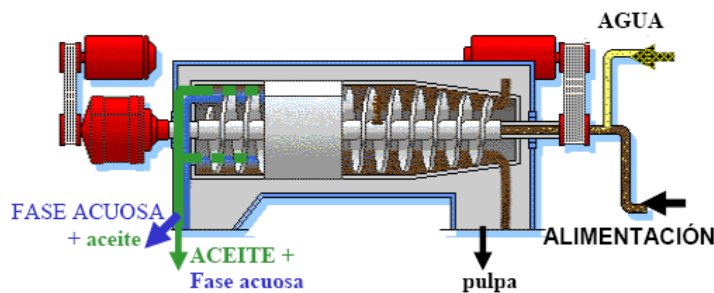


Figura. Diagrama de un decantador de tres fases

[28] P. Amirante, P. Catalano. "Analisi teorica e sperimentale dell´estrazione dell´olio d´oliva per centrifugazione". *Riv. Ital. Sost. Grasse* 70 (1993) 329-335.
[29] P. Amirante, L. Di Giovacchino, G. C. Di Renzo. "Riciclo delle acque di vegetazione nell'estrazione centrifuga: aspetti qualitativi e quantitativi" en: Proceedings of Intern. Congress on "Olive Oil Quality". Ed. Regione Toscana, Firenze (Italy). (1992) Pp. 115–126.

Ésta consiste en un rotor cilíndrico-cónico y un rascador helicoidal de eje hueco, que gira coaxialmente en el interior del mismo. La diferencia de la velocidad entre uno y otro hace que los sólidos se adosen a la pared interior del rotor y sean arrastrados hacia un extremo por el tornillo sinfín. Los líquidos (el aceite y la fase acuosa) forman anillos concéntricos más interiores según su densidad y salen al exterior por diferentes conducciones. Para obtener una mejor separación de los componentes es necesario agregar agua a la pasta que viene de la batidora.

Del decantador centrífugo salen dos tipos de líquidos: uno de color verde formado por el aceite y algo de fase acuosa (agua de vegetación y la agregada en el proceso) y el otro (en un volumen mayor) de color marrón constituido principalmente por la fase acuosa con algo de aceite.

Centrifugación de los líquidos

Ambos líquidos se someten a una centrifugación en una centrífuga de platos.



Figura. Centrífugas

De esta manera, se recupera la fracción de aceite que acompaña a la fase acuosa (la de color marrón) y mediante la adición de una cierta cantidad de agua se retira parte de la humedad y se limpia el aceite de la fase oleosa.

En general, los procesos de centrifugación han ayudado ha aumentar la cantidad de aceite producido en áreas donde el rendimiento era mediocre o pobre. Veamos ahora el proceso de dos fases.

## **Proceso de dos fases**

En 1992 algunas empresas fabricantes de maquinaria para las industrias oleícolas lanzaron al mercado nuevos modelos de decantadores centrífugos horizontales. Estos eran capaces de separar la fase aceitosa de la pasta de aceituna sin requerir la adición de agua caliente. Esto implicaba que no se producían vertidos líquidos, ya que el agua de vegetación de las aceitunas permanecía junto con la pulpa, teniendo por tanto el residuo sólido un mayor contenido en humedad [30].

Este proceso se realiza en plantas modulares que trabajan en continuo y en las mismas se obtiene separadamente, por una parte, el aceite y, por otra, una pasta fluida ("alperujo") que contiene el agua de vegetación y la pulpa.

Las dos primeras fases de este proceso se realizan de forma similar a las comentadas para el sistema de tres fases. La única la diferencia se encuentra en la centrifugación de la pasta y la subsiguiente centrifugación del aceite resultante.

### Centrifugación de la pasta

En este proceso las fase aceitosa se separa de la aceituna (pulpa + agua de vegetación) por efecto de la fuerza centrífuga que aumenta las diferencias entre las densidades específicas del aceite y el "alperujo" (materia sólida más el agua de vegetación).

Esta operación se realiza en un decantador centrífugo horizontal similar al empleado en el sistema de tres fases (Figura).
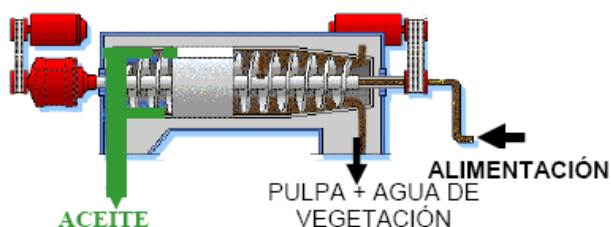


*Figura*. Diagrama de un decantador de dos fases.

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[ 30 ] M. Hermoso, J. González, M. Uceda, A. García-Ortiz, J. Morales, L. Frías, A. Fernández. "Elaboración de aceites de oliva de calidad. Obtención por el sistema de dos fases". Apuntes, núm. 11/94. Junta de Analucía. Sevilla. (1995).

El valor de "*G*" aplicado es superior en la centrífuga de dos fases (3000-3600) que en el proceso de tres fases (2000-2600). Este valor "*G*" depende de la velocidad de rotación y del diámetro interior del rotor. En este caso no es necesario añadir agua para conseguir una mejor separación del aceite como ocurría en el proceso de dos fases.

Centrifugación de los líquidos

La fase oleosa se somete a una centrifugación en una centrífuga de platos. En esta operación se añade una cierta cantidad de agua al aceite con objeto de lavarlo y poder retirarle parte de la humedad que traía del decantador. Este agua es el único vertido que se genera en este proceso de extracción del aceite [31].

**Comparación entre el proceso de extracción de tres y dos fases**

Lógicamente, la calidad del aceite obtenido depende del sistema usado para procesar las aceitunas. En el proceso de tres fases se reduce la cantidad de antioxidantes naturales presentes en el aceite debido al agua agregada para diluir la pasta de aceituna. El agua disuelve una parte de los polifenoles del aceite, disminuyendo el tiempo de estabilidad oxidativa, que se correlaciona con la resistencia a la autooxidación. Sin embargo, el contenido en pigmentos clorofílicos es más alto en el aceite obtenido en las plantas modulares debido a que en la molienda en el molino de martillo se liberan más clorofilas que en el de piedras, disolviéndose en gran cantidad en el aceite.

El mayor problema del proceso de tres fases es el elevado volumen de agua residual que se genera ("alpechín") (1,2-1,3 litros/kilogramo de aceituna procesada). Ello es debido a la adición de agua que se realiza a la pasta de aceitunas antes de entrar en el decantador horizontal.

Por otra parte, el rendimiento en el proceso de extracción de dos fases es superior que el que se tiene en el de tres fases. Ello se debe fundamentalmente al hecho de que en el nuevo sistema no se añade agua a la pasta y se evita la formación de emulsiones aceite/agua.

Este proceso no produce apenas vertidos, sólo se origina un pequeño volumen del agua añadida en el lavado del aceite en la centrifugación. Con ello, se evitan los problemas

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[31] L. Di Giovacchino, N. Costantini. M. Di Febo. "La centrifugazione della paste di olive senza acqua di diluizione". *Riv. Ital. Sost. Grasse* 71 (1994) 555-559.

ambientales que se originan por las grandes dimensiones que deben tener las balsas de evaporación en las que se debería verter el agua residual que se produce en los otros dos sistemas de extracción y el consiguiente aumento de los costos que ello origina.

La calidad del aceite producido es superior a la obtenida en el proceso de tres fases [32]. Ello es debido a la mayor concentración de polifenoles y o-difenoles que se tiene con el nuevo sistema. Ello implica que dicho aceite es más estable durante el almacenamiento como lo indican los elevados valores del tiempo de estabilidad oxidativa que se tienen en los aceites obtenidos por este sistema.

Cuáles son las ventajas de cada uno de los sistemas de extracción, así como la influencia de otras muchas variables que toman parte en el proceso de elaboración del aceite, sirven de argumento para muchos trabajos de investigación desarrollados recientemente [33,34,35,36,37,38].

### Almacenamiento del aceite de oliva

Los tanques para almacenar el aceite de oliva deberían construirse con materiales totalmente impermeables e inatacables. El material usado para la construcción debe presentar la inercia mayor con respecto al aceite, para que éste no absorba olores y sabores defectuosos y no se disuelvan sustancias que podrían contaminar o producir fenómenos de oxidación (presencia de metales) en los aceites.

El tanque debe proteger al aceite de la luz y el aire, ya que estos factores aceleran la alteración del producto. Además, debe mantener el aceite a una temperatura casi constante (sobre 15-18 ºC), evitando cambios bruscos. Temperaturas bajas pueden

[32] L. Di Giovacchino, M. Solinas, M. Miccoli. "Effect of extraction systems on the quality of virgin olive oil". *J. Am. Oil Chem. Soc*. 71 (1994) 1189-1194.

[33] L. Di Giovacchino, N. Costantini, A. Serraiocco, G. Surricchio, C. Basti. "Natural antioxidants and volatile compounds of virgin olive oils obtained by two or three-phases centrifugal decanters". *Eur. J. Lipid Sci. Technol.* 103 (2001) 279–285.

[34] G. De Stefano, P. Piacquadio, M. Servili, L. Di Giovacchino, V. Sciancalepore. "Effect of extraction systems on the phenolic composition of virgin olive oils". *Fett/Lipid* 101 (1999) 328–332.

[35] A. Cert, J. Alba, M. C. Pérez-Camino, A. Ruiz-Gomez, F. Hidalgo, W. Moreda, M. J. Moyano, F. Martínez, R. Tubaileh, J.M. Olias. "Influencia de los sistemas de extracción sobre las características y componentes menores del aceite de oliva virgen extra". *Olivae* 79 (1999) 41–50.

[36] M. Uceda, A. Jiménez, G. Beltrán. "Olive oil extraction and quality". *Grasas Aceites* 57 (2006) 25-31.

[37] A. Ranalli, L. Lucera, S. Contento, N. Simone, P. Del Re. "Bioactive constituents, flavors and aromas of virgin oils obtained by processing olives with a natural enzyme". *Eur. J. Lipid Sci. Tecnol*. 106(3) (2004) 187-197.

[38] V. Sciancalepore, G. De Stefano, P. Piacquadio. "Effects of the cold percolation system on the quality of virgin olive oil". *Eur. J. Lipid Sci. Tecnol*. 102 (2000) 680-683.

provocar la congelación del aceite y altas contribuir a su oxidación. Hasta hace unos años los tanques que mejor cumplían estas condiciones eran los depósitos enterrados con un adecuado recubrimiento (generalmente azulejos refractarios). Hoy en día en España, se emplean depósitos de acero inoxidable de diferentes capacidades (generalmente de 50 toneladas), parecidos a los empleados en la industria del vino.



*Figura*. Bodega con depósitos de almacenamiento de aceite

Muchas son las publicaciones que recogen relevante información sobre el almacenamiento del aceite de oliva [39,40,41,42,43].

A continuación se presenta un esquema que ilustra el proceso de elaboración del aceite de oliva, no se contempla en él una distinción profunda entre los tipos de aceite puesto que esto nos ocupará en la sección siguiente sección:

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[39] G. Luna, M. T. Morales, R. Aparicio. "Changes induced by UV radiation during virgin olive oil storage". *J. Agric.Food Chem*. 54(13) (2006) 4790-4794.

[40] R. Rakesh, P. C. Sharma, K. B. B. Lal. "Evaluation of storage stability of olive (Olea europaea L.) oil from promising cultivars in Himachal Pradesh". *J. Food Sci. Technol-Mysore* 43(3) (2006) 259-262.

[41] A. Bendini, L. Cerretani, S. Vecchi, A. Carrasco-Pancorbo, G. Lercker. "Protective effects of extra virgin olive oil phenolics on oxidative stability in the presence or absence of copper ions". *J. Agric. Food Chem.* 54(13) (2006) 4880-4887.

[42] V. Lavelli, G. Fregapane, M. D. Salvador. "Effect of storage on secoiridoid and tocopherol contents and antioxidant activity of monovarietal extra virgin olive oils". *J. Agric. Food Chem.* 54(8) (2006) 3002-3007.

[43] A. Del Caro, V. Vacca, M. Poiana, P. Fenu, A. Piga. "Influence of technology, storage and exposure on components of extra virgin olive oil (Bosana cv) from whole and de-stoned fruits". *Food Chem.* 98 (2006) 311-316.

## ELABORACION DEL ACEITE DE OLIVA

**PRENSA**
Prensado
Decantación

**MOLINO**
Lavado
Triturado
Molturación

**ACEITUNA**

**OLIVO**

**DECANTADOR**
Procesos de 3 y 2 fases

→ Aceites de oliva vírgenes

→ Aceites que necesitan otra serie de procesos previos a su consumo
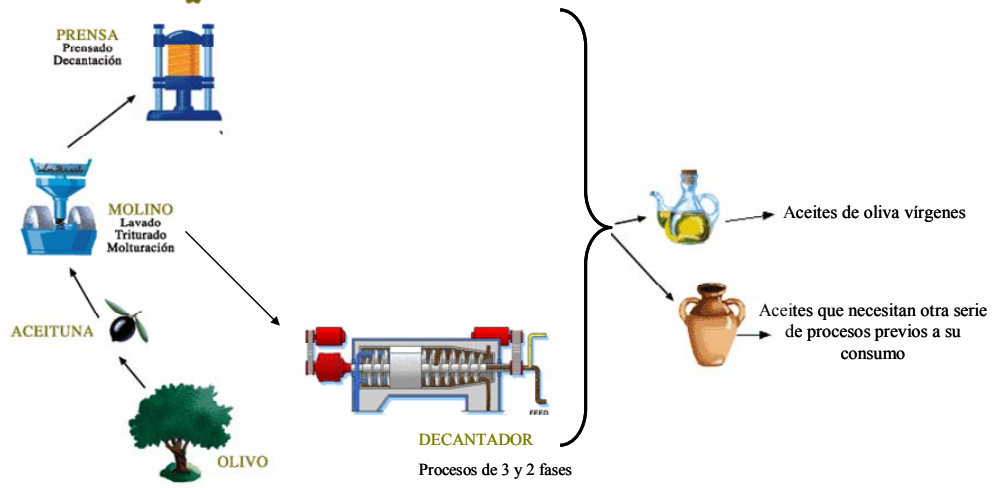
## Tipos de aceite de oliva

A modo general podemos decir que son tres los organismos encargados de controlar toda la legislación referente al aceite de oliva:

- Unión Europea
- Consejo Oleícola Internacional
- Codex Alimentarius (FAO OMS)

A fin de entender las fuentes de la legislación europea, es importante saber que éstas pueden ser clasificadas como legislación primaria -Tratados- y legislación secundaria -Leyes. Los tratados establecen los objetivos y los poderes asignados a las instituciones de la Unión Europea. Las dos fuentes principales de la legislación secundaria son las Directivas y los Reglamentos. La mayor parte de legislación está en forma de Directivas, que establecen objetivos comunes y plazos para que los Estados Miembros los apliquen a la legislación nacional apropiada. Los Reglamentos, al contrario, tienen una aplicabilidad directa, lo que significa que no tienen que ser transformadas en leyes nacionales: imponen directamente derechos y obligaciones a todos los ciudadanos de la Unión [44].

Las industrias del aceite de oliva tienen que cumplir con toda la legislación europea vigente para la industria alimentaria, así como con la legislación específica del sector. A continuación se menciona la legislación específica de la industria olivarera más importante y se recoge una clasificación de los aceites de oliva.

Para conseguir completa armonía entre los Reglamentos europeos y las normas internacionales del Consejo Oleícola Internacional y del *Codex Alimentarius*, fue necesario revisar determinados valores límite relativos a las características de los aceites de oliva y de los aceites de orujo de oliva incluidos en el Reglamento (CEE) Nº 2568/91.

El Reglamento CE Nº 1989/2003 de la Comisión de 6 de Noviembre de 2003 que modifica el Reglamento (CEE) Nº 2568/1991, relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis,

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[44] TDC.Olive. "Enciclopedia del Olivo". Capítulo: "Legislación Europea Relacionada con el Sector Olivarero y APPCC", elaborado por Biozoon GmbH y traducido al español por CINDOC_CSIC.

define las características físicas, químicas y organolépticas de los aceites de oliva y de orujo de oliva, así como los métodos de valoración de esas características. Así, contempla a los <u>aceites de oliva vírgenes</u> como: los obtenidos a partir del fruto del olivo únicamente por procedimientos mecánicos u otros procedimientos físicos, en condiciones que no ocasionen la alteración del aceite y que no hayan sufrido tratamiento alguno distinto del lavado, la decantación, el centrifugado y la filtración, con exclusión de los aceites obtenidos mediante disolvente, mediante coadyuvante de acción química o bioquímica, o por procedimiento de reesterificación y de cualquier mezcla de aceites de otra naturaleza.

Desaparece pues, desde el 01/11/03 y de acuerdo con la reglamentación comunitaria, la categoría denominada "aceite de oliva virgen corriente" y se modifican los límites máximos de acidez en las categorías virgen extra, virgen, aceite de oliva y aceite de orujo de acuerdo con la siguiente descripción. El nuevo reglamento incluye la siguiente clasificación:

• **Aceite de Oliva Virgen Extra**: Aceite de oliva virgen cuya acidez libre, expresada en ácido oleico, no supera 0,8 g por cada 100 g y cuyas demás características son conformes a las establecidas para la categoría.

• **Aceite de Oliva Virgen**: Aceite de oliva virgen cuya acidez libre, expresada en ácido oleico, no supera 2 g por cada 100 g y cuyas demás características son conformes a las establecidas para la categoría. (En fase de producción y comercialización al por mayor podrá emplearse el término de "fino")

• **Aceite de Oliva Virgen Lampante**: Aceite de oliva cuya acidez libre, expresada en ácido oleico, es superior a 2 g por cada 100 g y cuyas demás características son conformes a las establecidas para la categoría.

**Aceite de Oliva Refinado**: Aceite de oliva obtenido mediante refino de aceites de oliva vírgenes, cuya acidez libre, expresa en ácido oleico, no podrá ser superior a 0,3 g por 100 g y cuyas otras características son conformes a las establecidas para esta categoría.

**Aceite de oliva**: Contiene exclusivamente aceites de oliva refinados y aceites de oliva vírgenes distintos del lampante, cuya acidez libre, expresada en ácido oleico no podrá ser superior a 1 g por 100 g y cuyas demás características son conformes a las establecidas para la categoría.

Por otro parte, el Reglamento anteriormente mencionado incluye también aceites de orujo de oliva. El orujo de oliva es el residuo sólido o pasta que se obtiene en el proceso de elaboración del aceite de oliva (durante el prensado o centrifugado). Este residuo se trata con solventes para extraer el aceite que contiene, obteniéndose así el **Aceite de Orujo Crudo**, que al no ser comestible debe someterse a un proceso de refinado similar al de los aceites de oliva vírgenes lampantes obteniendo de este modo el **Aceite de Orujo Refinado**. Este aceite, desprovisto de sabor, olor y color, se enriquece con aceites de oliva vírgenes comestibles (distintos del lampante) logrando así el denominado **"Aceite de Orujo de Oliva"**. Tal y como hemos hecho con los aceite de oliva, vamos a clasificar los tipos de aceite de orujo de oliva:

- **Aceite de orujo de oliva crudo**: Aceite obtenido a partir de orujo de oliva mediante tratamiento con disolvente o por medios físicos, o que corresponda, con excepción de algunas características determinadas, a un aceite de oliva lampante; con exclusión de los aceites obtenidos por procedimientos de reesterificación y de cualquier mezcla con aceites de otra naturaleza y cuyas otras características son conformes a las establecidas para esta categoría.
- **Aceite de orujo de oliva refinado**: Aceite obtenido mediante refino de aceite de orujo de oliva crudo, cuya acidez libre, expresada en ácido oleico, no podrá ser superior a 0,3 g por 100 g y cuyas otras características son conformes a las establecidas para esta categoría.
- **Aceite de orujo de oliva**: Aceite constituido por una mezcla de aceite de orujo de oliva refinado y de aceites de oliva vírgenes distintos del lampante, cuya acidez libre, expresada en ácido oleico, no podrá ser superior a 1 g por 100 g y cuyas otras características son conformes a las establecidas para esta categoría.

De los mencionados anteriormente, sólo el aceite de oliva virgen extra, el aceite de oliva virgen, el aceite de oliva y el aceite de orujo de oliva se presentan directamente al consumidor final. El aceite Lampante por su parte, se comercializa solamente al por mayor.
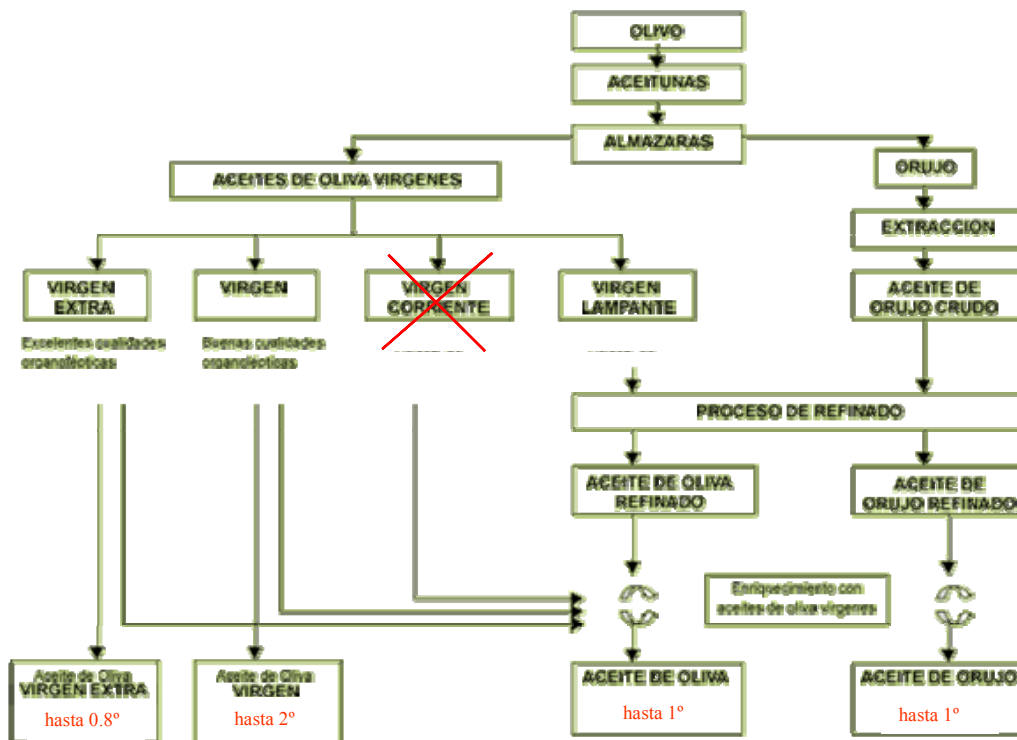
*Figura*. Esquema que incluye los distintos tipos de aceites, tanto vírgenes como de orujo, y los límites de acidez de cada categoría.

La siguiente tabla recoge todas las características de cada una de las categorías de los aceites de oliva (Reglamento CE Nº 1989/2003; L295/60-L295/61)

CARACTERÍSTICAS DE LOS ACEITES DE OLIVA

| Categoría | Acidez (%) (*) | Índice de peróxidos mEq O$_2$/kg (*) | Ceras mg/kg (**) | Ácidos saturados en posición 2 de los triglicéridos (%) | Estigmastadieno mg/kg [1] | Diferencia entre ECN42 HPLC y ECN42 (cálculo teórico) | K$_{232}$ (*) | K$_{270}$ (*) | Delta-K (*) | Evaluación organoléptica Mediana del defecto (Md) (*) | Evaluación organoléptica Mediana del atributo frutado (Mf) (*) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. Aceite de oliva virgen extra | ≤ 0,8 | ≤ 20 | ≤ 250 | ≤ 1,5 | ≤ 0,15 | ≤ 0,2 | ≤ 2,50 | ≤ 0,22 | ≤ 0,01 | Md = 0 | Mf > 0 |
| 2. Aceite de oliva virgen | ≤ 2,0 | ≤ 20 | ≤ 250 | ≤ 1,5 | ≤ 0,15 | ≤ 0,2 | ≤ 2,60 | ≤ 0,25 | ≤ 0,01 | Md ≤ 2,5 | Mf > 0 |
| 3. Aceite de oliva lampante | > 2,0 | — | ≤ 300 [3] | ≤ 1,5 | ≤ 0,50 | ≤ 0,3 | — | — | — | Md > 2,5 [2] | — |
| 4. Aceite de oliva refinado | ≤ 0,3 | ≤ 5 | ≤ 350 | ≤ 1,8 | — | ≤ 0,3 | — | ≤ 1,10 | ≤ 0,16 | — | — |
| 5. Aceite de oliva compuesto exclusivamente por aceites de oliva refinados y aceites de oliva vírgenes | ≤ 1,0 | ≤ 15 | ≤ 350 | ≤ 1,8 | — | ≤ 0,3 | — | ≤ 0,90 | ≤ 0,15 | — | — |
| 6. Aceite de orujo de oliva crudo | — | — | > 350 [4] | ≤ 2,2 | — | ≤ 0,6 | — | — | — | — | — |
| 7. Aceite de orujo de oliva refinado | ≤ 0,3 | ≤ 5 | > 350 | ≤ 2,2 | — | ≤ 0,5 | — | ≤ 2,00 | ≤ 0,20 | — | — |
| 8. Aceite de orujo de oliva | ≤ 1,0 | ≤ 15 | > 350 | ≤ 2,2 | — | ≤ 0,5 | — | ≤ 1,70 | ≤ 0,18 | — | — |

[1] Suma de isómeros que podrían separarse (o no) mediante columna capilar.
[2] O cuando la mediana de los defectos es inferior o igual a 2,5 y la mediana del atributo frutado es igual a 0.
[3] Los aceites con un contenido de ceras comprendido entre 300 y 350 mg/kg se consideran aceite de oliva lampante si el contenido de alcoholes alifáticos totales es inferior o igual a 350 mg/kg o si el porcentaje de eritrodiol y uvaol es inferior o igual a 3,5.
[4] Los aceites con un contenido de ceras comprendido entre 300 y 350 mg/kg se consideran aceite de orujo de oliva crudo si el contenido de alcoholes alifáticos totales es superior à 350 mg/kg y si el porcentaje de eritrodiol y uvaol es superior a 3,5.

| Categoría | Contenido de ácidos grasos (¹) | | | | | | Sumas de los isómeros trans-oleicos (%) | Sumas de los isómeros trans-linoleicos + trans-linolénicos (%) | Composición de esteroles | | | | | | Esteroles totales (mg/kg) | Eritrodiol y uvaol (%) (**) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mirístico (%) | Linolénico (%) | Araquídico (%) | Eicosenoico (%) | Behénico (%) | Lignocérico (%) | | | Colesterol (%) | Brasicasterol (%) | Campesterol (%) | Estigmasterol (%) | Beta-sitosterol (%) (²) | Delta-7-estigmastenol (%) | | |
| 1. Aceite de oliva virgen extra | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,2 | ≤ 0,2 | ≤ 0,05 | ≤ 0,05 | ≤ 0,5 | ≤ 0,1 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 000 | ≤ 4,5 |
| 2. Aceite de oliva virgen | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,2 | ≤ 0,2 | ≤ 0,05 | ≤ 0,05 | ≤ 0,5 | ≤ 0,1 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 000 | ≤ 4,5 |
| 3. Aceite de oliva lampante | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,2 | ≤ 0,2 | ≤ 0,10 | ≤ 0,10 | ≤ 0,5 | ≤ 0,1 | ≤ 4,0 | — | ≥ 93,0 | ≤ 0,5 | ≥ 1 000 | ≤ 4,5 (³) |
| 4. Aceite de oliva refinado | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,2 | ≤ 0,2 | ≤ 0,20 | ≤ 0,30 | ≤ 0,5 | ≤ 0,1 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 000 | ≤ 4,5 |
| 5. Aceite de oliva compuesto exclusimente por aceites de oliva refinados y aceites de oliva vírgenes | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,2 | ≤ 0,2 | ≤ 0,20 | ≤ 0,30 | ≤ 0,5 | ≤ 0,1 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 000 | ≤ 4,5 |
| 6. Aceite de orujo de oliva crudo | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,3 | ≤ 0,2 | ≤ 0,20 | ≤ 0,10 | ≤ 0,5 | ≤ 0,2 | ≤ 4,0 | — | ≥ 93,0 | ≤ 0,5 | ≥ 2 500 | > 4,5 (⁴) |
| 7. Aceite de orujo de oliva refinado | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,3 | ≤ 0,2 | ≤ 0,40 | ≤ 0,35 | ≤ 0,5 | ≤ 0,2 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 800 | > 4,5 |
| 8. Aceite de orujo de oliva | ≤ 0,05 | ≤ 1,0 | ≤ 0,6 | ≤ 0,4 | ≤ 0,3 | ≤ 0,2 | ≤ 0,40 | ≤ 0,35 | ≤ 0,5 | ≤ 0,2 | ≤ 4,0 | < Camp. | ≥ 93,0 | ≤ 0,5 | ≥ 1 600 | > 4,5 |

(¹) Contenido de otros ácidos grasos (%): palmítico 7,5-20,0; palmitoleico 0,3-3,5; heptadecanoico ≤; heptadecenoico ≤ 0,3; esteárico 0,5-5,0; oleico 55,0-83,0; linoleico 3,5-21,0.

(²) Suma de: Delta-5-23-estigmastadienol + clerosterol + beta-sitosterol + sitostanol + delta-5-avenasterol + delta-5-24-estigmastadienol.

(³) Los aceites con un contenido de ceras comprendido entre 300 y 350 mg/kg se consideran aceite de oliva lampante si el contenido de alcoholes alifáticos totales es inferior o igual a 350 mg/kg o si el porcentaje de eritrodiol y uvaol es inferior o igual a 3,5.

(⁴) Los aceites con un contenido de ceras comprendido entre 300 y 350 mg/kg se consideran aceite de orujo de oliva crudo si el contenido de alcoholes alifáticos totales es superior a 350 mg/kg y si el porcentaje de eritrodiol y uvaol es superior a 3,5.

Notas:

a) Los resultados de los análisis deberán expresarse con el mismo número de decimales que el previsto para cada característica. La última cifra expresada deberá redondearse hacia arriba si la cifra siguiente es superior a 4.

b) Para cambiar de categoría un aceite o declararlo no conforme en cuanto a su pureza, basta con que una sola de las características no se ajuste a los límites fijados.

c) Las características indicadas con asterisco (*), relativas a la calidad del aceite, implican lo siguiente:

— en el caso del aceite de oliva lampante, los límites correspondientes pueden no respetarse simultáneamente,

— en el caso de los aceites de oliva vírgenes, el incumplimiento de uno de los límites supondrá un cambio de categoría, aunque seguirán clasificados dentro de una de las categorías de los aceites de oliva vírgenes.

d) Las características indicadas con dos asteriscos (**) implican que, en el caso de todos los aceites de orujo de oliva, los límites correspondientes pueden no respetarse simultáneamente.»

Dentro de la categoría de aceites de oliva virgen extra, sería posible hacer una sub-clasificación. Dicha clasificación resultará útil para entender parte del trabajo experimental incluido en esta tesis doctoral, ya que la elección de las muestras en ciertos casos atendió a las siguientes categorías:

- Monovarietales: Elaborados en bases 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 (donde, asimismo, el aceite debe ser elaborado y embotellado) y oficialmente reconocida.

## Variedades españolas de aceitunas de almazara

Como varias de las aplicaciones de los métodos desarrollados en la presente memoria fueron llevadas a cabo utilizando aceites monovarietales, nos parece interesante describir un poco las características principales de las variedades empleadas. De ahí que se haya incluido esta sección en la tesis.

Existen muchos tipos y variedades de aceituna, tanto como para el consumo en verde como para la producción de aceite [45]. En España, como en muchos otros países, la olivicultura nacional está formada por numerosas variedades, de las más diversas características. No obstante, con sólo 4 variedades se tiene más del 60% de la olivicultura, y una sola de estas variedades produce prácticamente la mitad del aceite español [46].

Sería muy difícil recoger en la presente memoria las características principales de todas las variedades españolas, por eso sólo se dará una mayor información de las variedades que han sido sometidas a estudio, que son siete. El por qué de esta elección se halla en el hecho de que hoy en día resulta enormemente difícil conseguir aceites monovarietales, y es de estas seis variedades de las que se logró disponer de aceites con garantía de que provenían de un solo tipo de aceituna.

En España, entre otras, encontramos las siguientes variedades: Arbequina, Cornicabra, Farga, Hojiblanca, Manzanilla, Picual, Gordal, Lechín de Sevilla, Empeltre, Picudo, Verdial de Vélez-Málaga, Lechín de Granada, Verdial de Huévar, Morisca, Manzanilla Cacereña, Blanqueta…

A continuación, se comentan las características generales de las variedades Picual, Cornicabra, Arbequina, Lechín de Granada, Lechín de Sevilla, Hojiblanca y Picudo:

(este listado de características generales no hay que tomarlo como "aplicable" en todos los casos; veremos más adelante que hay muchísimos factores responsables de la composición y otras características de los aceites que pueden provocar que aceites de una misma variedad sean muy diferentes)

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[45] 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).

[46] J. Humanes Guillén, M. Civantos López-Villalta. "Producción de aceite de oliva de calidad. Influencia del cultivo". Junta de Andalucía; Consejería de Agricultura y Pesca. (1993).

**Picual**

**Origen**: Jaén.
**Área de cultivo**: Provincia de Jaén y zonas limítrofes de Granada, Córdoba, Ciudad Real.
**Planta**

Vigor: Bueno.
Vegetación: Ramos algo cortos, ramificados, con tendencia a producir brotes y chupones. Copas vigorosas que tienden a cerrarse, con gran desarrollo foliáceo.
Color madera joven: Verde grisáceo.

**Hoja**

Forma: Algo alargada, ensanchada en su mitad superior.
Color: Haz verde oscuro, envés plateado verdoso.

**Drupa**

Forma: Elipsoidal apuntada por el ápice.
Volumen: Medio a grueso.
Peso medio: de 2.14 a 3.66 g.
Rendimiento graso: 23.8 a 27.7%
Pulpa (porcentaje): 78.7 a 85.5%

La variedad "Picual" o "Marteña", se cultiva en una extensión de alrededor de 600,000 Has, que es aproximadamente el 30% de la superficie olivarera. Como se ha dicho antes, produce la mitad del aceite nacional.

En Jaén, ocupa casi la totalidad del olivar, y se extiende su cultivo a las provincias limítrofes de Granada y Córdoba, en superficies importantes. Se cultiva también en otras provincias andaluzas y en un buen número de las provincias olivareras nacionales.

Los olivos picuales tienen una alta productividad, siendo ésta una de las razones por las que se han intensificado tanto sus plantaciones. Es un tipo de árbol que se adapta a diversas condiciones de clima y suelo y es tolerante a las heladas, pero poco resistente a la sequía y a los terrenos muy calizos. La maduración de sus frutos transcurre desde la segunda semana de noviembre hasta la tercera de diciembre.

En relación a las características de calidad de los aceites de picual cabe destacar su gran estabilidad (171.9 E.R) y su riqueza en ácido oleico (78.93%). Su alto contenido en polifenoles, además de corroborar su alta estabilidad, nos indica la existencia de unos aceites muy afrutados y de gran personalidad.

Para conseguir estos aceites afrutados es necesario elegir una buena época de recolección y realizar esta operación de forma esmerada y teniendo muy presente la necesidad de recoger por separado la aceituna del árbol de la del suelo dada la tendencia de esta variedad a la caída natural de los frutos.

Esta variedad está amparada en las DOPs de Sierra Mágina y Sierra Segura (en estas dos como variedad principal). También en Priego de Córdoba, Sierra de Cazorla y Montes de Granada.

## Cornicabra



**Origen**: Mora de Toledo.
**Área de cultivo**: Provincias de Toledo y Ciudad Real.
**Planta**
  Vigor: Medio.
  Vegetación: Ramos de longitud media, con frecuencia dicotómicamente ramificados, con escasa formación de brindillas y chupones.
  Color madera joven: Gris claro algo ocráceo.
**Hoja**
  Forma: Larga, lanceolada, simétrica.
  Color: Haz verde claro, envés gris verdoso.
**Drupa**
  Forma: Alargada, algo encurvada, asimétrica bombeada y plana por el dorso, vientre en forma de cuerno en las formas típicas.
  Volumen: Medio.
  Peso medio: 3.06 a 3.51 g.
  Rendimiento graso: 21.8 a 27%
  Pulpa (porcentaje): 80.9 a 83.6%

Esta variedad es, en número de hectáreas cultivadas, la segunda en importancia, pero la tercera en producción. Originaria de Mora de Toledo, su área de cultivo abarca las provincias de Toledo y Ciudad Real, en la Comunidad de Castilla la Mancha. Su nombre proviene de la característica forma de cuerno de su fruto. También llamada cornezuelo, es variedad principal de la DOP Montes de Toledo.

Tiene una gran resistencia a las sequías, heladas y fríos invernales, lo que nos indica que siendo árbol mediterráneo se ha adaptado perfectamente a un clima continental. Al final de su maduración se caracteriza por un intenso color violáceo.

El aceite de Cornicabra tiene un bajo contenido en tocoferoles, lo que puede hacer pensar en una falta de estabilidad, pero su alto contenido en polifenoles probablemente la compensa sobradamente y, en realidad, posee un alto índice de estabilidad en el Rancimat, muy próximo al del aceite de la variedad picual. Son aceites estables debido también a su alto contenido en ácidos grasos monoinsaturados.

Son aceites afrutados y aromáticos, mostrando valores medios de amargo y picante. Cuando se obtienen de aceitunas más maduras, al final de la cosecha, es característica la aparición de distintos sabores y texturas a frutos exóticos como el aguacate. Los aceites de Cornicabra presentan un notable equilibrio entre el dulce a la entrada, amargo a hojas verdes y el picante de intensidad media.



**Hojiblanca**

**Origen**: Lucena (Córdoba).
**Área de cultivo**: Provincias de Córdoba y Málaga.
**Planta**
    Vigor: Bueno a medio.
    Vegetación: Ramos fructíferos, más bien largos y algo péndulos. Copas de densidad media y superficie foliar regular.
    Color madera joven: Gris claro verdoso.
**Hoja**
    Forma: Alargada, algo ensanchada, poco acanalada.
    Color: Haz verde grisáceo, envés plateado.
**Drupa**
    Forma: Oblonga, poco asimétrica.
    Volumen: Medio
    Peso medio: 1.4 a 4.3 g.
    Rendimiento graso: 23.5 a 28.6%
    Pulpa (porcentaje): 83.5 a 87.1%

Su área de influencia se extiende por Andalucía, en concreto por el este de la provincia de Sevilla, el sur de Córdoba y todo el norte de la provincia de Málaga. Puede suponer

el 16 % del olivar andaluz. También se la conoce en Andalucía bajo el nombre de Lucentino, por los reflejos metalizados que tienen sus hojas cuando les da el sol. Las aceitunas hojiblancas sirven tanto para aceitunas de mesa negra por la firme textura de su pulpa, como para la producción de aceite.

La maduración de sus frutos es algo tardía, desde finales de noviembre a finales de diciembre y una vez maduro el fruto presenta resistencia al desprendimiento, con lo que su recolección es dificultosa. Es muy vecero en su producción como consecuencia de su tardía recolección (marzo-abril).

Presenta una composición de ácidos grasos muy equilibrada con ácidos saturados relativamente más bajos que en el resto de los aceites de otras variedades. La estabilidad ante la oxidación no es elevada y se recomienda mantener estos aceites al amparo de la luz y sin excesiva oxigenación durante el almacenamiento.

Desde el punto de vista organoléptico, presentan una inmensa gama de flavores, aunque predominan los sabores vegetales. Son valores comunes los atributos de dulzura al inicio de la cata, frutado de hierba fresca en el aroma, ligero amargor a fruta verde y otras frutas que a veces recuerdan a una macedonia, ligero picante en garganta y regusto final almendrado.

**Lechín**

**Origen:** Entre Córdoba y Sevilla.
**Área de cultivo:** Provincias de Sevilla y Granada.
**Planta**

Vigor: Medio.
Vegetación: Ramos más bien cortos, con mediana producción de brindillas y chupones en madera vieja.
Color madera joven: Gris ocráceo.

**Hoja**

Forma: Corta, poco ensanchada en su mitad, casi plana.
Color: Verde amarillento en el haz, envés verde grisáceo.

**Drupa**

Forma: Elipsoidal, un poco bombeada por el dorso.
Volumen: Medio-grueso.
Peso medio: 3.58 a 3.80 g.
Rendimiento graso: 23.5 a 26.8%
Pulpa (porcentaje): 79.1 a 85.4%

Dentro de esta variedad Lechín, podemos hacer la distinción entre Lechín de Sevilla y Lechín de Granada.

### Lechín de Sevilla

Esta variedad se extiende por las provincias de Sevilla, Córdoba, Cádiz, Málaga y Huelva. Su nombre corresponde al color blanquecino de su pulpa y de su mosto oleoso (mezcla de agua de vegetación y aceite). Es una variedad vigorosa. Es capaz de soportar bien las sequías y los fríos invernales y se adapta a los terrenos calizos y pobres.

Desde el punto de vista organoléptico, es un aceite fluído de sabores vegetales, amargor medio y un postgusto a almendra verde en boca. Por lo general, no se suelen comercializar aceites monovarietales de Lechín, pero sí interviene para complementar a otras variedades como la Hojiblanca y Picual.

**Lechín de Granada**

Esta variedad se cultiva en las provincias de Granada, Almería y algo en Málaga. Ocupa una superficie de 15.000 hectáreas.

Es variedad productiva y vecera, con gran capacidad de adaptación, tolera el frío y la sequía. Su época de maduración es tardía, y sus frutos pequeños ofrecen gran resistencia al desprendimiento, por lo que su recolección es difícil y costosa. Tiene un buen rendimiento graso.

Esta variedad se asocia con la Gordal de Granada en el Valle de Lecrín y en Órjiva, en donde se pueden obtener excelentes aceites si se cuida el cultivo y la elaboración. Son aceites de color amarillo pálido, sabor dulce y muy fluídos. Han sido tradicionalmente muy nombrados en Granada y Almería.

## Arbequina

**Origen**: Arbeca (Lleida).
**Área de cultivo**: Provincia de Lleida y Tarragona.
**Planta**

Vigor: Poco.
Vegetación: Brotes más bien largos, poco ramificados, sin ramos adventicios ni chupones.
Color madera joven: Gris verde oscuro.

**Hoja**

Forma: Acanalada con bordes no espesados, ensanchada por el ápice, mucrón pequeño.
Color: Haz verde ocráceo, envés gris amarillo verdoso.

**Drupa**

Forma: Ovalada, corta, casi simétrica.
Volumen: Pequeño.
Peso medio: 0.80 a 1.20 g.
Rendimiento graso: 17.2 a 19.5%
Pulpa (porcentaje): 67 a 76%

Las Arbequinas son aceitunas pequeñas, pero muy apreciadas por su precoz entrada en producción, elevada productividad y aceptable rendimiento graso.

Son aceites que presentan un olor afrutado fresco con aromas a almendras y otras frutas. Amargan y pican muy poco sino nada, la nota de astringencia no aparece nunca y de entrada presentan una gran suavidad y ofrecen una sensación táctil de almendrado muy agradable y delicada. Los podríamos describir como aceites de características armoniosas, suaves, ligeros, delicados, dulces, casi siempre almendrados y con un aroma a frutos maduros (papilla de frutas y manzana), en los que a veces se atisban aromas exóticos.

Sin embargo, también se da el tipo de aceite afrutado ligeramente verde y medianamente amargo, picante y dulce. Este aceite corresponde al de principio de campaña, cuando las aceitunas están todavía verdes y esta característica se refleja lógicamente en el perfil organoléptico del aceite.

Por su composición son algo más delicados que otras variedades frente a la oxidación y una vez envasados es muy importante que estén al resguardo de la luz y el calor.

## Picudo

**Origen**: Córdoba
**Área de cultivo**: muy difundida en las provincias de Córdoba, Granada, Málaga y Jaén, con mayor densidad en la zona de Denominación de Origen Baena, al sudeste de Córdoba.

**Planta**

Vigor: Medio-alto.
Vegetación: de porte abierto y de densidad de copa muy espesa. Los ramos fructíferos son de color verde grisáceo.
Color madera joven: Verde grisáceo.

**Hoja**

Forma: de tamaño grande, larga y ancha.
Color: El color del haz es de un verde muy oscuro. Desde lejos se puede distinguir el árbol por presentar el aspecto más oscuro de todos.

**Drupa**

Forma: Fruto con ápice apuntado y curvado.
Volumen: Medio a grueso.
Peso medio: 3.80 a 4.80 g.
Rendimiento graso: 18.2 a 21.5%
Pulpa (porcentaje): 72 a 86%

La Picudo o Picuda, también conocida como Carrasqueña de Córdoba, es seguramente la variedad más emblemática de Córdoba y está amparada por la DOP de Baena y Priego de Córdoba. En el pueblo de Luque se le llama "pajarero". Este nombre le viene porque se dice que su aceite es tan dulce que en el momento de la maduración los pájaros pican los frutos. Esta variedad se encuentra muy difundida en las provincias de Córdoba, Granada, Málaga y Jaén.

La maduración de sus frutos transcurre entre la cuarta semana de noviembre hasta final de diciembre y el rendimiento graso es bueno, sin llegar a los valores de la Picual, pero con cifras cercanas al 20%. Las aceitunas Picudo también se destinan para aceitunas de mesa.

Las características organolépticas de la variedad picudo son muy buenas, con un equilibrio y dulzura inmejorables, sin sabores duros. A veces se puede encontrar ligeros sabores y aromas que recuerdan a frutas exóticas, así como manzana y almendrados. Por su composición en ácidos grasos, la Picudo se coloca en la gama de aceites delicados ante la oxidación, por lo que se complementa con otras variedades como la Picual.

## La cata del aceite de oliva virgen

El análisis sensorial se aprovecha de la capacidad de los sentidos para reaccionar ante estímulos químicos, físicos y fisicoquímicos. El sistema nervioso periférico permite la interconexión entre el entorno y el cerebro, que al estar dentro del cráneo obviamente no puede interactuar directamente con el mundo exterior.

Los cinco sentidos permiten evaluar las siguientes propiedades sensoriales:

- Apariencia, color y forma mediante la vista.
- Consistencia y las características relacionadas (fluidez, viscosidad, dureza, fibrosidad, crujiente, flexibilidad) mediante el tacto y el oído.
- Aroma mediante el olfato
- Sensaciones gustativas mediante el gusto
- Sabor mediante una combinación del olfato, gusto y tacto.

Un Análisis Descriptivo Cuantitativo, conocido como Panel de cata [47], es el mejor método para evaluar las características sensoriales del aceite de oliva virgen. Fue desarrollado por el Comité Oleícola Internacional (COI o IOOC: Internacional Olive Oil Council) durante muchas reuniones de expertos de la cuenca mediterránea. Al aplicar procedimientos estadísticos a los datos provenientes de los expertos se obtuvieron unos resultados con una fiabilidad similar, debido a sus niveles significativos, a la de otros métodos normalmente utilizados en otros campos científicos. El panel de cata tiene como fin sustituir un juicio individual por el criterio medio de un grupo de catadores.

Los paneles de cata son los encargados de clasificar los aceites de oliva vírgenes. Son los que deciden a través del análisis sensorial cuándo un aceite puede llevar la etiqueta de "virgen extra". Delimitar la frontera entre el virgen extra y el virgen es un trabajo crítico. La cata no es una ciencia exacta, pero de su seriedad depende la confianza del mercado. Comprende las siguientes fases:

- Análisis visual
- Análisis olfativo
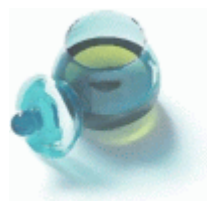
[47] Reglamento (CE) n° 796/2002 de la Comisión, de 6 de mayo de 2002 (DO L 128 de 15.5.2002), por el que se modifica el Reglamento (CEE) n° 2568/91 relativo a las características de los aceites de oliva y de los aceites de orujo de oliva y sobre sus métodos de análisis, así como las notas complementarias que figuran en el anexo del Reglamento (CEE) n° 2658/87 del Consejo relativo a la nomenclatura arancelaria y estadística y al arancel aduanero común.

- Análisis gustativo
- Análisis táctil
- Equilibrio-armonía

**Instrumento**

La metodología, incluida en las regulaciones de la Unión Europea desde 1991, determina como instrumento de medida a un grupo de personas, de 8 a 12, seleccionados de una manera regulada y entrenado convenientemente para identificar y medir la intensidad de las diferentes sensaciones positivas y negativas percibidas por sus sentidos. La elección de un grupo de personas permite promediar las diferencias que existen en los umbrales de algunos olores dependiendo de las personas, probablemente relacionadas con factores genéticos, culturales y ambientales, y así el resultado final representa a todos los consumidores.

**Vocabulario**

Para solucionar el problema derivado del hecho de que las percepciones varían de una persona a otra, estando esto relacionado con las propias experiencias de cada sujeto, y que por ello los conceptos son subjetivos, los catadores deben usar el mismo vocabulario. Una parte de este vocabulario es común a todos los alimentos, es el denominado "vocabulario general". El "vocabulario específico" fue desarrollado por los expertos del COI para el análisis del aceite de oliva virgen.

En el mencionado vocabulario general se incluyen términos como: aspecto, atributo, panel, percepción, sensibilidad, catador, respuesta, fatiga sensorial, estímulo, aroma, sabor, textura…Por otra parte, el vocabulario específico recoge términos que a una persona no experta, lógicamente, les resultarán menos intuitivos. Como algunos de ellos han sido de utilidad en partes del trabajo experimental de esta memoria, se añade la siguiente tabla:

*Tabla*. Vocabulario específico de atributos sensoriales que han de conocer los miembros del panel de cata.

| Atributos negativos | |
|---|---|
| Mohoso | Sabor característico del aceite obtenido de olivas almacenadas en pilas |
| Mohoso-húmedo | Sabor característico del aceite obtenido de olivas almacenadas en condiciones húmedas durante varios días. |
| Sedimento fangoso | Sabor característico del aceite que ha estado en contacto con los sedimentos depositados en los tanques subterráneos y las tinajas. |
| Avinado-Avinagrado | Sabor característico de algunos aceites que recuerda al vino o al vinagre. Esto es debido a la fermentación de las aceitunas que hace que se forma ácido acético, acetato etílico y etanol |
| Metálico | Sabor que recuerda al metal característico de los aceites que se obtienen en plantas de procesamiento nuevas u obtenidos al principio de la cosecha |
| Rancio | Sabor resultado de un proceso oxidativo al estar en contacto con el aire. |
| | |
| **Atributos positivos** | |
| Afrutado | Serie de sensaciones olfativas que dependen de la variedad de la oliva. Se perciben por medio de la nariz |
| Amargo | Sabor característico del aceite que se obtiene sobre todo de aceitunas verdes |
| Picante | Sensación característica de los aceites producidos al principio de la cosecha, principalmente con aceitunas sin madurar |
| **Otros atributos negativos** | |
| Quemado | Sabor característico de los aceites que son calentados excesivamente durante el proceso, especialmente cuando la pasta se mezcla térmicamente si se hace en condiciones inadecuadas. |
| Heno-madera | Sabor característico de los aceites producidos con olivas que se han secado. |

## Instalaciones

La selección de las condiciones ambientales siguió criterios centrados en la comodidad del catador. Han de controlarse el volumen y la temperatura de la muestra de aceite, la forma y las dimensiones de la copa para la prueba y el color del cristal. La temperatura de cata del aceite de oliva es de 28°C. Es esta temperatura la que permite la volatilidad de los compuestos aromáticos en un líquido denso y graso.

Para el aceite de oliva se llevan a cabo los mismos pasos analíticos que en la cata de otros productos líquidos como, por ejemplo, el vino: se coloca cada muestra en un copa diferente, se tapa, se huele y se degusta. Entre cada cata de aceite, para quitar el gusto de la muestra anterior, se come un pedazo de manzana y se bebe un sorbo de agua.

La sala de cata tiene una serie de cabinas. Cada catador realiza la prueba en privado en una cabina, regulada en su tamaño y equipamiento.

## Preparación de la muestra

La presentación de las muestras se hace al azar para evitar el efecto memoria y su número es normalmente bajo para eliminar la fatiga.

**Hoja de perfil**

Los catadores utilizan una hoja de perfil (figura) que contiene los defectos sensoriales que se pueden encontrar normalmente en los aceites de oliva virgen, y de entre las percepciones positivas sólo los atributos que caracterizan el sabor del aceite, afrutado, amargo y picante.

El afrutamiento, la sensación evocadora de la aceituna convenientemente madura, se evalúa mediante la inhalación directa, mientras que el resto de sensaciones se perciben por vía retronasal, ya que su identificación es más precisa al tardar el estímulo más tiempo en desaparecer.



**Escala**

Es adecuado adoptar una escala, que normalmente es sencilla de usar por parte de personas expertas e inexpertas, ya que permite cuantificar los diferentes estímulos y procesar los datos estadísticos.

Se utiliza una escala de 10 cm de longitud para medir la intensidad de las notas sensoriales definidas en el vocabulario específico. Los datos de intensidad, expresados en cm, proporcionados por los catadores se tratan estadísticamente.

La evaluación sensorial de los aceites de oliva virgen se puede justificar por una de las siguientes 5 razones [48]:

1) establecer una calidad básica del producto que verifique la ausencia o presencia y la intensidad de los defectos sensoriales.

2) determinar la pertenencia de un aceite a una denominación de origen protegida (DOP).

3) poner de manifiesto modificaciones de los perfiles sensoriales en relación con la variedad, origen geográfico, tecnología y tiempo de vida del producto.

4) encontrar características sensoriales críticas en la preferencia de los consumidores.

5) evaluar, en términos sensoriales, las diferencias en preferencia entre los consumidores habituales y potenciales.

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[48] F. Angerosa. "Calidad sensorial de los aceites de oliva" en R. Aparicio, J. Hardwood. "Manual del aceite de oliva". Ed. Mundi-Prensa. Madrid. (2003) 345-380.

# 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; en otras palabras, en la composición del aceite hallamos dos fracciones, una saponificable y otra insaponificable. La segunda, presente en una mucho menor proporción, contiene constituyentes menores que son muy importantes para la estabilidad, sabor y aroma del aceite de oliva.
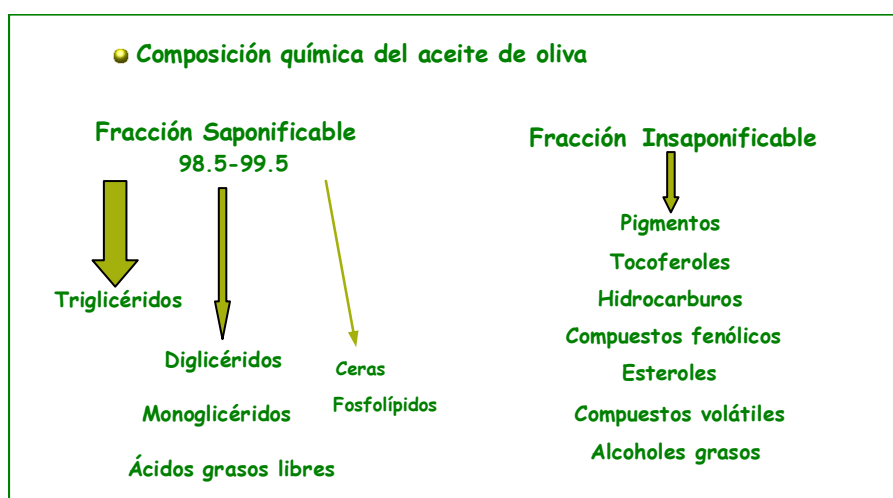


*Cuadro*. Esquema de la composición química del aceite de oliva (fracciones saponificable e insaponificable y cada una de las familias que éstas contienen.).

## 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.

### Ácidos grasos

Los ácidos grasos se encuentran en su mayoría combinados con glicerina, en forma de triglicéridos, o libres confiriéndole al aceite su carácter más o menos ácido, dependiendo de la mayor o menor concentración de estos. Su proporción depende del

grado de hidrólisis de los triglicéridos, y además su composición varía según la variedad, las condiciones climáticas y la localización geográfica del olivar [49,50].

Los ácidos grasos están formados por una cadena de átomos de carbono e hidrógeno y un grupo carboxilo que le confiere su función ácida. Los ácidos grasos se dividen en saturados e insaturados, según si los átomos de carbono se unen por enlaces simples o dobles. Si sólo existe un enlace doble, es *monoinsaturado* y si existen más, *polinsaturado*. La presencia del enlace doble confiere a la grasa mayor fluidez. Las grasas animales, como la mantequilla o la manteca de cerdo, son sólidas por contener ácidos grasos *saturados*.

En cambio, los aceites vegetales están formados mayoritariamente por ácidos grasos *insaturados*. El porcentaje de ácidos saturados en el aceite de oliva es moderado. En el siguiente gráfico se puede observar el porcentaje medio de los mismos.

*Figura*. Porcentaje de ácidos grasos (AG) en el aceite de oliva.
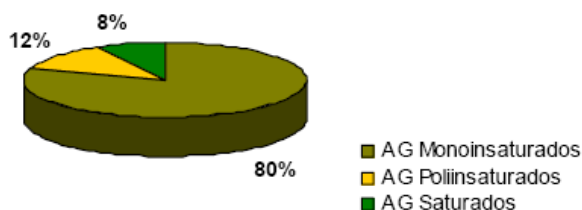


Los ácidos grasos presentes en el aceite de oliva son: mirístico (C14:0), palmítico (C16:0), palmitoleico (C16:1), heptadecanoico (C17:0), heptadecenoico (C17:1), esteárico (C18:0), oleico (C18:1), linoleico (C18:2), linolénico (C18:3), araquídico (C20:0), eicosenoico (C20:1), behénico (C22:0), y lignocérico (C24:0).

**Triglicéridos**

Los triglicéridos (triacilgliceroles) constituyen el 98-99% del aceite de oliva [51]. Son ésteres provenientes de la unión del trialcohol glicerina (1,2,3-propanotriol) con ácidos grasos.

[49] R. Aparicio, V. Alonso. "Characterization of virgin olive oils by SEXIA Expert System". *Prog. Lipid Res*. 33 (1994) 29-38.
[50] R. Aparicio, V. Alonso, M. T. Morales. "Detailed and exhaustive study of the authentication of European virgin olive oils by SEXIA expert system" *Grasas Aceites*, 45 (1994) 241-252.
[51] E. Fedeli. "Lipids of olives" en "Progress on Chemistry of fats and other lipids". Ed. E. Ralph, T. Holman. Pergamon Press. Paris. (1997) Pp 15-74.

De los triglicéridos que se encuentran presentes en el aceite en cantidades más significativas destacan: OOO (40-59%), POO (12-20%), OOL (12.5-20%), POL (5.5-7%) y EOO (3-7%) (donde O: ácido oleico; P: ácido palmítico; L: ácido linoleico; E: ácido esteárico) [52].

**Mono- y di-acilgliceroles**

Los aceites, además de contener mayoritariamente triglicéridos, también contienen glicéridos parciales. La presencia en los aceites de mono-acilgliceroles (monoglicéridos) y di-acilgliceroles (diglicéridos) es en parte debida a una biosíntesis incompleta y, principalmente, a una hidrólisis del aceite. Cuando los di-acilgliceroles son de estructura 1,2-diacilo, son productos intermedios en la síntesis de los triglicéridos; mientras que, cuando son de estructura 1,3-diacilo, son productos provenientes de la hidrólisis de los tri-acilgliceroles (triglicéridos) [53].

Se puede usar la determinación de di-acilgliceroles para evaluar la calidad de los aceites, ya que según un estudio llevado a cabo por Mariani y col. [54] la presencia de estos compuestos en el aceite de oliva es indicativa de que éste es de baja calidad.

**Ceras**

Las ceras son ésteres de alcoholes grasos con ácidos grasos. Las principales ceras de los aceites de oliva son de número de carbono par, es decir, ésteres C-36 a C-46. El contenido de ceras en aceite es muy bajo, no superando los 35 mg/100 g [55].

Las ceras se producen mediante una esterificación entre los alcoholes presentes en el aceite y los ácidos grasos libres. Cuando se produce una hidrólisis de los triglicéridos se aumenta el contenido de ácidos grasos libres, originándose un aumento en la velocidad de la reacción de esterificación.

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[52] F. C. Phillips, W. L. Erdahl, J. A. Schmit, O. S. Privett. "Quantitative analysis of triglyceride species of vegetable by high performance liquid chromatography via a flame ionization detector". *Lipids* 19 (1984) 880-887.

[53] M. C. Pérez-Camino, W. Moreda, A. Cert. "Determination of diacylglycerol isomers in vegetable oils by solid-phase extraction followed by gas chromatography on a polar phase". *J. Chromatogr. A* 721 (1996) 305-314.

[54] C. Mariani, E. Fedeli. "Determinazione delle forme gliceridiche presenti negli oli alimentari, Nota I: caso dell`olio di oliva". *Riv. Ital. Sost. Grasse* 62 (1985) 3-7.

[55] C. Mariani, S. Venturini, P. Bondioli, E. Fedeli, K. Grob. "Valutazione delle variazioni indotte dalla decolorazione sui principali componenti minori liberi ed esterficati dell'olio di oliva (Evaluation of the variations produced by bleaching process on more meaningful minor components free and esterified in olive oil)". *Riv. Ital. Sost. Grasse* 69 (1992) 393-399.

**Fosfolípidos**

No hay mucha bibliografía ni trabajo experimental que contemple esta familia como su objeto de estudio. Los fosfolípidos están en pequeñas cantidades en aceites de oliva recién producidos, y aún en cantidades más pequeñas en aceites más viejos [56]. Entre los fosfolípidos que se han encontrado en el aceite, podemos mencionar: fosfadidilcolina, fosfadidiletanolamina, fosfatidi-linositol y fosfatidilserina [57].

[56] M. Vitagliano. "Minor constituents of vegetable oils". *Riv. Ital. Sost. Grasse* 36 (1961) 46-55.
[57] M. Alter, T. Gutfinger. "Phospholipids in several vegetable oils" *Riv. Ital. Sost. Grasse* 59 (1982) 14-18.

# Fracción insaponificable

La fracción insaponificable constituye entre el 0.5 y el 1.5% de los aceites. Incluye clases de compuestos que no están relacionados químicamente con los ácidos grasos, como son hidrocarburos, esteroles, tocoferoles, pigmentos, alcoholes grasos, compuestos volátiles y aromáticos y compuestos fenólicos.

## Hidrocarburos

El escualeno, precursor bioquímico de los esteroles, es un importante hidrocarburo de los aceites de oliva vírgenes y refinados. El aceite de oliva contiene la mayor cantidad de escualeno (2500-9250 µg/g) en comparación con otros aceites comestibles (16-370 µg/g) [58]. En el aceite de oliva se encuentran también otros hidrocarburos como los policíclicos aromáticos, por ejemplo, fenantreno, pireno, fluorantreno, 1,2-benzantraceno, criseno, etc. [51]. Además, es posible hallar en el aceite de oliva otros hidrocarburos tales como las parafinas con un número par e impar de carbonos ($C_{11}$ a $C_{30}$).

El β-caroteno, que por su estructura química podría incluirse en la familia de hidrocarburos terpénicos, lo citaremos en la sección de pigmentos.

## Esteroles

Los esteroles constituyen una huella analítica que permite la autentificación del aceite de oliva. Hay cuatro clases de esteroles en el aceite de oliva: esteroles comunes (4α-desmetilesteroles), 4α-metilesteroles, 4,4-dimetilesteroles (alcoholes triterpénicos) y dialcoholes triterpénicos. Los valores normales para el contenido total de los esteroles son 100-220 mg/100 g.

### Desmetilesteroles (esteroles comunes)

Los principales esteroles en el aceite de oliva son β-sitosterol, $\Delta^5$-avenasterol y campesterol. Numerosos estudios muestran que el β-sitosterol representa el 75-90% de la fracción total de esteroles [59,60].

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[58] J. Gutfinger, A. Letan. "Studies of unsaponifiables in several vegetable oils". *Lipids* 9 (1974) 658-663.

[59] L. S. Conte, M. F. Caboni, G. Lercker. "Olive oils produced in Romagnia", Note I. *Riv. Ital. Sost. Grasse* 70 (1993) 175-180.
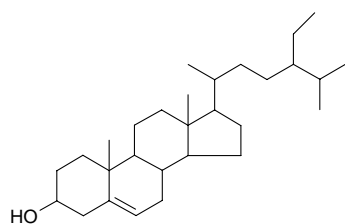
*Figura*. Estructura molecular del β-sitosterol.

Otros esteroles presentes, aunque en menores cantidades, son estigmasterol, colesterol, 24-metilencolesterol, $\Delta^7$-campesterol, $\Delta^{5,23}$-estigmastadienol, sitostanol, $\Delta^{5,24}$-estigmastadienol, $\Delta^7$-estigmastenol y $\Delta^7$-avenasterol [61,62,63].

El campesterol y el estigmasterol se encuentran en 1-4% y 0.5-2%, respectivamente [59, 64], aunque en ocasiones se han llegado a determinar valores más elevados [Reglamento (CEE) Nº 2568/1991]. En los aceites de oliva originales, el porcentaje de estigmasterol es siempre inferior al del campesterol [Reglamento (CEE) Nº 2568/1991].

*4α-metilesteroles*

El aceite de oliva contiene los siguientes 4α-metilesteroles: 4α-metil-24-metilen-$\Delta^7$-colesten-3β-ol (gramisterol); 4α,14α-dimetil-24-metilen-$\Delta^8$-colesten-3β-ol (obtusifoliol); 4α,14α-dimetil-9,19-ciclopropan-24-metilen-colestan-3β-ol (cicloeucalenol), 4α-metil-(24Z)-24-etiliden-$\Delta^7$-colestan-3β-ol (citrostadienol).

*4,4-dimetilesteroles (alcoholes triterpénicos)*

Los principales 4,4-dimetilesteroles (alcoholes triterpénicos) presentes en el aceite de oliva son α y β-amirina, butirospermol, cicloartenol, 24-metilencicloartanol, taraxerol, dammaradienol y 24-metilen-24-dihidroparkeol [65, 66]. El contenido de alcoholes triterpénicos oscila entre 100-150 mg/100 g de aceite [67].

[ 60 ] R. Calapaj, S. Chiricosta, G. Saija, V. Binova. "Evaluation of gas chromatographic and spectrophotometric analytical results to check the presence of seed oils in olive samples". Riv. Ital. Sost. Grasse 70 (1993) 5X5-5X10.

[61] D. Boskou, I. D. Morton. "Changes in the sterol composition of olive on heating". *J. Sci. Food Agric*. 26 (1975) 1149-1153.

[62] D. Boskou. "Stability of natural terpenoids in heated olive oil". *Grasas Aceites* 29 !1978) 193-195.

[63] I. Itoh, K. Yoshida, T. Yatsu, T. Tamura, T. Matsumoto, G. F. Spencer. "Triterpene alcohols and sterols of Spanish olive oil". *J. Am. Oil Chem. Soc*. 58 (1981) 545-550.

[64] V. Paganuzzi. "Influence of origin and conservation on the sterolic composition of nontreated olive oils", III. Riv. Ital. Sost. Grasse 62 (1985) 399-410.

[65] A. Kiritsakis, W. W. Christie. "Análisis de aceites comestibles" en en R. Aparicio, J. Hardwood. "Manual del aceite de oliva". Ed. Mundi-Prensa. Madrid. 135-162 (2003).

Los alcoholes triterpénicos (24-metilen-cicloartenol, junto con cicloartenol, α-amirina y β-amirina) son especialmente importantes desde el punto de vista analítico, en la detección de la presencia de aceite de orujo de oliva.

*Dialcoholes triterpénicos*

Los dos principales dialcoholes triterpénicos son el eritrodiol (homo-olestranol, 5α-olean-12-en-3β,28-diol) y el uvaol (Δ-12-ursen-3β,28-diol). Las cantidades totales de eritrodiol más uvaol en aceite de oliva varían desde 1 a 20 mg/100 g de aceite, hasta valores que llegan a 280 mg/100 g de aceite β-residual [68].

## Tocoferoles

Los tocoferoles son compuestos que contribuyen de modo importante a dar estabilidad al aceite de oliva, y tienen un papel biológico beneficioso como antioxidantes [42,69,70,71].

El aceite de oliva contiene α-tocoferol en cantidades mayores que los otros tocoferoles. Dicho α-tocoferol es el principal homólogo de las formas de la vitamina E y representa el 95% del total de tocoferoles.
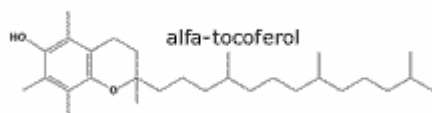


*Figura*. Estructura del tocoferol mayoritario (α-tocoferol).

El otro 5% es β+γ tocoferoles [72, 73]. Todos ellos tienen un grupo de cromano sustituído por grupos fenólicos y metilos y una cadena lateral saturada de 16 átomos de

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[66] T. Itoh, T. Tamura, T. Matsumoto. "Methylsterol compositions of 19 vegetable oils". J. Am. Oil Chem. Soc. 50(8) (1973) 300-303

[67] B. Kiosseoglou, L. Vlachopoulou, D. Boskou. "Esterified 4-monomethyl- and 4,4-dimethyl-sterols in some vegetable oils". Grasas Aceites 38 (1987) 102-103.

[68] C. Mariani, E. Fedeli, G. Morchio. "Absolute erythrodiol content as a possibility to detect olive husk oil on olive oil". *Riv. Ital. Sost. Grasse* 64 (1987) 359-363.

[69] R. Mateos, M. Trujillo, M. C. Pérez-Camino, W. Moreda, A. Cert. "Relationships between oxidative stability, triacylglycerol composition, and antioxidant content in olive oil matrices". *J. Agric. Food Chem.* 53(14) (2005) 5766-5771.

[70] D. Beardsell, J. Francis, D. Ridley, K. Robards. "Health promoting constituents in plant derived edibles oils". *J. Food Lipids* 9 (2002) 1-34.

[71] D. Giugliano. "Dietary antioxidants for cardiovascular prevention". *Nutrition Metabolism Cardiovascular Diseases* 10 (2000) 30-44.

carbono con tres esqueletos de isopreno. Se diferencian unos de otros en la posición de los grupos metilos sustituyentes.

El contenido de tocoferol depende mucho de la variedad de la aceituna. Sus concentraciones varían desde 5 a 300 ppm [74]. En los aceites de oliva de buena calidad, el contenido suele estar entre 100 y 300 ppm [72]. En los aceites comerciales de alta acidez es donde se han encontrado valores más bajos (aprox. 5 ppm).

### Pigmentos

El aceite de oliva tiene un color que va desde el verde-amarillo hasta el dorado, dependiendo de la variedad y del estado de madurez del fruto. La composición y el contenido total de pigmentos presentes de forma natural en el aceite de oliva, son importantes parámetros para la determinación de su calidad, ya que están relacionados con el color, que es uno de los atributos básicos para evaluar la calidad del aceite de oliva. Los pigmentos también están involucrados en mecanismos de autooxidación y fotooxidación.

Podemos distinguir dos clases de pigmentos naturales en el aceite de oliva [75]:

-clorofilas y feofitinas

-carotenoides

Las clorofilas y las feofitinas pueden ser a o b y son las responsables del color verde del aceite. Las estructuras moleculares de la clorofila y de la feofitina contienen cuatro grupos pirrólicos con un átomo de magnesio, en el caso de la clorofila, y de dos átomos de hidrógeno, en el de la feofitina. En la fracción de pigmentos clorofílicos predomina la feofitina a, ya que su concentración representa el 70-80% del total [76,77,78].

[72] A. J. Speek, J. Schrijver, W. H. P. Schreure. "Vitamin E composition of some seed oils as determined by high-performance liquid chromatography with fluorimetric detection". *J. Food Sci.* 50 (1985) 121-124.

[73] N. K. Andrikopoulos, M. N. Hassapidou, A. G. Manoukas. "The tocopherol content of Greek olive oils". *J. Sci. Food Agric.* 46 (1989) 503-509.

[74] J. L. Perrin. "Les composes mineurs et les antioxygenes naturels de l`olive et de son huile". *Rev. Franc. Corps Gras.* 39 (1992) 25-32.

[75] M. I. Minguez-Mosquera, B. Gandul-Rojas, J. Garrido-Fernández, L. Gallardo-Guerrero. "Pigments present in virgin olive oil". *J. Am. Oil Chem. Soc.* 67 (1990) 192-196.

[76] A. Serani, D. Piacenti. "Kinetics of pheophitin-A photodecomposition in extra-virgin olive oil". *J. Am. Oil Chem. Soc.* 69 (1992) 469-470.

[77] A. Cichelli, G. P. Pertesana. "High-performance liquid chromatographic analysis of chlorophylls, pheophytins and carotenoids in virgin olive oils: chemometric approach to variety classification". *J. Chromatogr. A* 1046 (2004) 141-146.
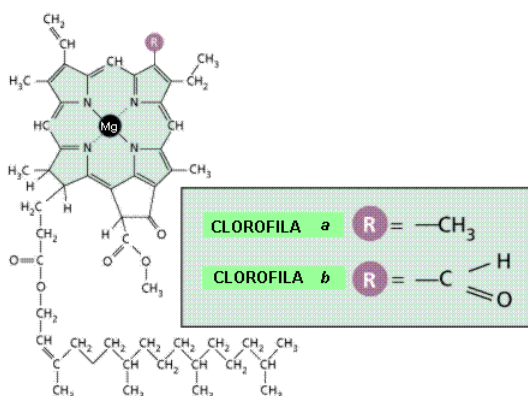
*Figura*. Estructura molecular de las clorofilas a y b.

Por otra parte, los principales carotenoides presentes en el aceite de oliva son: luteína, β-caroteno, violaxantina y neoxantina. El principal componente de la fracción carotenoide del aceite de oliva es la luteína.
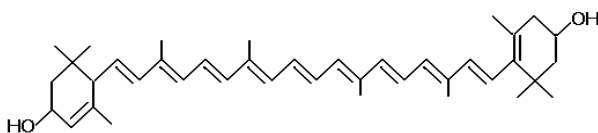


*Figura*. Luteína, principal compuesto de la fracción carotenoide del aceite de oliva.

Normalmente, el contenido de carotenoides varía entre 1 y 20 ppm en el aceite de oliva. El β-caroteno se encuentra en concentraciones que varían entre 0.5 y 4 ppm [79,80].

### Alcoholes grasos

Los alcoholes grasos son unos constituyentes menores pero importantes en el aceite de oliva, ya que pueden ser utilizados para diferenciar los distintos tipos de aceites [Reglamento 2568/91].

Los alcoholes grasos pueden ser lineales (alifáticos) o triterpénicos. También dentro de esta fracción alcohólica se han encontrado lo que se denominan diterpenoides.

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[78] A. J. Márquez. "Monitoring carotenoid and chlorophyll pigments in virgin olive oil by visible-near infrared transmittance spectroscopy. On-line application". *J. Near Infrared Spectroscopy* 11 (2003) 219-226.

[79] M. I. Minguez-Mosquera, L. Rejano-Navarro, B. Gandul-Rojas, A. H. Sánchez-Gómez, J. Garrido-Fernández. "Color pigment correlation in virgin olive oil". *J. Am. Oil Chem. Soc.* 68 (1991) 332-336.

[80] F. Gutiérrez-Rosales, J. Garrido-Fernández, L. Gallardo-Guerrero, B. Gandul-Rojas, M. I. Minguez-Mosquera. "Action of clhorophylls on the stability of virgin olive oil". *J. Am. Oil Chem. Soc.* 69 (1992) 866-871.

*Alcoholes alifáticos*

Los alcoholes alifáticos son compuestos de estructura lineal. Según el número de átomos de carbono, estos alcoholes pueden ser el monocosanol (C-21), dicosanol (C-22), tricosanol (C-23), tetracosanol (C-24), pentacosanol (C-25), hexacosanol (C-26), heptacosanol (C-27) y octacosanol (C-28). Normalmente en los aceites de oliva los alcoholes en cantidades más relevantes son los de número par, mientras que los impares sen encuentran en cantidades más pequeñas [81,82].

Los alcoholes alifáticos son precursores de la formación de ceras. Así, un alto contenido de alcoholes puede traer como consecuencia que el contenido de ceras aumente a lo largo del tiempo.

*Alcoholes triterpénicos*

Los alcoholes triterpénicos son también denominados 4,4-dimetilesteroles; en la presente memoria vienen incluidos en la sección que versa sobre los esteroles, pero se mencionan aquí para evitar confusiones.

*Diterpenoides*

Dentro de la fracción alcohólica se han encontrado lo que se denominan diterpenoides acíclicos, concretamente han sido hallados dos: el fitol (que probablemente proviene de la clorofila) y el geranilgeraniol [55,83]. Paganuzzi ha recogido en alguna de sus publicaciones valores de concentración de fitol que van desde 120-180 ppm [83].

## Compuestos volátiles y aromáticos

El aroma y el flavor ("mezcla" entre el sabor y el olor) son características distintivas del aceite de oliva virgen cuando se compara con otros aceites comestibles. Los compuestos volátiles y aromáticos han llamado la atención de muchos investigadores que han obtenido resultados más que notables [84,85,86,87,88]. Se han identificado

[81] D. Boskou, G. Stephanou, M. Konstantinidis. "Tetracosanol and hexacosanol content of greek oilve oils". *Grasas Aceites* 34 (1983) 402-408.

[82] N. Frega, F. Bocci, G. Lercker. "Direct gas chromatographic analysis of the unsaponificable fraction of different oils with a polar capillary column". *J. Am. Oil. Chem. Soc.* 69 (1992) 447-450

[83] V. Paganuzzi. "Composition of Iranian olive oil". *J. Am. Oil. Chem. Soc.* 56 (1979) 925-930.

[84] J. M. Olías-Jiménez, F. Gutiérrez-Rosales, C. Dobarganes-García. "Componentes volátiles en el aroma del aceite de oliva virgen. IV. Su evolución e influencia en el aroma durante el proceso de maduración de los frutos de las variedades Picual y Hojiblanca". *Grasas Aceites* 31 (1980) 391-402.

más de cien componentes como hidrocarburos, alcoholes, aldehídos, ésteres, fenoles, terpenos y derivados del furano. Los aldehídos son los componentes volátiles mayoritarios en este grupo.

[85] G. Montedoro, M. Bertuccioli, F. Anichini. "Aroma análisis of virgin olive oil by head space and extraction techniques" en "Flavor of food and beverages". Ed. G. Charalambous, Academia Press. Pp 247-281 (1998).

[86] F. Bocci, N. Frega, G. Lercker. "Studio preliminare sui componenti volatili di oli di oliva extravergini (Preliminary research on volatile extra virgin olive oil components)". *Riv. Ital. Sost. Grasse*. 69 (1992) 611-613.

[87] H. Guth, W. Grosh. "A comparative study of the potents odorants of different virgin olive oils". *Fat. Sci. Techn*. 93 (1991) 335-341.

[88] R. Gutiérrez, M. C. Dobarganes, F. Gutiérrez, M. Olías. "Volatile components in the aroma of virgin olive oil". *Grasas Aceites* 32 (1981) 299-303.

# Compuestos fenólicos del aceite de oliva

Si alguna de las familias requiere tal vez una atención especial en la presente memoria, ésta es la familia de los compuestos fenólicos.

Los compuestos fenólicos se encuentran principalmente en el mesocarpio de la aceituna y, aunque son solubles en agua y se pierden en gran cantidad en el proceso de elaboración del aceite con las aguas de lavado, también se encuentran formando parte de la composición 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 [89,90]. De acuerdo con Harborne [89], los compuestos fenólicos pueden ser agrupados en varias categorías:

1. Fenoles, ácidos fenólicos, ácidos fenilacéticos
2. Ácidos cinámicos, cumarinas, isocumarinas y cromonas
3. Lignanos
4. Varios grupos de flavonoides
5. Ligninos
6. Taninos
7. Benzofenonas, xantonas, estilbenos
8. Quinonas
9. Betacianinas

Para particulizar en la matriz que nos ocupa, el aceite de oliva, podemos decir que cuando hablamos de polifenoles casi siempre nos referimos a los productos de hidrólisis de la oleuropeína y el ligustrósido, sus agliconas y productos derivados.

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.

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[89] J. B. Harborne, P. M. Dey. "Methods in Plant Biochemistry". J. B. Harborme (Ed.). Academic Press. London. (1989).
[90] A. Escarpa, M. C. González. "An overview of analytical chemistry of phenolic compounds in foods". *Critical Reviews in Analytical Chemistry* 31 (2001) 57-139.

El estudio de esta fracción del aceite de oliva se lleva desarrollando desde hace más de 50 años. Podríamos indicar a Cantarelli y Montedoro como pioneros en este campo [91,92]. Desde el comienzo de las investigaciones, estos científicos tuvieron claros los objetivos a perseguir:
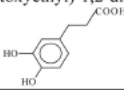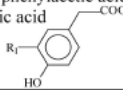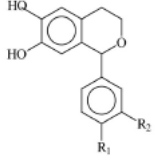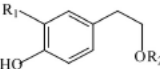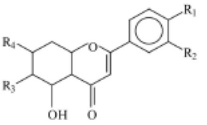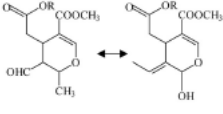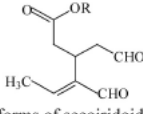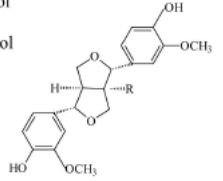
-Desarrollar procedimientos analíticos que permitieran cuantificar los compuestos fenólicos en los aceites de oliva.

-Hacer una estimación de los niveles de concentración de compuestos fenólicos en aceites vegetales.

-Establecer posibles conexiones entre los compuestos fenólicos y algunas de las características del fruto (variedad, grado de madurez…).

-Estudiar la influencia de la técnica de extracción y proceso de refinado en los niveles de polifenoles en un aceite.

-Ahondar en la importancia de los compuestos fenólicos como antioxidantes.

-Descubrir el papel que desempeñan este tipo de compuestos que pueda justificar el hecho de que aceites con alto valor de peróxidos tengan una estabilidad considerable.

Los objetivos que Cantarelli y Montedoro se propusieron, no han sido en absoluto fáciles de conseguir, de hecho, aún hoy en día hay muchos grupos de investigación que se dedican al estudio de alguno de estos puntos. Sin embargo, hay que decir que sí se han conseguidos logros importantes en el campo, que se conoce mucho más en profundidad la composición de esta compleja fracción y que se han llevado a cabo estudios sistemáticos sobre algunas de las familias que conforman esta fracción de modo individualizado.

[91] C. Cantarelli. "Sui polifenoli presenti nella drupa e nell'olio di oliva". *Riv. Ital. Sost. Grasse* 38 (1961) 69–72.
[92] G. F. Montedoro, C. Cantarelli. "Indagine sulle sostanze fenoliche presenti nell'olio di oliva". *Riv. Ital. Sost. Grasse* 46 (1969) 115–124.

La Tabla que se muestra a continuación contiene de modo esquemático las distintas categorías que podemos distinguir entre los compuestos fenólicos del aceite de oliva [93].



Phenolic compounds in virgin olive oil

**Phenolic acids**
**Benzoic acid derivatives**
Gallic acid
Gentisic acid
Benzoic acid
Vanillic acid
Protocatecuic acid
p-Hydroxybenzoic acid
Syringic acid
**Cinnamic acid derivatives**
Caffeic acid
p-Coumaric acid
o-Coumaric acid
Ferulic acid
Cinnamic acid
Sinapinic acid
**Other phenolic acids and derivatives**
4-(Acetoxyethyl)-1,2-dihydroxybenzene
Dopac (3,4-dihydroxyphenylacetic acid)
4-hydroxyphenylacetic acid

**Hydroxy-isocromans**
1-phenyl-6,7-dihydroxy-isochroman
1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman

**Phenolic alcohols**
(3,4-Dihydroxyphenyl)ethanol (3,4-DHPEA)
p-Hydroxyphenyl)ethanol (p-HPEA)
(3,4-Dihydroxyphenyl)ethanol-glucoside
2-(4-hydroxyphenyl)ethyl acetate

**Secoiridoids**
Oleuropein
Dialdehydic form of oleuropein aglycon
Dialdehydic form of ligstroside aglycon
Ligstroside aglycon
Oleuropein aglycon (3,4-DHPEA-EA)
Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA)
Dialdehydic form of decarboxymethyl elenolic acid linked to p-HPEA (p-HPEA -EDA)

Dialdehydic forms of secoiridoids derivatives

**Flavonoids**
**Flavones**
Apigenin
Luteolin
**Flavanonol**
(+)-Taxifolin

**Lignans**
(+)-1-Acetoxypinoresinol
(+)-Pinoresinol
(+)-1-Hydroxypinoresinol

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[93] Tabla tomada de una de las publicaciones derivadas del trabajo desarrollado en esta tesis (Review que aparece en la presente memoria como Capítulo 1).

Los ácidos fenólicos con la estructura básica C6-C1 (ácidos benzoicos) y C6-C3 (ácidos cinámicos), como los ácidos cafeico, vanílico, siríngico, *p*-cumárico, *o*-cumárico, protocatecuico, sinápico y *p*-hidroxibenzoico, fueron el primer grupo de fenoles que se determinaron en aceite [94,95]. Posteriormente varios autores confirmaron la presencia de los ácidos fenólicos como componentes minoritarios del aceite de oliva [96,97,98,99,100].

Los compuestos fenólicos que están en una mayor concentración en el aceite son los secoiridoides, que se caracterizan por la presencia en su estructura del ácido elenólico o alguno de sus derivados [101]. Estos compuestos (oleuropeína, demetiloleuropeína y ligustrósido, etc.) son derivados de las formas glucosídicas de los secoiridoides de las aceitunas. Los productos originados a partir de la rotura de los dos compuestos fenólicos más abundantes en la aceituna (oleuropeína (Ol) y ligustrósido (Lig)) forman la mayoría de esta fracción.

Así, los secoiridoides más abundantes en el aceite de oliva virgen son la forma dialdehídica del ácido elenólico (EA) unida al hidroxitirosol (HYTY) o tirosol (TY) (3,4-DHPEA-EDA (HYTY-EDA) o p-HPEA-EDA (TY-EDA)) y un isómero de la oleuropeína aglicona (3,4-DHPEA-EA (HYTY-EA)). Estos compuestos fueron hallados

[94] 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.
[95] A. Vázquez Roncero. "Les polyphenols de l'huile d'olive et leur influence sur les characteristiques de l'huile". *Rev. Fr. Corps Gras*. 25 (1978) 21–26.
[96] M. Solinas, A. Cichelli. "Sulla deteminazione delle sostanze fenoliche dell'olio di oliva". *Riv. Ital. Sostanze Grasse* 58 (1981) 159–164.
[97] N. Cortesi, E. Fedeli. "Polar components of virgin olive oil. Note 1". Riv. Ital. Sostanze Grasse 60 (1983) 341–351.
[98] G.F. Montedoro, M. Servili, M. Baldioli, E. Miniati. "Simple and hydrolyzable compounds in virgin olive oil. 1. Their extraction, separation and quantitative and semiquantitative evaluation by HPLC". *J. Agric. Food. Chem.* 40 (1992) 1571–1576.
[99] M. Tsimidou, M. Lytridou, D. Boskou, A. Paooa-Lousi, F. Kotsifaki, C. Petrakis. "On the determination of minor phenolic acids of virgin olive oil by RP-HPLC". *Grasas Aceites* 47 (1996) 151–157.
[100] 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.
[101] M.J. Garrido Fernández Díez, M. R. Adamos. "Table olives". Chapman & Hall, London (UK). Pp 67-109 (1997).

por primera vez por Montedoro y sus colaboradores [98,102]. Recientemente, han sido también encontrados en aceite la oleuropeína aglicona (Ol Agl) y el ligustrósido aglicona (Lig Agl) [103,104,105].



R=H: **Lig Agl**
R=OH: **Ol Agl**

R=H: **forma dialdehídica-Lig Agl**
R=OH: **forma dialdehídica-Ol Agl**

R=H: **forma dialdehídica decarboxilada-Lig Agl**
R=OH: **forma dialdehídica decarboxilada-Ol Agl**

**10-H-Ol Agl**

R=H: **decarboxi-Lig Agl**
R=OH: **decarboxi-Ol Agl**

**EA**
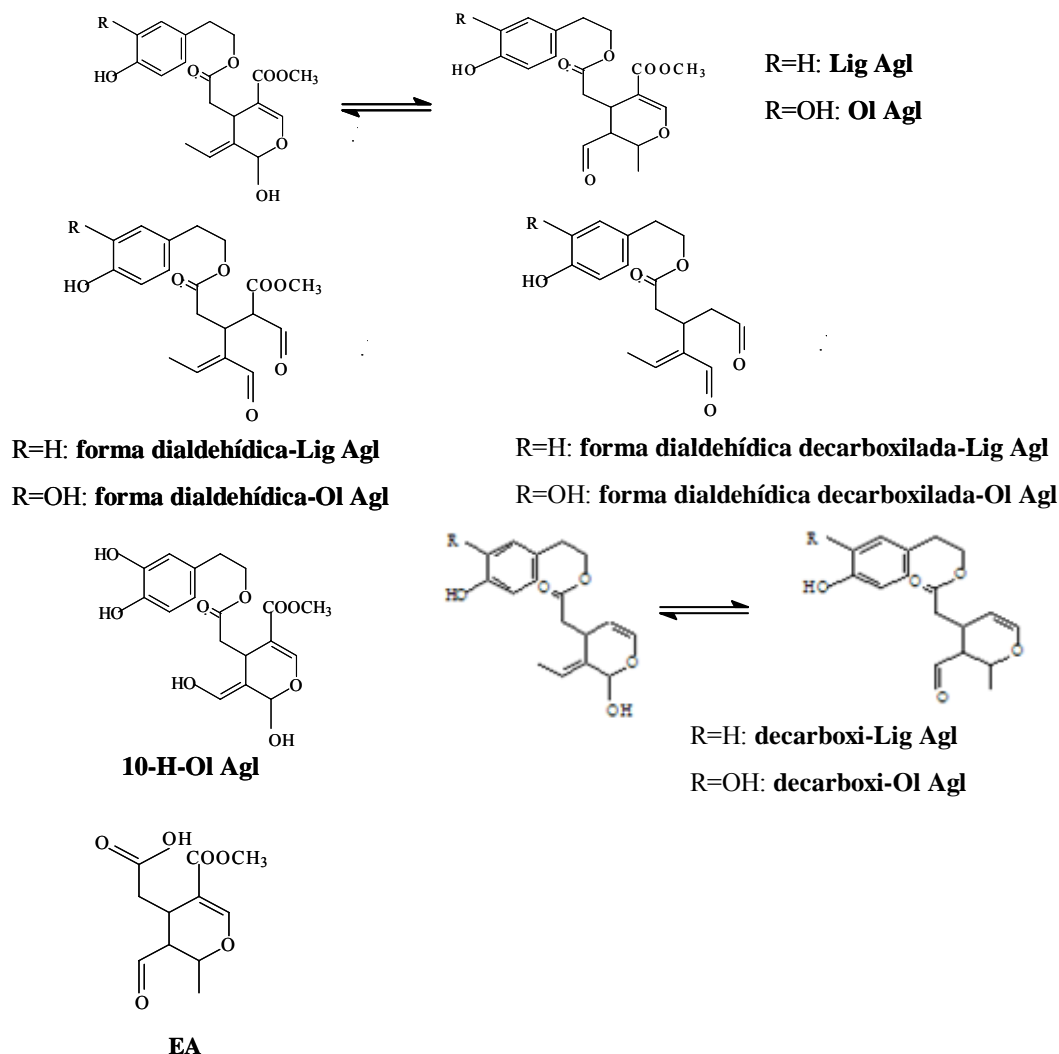
*Figura*. Estructuras moleculares de varios de los compuestos pertenecientes a la familia de fenoles complejos (secoiridoides) y otros muy relacionados con dicha familia (EA= ácido elenólico).
Nuevas abreviaturas: 10-H-Ol Agl: 10-hidroxioleuropeína aglicona.

[102] G.F. Montedoro, M. Servili, M. Baldioli, E. Miniati. "Simple and hydrolyzable compounds in virgin olive oil. Note 2: Initial characterization of the hydrolizable fraction". *J. Agric. Food. Chem.* 40 (1992) 1577–1580.
[103] R.W. Owen, W. Mier, A. Giacosa, W.E. Hull, B. Spiegelhalder, H. Bartsch. Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignans and squalene". *Food Chem. Toxicol.* 38 (2000) 647–659.
[104] E. Perri, A. Raffaelli, G. Sindona. "Quantification of oleuropein in virgin olive oil by ionspray mass spectrometry-selected reaction monitoring". *J. Agric. Food Chem.* 47 (1999) 4156–4160.
[105] En publicaciones científicas en castellano es posible encontrar la denominación "aglicona" o "aglucona" para las formas de aquellos secoiridoides que han perdido el grupo glucósido.

Hidroxitirosol (HYTY) y tirosol (TY) son los alcoholes fenólicos más importantes, aunque también se pueden mencionar dentro de esta familia el hidroxitirosol acetato (HYTY-Ac) [106], tirosol acetato [107] y una forma glucosídica del HYTY [108].



**TY**  **HYTY**  **HYTY-Ac**
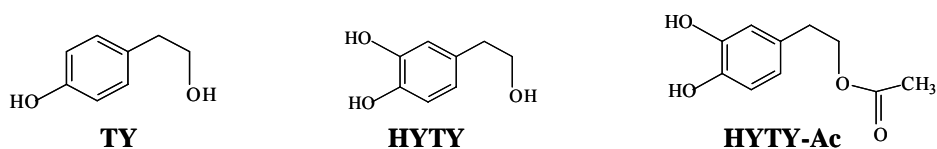
*Figura*. Fórmulas de tres de los fenoles simples o alcoholes fenólicos más relevantes.

Varios autores han recogido en sus trabajos de investigación que los flavonoides (luteolina (Lut) y apigenina (Apig)) pueden ser considerados componentes fenólicos presentes en el aceite de oliva [109,110]. Un compuesto cercano a ellos, (+)-taxifolin, un flavanonol, ha sido encontrado recientemente en aceites de oliva españoles [100].



R=H: **Apig**
R=OH: **Lut**
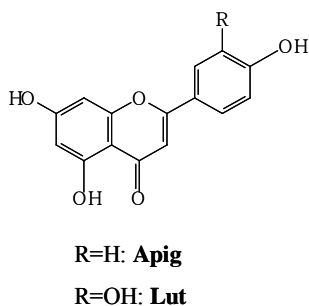
*Figura*. Flavonoides hallados en aceite de oliva.

[106] M. Brenes, A. García, P. García, J.J. Ríos, A. Garrido. "Phenolic compounds in Spanish olive oils". *J. Agric. Food Chem*. 47 (1999) 3535–3540.

[107] 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.

[108] 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.

[109] P. Rovellini, N. Cortesi, E. Fedeli. "Analysis of flavonoids from *Olea europaea* by HPLC-UV and HPLC-electrospray-MS". *Riv. Ital. Sostanze Grasse* 74 (1997) 273–279.

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

Otro grupo importante a tener en cuenta son los lignanos; Owen y col. [103,111] y Brenes y su grupo de investigación [ 112 ] han aislado y caracterizado (+)-acetoxipinoresinol (Ac Pin), (+)-pinoresinol (Pin) y (+)-1-hidroxipinoresinol (H-Pin) como los lignanos hallados más frecuentemente en aceite.
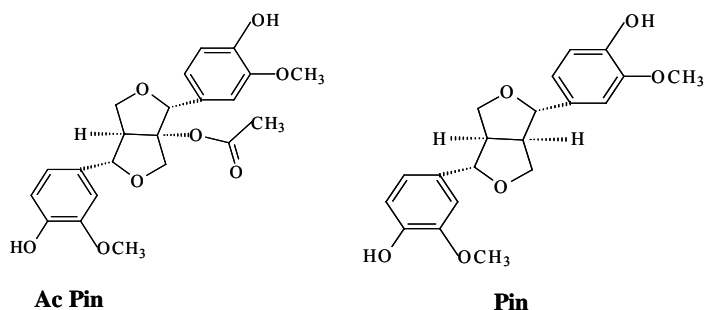


*Figura*. Estructura de los dos compuestos más representativos de la familia de los lignanos en aceite de oliva.

Una nueva clase de compuestos fenólicos, hidroxi-cromans, se ha encontrado en varias muestras diferentes de aceite de oliva. En particular, la presencia de dos de estos compuestos, 1-fenil-6,7-dihidroxi-isocroman y 1-(3´-metoxi-4´-hidroxi)fenil-6,7-dihidroxi-isocroman ha sido demostrada [113].

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[111] 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.
[112] M. Brenes, F.J. Hidalgo, A. García, J.J. Ríos, P. García, R. Zamora, A. Garrido. "Identification of lignans as major components in the phenolic fraction of olive oil". *J. Am. Oil Chem. Soc*. 77 (2000) 715–720.

[113] A. Bianco, F. Coccioli, M. Guiso, C. Marra. "The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans". *Food Chem*. 77 (2001) 405–411.

# ¿Por qué son importantes los compuestos fenólicos?: Sus efectos como antioxidantes, posibles propiedades saludables e influencia en las propiedades sensoriales del aceite

El potencial antioxidante de los compuestos fenólicos presentes en el aceite de oliva ha sido una cuestión que ha despertado gran interés, tanto por su efecto quimio-protectivo en seres humanos [114,115,116,117,118,119,120], como por ser uno de los factores más importantes en lo que a estabilidad oxidativa (shelf-life) de los aceites se refiere [118,121,121,122,123]. La actividad antioxidante de los componentes del aceite de oliva virgen se ha relacionado con la protección frente a importantes enfermedades crónicas y degenerativas tales como las enfermedades coronarias (CHD), las enfermedades de envejecimiento neuro-degenerativo y tumores localizados en diversas zonas [124,125,126]. Entre todos estos efectos protectores, probablemente se puede

[114] R. Leenen, A.J.C. Roodenburg, M.N. Vissers, J.A.E. Schuurbiers, K.P.A.M. van Putte, S.A. Viseman, F.H.M.M. van de Put. "Supplementation of plasma with olive oil phenols and extracts: Influence on LDL oxidation". *J. Agric. Food Chem*. 50 (2002) 1290–1297.

[115] M.N. Vissers, P.L. Zock, R. Leenen, A.J.C. Roodenburg, K.P.A.M. van Putte, M.B. Katan. "Effect of consumption of phenols from olives and extra virgin olive oil on LDL oxidizability in healthy humans". *Free Radical Res*. 35 (2001) 619–629.

[116] R. Briante, F. La Cara, M.P. Tonziello, F. Frebbraio, R. Nucci. "Antioxidant activity of the main bioactive derivatives from oleuropein hydrolysis by hyperthermophilic beta-glycosidase". *J. Agric. Food Chem*. 49 (2001) 3198–3203

[117] 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.

[118] F. Caponio, V. Alloggio, T. Gomes. "Phenolic compounds of virgin olive oil: influence of paste preparation techniques". *Food Chem*. 64 (1999) 203–209.

[119] E. Tripoli, M. Giammanco, G. Tabacchi, D. Di Majo, S. Giammanco, M. La Guardia. "The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health ". *Nutrition Research Reviews* 18 (2005) 98-112.

[120] F. Caponio, T. Gomes, A. Pasqualone. "Phenolic compounds in virgin olive oils: influence of the degree of olive ripeness on organoleptic characteristics and shelf-life". *Eur. Food Res. Technol*. 212 (2001) 329–333.

[121] M. Tsimidou. "Polyphenols and quality of virgin olive oil in retrospect". *Ital. J. Food Sci*. 10 (1998) 99–116.

[122] M. Baldioli, M. Servili, G. Perreti, G. F. Montedoro. "Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil". *J. Am. Oil Chem. Soc*. 73 (1996) 1589–1593

[123] J. Velasco, C. Dobarganes. "Oxidative stability of virgin olive oil". *Eur. J. Lipid Sci. Technol*. 104 (2002) 661–676.

[124] M. Soler, L. Chatenaud, C. La Vecchia, S. Franceschi, S. Negri. "Diet, alcohol, coffee and pancreatic cancer: final results from an Italian study". *Eur. J. Cancer Prev*. 7 (1998) 450-461.

[125] S. Franceschi, A. Favero, E. Conti, R. Salamini, R. Volpe, E. Negri, L. Barman, C. La Vecchia. "Food groups, oils and butter, and cancer of the oral cavity and pharynx". *British J. Cancer* 80 (1999) 614–620.

[126] E. Hodge, D. R. English, M. R. E. McCredie, G. Severi, P. Boyle, J.L. Hopper, G.G. Giles. "Foods, nutrients and prostate cancer". *Cancer Causes&Control* 15 (2004) 11–22.

resaltar, por ejemplo, la oxidación de las lipoproteínas de baja densidad (LDL) [127], la reducción del daño oxidativo de los eritrocitos humanos por el HYTY [128] y la reducción de la producción de radicales libres en la matriz fetal [129]. Además, 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 [103,129,130]. Los radicales libres, en general, se han relacionado con enfermedades del corazón, cáncer y envejecimiento.

Entre los compuestos fenólicos presentes en el aceite de oliva, tienen especial interés aquellos que poseen grupos orto-difenólicos, principalmente la oleuropeína y el HYTY, por ser grandes inhibidores de la oxidación de la LDL *in vitro* [131]. Estos dos compuestos, secuestradores de radicales libres, han demostrado tener una capacidad antioxidante igual o superior a la de otros antioxidantes ampliamente estudiados, como la vitamina E, C y el hidroxitolueno butilado [132]. Recientemente se ha publicado que los lignanos, compuestos presentes también en esta fracción, son potentes antioxidantes *in vitro* y presentan la más alta correlación con la capacidad antioxidante, seguidos por algunos secoiridoides, HYTY y TY [133]. Por otro lado, en estudios con animales se ha atribuido a los lignanos la capacidad de inhibir la peroxidación lipídica *in vivo* [134].

Además de todas estas acciones preventivas, el efecto anti-inflamatorio que exhibe un compuesto fenólico presente en el aceite de oliva llamado "oleocanthal" ha sido puesto de manifiesto por Beauchamp y col. [135] y publicado en la revista Nature. Este grupo

[127] F. Visioli, G. Bellomo, G. F. Montedoro, C. Galli. "Low-density-lipoprotein oxidation is inhibited in-vitro by olive oil constituents". *Atherosclerosis* 117 (1995) 25–32.

[128] C. Manna, V. Galletti, P. Cucciolla, G.F. Montedoro, V. Zappia. "Olive oil hydroxytyrosol protects human erythrocytes against oxidative damages". *J. Nutr. Biochem*. 10 (1999) 159–165.

[129] R. W. Owen, A. Giacosa, W. E. Hull, R. Haubner, G. Wurtele, B. Spiegelhaldar, H. Bartsch. "Olive-oil consumption and health: the possible role of antioxidants". *Lancet Oncol*. 1 (2000) 107–112.

[130] 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.

[131] F. Visioli, C. Galli. "Free radical-scavenging actions of olive oil phenolics". *Lipids* 34Suppl. (1999) S315-S315.

[132] F. Visioli, C. Galli. "The effects of minor constituents of olive oil on cardiovascular disease: new findings". *Nutr. Rev*. 56 (1998) 142-147.

[133] R. R. Owen, A. Giacosa, W. E. Hull, R. Haubner, B. Spiegelhalder, H. Bartsch. "The antioxidant/anticancer potential of phenolic compounds isolated from olive oil". *Euro. J. Cancer* 36 (2000) 1235-1247.

[134] M. H. Kang, M. Natio, N. Tsujihara, T. Osawua. "Sesamolin inhibits lipid peroxidation in rat liver and kidney". *J. Nutr*. 128 (1998) 1018-1022.

[135] 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

de investigación declara que el (-)-oleocanthal es tanto un potente agente anti-inflamatorio (similar al ibuprofeno) como un poderoso antioxidante (parecido al α-tocoferol) [136]. Es, no obstante, importante resaltar, en mi opinión, que en una reciente carta al editor de Mol. Nutr. Food Res., dos científicos italianos [137] comentaban que atribuir los efectos saludables de una dieta a un compuesto de manera individual es realmente arriesgado, y esto podría ser particularmente cierto en el caso del oleocanthal, que está presente en el aceite de oliva en pequeña cantidad.

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" [121,138,139,140], así como responsables de características organolépticas en general [141]. Menos conocida es su faceta "picante" asociada a sensaciones que "queman" al gusto [121,142,143]. 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 [144], mientras que otros estudios no demostraban ninguna relación entre la sensación de "amargor" y el contenido en ácido fenólicos de un aceite [145]. 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 3,4-

[136] A. B. Smith, Q. Han, P. A. S. Breslin, G. K. Beauchamp. "Synthesis and assignment of absolute configuration of (-)-oleocanthal: a potent, naturally occurring non-steroidal anti-inflammatory and anti-oxidant agent derived from extra virgin olive oils". *Org. Lett.* 22 (2005) 5075–5078.
[137] V. Fogliano, R. Sacchi. "Oleocanthal in olive oil: between myth and reality". *Mol. Nutr. Food Res.* 50 (2006) 5-6.
[138] 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.
[139] G.F. Montedoro, M. Baldioli, M. Servili. "I composti fenolici dell'olio di oliva e la loro importanza sensoriale, nutrizionale e merceologica". *Giornale Ital. Nutriz. Clin. Prev*. 1 (1992) 19–32.
[140] F. Gutiérrez-Rosales, J. J. Ríos, M. L. Gómez-Rey, J. Agric. Food Chem. 2003, 51, 6021–6025.
[141] D. Ryan, K. Robards. "Phenolic compounds in olives". *Analyst* 123 (1998) 31R–44R.
[142] M. Servili, G. Montedoro. "Contribution of phenolic compounds to virgin olive oil quality". *Eur. J. Lipid Sci. Technol*. 104 (2002) 602–613.
[143] 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.
[144] 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.
[145] N. Uccella. A. H. Spanier, F. Shahidi, T. H. Parliment, C. J. Mussinan, C. T. Ho, E. Tratas Conti, (Eds.), "Food Flavours and Chemistry: Advances of the New Millenium". The Royal Society of Chemistry Publishers, Cambridge, UK, Pp 253 (2001).

DHPEA-EDA (HYTY-EDA) y *p*-HPEA-EA (TY-EDA) [146,147]. En este caso, García y col. [147] 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 [148] y el contenido de la forma aldehídica de la Ol Agl [149].

[146] A. K. Kiritsakis. "Flavor components of olive oil - A review". *J. Am. Oil Chem. Soc*. 75 (1998) 673–681.
[147] 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.
[148] 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.
[149] 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.

# Influencia de los factores agronómicos y tecnológicos sobre la calidad y composición del aceite de oliva

La composición del aceite de oliva y, en particular, de los compuestos fenólicos del mismo, es el resultante de una serie de interacciones entre factores agronómicos y tecnológicos que marcan tanto la fase de desarrollo y de maduración del fruto como su transformación [150].

## Parámetros agronómicos

Varios parámetros agronómicos pueden modificar la concentración de compuestos fenólicos en el aceite de oliva. Los aspectos más estudiados en relación con este apartado incluyen la variedad de aceituna, el grado de maduración, las condiciones pedoclimáticas de producción y algunas otras técnicas agronómicas como la irrigación [147,148,151].

### Influencia de la variedad y del grado de maduración de la oliva

El aceite de oliva, como producto del metabolismo del olivo está fuertemente influenciado por la variedad. Esta influencia podría considerarse como directa si se hace referencia a las variaciones específicas de los constituyentes individuales, con un grado de madurez igual, y como indirecta si va unida a variaciones del modelo de maduración de los frutos o a interacciones en condiciones de crecimiento diversas.

La variedad tiene una notable influencia sobre los compuestos fenólicos [152,153,154,155], aunque existen otros factores a tener en cuenta. Se ha señalado que

[150] G. Montedoro, M. Servili. "Chimica e qualità dell' olio di oliva: i fattori che la condizionano" in Atti Convegno "L' olio di oliva ed il suo futuro" Spoleto 6-7 diciembre. (1991) 33-55.

[151] M. Servili, R. Selvaggini, S. Esposto, A. Taticchi, G. F. Montedoro, G. Morozzi. "Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil". *J. Chromatogr. A* 1054 (2004) 113-127.

[152] 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.

[153] P. Pinelli, C. Galardi, N. Mulinacci, F.F. Vinceri, A. Cimato, A. Romani. "Minor polar compound and fatty acid analyses in monocultivar virgin olive oils from Tuscany". *Food Chem.* 80 (2003) 331-336.

[154] S. Gómez-Alonso, M.D. Salvador, G. Fregapane. "Phenolic compounds profile of Cornicabra virgin olive oil". *J. Agric. Food Chem.* 50 (2002) 6812-6817.

[155] M. D. Salvador, F. Aranda, G. Fregapane. "Contribution of chemical components of *Cornicabra* virgin olive oils to oxidative stability. A study of three successive crop Seasons". *J. Am. Oil Chem. Soc.* 76 (199) 427-432.

el perfil fenólico de un aceite, desde el punto de vista cualitativo, es característico en función de la variedad de oliva de origen [106,156]; después, a medida que madura, la oliva va experimentando cambios fisiológicos, modificándose su textura, color y composición por lo que las características del aceite varían en función de la fase de madurez en que han sido recogidas las olivas. Así, a lo largo del proceso de maduración, una vez finalizada la lipogénesis o proceso de formación del aceite, se producen cambios en la composición del mismo.

Por ejemplo, se ha observado una disminución del contenido de tocoferoles, polifenoles totales y pigmentos del aceite de diversas variedades españolas al avanzar el estado de maduración de las olivas [157, 158]. Otros estudios han mostrado un aumento del contenido de polifenoles en los aceites procedentes de olivas en las fases de maduración desde verde a envero y una disminución en las fases de envero a maduro [159].

### Influencia de los factores ambientales

El clima ejerce una gran influencia sobre la composición química y la calidad del aceite, se ha observado que existe una notable influencia de las condiciones metereológicas sobre la evolución en el crecimiento del fruto y su maduración [160]. En un estudio sobre los efectos de los factores agronómicos y estacionales en la producción del olivo y las características cualitativas del aceite se puso de manifiesto que las condiciones climáticas, en particular las precipitaciones, influían en la composición química del aceite de oliva. Los compuestos que más se veían influidos eran los alcoholes alifáticos, los compuestos fenólicos y los componentes del espacio de cabeza, de particular importancia en lo que respecta a las características organolépticas y de calidad del aceite [161].

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[156] M. Solinas. "Analisis HRGC delle sostanze fenoliche di oli vergine di oliva in relazione al grado di maturazione e alla varietà delle olive". *Riv. Ital. Sost. Grasse* 64 (1987) 255-262.

[157] A. Agramont, M. C. López, J. Boatella, M. C. de la Torre. "Évolution de la teneur en tocophérols et en tocotrienols pendant le mûrissage des olives". *Riv. Ital. Sost. Grasse* 63 (1986) 443-447.

[158] F. Gutiérrez, B. Jiménez, A. Ruíz, M. A. Albi. "Effect of olive ripeness on the oxidative stability of virgin olive oil extracted from the varieties Picual and Hojiblanca and on the different components involved". *J. Agric. Food Chem.* 47 (1999) 121-127.

[159] J. J. Sánchez, C. de Miguel, J. Marín. "La calidad del aceite de oliva procedente de variedades cultivadas en Extremadura en relación con la composición y maduración de la aceituna". *Olivae* 75 (199) 31-36.

[160] A. Jacoboni, M. Pinnola Ida, A. Baltadori. "The production of *Olea europaea* L. in Viterbo in relation to the climatological factors". *Acta Horticulturae* 474 (1999) 229-232.

[161] G. Pannelli, M. Servili, M. Selvaggini, M. Baldioli, G. F. Montedoro. "Effect of agronomic and seasonal factor on olive (*Olea europaea* L.) production and on the qualitative characteristics of the oil". *Acta Horticulturae* 356 (1994) 239-243.

En un trabajo realizado por Ranalli y col [162] se observa que aceites provenientes de la misma variedad y cultivados en distintas áreas geográficas presentan características significativamente diferentes respecto a la composición aromática, concentración de compuestos fenólicos y composición acídica, mostrando que la tipicidad de los aceites está definida también por la zona de producción y consecuentemente por factores climáticos y edafológicos.

### Influencia del riego

Diversos investigadores han estudiado la influencia que tiene la aplicación de riego al olivo sobre la composición química y las características organolépticas del aceite de oliva, aunque los resultados obtenidos no siempre han sido concordantes.

La mayoría de los trabajos publicados coinciden en afirmar que la concentración de los compuestos fenólicos en el aceite disminuye a medida que aumenta la dosis de riego [163,164,165,166], aunque los trabajos de Dettori y Russo [167] e Inglese y col [168] muestran un mayor contenido de polifenoles en los aceites de árboles más regados.

### Parámetros tecnológicos

Aunque la presencia de compuestos fenólicos en el aceite de oliva está estrictamente relacionada con la actividad de varias enzimas endógenas de la aceituna, su concentración se ve bastante afectada por la condiciones de extracción empleadas durante la producción del aceite. La molienda del fruto y el batido son los puntos más críticos en el proceso de extracción mecánica del aceite.

[162] A. Ranalli, G. de Mattia, M. L. Ferrante, L. Giansante. "Incidence of olive cultivation area on the analytical characteristics of the oil. Note 1". *Riv. Ital. Sost. Grasse* 74 (1997) 501-508.

[163] G. Beltrán, A. Jiménez, M. Uceda. "Efecto del régimen hídrico del cultivo sobre la fracción fenólica del aceite de oliva de la variedad Arbequina". I Simposio del olivo Arbequino en Cataluña, Borges Blanques. (1995) 153-155.

[164] J. Salas, M. Pastor, J. Castro, V. Vega. "Influencia del riego sobre la composición y características organolépticas del aceite de oliva". *Grasas y Aceites* 48 (1997) 74-82.

[165] M. Patumi, R. d'Andria, G. Fontanazza, G. Morelli, P. Giorio, G. Sorrentino. "Yield and oil quality of intensively trained trees of three cultivars of olive (*Olea europaea* L.) under different irrigation regimes". *J. Hort. Science & Biotech.* 74 (1999) 729-737.

[166] J. M. Faci, M. J. Berenguer, S. Gracia, J. L. Espada. "Effect of variable water irrigation supply in olive (*Olea europaea* L.) cv. *Arbequina* in Aragón (Spain). II. Extra virgin oil quality parameters". Actas del IV International congress on Olive growing. Bari, Italy. (2000) 4-87.

[167] S. Dettori, G. Russo. "Influencia del cultivar y del régimen hídrico sobre el volumen y la calidad del aceite de oliva". *Olivae* 49 (1993) 36-43.

[168] P. Inglese, E. Barone, G. Gullo. "The effect of complementary irrigation on fruit growth, ripening pattern and oil characteristics of olive (*Olea europaea* L.) cv. Carolea". *J. Hort. Sci.* 71 (1996) 257-263.

**Influencia de la tecnología de extracción del aceite**

La tecnología de extracción tiene una gran influencia sobre las características del aceite de oliva. La molienda del fruto afecta a la composición del aceite, principalmente al color y a los compuestos fenólicos. Las formas "agliconas" de los secoiridoides, por ejemplo, se forman durante la molienda del fruto por hidrólisis de la oleuropeína, demetiloleuropeína y ligustrósido; la reacción es catalizada por β-glucosidasas endógenas, de acuerdo con el mecanismo que se recoge en la siguiente figura [169].

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[169] Nos parece muy interesante recoger el mecanismo de formación de varias formas de los secoiridoides, ya que puede ayudar a entender la complejidad estructural de esta familia, así como la existencia de distintas formas isoméricas a las que se alude en la presente memoria.
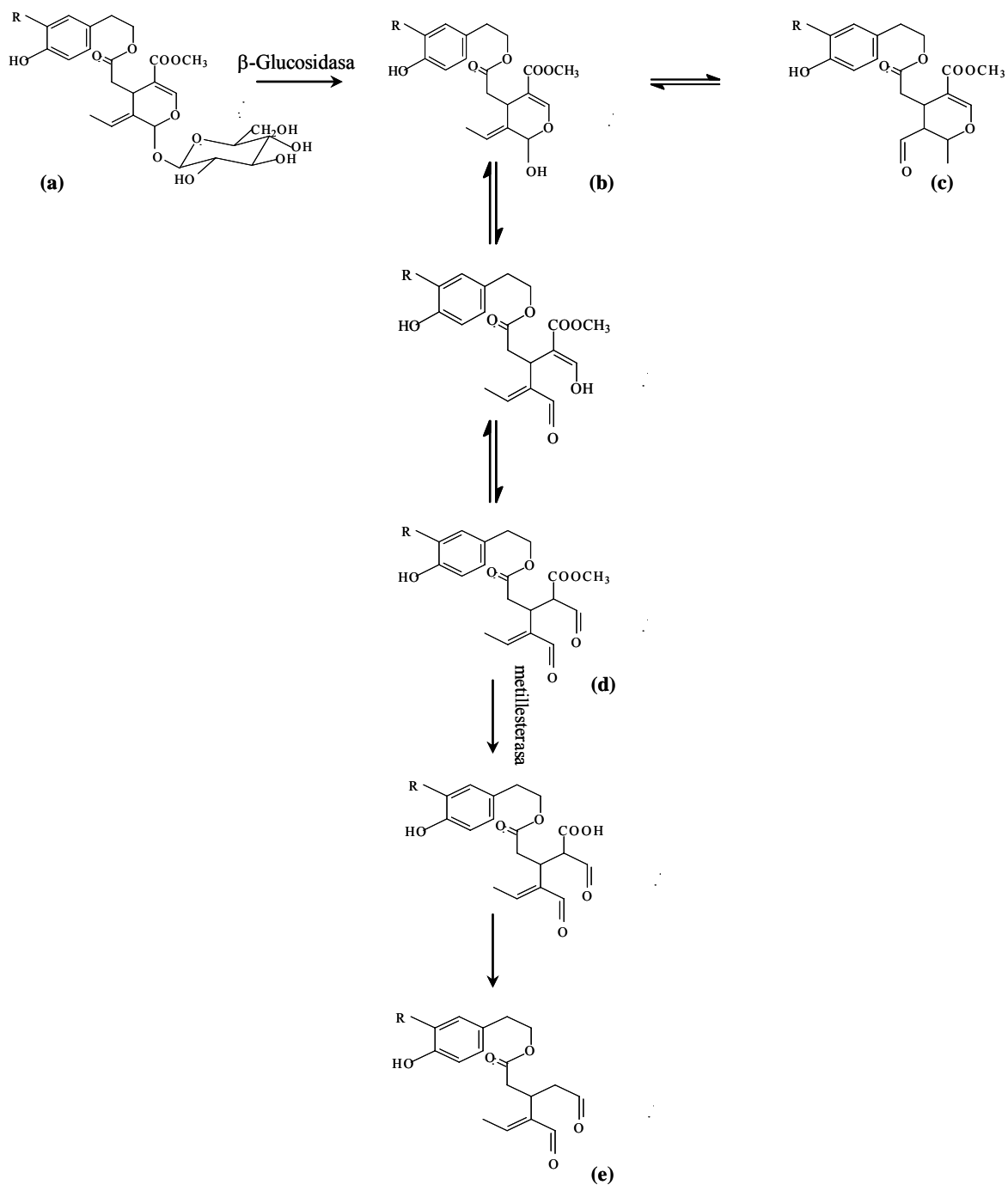
*Figura*. Mecanismo bioquímico propuesto para la formación de derivados de los secoiridoides: **(a)** R= H: ligustrósido; R= OH: oleuropeína; **(b)** R= H: Lig Agl; **(c)** R= OH: Ol Agl; **(d)** R= H: forma dialdehídica de Lig Agl; R= OH: forma dialdehídica de Ol Agl; **(e)** R= H: forma dialdehídica decarboxilada-Lig Agl ; R=OH: forma dialdehídica decarboxilada -Ol Agl .

Alloggio y Caponio [170] llegaron a la conclusión de que los molinos de martillos mostraban, en general, una mayor capacidad de extracción de los compuestos fenólicos que los molinos de empiedro.

Cualquiera que sea el sistema de elaboración del aceite, el tiempo de batido y las temperaturas que se alcanzan durante todo el proceso, constituyen variables de regulación de gran importancia en su control en lo que se refiere a las características del aceite obtenido. Se ha evidenciado que un aumento en la temperatura favorece la disolución de los compuestos fenólicos de la pasta en el aceite, mientras que un aumento en el tiempo de batido implica una disminución del contenido de polifenoles independientemente de la temperatura de batido, debido probablemente a la intervención de la fenoloxidasa que cataliza la oxidación de los fenoles a quinonas y después a polímeros [171,172]. De ahí que el control de la concentración de $O_2$ durante el procesado de la pasta pueda ser optimizado para controlar la cantidad de compuestos fenólicos en el aceite de oliva virgen [173].

En la fase de separación sólido-líquido hay que tener en cuenta que el sistema de centrifugación necesita adición de agua a la pasta de la oliva para facilitar la separación del aceite de las otras fases, mientras que los sistemas de presión y de percolación no necesitan esa adición de agua.

Esto determina diferencias en algunas características del aceite de oliva, como el contenido en polifenoles totales y pigmentos clorofílicos [174]. El contenido de polifenoles totales es superior en los aceites obtenidos con los sistemas de presión y de percolación que en los extraídos con el sistema de centrifugación. El agua utilizada para diluir la pasta en la centrífuga disminuye la concentración de las sustancias fenólicas de

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[170] V. Alloggio, F. Caponio. "Influenza delle tecniche di preparazione della pasta di oliva sulla qualità dell' olio. Nota II. Evoluzione delle sostanze fenoliche e di alcuni parametri di qualità in funzione della maturazione delle drupe in olio d' oliva vergine della cv. Coratina". *Riv. Ital. Sost. Grasse* 74 (1997) 443-447.

[171] M. Solinas, L. di Giovacchino, A. Mascolo. "I polifenoli delle olive e dell' olio d' oliva. Nota III. Influenza della temperatura e della durata de la gramolatura sul contenuto in polifenoli degli oli". *Riv. Ital. Sost. Grasse* 55 (1978) 19-23.

[172] A. Jiménez, M. Hermoso, M. Uceda. "Elaboración del aceite de oliva virgen mediante sistema continuo de dos fases. Influencia de las diferentes variables del proceso en algunos parámetros relacionados con la calidad del aceite". *Grasas y Aceites* 46 (1995) 299- 303.

[173] A. García, M. Brenes, F. Martínez, J. Alba, P. García, A. Garrido. "High-performance liquid chromatography evaluation of phenols in virgin olive oil during extraction at laboratory and industrial scale". *J. Am. Oil Chem. Soc*. 78 (2001) 625-629.

[174] L. di Giovacchino. "Influencia de los sistemas de extracción en la calidad del aceite de Oliva". *Olivae* 63 (1996) 52-63.

la fase acuosa por dilución, y según la ley del equilibrio entre fases, provoca asimismo la disminución de la concentración de dichas sustancias en la fase oleosa inmiscible.

Los aceites de percolación o primera extracción, se caracterizan por niveles superiores de polifenoles totales, tocoferoles y componentes volátiles, y niveles inferiores de clorofilas, feofitinas, esteroles y alcoholes alifáticos y terpénicos [175].

El aceite obtenido por el sistema de 2 fases, en relación con el de 3 fases, presenta un mayor contenido de polifenoles, tocoferoles, *trans*-hexenal y compuestos aromáticos y un menor contenido de pigmentos [176, 177]. La mayor cantidad de agua que se adiciona a la pasta favorece que los compuestos fenólicos, de naturaleza hidrosoluble, pasen a la fase acuosa y se pierdan en los alpechines.

El esquema que se presenta a continuación recoge los factores que afectan a la calidad (y composición) del aceite de oliva. Incluye tanto los aspectos agronómicos y tecnológicos mencionados en el presente apartado, como la conservación del aceite, proceso que puede afectar de manera muy drástica a su calidad.

[175] A. Ranalli, M. L. Ferrante, G. de Mattia, N. Costantini. "Analytical evaluation of virgin olive oil of first and second extraction". *J. Agric. Food Chem.* 47 (1999) 417-424.

[176] A. Ranalli, F. Angerosa. "Integral centrifuges for olive oil extraction. The qualitative characteristics of products". *J. Am. Oil Chem. Soc.* 73 (1996) 417-422.

[177] M. J. Motilva, I. Jaria, I. Bellart, M. P. Romero. "Estudio de la calidad del aceite de oliva virgen de la Denominación de Origen " Les Garrigues" (Lleida) durante la campaña 1995/96". *Grasas y Aceites* 49 (1998) 425-433.

**Calidad del aceite de oliva**

- Ambiente
  - clima
  - terreno
- Técnicas culturales
  - riego
  - poda
  - defensa fitosanitaria
  - abono
  - recogida
- Variedad
- Método de extracción
  - tradicional a presión
  - continuo a 2 o 3 fases
  - percolación
- Conservación
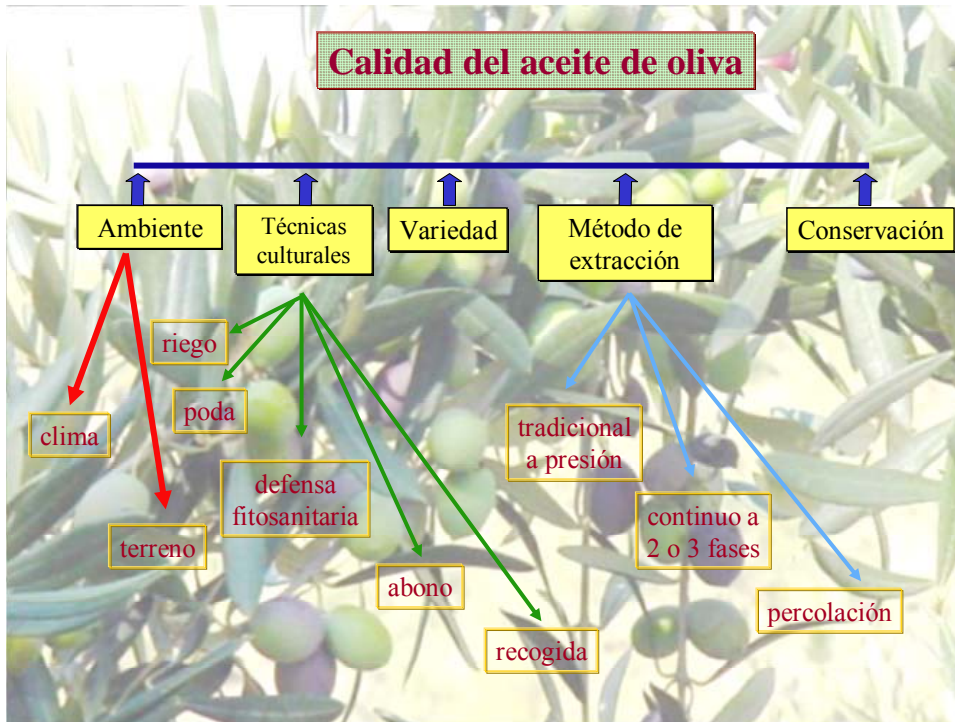
*Esquema*. Factores que afectan a la calidad (y composición) del aceite de oliva.

**Consideraciones sobre las
técnicas de separación utilizadas
en esta memoria**

La matriz del aceite de oliva posee una composición muy rica y compleja. Para poder llevar a cabo nuestros objetivos centrados en el estudio de la fracción polifenólica del aceite de oliva del modo más satisfactorio posible pensamos que sería útil utilizar no solo una técnica separativa, y por ello, se emplearon tanto la electroforesis capilar, como la cromatografía líquida de alta resolución. Del mismo modo, se acoplaron a estas técnicas varios sistemas de detección (absorción UV-Vis y espectrometría de masas). Así, la espectrometría de masas, podría complementar la información proporcionada por la detección óptica, dándonos información estructural muy valiosa.

Pasamos a comentar los aspectos más relevantes relacionados con la electroforesis capilar:

# Electroforesis Capilar

## Introducción y definición de la electroforesis capilar

El proceso electroforético fue antaño definido como "el movimiento diferencial o migración de iones en disolución por atracción o repulsión en un campo eléctrico". En términos prácticos, un electrodo positivo (ánodo) y uno negativo (cátodo) se colocan en una disolución conteniendo iones. Luego, cuando se aplica un voltaje a los electrodos, los iones de diferente carga, aniones con carga negativa y cationes con carga positiva, se mueven a través de la disolución hacia el electrodo de carga opuesta.

La electroforesis capilar (CE) es, por tanto, una técnica que desarrolla lo anteriormente dicho en estrechos capilares, normalmente de 25 a 100 μm de diámetro interno (i.d.), rellenos de una disolución de separación (o disolución electroforética de trabajo) que suele ser una disolución reguladora.

Las primeras experiencias separativas utilizando los principios electroforéticos fueron realizadas por Tiselius [178] usando un tubo en U que contenía disoluciones libres o no soportadas separadas por delimitadores que se movían por el voltaje impuesto. Por el desarrollo del primer aparato denominado celda electroforética en 1937, Tiselius fue galardonado con el premio Nobel en el 1948. Los posteriores estudios sobre electroforesis fueron llevados a cabo en columnas y en soportes planos humedecidos con líquido, generalmente disoluciones acuosas. Los soportes o materiales planos pueden ser papel o sílica (parecido a cromatografía en capa fina), o una variedad de geles como es el almidón o el poliacrilato. Las técnicas planas pueden ser denominadas con una gran variedad de nombres, incluyendo electroforesis en zona o electroforesis en gel. Estas separaciones por electroforesis convencional, han sido y siguen siendo muy eficaces y de muy extensa aplicación, principalmente en la identificación o cuantificación de macromoléculas, especialmente proteínas, pero dichas separaciones presentan una serie de inconvenientes:

- Son técnicas lentas y laboriosas.
- Tienen tendencia a ser poco reproducibles.
- No permiten la automatización.

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[178] A. Tiselius. "A new apparatus for electrophoretic analysis of colloidal mixtures". *Trans. Faraday Soc*. 33 (1937) 524-531.

El uso de capilares en vez de superficies planas en electroforesis supuso una gran novedad ya que se hizo viable eliminar el calentamiento por efecto Joule que acompaña a los fenómenos electroforéticos; fue entonces cuando comenzó una nueva forma de electroforesis que se denominó electroforesis capilar.

Esta técnica separativa fue descrita originalmente como electroforesis libre a finales de los años 60, concretamente en 1967, cuando Hjertén [179] utilizó capilares de 3 mm de diámetro interno empleando campos eléctricos altos. Pero no fue hasta 1974 cuando Virtanen [180] describió las ventajas de usar capilares con diámetros tan pequeños.

Todos estos primeros trabajos sobre CE se llevaron a cabo con instrumentación adaptada de la electroforesis convencional y no fue hasta 1979 cuando Mikkers y col. [181], demostraron el uso de capilares de teflón con diámetros internos de 200 μm para la electroforesis libre.

Todas las expectativas que en su día se generaron, se han logrado y como resultado hoy día la CE se ha convertido en una técnica popular complementaria a la cromatografía de líquidos de alta eficacia (HPLC). La CE ofrece la ventaja de que el desarrollo de los métodos es mucho más predecible que en HPLC; la migración electroforética sigue las reglas concretas y simples, mientras que las interacciones moleculares en HPLC son mucho más complejas. Se puede decir que la CE combina el poder de separación de la electroforesis convencional con la automatización propia de la HPLC.

Las principales características de la CE son:

-Elevada rapidez de análisis.

-Elevadas eficacias, normalmente en el intervalo de $10^5$-$10^6$ platos teóricos por metro de columna.

-Requerimiento de pequeños volúmenes de muestra (del orden de nanolitros) y reactivos.

-Gran variedad de aplicaciones.

-Facilidad de automatización.

[179] S. Hjertén. "High performance electrophoresis". *Chromatogr. Rev*. 9 (1967) 122-219.
[180] R. Virtanen. "Zone electrophoresis in a narrow-bore tube employing potentiometric detection". *Acta Polytech. Scand*. 123 (1974) 1-67.
[181] F. Mikkers, F. Everaerts, T. Verheggen. "High performance zone electrophoresis". *J. Chromatogr*. 169 (1979) 11-20.

## Instrumentación

La instrumentación requerida para CE se basa en un diseño bastante simple tal y como se puede ver en la siguiente figura.
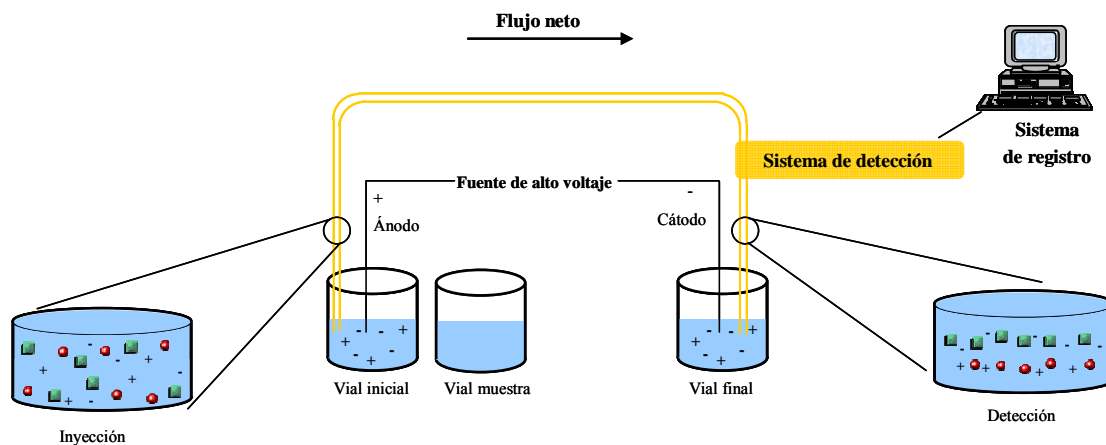


*Figura*. Esquema básico de un instrumento de CE.

La CE es muy fácil de automatizar y existen equipos comerciales equiparables a los ampliamente conocidos de HPLC. Los componentes básicos de estos equipos consisten en un automuestreador, un módulo de detección, una fuente de alto voltaje, el capilar y, por supuesto, un ordenador para controlar el instrumento. La fuente de alto voltaje es equivalente a las bombas en HPLC y los capilares (normalmente de sílice fundida recubiertos de poliimida) equivalen a la columna, por lo que la instrumentación es bastante semejante y esto se hace especialmente evidente en los programas usados para controlar los equipos de CE que están basados principalmente en los "softwares" existentes para HPLC.

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 capaz de alcanzar voltajes de hasta 30 kV (existen incluso aplicaciones desarrolladas a voltajes superiores). La muestra se inyecta dentro del capilar sustituyendo temporalmente el vial inicial con disolución de separación (usualmente el del ánodo) por un vial que contiene la muestra a separar y aplicando un potencial

eléctrico o una presión externa durante unos segundos. A la primera forma se le denomina inyección electrocinética y a la segunda hidrodinámica. Después se vuelve a reemplazar el vial de muestra por el inicial conteniendo la disolución de separación 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-.

La separación en CE se produce al migrar los solutos a través del capilar después de la aplicación de un determinado voltaje, pero posteriormente es necesario determinar y cuantificar los componentes que, previamente, se han separado.

En los últimos años las metodologías y las aplicaciones electroforéticas han progresado de un modo espectacular; sin embargo la principal limitación ha sido las posibilidades en cuanto al acoplamiento de diferentes sistemas de detección a la separación electroforética, debido principalmente a las pequeñas dimensiones del capilar utilizado, así como los pequeños volúmenes de muestra usados que hacen que la sensibilidad en la detección de las zonas ocupadas por los solutos de interés se vea afectada por la dispersión en la detección on-line, provocando alteraciones en la señal que, en ocasiones, son cruciales para la efectividad de la detección [182].

La pared interna del capilar tiene grupos silanoles que, en contacto con el tampón de separación, si 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 esto modo, la pared cargada negativamente atrae a los iones con carga positiva del tampón creando una doble capa eléctrica. Esta doble capa tiene dos zonas, una compacta próxima a la pared del capilar y en la cual las interacciones que se dan entre los grupos cargados negativamente de la sílice y los contraiones del tampón son tan grandes que compensan la agitación térmica; y otra más alejada de la pared, denominada difusa, con interacciones más débiles con los silanoles cargados,

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[182] A. Fernández Gutiérrez, A. Segura Carretero, A. Carrasco Pancorbo. "Fundamentos teóricos y modos de separación" en "Electroforesis capilar: aproximación según la técnica de detección". Ed. Univ. Granada. (2005) Pp 11-54.

pudiéndose producir agitación térmica. Bajo la acción del campo eléctrico, las cargas positivas de la zona difusa se desplazan hacia el cátodo y arrastran con ellas el agua de solvatación que llevan asociada. El resultado de este fenómeno es un moviendo global de todas las especies en el interior del capilar, que viene determinado por la movilidad electroosmótica [183,184,185]:

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

donde $\varepsilon$ es la constante dieléctrica del tampón, $\eta$ su viscosidad y $\zeta$ (llamado potencial zeta) el potencial que se genera aproximadamente entre la superficie del capilar y el tampón. Al emplear electrolitos con concentraciones muy elevadas, el espesor de la capa difusa de la doble capa eléctrica disminuye y el de la zona compacta aumenta, el potencial zeta entonces disminuye, y en la misma proporción lo hace $\mu_{eo}$. El flujo electroosmótico (FEO) 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. Una de las características más importantes de este flujo es que tiene perfil prácticamente plano, lo que hace posible la obtención de elevadas eficacias.

Bajo la presencia de un campo eléctrico, además, las sustancias cargadas sufren el proceso de electromigración, por el cual, los analitos en el interior del capilar tienden a migrar hacia su polo correspondiente (cationes al cátodo, aniones al ánodo), al verse sometidos a la fuerza generada por el campo eléctrico. Por otro lado, aparece también una fuerza de rozamiento que se opone a la eléctrica. Durante la separación ambas fuerzas se igualan, de modo que los iones adoptan un movimiento rectilíneo uniforme, cuya velocidad de migración puede ser expresada como:

$$\nu_e = \mu_e E$$

donde $\nu_e$ es la velocidad de migración de cada ion (m s$^{-1}$), $\mu_e$ es la movilidad electroforética (m$^2$V$^{-1}$s$^{-1}$) y E es el campo eléctrico aplicado (Vm$^{-1}$). La intensidad del campo eléctrico es función del voltaje aplicado dividido por la longitud total del capilar.

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

La movilidad electroforética es un factor que indica cómo de rápido se mueve un ión o soluto a través de la disolución de separación. Ésta 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. 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. El radio de ión, r, puede estar afectado por el contraión presente o por los agentes complejantes utilizados. 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. Elevada carga y pequeño tamaño confieren una gran movilidad, mientras que una baja carga y un gran tamaño confieren una baja movilidad.

En los capilares sin recubrimiento interno, se dan simultáneamente la migración electroforética y la migración electroosmótica de los componentes de la muestra. En estas condiciones, la velocidad que van a adoptar las sustancias dentro del capilar va a ser suma (o tal vez resta) resultante, de los vectores electroosmótico y electroforético que van a depender de las condiciones de separación y de la carga y volumen de los analitos) de estos dos factores:

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

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.

## Modos en CE

Es ampliamente reconocido que la CE es una técnica muy versátil, y esto es causado en parte por los distintos modos de separación disponibles. Los modos de CE más comunes están resumidos en la siguiente tabla junto con el principio de separación de cada uno de ellos. 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.

Una de las grandes ventajas de esta técnica es que para cambiar de modo puede bastar simplemente con variar la composición de la disolución reguladora empleada. A efectos prácticos, esto quiere decir que dos modos electroforéticos pueden ser utilizados en análisis sucesivos usando el mismo capilar. Este hecho contrasta con HPLC, donde para cambiar de modo normalmente hemos de cambiar tanto la fase móvil, como la columna.

*Tabla*. Modos de separación en electroforesis capilar.

| Modo de separación | Acrónimo-Simbología | 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 |

**Electroforesis capilar en zona (CZE)**

El más versátil y simple de los modos en electroforesis capilar es conocido como electroforesis capilar en zona (CZE), denominada también electroforesis capilar en zona libre. Es por ello por lo que este modo es el más empleado, con un amplio rango de aplicaciones.

El mecanismo de separación se basa en las diferencias en la relación carga/masa de las diferentes sustancias que componen una muestra. Estas diferencias implican distintas movilidades electroforéticas y, por tanto, diferentes velocidades de migración de las especies iónicas en el medio electroforético que contiene el capilar. Este mecanismo de separación permite la separación de mezclas de aniones y cationes, aunque las especies neutras no podrán ser separadas.

Cuando la muestra ha sido inyectada, se aplica el voltaje de separación, y dicha muestra se moverá a través del capilar a causa del FEO, mientras que los componentes individuales se separarán en zonas de diferente migración y su separación tendrá lugar a medida que se mueven dentro del capilar debido a las diferencias en sus movilidades electroforéticas.

El orden de elución es determinado por la relación carga/tamaño de los analitos, siendo: cationes, sustancias neutras y aniones, aunque como se indicó con anterioridad las sustancias neutras no se separan.

*Figura.* Representación de un electroferograma por CZE indicando el orden de elución
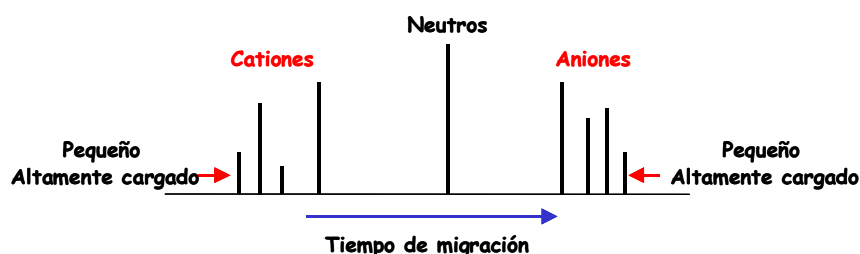


Los cationes se mueven a través del capilar en la misma dirección que el FEO, por lo que sus velocidades de migración serán más rápidas que el propio FEO. Las moléculas neutras, que se mueven a través del capilar empujadas sólo por el FEO, eluyen después de los cationes, pero sin separarse. Los aniones, finalmente, al poseer carga negativa, tenderán a moverse hacia el ánodo en sentido opuesto al FEO, 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. La figura mostrada a continuación representa un esquema de lo que sería una separación en CZE.

Figura. Representación esquemática de una separación empleando electroforesis capilar en zona (CZE).
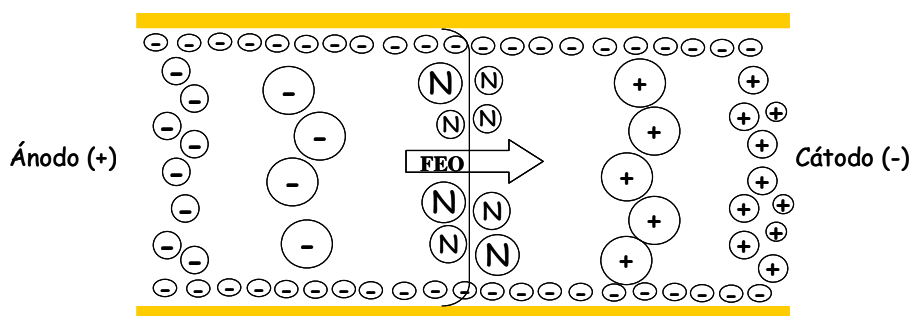


Hay numerosas aplicaciones de este modo de separación, incluyendo aniones y cationes inorgánicos [186- 191], moléculas pequeñas contenidas en productos farmacéuticos o biomoléculas grandes [ 192 - 198 ] y en otros campos de la Química Analítica [199,200,201,202,203,204,205,206].

[186] M. P. Harrold, M. J. Wajtusik, J. Riviello, R. Henson. "Parameters influencing separation and detection of anions by capillary electrophoresis". *J. Chromatogr.* 640 (1993) 463-471.

[187] A. Fernández, C. Cruces, S. Cortacero, A. Segura. "Sensitive determination of inorganic anions at trace levels in samples of snow water from Sierra Nevada (Granada, Spain) by capillary ion electrophoresis using calix[4]arene as selective modifier". *Chromatographia.* 52 (2000) 413-417.

[188] P. Y. Jandik, W. R. Jones. "Optimization of detection sensitivity in the capillary electrophoresis of inorganic anions". *J. Chromatogr.* 546 (1991) 431-443.

[189] J. Xu, P. Che, Y. Ma. "More sensitive way to determine iron using an iron(II)-1,10-phenanthroline complex and capillary electrophoresis". *J. Chromatogr. A.* 749 (1996) 287-294.

[190] M. A. Friedberg, M. E. Hinsdale, Z. K. Shihabi. "Analysis of nitrate in biological fluids by capillary electrophoresis". *J. Chromatogr. A.* 781 (1997) 491-496.

[191] K. E. Ferslew, A. N. Hagardorn, M. T. Harrison, W. F. McCormick. "Capillary ion analysis of potassium concentrations in human vitreous humor". *Electrophoresis.* 19 (1998) 6-10.

[192] R. Lehmann, M. Koch, W. Voelter, H. U. Haring, H. M. Liebich. "Routine serum protein analysis by capillary zone electrophoresis: Clinical evaluation". *Chromatographia.* 45 (1997) 390-395.

[193] R. D. Mcfarlane, P. V. Bondarenko, S. L. Cockrill, I. D. Cruzado, W. Doss, C. J. Mcneal, A. M. Spiekerman, L. K. Watkins. "Development of a lipoprotein profile using capillary electrophoresis and mass spectrometry". *Electrophoresis.* 18 (1997) 1796-1806.

[194] C. R. Jolliff, C. R. Blessum. "Comparison of serum protein electrophoresis by agarose gel and capillary zone electrophoresis in a clinical setting". *Electrophoresis.* 18 (1997) 1781-1784.

[195] Y. Henskens, J. De Winter, M. Pedelharing, G. Ponjee. "Detection and identification of monoclonal gammopathies by capillary electrophoresis". *Clin. Chem.* 44 (1998) 1184-1190.

[196] J. Bienvenu, M. S. Graziani, F. Arpin, H. Bernon, C. Blessum, C. Marchetti, G. Roighetti, M. Somenzini, G. Verga, F. Aguzzi. "Multicenter evaluation of the Paragon CZE (TM) 2000 capillary zone electrophoresis system for serum protein electrophoresis and monoclonal component typing". *Clin. Chem.* 44 (1998) 599-605.

[197] C. C. Liu, J. S. Huang, D. L. J. Tyrrell, N. J. Dovichi. "Capillary electrophoresis-electrospray-mass spectrometry of nucleosides and nucleotides: Application to phosphorylation studies of anti-human imminodeficiency virus nucleosides in a human hepatoma cell line". Electrophoresis. 26 (2005) 1424-1431.

[198] H. Siren, R. Kuldvee, T. Karla, T. Ekstrom, M. L. Riekkola. "Capillary zone electrophoresis of cationic and anionic drugs in methanol". J. Chromatogr. A. 1068 (2005) 89-97.

[199] C. W. Klampfl. "Analysis of organic acids and inorganic anions in different types of beer using capillary zone electrophoresis". *J. Agr. Food Chem.* 47 (1999) 987-990.

Un problema de la CZE es la tendencia de algunos cationes de adsorberse sobre la pared del capilar cargado negativamente. Este efecto supone un handicap en la separación de proteínas. Para minimizarlo, las separaciones de proteínas son llevadas a cabo a menudo a bajos valores de pH donde la carga del capilar es mínima [207]. Alternativamente, los capilares pueden ser recubiertos con aditivos [208,209,210].

La fase móvil o disolución de separación es usualmente, en esta metodología, una disolución reguladora acuosa, aunque ya existen numerosos estudios de separaciones usando medios no acuosos (se comenta a continuación).

El modo descrito en este apartado ha sido el que se ha utilizado en todos los métodos electroforéticos puestos a punto en el trabajo que aquí se expone. Comentaremos muy resumidamente otros de los modos electroforéticos empleados con frecuencia.

*Electroforesis capilar en zona con medios no acuosos*

[200] D. Kaniansky, M. Masár, V. Madajová, J. Marák. "Determination of sorbic acid in food-products by capillary electrophoresis in a hydrodynamically closed separation compartment". *J. Chromatogr. A.* 677 (1994) 179-185.

[201] I. Recio, M. Ramos, L. Amigo. "Study of the polymorphism of ovine alpha(s1)- and alpha(s2)-caseins by capillary electrophoresis". *J. Dairy Res.* 64 (1997) 525-534.

[202] T. Soga, I. Tajima, D. N. Heiger. "Capillary electrophoresis for the determination of forensic anions in adulterated foods and beverages". *American Lab.* 2000 Agilent Tecnologies N. 5968-9463E.

[203] S. Pozdniakova, A. Padarauskas, G. Schwedt. "Simultaneous determination of iron(II) and iron(III) in water by capillary electrophoresis". *Anal. Chim. Acta.* 351 (1997) 41-48.

[204] E. Dabek-Zlotorzynska, J. F. Dlouhy, N. Houle, M. Piechowski, S. Ritchie. "Comparison of capillary zone electrophoresis with ion chromatography and standard photometric methods for the determination of inorganic anions in atmospheric aerosols". *J. Chromatogr. A.* 706 (1995) 469-478.

[205] A. Bendini, M. Bonoli, L. Cerretani, B. Biguzzi, G. Lercker, T. G. Toschi. "Liquid-liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods". *J. Chromatogr. A.* 985 (2003) 425-433.

[206] A. M. Gómez Caravaca, A. Carrasco Pancorbo, B. Cañabate Díaz, A. Segura Carretero, A. Fernández Gutiérrez. "Electrophoretic identification and quantification of compounds in polyphenolic fraction of Extra-Virgin Olive Oil". *Electrophoresis* 26 (2005) 3538-3551.

[207] H. Stutz. "Advances in the anlysis of proteins and peptides by capillary electrophoresis with matrix-assisted laser desorption/ionization and electrospray-mass spectrometry", *Electrophoresis.* 26 (2005) 1254-1290.

[208] T. T. Razunguzwa, M. Warrier, A. T. Timperman. „ESI-MS compatible permanent coating of glass surfaces using poly(ethylene glycol)-terminated alkoxysilanes for capillary zone electrophoretic protein separations". Anal. Chem. 78(13) (2006) 4326-4333.

[209] M. Kato, E. Imamura, K. Sakai-Kato, T. Nakajima, T. Toyo´oka. "Cationic amylopectin derivatives as additives for analysis of proteins in capillary electrophoresis". Electrophoresis 27(10) (2006) 1895-1899.

[210] J. Horvath, V. Dolnik. "Polymer wall coatings for capillary electrophoresis". Electrophoresis 22(4) (2001) 644-655.

La CZE con disoluciones de separación no acuosas está basada en el uso de disoluciones de electrolito preparadas en disolventes orgánicos puros o mezclas de estos, y hoy día es una alternativa muy interesante al uso de disoluciones reguladoras tradicionales acuosas, o acuosas con alguna proporción de disolvente orgánico [211]. Esta metodología se conoce como NACE (non-aqueous capillary electrophoresis).

En primer lugar, los disolventes orgánicos permiten solubilizar compuestos insolubles en agua y así afrontar la separación de sustancias apolares [212]. Además, otra de las características atractivas de la CZE en medios no acuosos es que proporciona una amplia gama de disolventes orgánicos que pueden ser usados en este modo electroforético como medio para la disolución de separación, pudiendo elegir por sus propiedades físicas y químicas condiciones que proporcionen una óptima separación.

**Electroforesis capilar en medios micelares**

Para mejorar la aplicabilidad de la técnica, y hacerla útil incluso para moléculas neutras, una segunda fase puede ser añadida y servir como una fase pseudoestacionaria en un sentido cromatográfico. Si dicha fase está basada en la formación de micelas la metodología se denomina electroforesis capilar en medios micelares (Micellar Electrokinetic Chromatography, MEKC; o bien, Cromatografía Capilar Electrocinética Micelar, CCEM).

MEKC puede ser aplicada para la separación no sólo de sustancias neutras, sino también de analitos cargados. Se basa en el reparto de los analitos entre dos fases, que son las micelas y la disolución tampón de separación. Los analitos se reparten entre la disolución acuosa y la pseudofase micelar, generando un segundo equilibrio en el proceso de separación.

*Electroforesis capilar en medios micelares con modificadores orgánicos*

Generalmente, la modalidad electroforética MECK se lleva a cabo sólo con un medio electroforético formado con la disolución reguladora y un surfactante. Sin embargo, hay casos en los que es necesario el empleo de otras sustancias que alteran la selectividad de la separación (modificadores o aditivos).

[211] J. L. Miller, M. G. Khaledi (Eds). "High Peformance Electrophoresis". Wiley, New York, 1998 (Chemical Analysis Series, vol. 146).
[212] M. Vaher, M. Koel. "Specific bacjground electrolytes for nonaqueous capillary electrophoresis", *J. Chromatogr. A*. 1068 (2005) 83-88.

Si bien es cierto que los disolventes orgánicos se podrían emplear como aditivos en un medio electroforético típico de la modalidad de CZE [213], la mayoría de los autores, incluyen el empleo de estos modificadores como un apartado dentro de la MECK.

De igual manera que en HPLC un disolvente orgánico miscible con el agua se puede emplear como aditivo de la disolución para manipular los factores de capacidad o de selectividad, en MECK se pueden emplear disolventes orgánicos para modificar la selectividad de la separación, pero a concentraciones no excesivamente elevadas, ya que si no se rompería la estructura micelar.

**Separaciones quirales**

La CE puede emplearse para llevar a cabo separaciones quirales. Realmente, este tipo se separaciones no conforma un modo distinto de electroforesis capilar, sino, más exactamente, el uso de aditivos específicos a los tampones utilizados en CZE o MECK para conseguir una selectividad quiral adicional [214,215]. Como es bastante similar a MECK, algunos autores consideran este modo como un subapartado dentro de la electroforesis capilar en medios micelares.

La aplicaciones más comunes de electroforesis capilar quiral emplean ciclodextrinas ("CDs") como aditivos, bien en CZE [216,217], o especialmente en MECK [218,219]. Aunque existen otros receptores estereoselectivos que también pueden ser usados, como son los éteres corona o las sales biliares.

En MECK, las CDs se emplean con frecuencia con micelas de SDS. Al ser la superficie de la CD hidrofílica, se puede asumir que ésta no se incorpora en la micela. Sin embargo, una molécula de surfactante se podría incluir en la cavidad de la ciclodextrina.

[213] A. Carrasco Pancorbo, A. Segura Carretero, A. Fernández Gutiérrez. "Co-electroosmotic capillary electrophoresis determination of phenolic acids in commercial olive oil" *J. Sep. Sci.* 28 (2005) 925-934.

[214] M. M. Rogan, K. D. Altria. "Introduction to the Theory and Applications of Chiral Capillary Electrophoresis". Part Number 726388. Beckman Instruments. Fullerton, CA, (1995).

[215] G. Gubitz, M. G. Schmid. "Recent advances in chiral separation principles in capillary electrophoresis and capillary electrochromatography". *Electrophoresis.* 25 (2004) 3981-3996.

[216] Y. J. Shi, C. H. Huo, H. J. Yao, R. F. Gao, Y. Y. Zhao, B. J. Xu. "Enantioseparation of 2-O-beta-D-glucopyranosyl-2H-1,4-benzoxazin-3(4H)-one and its 7-chloro-derivative by capillary zone electrophoresis using native and substituted beta-cyclodextrins as chiral additives". *J. Chromatogr. A.* 1072 (2005) 279-282.

[217] M. Salami, T. Jira, H. H. Otto. "Capillary electrophoretic separation of enantiomers of amino acids and amino acid derivatives using crown ether and cyclodextrin". *Pharmazie*, 60 (2005) 181-185.

[218] S. Terabe, Y. Miyashita, Y. Ishihama, O. Shibata. "Ciclodextrin-modified micellar electrokinetic chromatography: separation of hydrophobic and enantiomeric compounds". *J. Chromatogr.* 636 (1993) 47-55.

[219] S. A. C. Wren. "Chiral separation in capillary electrophoresis". *Electrophoresis.* 16 (1995) 2127-2131.

La separación se logra por las diferencias en la distribución de los solutos entre las CDs y las micelas. La cantidad de soluto dentro de una micela depende de su hidrofobicidad; mientras que la cantidad de soluto dentro de la CD depende de la capacidad de encajar dentro de la cavidad de la misma, así como de su hidrofobicidad.

**Cromatografía capilar electrocinética micelar con microemulsiones**

Las microemulsiones son líquidos totalmente transparentes formados por gotas de un disolvente orgánico lipofílico inmiscible (con un tamaño de nanómetros) en un sistema acuoso. En las ciencias separativas, las microemulsiones han sido empleadas como fase móvil en HPLC, y como disoluciones reguladoras en CE; este último uso da como resultado una modalidad electroforética muy parecida a MECK, la cromatografía capilar electrocinética micelar con microemulsiones (MEEKC). Las microemulsiones de aceite-en-agua se emplean normalmente en las separaciones en MEEKC, y contienen gotitas de aceite (por ejemplo, heptano u octano) suspendidas en una disolución reguladora acuosa. Las moléculas de surfactante, habitualmente SDS, son añadidas en una concentración mayor que su concentración micelar crítica, para facilitar la formación de las gotas al disminuir la tensión superficial. Un co-surfactante (alcohol de tamaño relativamente pequeño) se suele también añadir para que haga de puente en la interfase "aceite-agua", la cual disminuye aún más la tensión superficial y estabiliza el sistema de la microemulsión [220-222].

En MEEKC los solutos son separados por una combinación de mecanismos electroforéticos y reparto cromatográfico. El reparto se da entre las gotas de aceite y la continua fase acuosa. Los solutos insolubles en agua experimentan una tendencia hacia su inclusión en la gota de aceite en vez de en la fase acuosa; mientras que los analitos solubles en agua residen, principalmente, en la fase acuosa.

Su mayor diferencia con respecto a MECK es la adición del disolvente orgánico lipofílico inmiscible. Este disolvente se incluye en el núcleo de la micela formada por el surfactante para formar la microemulsión. Las micelas, en muchos casos, parecen convertirse en estructuras "más abiertas" que facilitarán una transferencia de masa más

[220] A. Marsh, B. Clark, M. Broderick, J. Power, S. Donegan, K. Altria. "Recent advances in microemulsion electrokinetic chromatography". *Electrophoresis*, 25 (2004) 3970-3980.

[221] S. H. Hansen. "Recent applications of microemulsion electrokinetic chromatography". *Electrophoresis* 24 (2003) 3900-3907.

[222] K. D. Altria, M. F. Broderick, S. Donegan, J. Power. "The use of novel water-in-oil microemulsion in microemulsion electrokinetic chromatography". *Electrophoresis*. 25 (2004) 645-652.

rápida. Los solutos, por tanto, pueden atravesar la superficie de las gotitas en MEEKC más fácilmente que en la micela más rígida en MEKC, cosa que permite que la modalidad que nos ocupa en este apartado pueda ser aplicada a un mayor rango de analitos.

**Electroforesis capilar por afinidad**

La electroforesis capilar por afinidad es un término amplio que se refiere a la separación por electroforesis capilar de sustancias que participan en interacciones selectivas y no-selectivas que tienen lugar en el interior de un capilar. Las interacciones moleculares pueden ser libres en disolución o inmovilizadas en un soporte sólido. Las dos sustancias que despiertan interés en la electroforesis capilar por afinidad son el ligando y el analito.

Las interacciones moleculares llevadas a cabo en un soporte sólido, sin embargo, se emplean para que el ligando "capture" al analito de interés, que puede estar presente en un amplio rango de concentraciones y en matrices tanto simples, como complejas. Cuando esto se lleve a cabo, se podrá conseguir que el analito aislado esté más concentrado y bastante más puro. Esta modalidad electroforética que emplea el ligando inmovilizado se puede utilizar, además, para realizar micro-reacciones en el medio electroforético.

Las ventajas de la electroforesis capilar por afinidad hicieron que muchos investigadores utilizaran los dispositivos "concentrator-microreactor" conectados a un equipo de CE, habiendo hoy en día un gran número de artículos referentes a este tema [223- 227]. En los últimos años ha aumentado notablemente el número de artículos que

[223] N. A. Guzmán, M. A. Trebilcock, J. P. Advis. "The use of a concentration step to collect urinary components separated by capillary electrophoresis and further characterization of collected analytes by mass-spectrometry". *J. Liq. Chromatogr.* 14 (1991) 997-1015.
[224] W. Nashabeh, Z. El Rassi. "Enzymophoresis of nucleic-acids by tandem enzyme reactor-capillary zone electrophoresis". *J. Chromatogr.* 596 (1992) 251-264.
[225] T. M. Philips, J. J. Chmielinska. "Immunoaffinity capillary electrophoretic analysis of cyclosporine in tears". *J. Biomed. Chromatogr.* 8 (1994) 242-246.
[226] C. B. Kundsen, J. H. Beattie. "On-line solid-phase extraction capillary electrophoresis for enhanced detection sensitivity and selectivity: application to the analysis of metallothionein isoforms in sheep fetal liver". *J. Chromatogr. A.* 792 (1997) 463-473.
[227] K. P. Bateman, R. L. White, P. Thibault. "Evaluation of adsorption preconcentration capillary zone electrophoresis nanoelectrospray mass spectrometry for peptide and glycoprotein analyses". *J. Mass Spectrom.* 33 (1998) 1109-1123.

emplean esta modalidad electroforética, la electroforesis capilar por afinidad, tanto con interacciones libres en disolución, como en soporte sólido [228- 234].

**Electroforesis capilar en gel (CGE)**

La electroforesis capilar en gel opera usando capilares rellenos de gel. El mecanismo de separación en CGE se basa en las diferencias de movilidad de los componentes de la muestra debido a su tamaño molecular para migrar a través de los poros del gel que rellena el capilar, es decir, esta separación está basada en un tamizado o criba molecular. Como el principio de separación en este caso es el tamaño, este modo será particularmente apropiado para separar moléculas cargadas que tengan diferentes tamaños.

Emplear este modo de separación conlleva ciertas ventajas:

- Se previene la adsorción del soluto a las paredes del capilar
- Son medios anticonvectivos
- El uso de los geles ayuda a eliminar el FEO
- Se minimiza la difusión del soluto

Según las características y tamaño de los componentes de una mezcla, se han de emplear distintos geles. Los dos tipos fundamentales de geles que se emplean en CGE

[228] M. Kinoshita, K. Kakehi. "Analysis of the interaction between hyaluronan and hyaluronan-binding proteins by capillary affinity electrophoresis: significance of hyaluronan molecular size on binding reaction". *J. Chromatogr. B*. 816 (2005) 289-295.

[229] K. Nakajima, T. Urashima, M. Suzuki, A. Suzuki, K. Kakehi. "Capillary affinity electrophoresis for the analysis of carbohydrates derived from milk and glycosphingolipids". *Glycobiology*. 14 (2004) 1190-1191.

[230] K. Nakajima, M. Kinoshita, Y. Oda, T. Masuko, H. Kaku, N. Shibuya, K. Kakehi. "Screening method of carbohydrate-binding proteins in biological sources by capillary affinity electrophoresis and its application to determination of Tulipa gesneriana agglutinin in tulip bulbs". *Glycobiology*. 14 (2004) 793-804.

[231] Y. Oda, T. Senaha, Y. Matsuno, K. Nakajima, R. Naka, M. Kinoshita, E. Honda, I. Furuta, K. Kakehi. "A new fungal lectin recognizing alpha(1-6)-linked fucose in the N-glycan". *J. Biolog. Chem*. 278 (2003) 32439-32447.

[232] R. Huhn, R. Frei, M. Christen. "Use of capillary affinity electrophoresis for the determination of lectin sugar interactions". *Anal. Biochem*. 218 (1994) 131-135.

[233] K. Uegaki, A. Taga, Y. Akada, S. Suzuki, S. Honda. "Simultaneous estimation of the association constants of glycoprotein glycofroms to a common protein by capillary electrophoresis". *Anal. Biochem*. 309 (2002) 269-278.

[234] N. A. Guzmán, R. J. Stubbs. "The use of selective adsorbents in capillary electrophoresis-mass spectrometry for analyte preconcentration and microreactions: A powerful three-dimensional tool for multiple chemical and biological applications". *Electrophoresis*. 22 (2001) 3602-3628.

son los geles físicos, dentro de los cuales destaca la agarosa; y los geles químicos, que se preparan por polimerización de la acrilamida en presencia de agentes que produzcan enlaces cruzados para dar una matriz de poliacrilamida. Estos geles de poliacrilamida pueden ser entrecruzados y lineales.

Esta modalidad electroforética está siendo ampliamente utilizada para la secuenciación de ADN [235-237].

**Isoelectroenfoque capilar**

Como su propio nombre sugiere, el modo de separación llamado isoelectroenfoque capilar (CIEF) separa los analitos atendiendo a las diferencias entre sus puntos isoeléctricos.

Generalmente, este modo electroforético se emplea para separar analitos de carácter anfótero, como las proteínas o los polipéptidos [238-240]. Los anfolitos son compuestos anfóteros que pueden existir como aniones o cationes dependiendo del pH de la disolución en la que se encuentren. El pH al cual el anfolito es neutro se denomina punto isoeléctrico y se representa por pI. A un valor de pH menor del pI el anfolito se encuentra como catión, y por encima del pI, como anión.

Es decir, a modo de resumen, podemos decir que la separación por CIEF se basa en la migración electroforética de sustancias anfóteras en un gradiente de pH. Para realizar una separación empleando este principio, el extremo anódico del capilar tendrá que estar sumergido en una disolución ácida, mientras que el catódico, en una solución básica. Cuando se aplica un campo eléctrico, la presencia de los anfolitos en el interior del capilar, provoca un gradiente de pH. Los componentes cargados de la muestra,

[235] D. Y. Chen, H. R. Harke, N. J. Dovichi. "Two-Label Peak-Height Encoded DNA sequencing bu capillary gel electrophoresis: Three examples". *Nucleic Acids. Res*. 20 (1992) 4873-4880.

[236] S. Bay, H. Starke, J. Z. Zhang, J. F. Elliot, L. D. Coulson, N. J. Dovichi. "Capillary gel electrophoresis for DNA sequencing of a template from the malaria genome by use of 4%T, 5%C polyacylamide and two-color peak-height encoded fluorescence detection". *J. Capillary Electrophoresis*. 1 (1994) 121-126.

[237] T. Manabe, N. Chen, S. Terabe, M. Yohda, L. Endo. "Effects of linear polyacrylamide concentrations and applied voltages on the separation of oligonucleotides and DNA sequencing fragments by capillary electrophoresis". *Anal. Chem.* 66 (1994) 4243-4252.

[238] P. G. Righetti, C. Gelfi. "Isoelectric focusing in capillaries and slab gels: A comparison". *J. Cap. Elec*. 1 (1994) 27-35.

[239] C. Schwer. "Capillary isoelectric focusing: a routine method for protein analysis?". *Electrophoresis*. 16 (1995) 2121-2126.

[240] A. B. Chen, C. A. Rickel, A. Flanigan, G. Hunt, K. G. Moorhouse. "Comparison of ampholites used for slab gel and capillary isoelectric focusing of recombinant tisuue-ttype plasminogen activator glycoforms". *J. Chromatogr. A*. 744 (1996) 279-284.

migran entonces a través del capilar hasta que lleguen a una región donde el pH sea igual que su pI, momento en el que se convierten en especies neutras y, por lo tanto, cesan de migrar. El resultado final serán una serie de zonas estrechas donde se agrupan ("enfocan") los solutos hasta conseguir una condición de estado de equilibrio.

Una vez que los solutos están enfocados en dichas zonas, hay que movilizarlos para que pasen a través del detector y obtener así el correspondiente electroferograma.

**Isotacoforesis capilar**

La isotacoforesis capilar (CITP) fue la primera variante de la electroforesis capilar que estuvo disponible comercialmente, aunque posteriormente su uso quedó algo estancado. Este modo puede ser empleado para separar especies iónicas, pero no es posible realizar la separación de aniones y cationes durante un mismo análisis. Se fundamenta en que las bandas de la muestra se desplazan en el interior del capilar de separación entre dos disoluciones tampón de diferente conductividad eléctrica, el llamado electrolito frontal y electrolito terminal. Es decir, la principal característica de este modo de electroforesis capilar es que tiene lugar en un sistema de tampón discontinuo. Se consigue que los distintos iones que componen la muestra sean acelerados o frenados hasta conseguir bandas estrechas donde se concentran cada uno de ellos, que se desplazan a la misma velocidad a través del capilar.

CITP opera normalmente con supresión del FEO, cosa que se consigue aumentando la viscosidad del medio, separando el capilar de los recipientes de la disolución tampón usando membranas semipermeables, o bien, revistiendo los capilares de sílice fundida.

La principal aplicación de este modo electroforético es como sistema de preconcentración de muestra previo a la separación por otros modos de CE [241-245],

[241] H. Okamoto, A. R. Timerbaev, T. Hirokawa. "Simultaneous determination of metal ions, amino acids, and other small biogenic molecules in human serum by capillary zone electrophoresis with transient isotacophoresis preconcentration". *J. Sep. Science*. 28 (2005) 522-528.

[242] M. Mazereeu, U. R. Tjaden, N. J. Reinhoud. "Single capillary isotacophoresis-zone electrophoresis: current practice and prospects, a review" *J. Chromatogr. Sci*. 33 (1995) 686-697.

[243] L. Krivankova, P. Gebauer, P. Bocek. "Some practical aspectsof utilizing the on-line combination of isotacophoresis and capillary zone electrophoresis" *J. Chromatogr. A*. 716 (1995) 35-48.

[244] D. T. Witte, S. Nagard, M. Larsson. "Improved sensitivity by on-line isotacophoretic preconcentration in the capillary zone electrophoretic determination of peptide-like solutes". *J. Chromatogr. A*. 687 (1994) 155-166.

aunque también existen numerosas aplicaciones que llevan a cabo separaciones electroféricas empleando este modo [246-247].

**Electrocromatografía capilar (CEC)**

La electrocromatografía capilar (CEC) es una técnica de separación híbrida que combina características tanto de CE, como de HPLC. Como consecuencia de esto, no puede ser estrictamente considerada como un modo de electroforesis capilar, aunque normalmente puede operar usando una instrumentación similar a la de CE.

En CEC el capilar es empaquetado con una fase estacionaria cromatográfica, con "fritas" en ambos extremos, que puede retener los solutos por medio de equilibrios de distribución normales asociados a cromatografía.

Como el líquido está en contacto tanto con la paredes de sílica, como con las partículas de sílica de la fase estacionaria, se genera flujo electroosmótico. Así, la CEC se ve favorecida por el perfil de flujo plano del FEO, el cual proporciona alta eficacia de separación. El mecanismo de separación en CEC depende en gran medida de la naturaleza de la muestra. Las moléculas neutras se separan en base a mecanismos puramente cromatográficos, mientras que los ácidos y las bases se separan por una combinación de mecanismos cromatográficos y electroféricos.

Se puede decir que este modo de CE es una cromatografía líquida en la cual se sustituye la bomba de alta presión por un generador de alto voltaje, que se basa en procesos electroféricos e interacciones con la fase estacionaria que pueden ser por adsorción, partición y gel permeable en un capilar estrecho y relleno.

Existe ya un gran número de interesantes aplicaciones desarrolladas en los laboratorios de investigación analítica [248- 253].

[245] N. A. Guzmán, M. A. Trebilcock, J. P. Advis. "The use of a concentration step to collect urinary components separated by capillary electrophoresis and further characterization of collected analytes by mass spectrometry". *J. Liq. Chromatogr*. 14 (1991) 997-1015.

[246] A. Zgola-Grzeskowiak, T. Grzeskowiak, J. Zembrzuska, M. Franska, R. Franski, Z. Lukaszewski. "Isotacophoretic determination of carboxilic acids in biodegradation samples". *J. Chromatogr. A*. 1068 (2005) 327-333.

[247] T. K. Natishan. "Recent progress in the anlysis of pharmaceuticals by capillary electrophoresis". *J. Liquid Chromatogr. Rel. Technolog*. 28 (2005) 1115-1160.

[248] R. Sakaguchi, Y. Kato, A. Ito, T. Tsuda, T. Yoshida. "Preparation of red blood cell column for capillary electrochromatography". *Analytical Sciences*. 21 (2005) 453-456.

[ 249 ] A. De Rossi, C. Desiderio. "Application of reversed phase short end-capillary electrochromatography to herbicides residues analysis". *Chromatographia*. 61 (2005) 271-275.

## Detectores empleados en CE

De forma general, cuando se acopla un detector a un sistema de separación, éste debe cumplir una serie de condiciones para el buen funcionamiento como son:

- Presentar una buena sensibilidad.

- Proporcionar límites de detección bajos. Para ello es necesaria la combinación de una alta sensibilidad con una baja fluctuación en la señal de fondo.

- A veces es necesario que el detector presente 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 del detector 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. Esto implica principalmente que las señales deben ser reproducibles y estables en el tiempo.

- Idealmente, la señal debe ser nula en ausencia de analito. La señal de fondo no debe perturbar la señal correspondiente a los analitos y conviene que sea lo más pequeña posible y/o constante posible.

- 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. Además, sería deseable que la relación entre la señal y la concentración fuera lineal.

[250] L. Yang, E. Guihen, J. D. Holmes, M. Loughran, G. P. O´Sullivan, J. D. Glennon. "Glod nanoparticle-modified etched capillaries for open-tubular capillary electrochromatography". *Anal. Chem.* 77 (2005) 1840-1846.

[251] Z. Aturki, G. D´Orazio, S. Fanali. "Rapid assay of vitamin E in vegetable oils by reversed-phase capillary electrochromatography". *Electrophoresis.* 26 (2005) 798-803.

[252] K. Ohyama, M. Wada, G. A. Lord, Y. Ohba, M. N. Nakashima, K. Nakashima, S. Akiyama, C. K. Lim, N. Kuroda. "Capillary electrochromatography of caffeine and its metabolites in rat brain microdialysate". *Electrophoresis.* 26 (2005) 812-817.

[253] T. Yokoyama, M. Zenki, M. Macka, P. R. Haddad. "Enhancement of separation capability of inorganic ions by capillary electrochromatography". *Bunseki Kagaku.* 52 (2005) 107-120.

Todas estas condiciones influyen en gran medida a la hora de decidir los sistemas de detección que se acoplan y además, los detectores que se pueden acoplar a CE deben ser sensibles a pequeñas cantidades de muestra y compatibles con las dimensiones físicas del capilar, aún en detrimento de otras cualidades.

Hasta ahora, se han adaptado una gran variedad de técnicas de detección a la electroforesis capilar con diferente éxito. En la tabla se muestran los distintos sistemas de detección y el rango de los límites de detección alcanzados en dicho acoplamiento.

*Tabla*. Sistemas de detección en CE y rangos de límites de detección alcanzados.

| Sistema de detección | Límite de detección (M) |
|---|---|
| **Técnicas ópticas** | |
| Absorción UV-Visible | |
| Directa | $10^{-5} - 10^{-7}$ |
| Indirecta | $10^{-4} - 10^{-6}$ |
| Con paso óptico aumentado | $10^{-7}$ |
| Fluorescencia | |
| Directa | $10^{-7} - 10^{-9}$ |
| Indirecta | $10^{-6} - 10^{-8}$ |
| Inducida por láser directa | $10^{-10} - 10^{-13}$ |
| Inducida por láser indirecta | $10^{-5} - 10^{-7}$ |
| Fosforescencia (RTPL) | $10^{-6} - 10^{-8}$ |
| Quimioluminiscencia | $10^{-5} - 10^{-11}$ |
| Infrarrojo | $10^{-7} - 10^{-9}$ |
| Resonancia Magnética Nuclear | $10^{-9} - 10^{-11}$ * |
| Dispersión Raman | $10^{-3} - 10^{-7}$ |
| Termoóptico | $10^{-7} - 10^{-8}$ |
| Índice de refracción | $10^{-5} - 10^{-7}$ |
| **Técnicas electroquímicas** | |
| Conductimetría | $10^{-7} - 10^{-8}$ |
| Potenciometría | $10^{-7} - 10^{-8}$ |
| Amperometría | $10^{-7} - 10^{-9}$ |
| **Otras técnicas** | |
| Espetrometría de masas | $10^{-7} - 10^{-9}$ |
| Radiométricos | $10^{-10} - 10^{-11}$ |

*Para RMN-[1]H (el núcleo más sensible de la tabla periódica desde el punto de vista de la RMN)

Como comentábamos anteriormente y se recogía en la última tabla mostrada, son varios los detectores que se pueden acoplar a la técnica de CE y, se podrían clasificar en tres categorías: técnicas ópticas, electroquímicas y una sección de "otras", que incluiría las técnicas radioquímicas y la espectrometría de masas. Los siguientes esquemas resumen esta idea de modo visual.
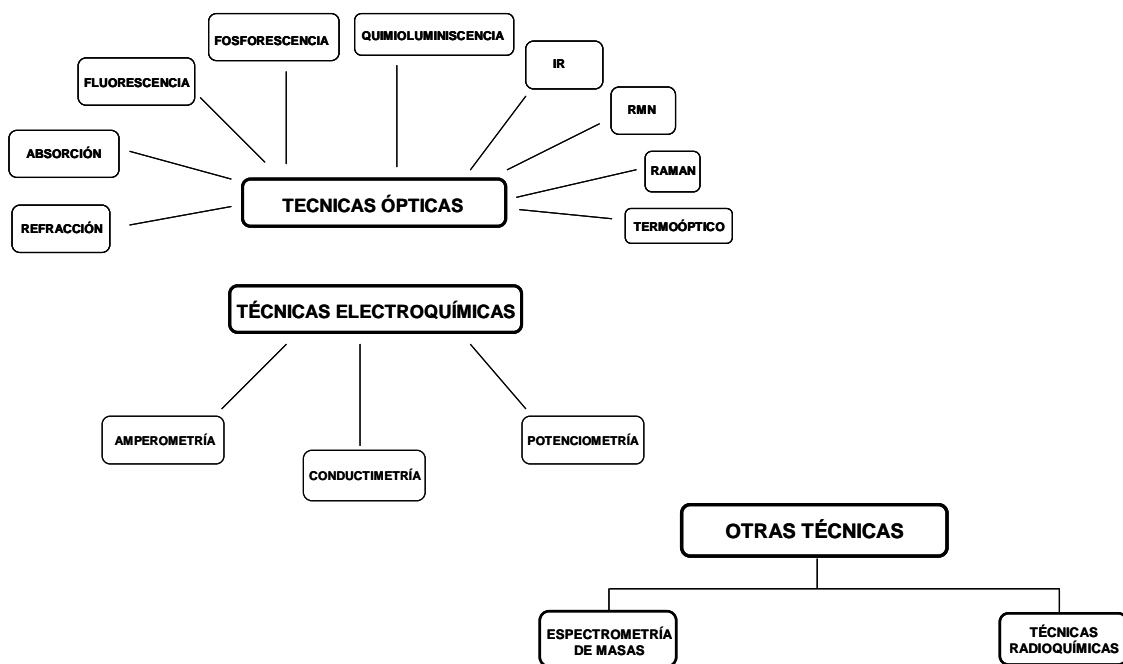
En el trabajo experimental que se recoge en la presente memoria, se han empleado la detección espectrofotométrica UV-Vis y la espectrometría de masas (MS). Se procederá a la descripción más detallada de estos sistemas de detección en próximos apartados.

**Absorción UV-Visible**

Los métodos basados en la absorbancia de la luz UV-Visible son los más utilizados en equipos comerciales de CE, debido a su posibilidad para determinar un gran número de compuestos y grupos funcionales y su facilidad de manejo, a pesar de que su sensibilidad es mucho menor que la de otros sistemas de detección, ya que depende del diámetro interno de la columna capilar y de las absortividades molares de los analitos. Su sencillez y relativo bajo coste son sus principales ventajas.

Este detector tiene su fundamento en la interacción entre la radiación UV-Visible (zona del espectro electromagnético comprendida entre 200 y 800 nm, aprox) y la materia, dando origen al fenómeno conocido como absorción de la radiación [254].

Los capilares de sílice suelen estar recubiertos en su parte exterior de una capa de protección de poliimida que previene la transmisión UV a través de los mismos y que le atribuye una cierta protección mecánica. Para usar los capilares en CE, la capa de imida debe ser eliminada en una pequeña sección del capilar cerca de su extremo y la detección se lleva a cabo a través de esta ventana. Esta capa de imida se elimina mediante calentamiento con una pequeña llama y/o un disolvente.

Son muchas las moléculas que pueden absorber radiación UV-Visible y ello hace que este modo de detección en CE pueda considerarse muy cercano al detector universal. Este comportamiento tiene ventajas pero también algunos inconvenientes. Ventajas porque al ser sensible a la gran mayoría de las especies químicas puede utilizarse para resolver, también, un número elevado de los problemas analíticos que puedan presentarse.

Como inconvenientes hay que señalar que, por una parte, la disolución portadora (electrolito de separación) tiene que ser cuidadosamente seleccionada de manera que todos sus componentes (disolventes y solutos) sean transparentes a la radiación UV-Visible empleada y, por otra parte, dado que los espectros moleculares de absorción son de bandas anchas, cualquier compuesto que coeluya con el analito interferirá en la cuantificación, muy probablemente, porque será muy difícil o imposible seleccionar una longitud de onda de medida de absorción del analito a la que el coeluyente sea totalmente transparente aunque tengan espectros bien diferenciados [255].

Consecuentemente, la utilización del detector UV-Visible en electroforesis capilar obliga a establecer métodos en los que el capilar tenga un adecuado poder de resolución del analito respecto de todos los compuestos absorbentes contenidos en la muestra problema.

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[254] J. J. Berzas Nevado, G. Castañeda Peñalvo. "La detección espectrofotométrica UV-Visible en Electroforesis Capilar" en "Electroforesis capilar: aproximación según la técnica de detección". Ed. Univ. Granada. (2005) Pp 157-187.
[255] A. L. Crego, M. L. Marina. "UV-Vis absorbance detection in capillary electrophoresis" en "Analysis and Detection by Capillary Electrophoresis". Ed. M. L. Marina, A. Ríos, M. Valcárcel. Elsevier. (2005) Pp 225-296.

Para llevar a cabo la identificación de los compuestos en un perfil electroforético utilizando la absorción UV-Vis como sistema de detección, habrá que disponer de patrones comerciales o patrones aislados mediante HPLC preparativa, y hacer un estudio riguroso de los espectros de absorbancia, tiempos de retención y llevar a cabo dopados en las muestras. Aun así, con este sistema, una identificación inequívoca es difícil de conseguir.

Estos detectores pueden ser de tres tipos:

-Longitud de onda fija: consta de una lámpara de mercurio a baja presión, que emite una radiación monocromática a 254 nm. A esta lámpara se le puede acoplar filtros para trabajar a otras longitudes de onda (280, 360, etc…).

-Longitud de onda variable: este detector trabaja en el rango del UV-Vis (190-650 nm). No hace muchos años, estos detectores estaban equipados con dos lámparas, una de deuterio para trabajar de 190-360 nm, y otra de tugsteno para hacerlo en el visible de 360-650, con un monocromador para seleccionar la longitud de onda deseada. Hoy en día, con una sola lámpara de deuterio se abarca todo el espectro de UV-Vis.

-Diodo array: este detector, que es el más moderno de los tres, conduce la luz mediante un sistema de diodos alineados y evita la dispersión. Un Fotodiodo array (PDA) es una sucesión lineal ordenada de fotodiodos discretos en un chip de circuitos integrados. Para espectroscopía se ponen en el plano de imagen de un espectrómetro para dejar que un rango de longitudes de onda se detecte simultáneamente.
Detectores de este tipo son especialmente útiles para recoger los espectros completos de absorción UV-Vis de muestras que pasan rápidamente por una celda. El PDA trabaja con el mismo principio que detectores fotovoltaicos simples.
Todos los detectores de este tipo van provistos de dos celdillas, una para la muestra y otra de referencia.

**Espectrometría de masas**

El acoplamiento entre la CE como técnica analítica de separación y la espectrometría de masas como sistema de detección (CE-MS) ha despertado un gran interés en los últimos años. El acoplamiento de un detector tan selectivo como MS a una técnica tan versátil y que proporciona eficacias tan elevadas como CE da como resultado una potentísima herramienta de análisis; se combinan así la rapidez del análisis, el alto poder de resolución, y un consumo muy pequeño de muestra, proporcionados por la CE, con la selectividad del análisis y la información estructural que proporciona la MS. Una de las ventajas del acoplamiento de la MS a cualquier técnica separativa es que proporciona una segunda dimensión de separación; en concreto, acoplada a la CE, además de la separación de los analitos según su relación carga/tamaño (en la modalidad CZE) se añade la separación en función de su relación masa/carga [256].

Su importancia queda probada cuando observamos el gran número de artículos, libros, "reviews" describiendo aplicaciones que se han publicado recientemente [257,258,259,260,261,262]. Es alentador ver que la naturaleza de los compuestos que se analizan mediante CE-MS se hace cada vez más extensa y variada y logra barrer un rango más amplio.

### Principios e instrumentación

Un esquema de lo que sería el acoplamiento CE-MS (o cualquier otra técnica separativa) se muestra en la siguiente figura:

[256] J. Hernández Borges, C. Simó, A. Cifuentes. "Principios de Electroforesis Capilar-Espectrometría de Masas: Aplicación al análisis de pesticidas". *Cromatografía y Técnicas afines* 24(2) (2003) 45-59.

[257] P. Schmitt-Kopplin, M. Frommberger, "Capillary electrophoresis-mass spectrometry: 15 years of developments and applications". *Electrophoresis* 24 (2003) 3837-3867.

[258] J.C. Severs, R.D. Smith. "Electrospray ionization mass spectrometry". R.B. Cole (Ed.). John Wiley & Sons, New York. (1997).

[259] J.F. Banks, "Recent advances in capillary electrophoresis/electrospray/mass spectrometry". *Electrophoresis* 18 (1997) 2255-2266.

[260] K.B. Tomer, L.J. Deterding, C.E. Parker. "High performance capillary electrophoresis. Theory, techniques and applications". M.G. Khaledi (Ed). John Wiley & Sons, New York. (1998).

[261] C. W. Huck, R. Bakry, G. K. Bonn. "Progress in capillary electrophoresis of biomarkers and metabolites between 2002 and 2005". *Electrophoresis* 27(1) (2006) 111-125

[262] W. F. Smyth. "Recent applications of capillary electrophoresis-electrospray ionisation-mass spectrometry in drug analysis". *Electrophoresis* 27(11) (2006) 2051-2062.
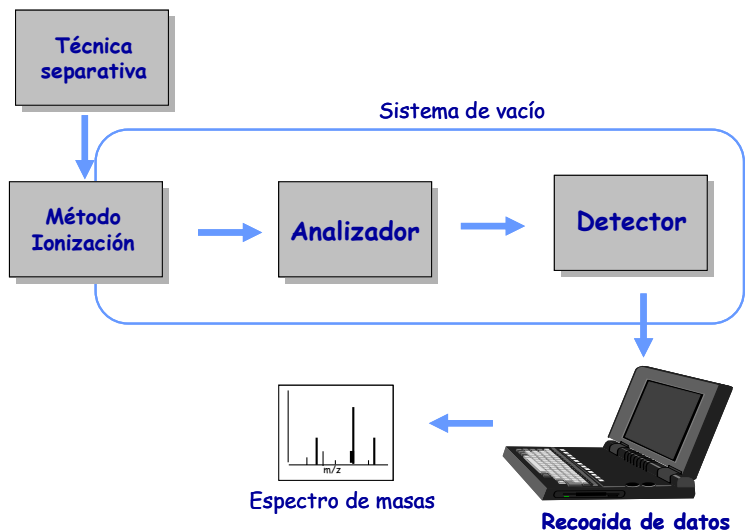
*Figura*. Esquema de un acoplamiento entre una técnica separativa y MS.

Lógicamente, la técnica separativa se coloca al inicio del esquema, seguida de la interfase, en la cual se produce la ionización de los analitos. Después los iones en fase gaseosa son transportados al analizador de masas, y una vez analizados, se detectan y se registran y procesan los datos a través de un sistema informático.

El siguiente esquema nos muestra que, tanto el método de ionización, como el analizador, pueden ser de varios tipos.



CF-FAB: FLUJO CONTINUO Y BOMBARDEO POR ÁTOMOS RÁPIDOS
ICP: PLASMA ACOPLADO INDUCTIVAMENTE
MALDI: IONIZACIÓN Y DESORCIÓN POR LÁSER ASISTIDA POR MATRIZ
APCI: IONIZACIÓN QUÍMICA A PRESIÓN ATMOSFÉRICA
ESI: IONIZACIÓN POR ELECTROSPRAY
ISP: ELECTROSPRAY ASISTIDO NEUMÁTICAMENTE

Q: CUADRUPOLO
IT: TRAMPA DE IONES
EBE: SECTOR MAGNÉTICO
TOF: TIEMPO DE VUELO
FT-ICR: TRANSFORMADA DE FOURIER-RESONANCIA CICLOTRÓNICA
HT-TOF: TRANSFORMADA DE HADAMARD-TIEMPO DE VUELO
Q-TOF: CUADRUPOLO-TIEMPO DE VUELO
IT-TOF: TRAMPA DE IONES-TIEMPO DE VUELO
Q-Q: DOBLE CUADRUPOLO
Q-Q-Q: TRIPLE CUADRUPOLO

Para poder acoplar CE con MS se han de solventar varias dificultades; la primera viene derivada de que la CE es una técnica que trabaja en fase líquida, y de que en un espectrómetro de masas las sustancias para ser analizadas deben entrar en fase gas. Esto obliga a la utilización de una adecuada interfase. Además, el acoplamiento debe hacer frente a otra segunda adversidad como es el cierre del circuito eléctrico en el que trabaja el instrumento de CE.

Se han descrito diversas interfases [263], entre ellas, la de flujo continuo y bombardeo con átomos rápidos (CF-FAB) [264]; la de ionización y desorción por láser asisitida por una matriz (MALDI) [265], que se utiliza principalmente en acoplamientos CE-MS *off-line*; la de ionización química a presión atmosférica (APCI) [266]; el plasma de acoplamiento inductivo (ICP, fundamentalmente utilizado para átomos metálicos y algunos no metálicos) [267]; la ionización por electrospray o electronebulización (ESI) [262,268]; el electrospray asistido reumáticamente o ISP…

En la figura, se muestra una comparativa entre los sistemas de ionización que han sido más utilizados en el acoplamiento CE-MS, teniendo en cuenta la polaridad y el peso molecular de los analitos en estudio.

[263] E. Gelpi, "Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments". *J. Mass Spectrom*. 37 (2002) 241-253.

[264] M.A. Moseley, L.J. Deterding, K.B. Tomer, J.B. Jorgenson. "Capillary-zone electrophoresis/fast-atom bombardment mass spectrometry: design of an online coaxial continuous-flow interface". *Rapid. Commun. Mass Spectrom*. 3 (1989) 87-93.

[265] J. Preisler, P. Hu, T. Rejtar, B.L. Karger. "Capillary electrophoresis-matrixassisted laser desorption/ionization time-of-flight mass spectrometry using a vacuum deposition interface". *Anal. Chem*. 72 (2000) 4785-4795.

[266] Y. Tanaka, K. Otsuka, S. Terabe. "Evaluation of an atmospheric pressure chemical ionization interface for capillary electrophoresis-mass spectrometry". *J. Pharm. Biomed. Anal*. 30 (2003) 1889-1895.

[267] M. Montes Bayón, D. Profrock, A. Sanz Medel, A. Prange. "Direct comparison of capillary electrophoresis and capillary liquid chromatography hyphenated to collision-cell inductively coupled plasma mass spectrometry for the investigation of Cd-, Cu- and Zn-containing metalloproteins". *J. Chromatogr. A* 1114 (2006) 138-144.

[268] J. A. Loo, R. R. O. Loo, K. J. Light, C. G. Edmonds, R. D. Smith. "Multiply charged negative-ions by electrospray ionization of polypeptides and proteins". *Anal. Chem*. 64 (1992) 81-88.
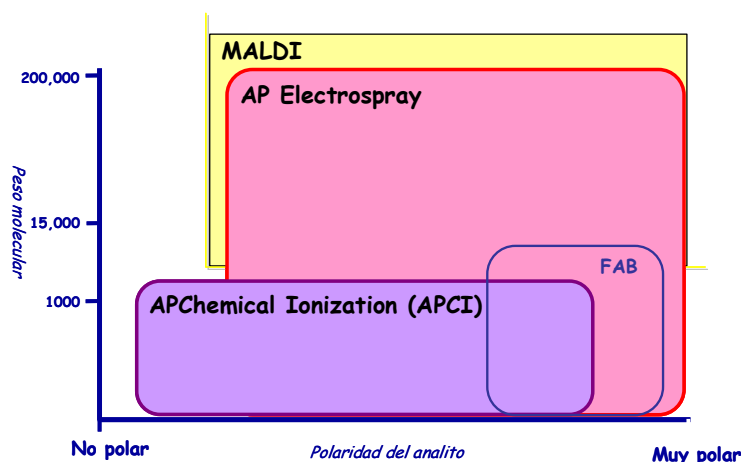
*Figura*. Rango al que trabajan los sistemas de ionización más comunes.
AP: Presión atmosférica

A pesar de la variedad de interfases desarrolladas para el acoplamiento CE-MS, la más utilizada actualmente 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.

**Ionización por electrospray (ESI)**

Corría el año 1912 cuando el científico J. J. Thomson (Premio Nobel en 1906) se las ingenió para crear el primer espectrómetro de masas. Pero el mérito no fue solo de él, ya que la espectrometría de masas comenzó a ver la luz en el año 1886 cuando Goldstein descubrió los iones positivos, siguió cogiendo forma con W. Wien que consiguió analizarlos por deflección magnética en 1898 y dio un paso definitivo cuando W. Kaufmam consiguió analizar los rayos catódicos usando campos eléctricos y magnéticos paralelos en 1901. Todos estos avances permitieron a la privilegiada mente de J. J. Thomson idear el primer espectrómetro de masas [269].

A partir de ese día se comenzó a usar en los laboratorios de química para separar iones atómicos y moleculares en función del cociente masa/carga con la unidad Thomson (Th) como unidad fundamental. Y aunque su avance era firme, podemos decir que su espectacular desarrollo no se produjo hasta el final de la decada de 1960, cuando Dole y

[269] D. A. Skoog, F. J. Hiller, T. A. Nieman. "Principios de Analisis Instrumental". Mc Graw Hill, 5ª Edición. Madrid. (2001) Pp 490.

col [270] describieron el primer uso analítico del electrospray, y alrededor de 1980, cuando Fenn y col [271] llevaron a cabo el principal desarrollo de la técnica ESI para su uso con espectrometría de masas, lo que le valió a este último la obtención del Premio Nobel.

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, procedente del capilar de separación, y 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 pronostican 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 [272,273].

[270] M. Dole, L.L. Mack, R.L. Hines, R.C. Mobley, L.D. Ferguson, M.B. Alice. "Molecular beams of macroions". *J. Chem. Phys*. 49 (1968) 2240-2249.
[271] M. Yamashita, J.B. Fenn. "Electrospray ion source. Another variation on the free-jet theme". *J. Phys. Chem*. 88 (1984) 4451-4459.
[272] C. Simó, A. Cifuentes. "Electroforesis Capilar: Detección mediante espectrometría de masas" en "Electroforesis capilar: aproximación según la técnica de detección". Ed. Univ. Granada. (2005) Pp 409-438.
[273] R. Martin Smith. "Instrumentation" en "Understanding Mass Spectra. A basic approach". Ed. K. L. Busch. John Wiley & Sons, Inc. (1999) 1-40.
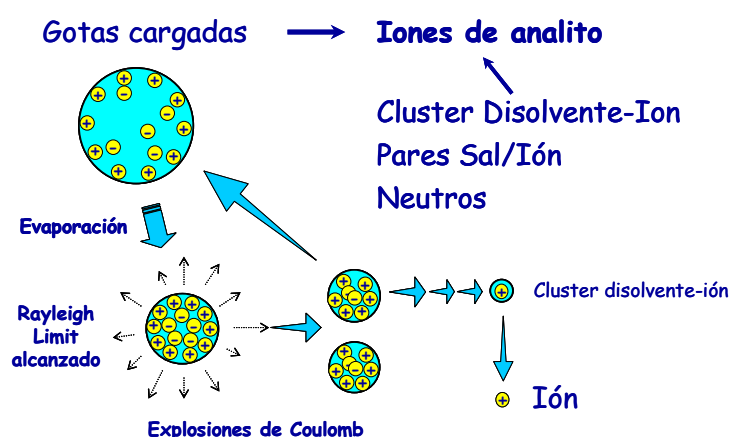
*Figura.* Proceso de formación del electrospray.

El hecho de que en el proceso de ionización se puedan formar iones mono- o multicargados, es una de las características que ha hecho que esta técnica haya revolucionado el campo de los acoplamientos entre técnicas analíticas de separación que trabajan en fase líquida y la espectrometría de masas. Un incremento en el número de cargas en una molécula disminuirá el valor de su relación masa/carga (m/z), que es el parámetro utilizado para el análisis por un espectrómetro de masas. De este modo, mediante la detección de iones multicargados, se va a poder detectar compuestos con pesos moleculares muy altos empleando analizadores de masas que trabajan con un intervalo limitado de valores m/z.

La ionización se puede llevar a cabo en 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 más utilizadas**

Como ya se adelantaba antes, 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). Para solucionar este problema se han propuesto dos estrategias, la primera de ellas es el uso de un flujo adicional, y la segunda, el uso de las llamadas interfases

micro- o nano-ESI, con las cuales se va a poder trabajar con flujos extremadamente pequeños.

El segundo 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. Otro problema que aparece en el acoplamiento CE-ESI-MS es la compatibilidad de los diferentes modos de electroforesis capilar con el espectrómetro de masas, ya que dependiendo del modo de CE, se va a requerir o no el empleo de sustancias poco compatibles con ESI (como iones borato, fosfato, detergentes para la formación de micelas, ciclodextrinas, etc.), las cuales por su escasa volatilidad contribuyen de forma significativa al incremento del ruido de fondo en la detección por MS, arruinando la sensibilidad, y llegando en algunos casos a obstruir y/o contaminar el sistema de detección.

Todas las dificultades indicadas del acoplamiento CE-MS han hecho que se hayan desarrollado diversos tipos de interfases para CE-ESI-MS [257,260,263,274,275,276,277,278,279] teniendo como objetivo la formación de un electrospray estable y el mantenimiento de la corriente eléctrica en el interior del capilar. Con esta idea, se han desarrollado fundamentalmente tres tipos de interfase ESI: sin flujo adicional, con flujo adicional y con unión líquida. En la tabla siguiente se muestran de forma resumida las principales características de todas ellas.

[274] 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(11) (2006) 2083-2090.

[275] C. Huhn, C. Neususs, M. Pelzing, U. Pyell, J. Mannhardt, M. Putz. "Capillary electrophoresis-laser induced fluorescence-electrospray ionization-mass spectrometry: A case study". *Electrophoresis* 26(7-8) (2005) 1389-1397.

[276] 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(3) (2006) 414-422.

[277] J.M. Ding, P. Vouros. "Advances in CE/MS. Recent developments in interfaces and applications". *Analytical Chemistry News & Features* 71 (1999) 378A- 385A.

[278] J. Cai, J. Henion. "Capillary electrophoresis-mass spectrometry". *J. Chromatogr. A* 703 (1995) 667-692.

[279] D. Figeys, R. Aebersold. "High sensitivity analysis of proteins and peptides by capillary electrophoresis-tandem mass spectrometry: recent developments in technology and applications". *Electrophoresis* 19 (1998) 885-892.

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

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

Explicaremos la interfase ESI con flujo adicional, que ha sido la empleada en el acoplamiento CE-MS en esta memoria.

**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.
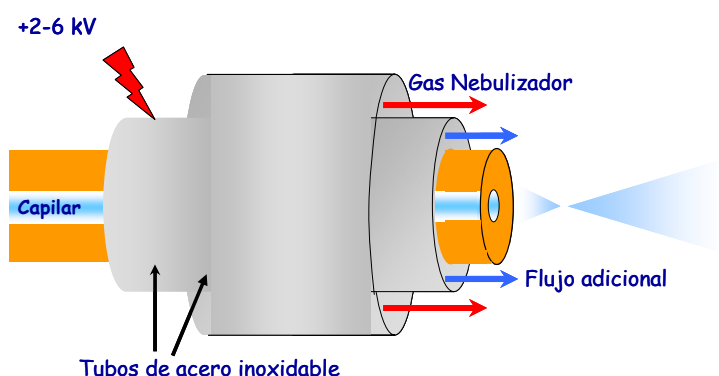


*Figura*. Esquema de una interfase ESI con flujo adicional.

Como se ha comentado anteriormente, para que una interfase ESI trabaje de modo estable requiere flujos del orden de ml/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 por ejemplo 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 [280], presión del gas nebulizador, situación del capilar con respecto al tubo concéntrico que lo rodea [281], 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 [282,283].

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:

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.

[280] J. Zheng, M. W. Jann, Y. Y. Hon, S. A. Shamsi. "Development of capillary zone electrophoresis-electrospray ionization-mass spectrometry for the determination of lamotrigine in human plasma". *Electrophoresis* 25(13) (2004) 2033-2043.
[281] L. Geiser, S. Rudaz, J. L. Veuthey. " Validation of capillary electrophoresis - mass spectrometry methods for the analysis of a pharmaceutical formulation". *Electrophoresis* 24(17) (2003) 3049-3056.
[282] 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 Spectrometry* 35(7) (2000) 919-924.
[283] 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.

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. Lógicamente, éstas son recomendaciones muy generales; determinadas aplicaciones pueden utilizar valores experimentales muy diferentes.

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.

**Analizadores de masas**

Una vez que en la interfase se ha llevado a cabo la transferencia de los iones procedentes del capilar desde la fase líquida a la fase gaseosa, los iones son dirigidos hacia el analizador de masas.

Los analizadores de masas permiten la separación, detección y cuantificación de los analitos en estudio con un grado de sensibilidad y selectividad muy elevado, proporcionando información sobre su masa molecular.

En los acoplamientos CE-MS se han utilizado todo tipo de analizadores como sencillos cuadrupolos (Q), trampas de iones (IT), sectores magnéticos (EBE), analizadores de tiempo de vuelo (TOF), e incluso los sistemas más complejos como los analizadores de Transformada de Fourier-Resonancia Ciclotrónica (FT-ICR), analizadores de Transformada de Hadamard-TOF (HT-TOF), y los formados por combinación de los anteriores, como por ejemplo analizadores Q-TOF, IT-TOF o triple cuadrupolo (QqQ) [284] (ver cuadro sinóptico de la página 118).

En los acoplamientos llevados a cabo entre CE y MS en el desarrollo experimental de esta memoria, han sido utilizados dos analizadores: Tiempo de vuelo y Trampa de iones; así que serán estos lo que se expliquen.

El analizador de <u>tiempo de vuelo (TOF)</u> separa masas sin un campo magnético. La manera en que TOF discrimina funciona obteniendo ventajas de las diferentes velocidades de los iones acelerados mediante un mismo potencial. Si los iones de masa diferente se aceleran a través del potencial y entonces se les permite pasar a través de un tubo de alto vacío con una determinada longitud, no todos llegarán al extremo contrario a la vez; es decir, se diferencian los iones basándose en la distinta velocidad que adquieren en el interior del analizador en función de su relación m/z. En primer lugar, los iones son extraídos de la cámara de ionización y acelerados hacia el tubo de vuelo mediante un campo electrostático que les aporta una elevada energía cinética. 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

[284] C. Simó, A. Cifuentes. "Mass spectrometry detection in capillary electrophoresis" en "Analysis and Detection by Capillary Electrophoresis". Ed. M. L. Marina, A. Ríos, M. Valcárcel. Elsevier. (2005) Pp 441-517.

mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energías de los iones formados en la fuente [273].

En la actualidad, se están reemplazando los espectrómetros TOF de trayectoria directa por aquellos que provocan la reflexión de los iones empleando un espejo de iones. Este instrumental recibe el nombre de TOF reflexivo [285].

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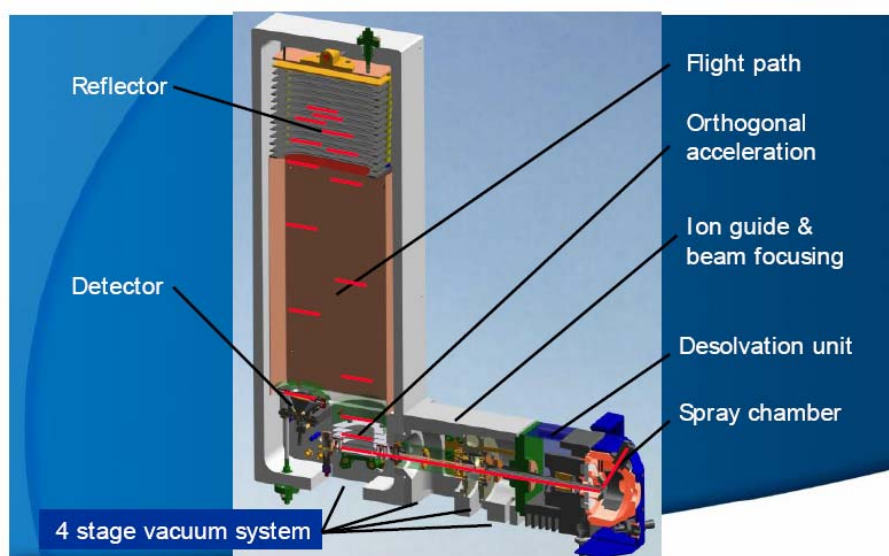


*Figura*. Esquema del interior de un MicroTOF de Bruker Daltonics.

Un esquema más detallado que el anteriormente presentado se muestra en la siguiente figura. Nos ayudará a ir entendiendo qué sucede en cada una de las partes del MicroTOF.

[285] K. A. Rubinson, J. F. Rubinson. "Espectrometría de masas" en "Análisis Instrumental". Ed. Pearson Education S. A. Madrid. (2000) Pp 522-577.
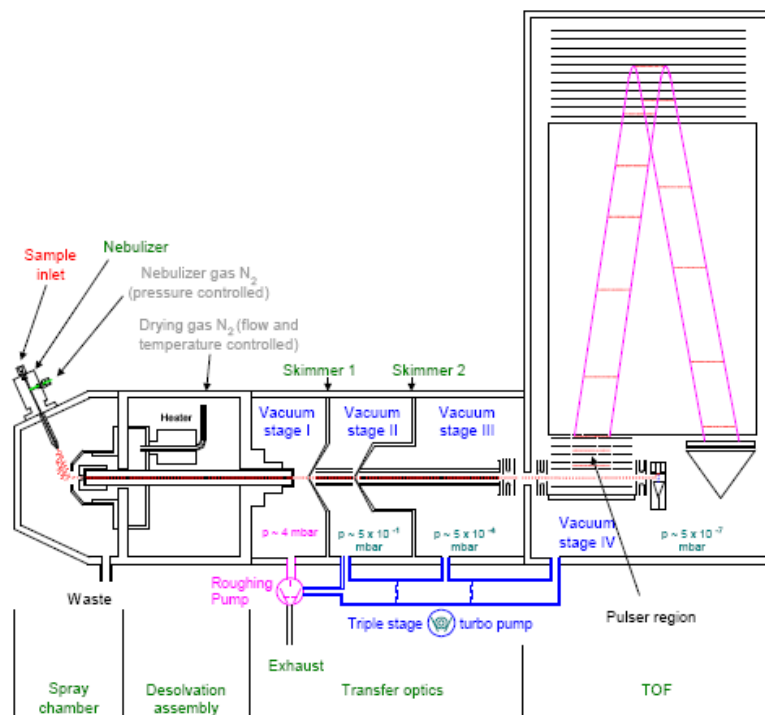
*Figura*. Esquema detallado de las partes de las que consta un ESI-MicroTOF.

En la primera de las partes del esquema, la cámara de formación del spray, ya sabemos lo que sucede (el inicio de la formación del electrospray) y cómo se produce (que dependerá de la interfase empleada). La unidad de desolvatación, separa las zonas que se encuentran a presión atmosférica de la primera zona a alto vacío, y consta de un calentador del gas de secado y un capilar de cristal. Se llega a través de ella al módulo de transmisión o transferencia óptica que consta de tres módulos que están a alto vacío.
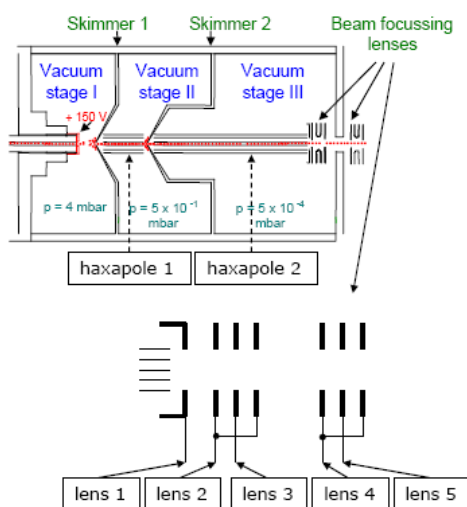


*Figura*. Esquema del módulo de transmisión con sus diferentes partes.

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 para medir el tiempo de vuelo.
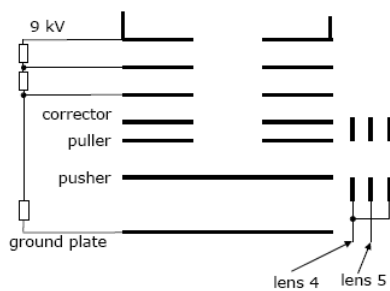


*Figura*. Imagen de la zona de aceleración ortogonal de un MicroTOF y esquema de sus componentes.

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, se repelen los iones pequeños con más facilidad. Asimismo, cada ión será repelido con distinto ángulo, aunque sea cual sea el ángulo, el ión incidirá en el detector.
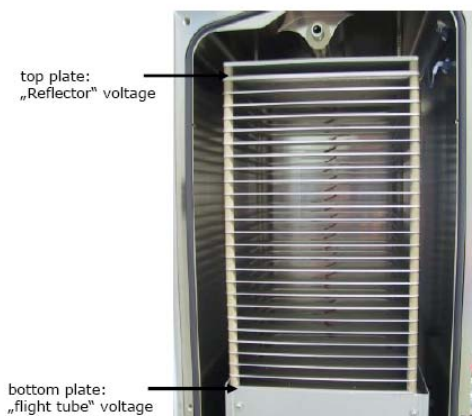


*Figura*. Imagen de de la parte final del tubo de vuelo donde se sitúa el reflector.

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. También hay un detector de referencia.

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. El diámetro aprox. del área activa del detector es de 25 mm.
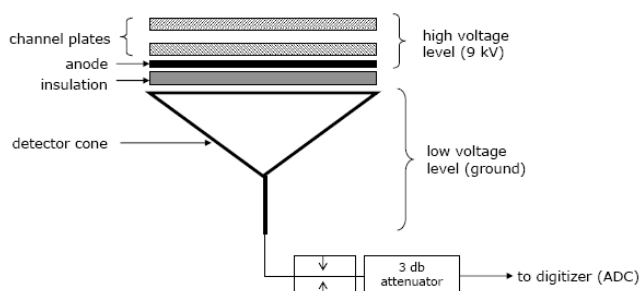


*Figura*. Esquema de detector de impacto electrónico.

Para concluir esta sección del analizador del tiempo de vuelo, se mencionarán algunas de sus especificaciones más notables en cuanto a rango de masas que puede analizar, resolución y exactitud:

-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 ppm con calibración externa.

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.

En la siguiente figura podemos ver el esquema de un espectrómetro de masas ESI-IT con sus distintas partes:
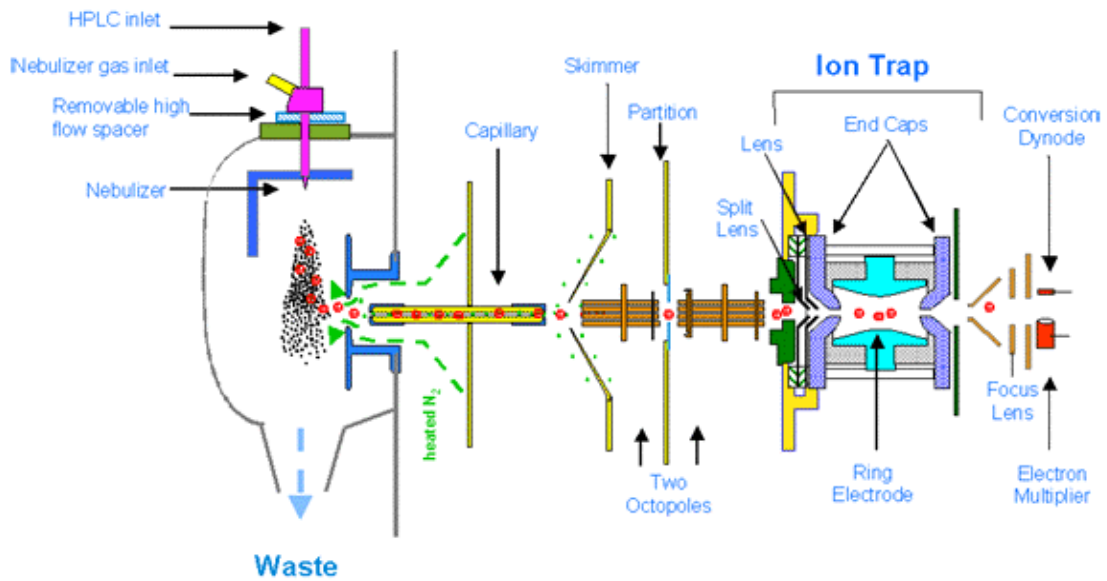
*Figura*. Esquema de un espectrómetro de masas ESI-IT.

Si observamos en orden cada una de las partes que éste posee, podemos encontrar: la interfase (ESI en este caso); la zona de "transporte y convergencia" de iones con los skimmers, optopolos y lentes; el analizador (IT); y el detector.

La primera zona es la cámara de formación del spray (la interfase). Ahí, como ya comentamos en la correspondiente sección, se nebuliza la solución de la muestra y se ioniza a través de un proceso de desolvatación. Un detalle de esta zona se muestra a continuación:
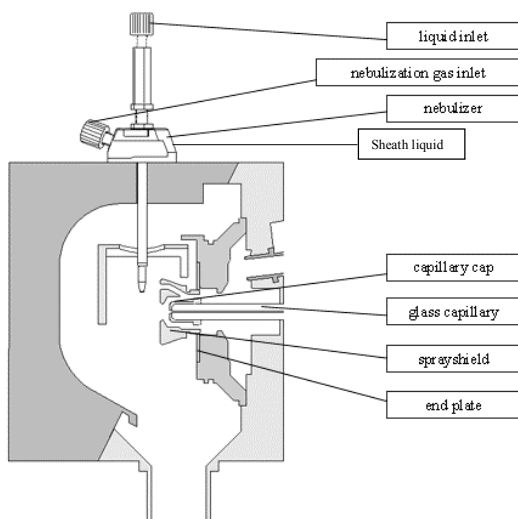


*Figura*. "Spray chamber" o cámara de formación del spray para CE.

La zona de "transporte y focalización" de iones posee cuatro zonas de alto vacío provocado por las bombas que hay en el sistema. Los iones a través del capilar de vidrio

pasan a la zona de transporte y focalización. El skimmer elimina el volumen del gas de secado; los iones pasan entonces al octopolo que los transporta y guía desde justo detrás del skimmer hasta el detector atravesando una serie de lentes.
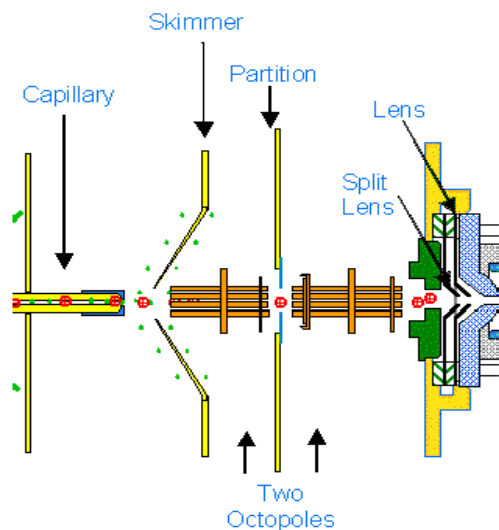


*Figura*. Detalle del skimmer, optopolos y lentes.

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. Durante la detección, los potenciales de los electrodos se alteran sometiéndolos a una rampa lineal de radiofrecuencia (RF) para provocar inestabilidad en las trayectorias de los iones y expulsarlos en la dirección axial en función de su relación m/z dando lugar a un espectro de masas.
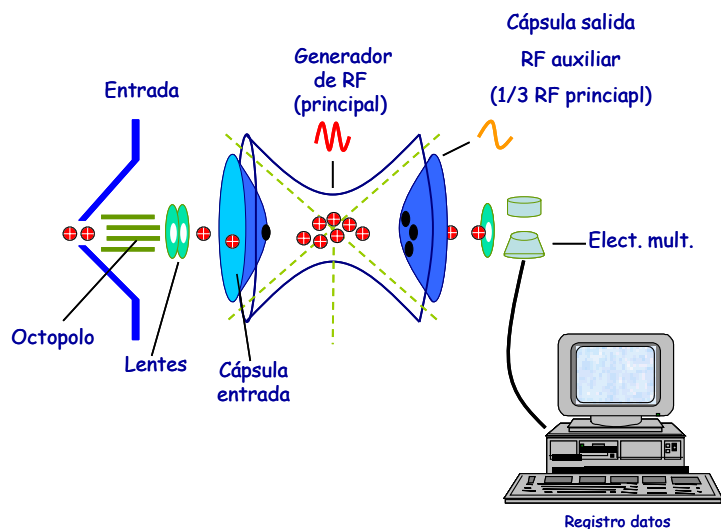
*Figura*. Esquema de un analizador de trampa de iones (y del optopolo y lentes previas al analizador).

Una vez que los iones se encuentran atrapados dentro de este analizador se puede llevar a cabo el análisis de sus masas (obteniéndose el espectro de MS) o 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 $MS^2$).

Después del analizador, los iones pasan al detector, que tiene también una serie de lentes y un sínodo que dirigen los iones hasta del propio detector.

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

-El rango de masas que puede analizar en modo es 50-2200 m/z.

-Resolución: podemos tener una resolución normal o máxima según la velocidad de escaneo.

-Posibilidad de llevar acabo análisis MS/MS.

Aunque en principio el acoplamiento CE-MS se puede llevar a cabo con cualquier tipo de espectrómetro de masas, hoy en día, los más utilizados son el cuadrupolo y la trampa de iones, fundamentalmente porque son equipos relativamente baratos y presentan menos dificultades técnicas. La trampa de iones, como ya hemos mencionado, 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. Los analizadores TOF (tiempo de vuelo) han experimentado un gran desarrollo en los últimos años. 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, lo que les hace muy compatibles para su acoplamiento con CE. De hecho, aunque los precios de estos analizadores de masas son superiores a los analizadores IT y Q, su acoplamiento directo con CE es también posible a través de ESI (o también APCI), existiendo ya equipos comerciales ESI-TOF.

En la tabla, se muestra un estudio comparativo en términos de rapidez de barrido, sensibilidad, resolución, posibilidad de llevar a cabo análisis MS-MS, cuantificación y precio de los principales analizadores de masas que han sido acoplados a un equipo de electroforesis capilar.

*Tabla*. Comparación de varios analizadores atendiendo a sus principales características.

| Analizador | Velocidad barrido | Sensibilidad Full Scan (SIM) | Resolución | MS/MS | Cuantificación | Coste | Aplicaciones principales |
|---|---|---|---|---|---|---|---|
| Q | - | - (+) | 0 | - | + | Bajo | Detector selectivo bajo precio |
| QqQ | - | - (+) | + | + | ++ | Alto | Cuantificación de compuestos "target" en matrices complejas |
| IT | + | + | + | ++ | + | Medio | Sustancias desconocidas en matrices complejas |
| TOF | ++ | + | ++ | (+) | + | Alto | Sustancias desconocidas en matrices complejas (Cuantificación) |
| FT-MS | + | + | +++ | + | + | Muy alto | Elucidación estructural en muestras complejas |

**Métodos de adquisición en espectrometría de masas**

La adquisición de datos en espectrometría de masas se puede llevar acabo de varias formas:

- **Scan (barrido)**: Proceso mediante el cual el analizador recorre todo el intervalo de masas seleccionado.
- **SIM (Single Ion Monitoring)**: Modo de registro de la señal de un solo ión o de varios seleccionados**.**

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Es decir, en el modo Scan se recoge "todo lo que entra al analizador", mientras que en el modo SIM sólo los iones de interés. Esto hace que la selectividad y la sensibilidad que se pueden obtener en el segundo de los modos sean mayores, si bien es cierto, que podríamos perder información adicional sobre nuestra muestra.
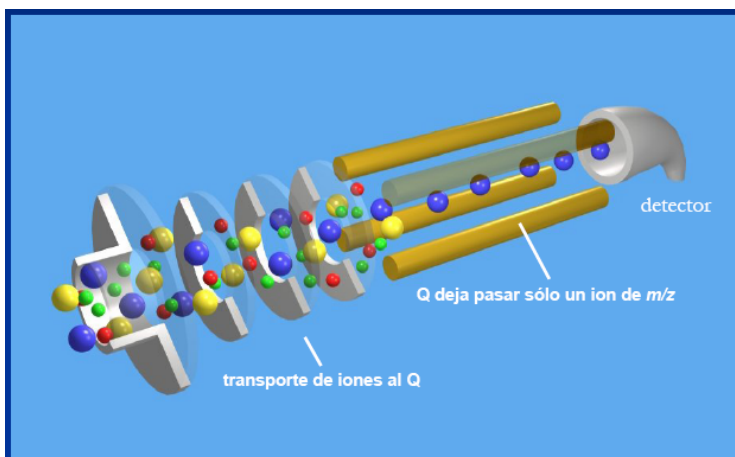


*Figura*. Esquema de un analizador de cuadrupolo que ilustra en qué consiste un método SIM de adquisición en MS.

A continuación se recogen las definiciones de varios conceptos muy útiles en lo que a la adquisición de datos en MS se refiere:

-**Rango de masas**: Intervalo de masas seleccionado.

-**Total Ion Current o Total Ion Chromatogram (TIC):** Modo de registro de la señal en el que se registra la corriente iónica total en cada instante. Éste es el modo más usual.

-**Base Peak Chromatogram o Electropherogram (BPC o BPE)**: Modo de registro de la señal que señala aquellos picos que contienen una única especie molecular predominante, restándole importancia a los picos muy heterogéneos y al ruido. TIC es la suma del ruido y la suma de la señal de cada barrido, mientras que BPC sólo muestra la contribución de los componentes más intensos con mayor resolución y mejor relación S/N.

-**Extract Ion Chromatogram o Electropherogram (EIC o EIE)**: Muy similar al BPC pero para una masa en particular seleccionada por el usuario.

## Desarrollo de métodos en CE

Podemos decir, en general, que a la hora de afrontar el estudio de una familia de compuestos en una matriz tan compleja como el aceite de oliva, hay que considerar simultáneamente varios aspectos:

-Compatibilidad de la familia de interés con CE

Hay que estar seguros de la potencialidad de la técnica CE para analizar los analitos de interés. Lo primero que se debe tener en cuenta son las propiedades físico-químicas de estos compuestos; de ellas, las más importantes son la solubilidad, el $pK_a$ (para deducir si van a presentar carga en las condiciones experimentales de trabajo) y la posibilidad de ser detectados.

Todo este conocimiento previo permitirá además seleccionar la mejor modalidad electroforética para llevar a cabo la separación y la cuantificación mediante CE.

-Detección

Se comentaba de soslayo en el punto anterior, pero es lo suficientemente importante como para ponerlo como un aspecto a tener en cuenta de por sí.

Los compuestos de interés deben ser lógicamente susceptibles de ser determinados por el sistema de detección acoplado a esta técnica separativa en cada caso.

-Extracción

En el caso particular que nos ocupa, un tercer aspecto, no menos importante, es que los compuestos a estudiar deben de estar presentes en una disolución en un disolvente compatible con la metodología electroforética elegida y a un nivel de concentración suficiente. Es decir, que tendremos que extraer los compuestos fenólicos de la matriz aceite intentando tener un extracto final lo más concentrado posible.

Conocida esta información, se realizará la optimización del método electroforético. Para el correcto desarrollo de una metodología CE han de seguirse las siguientes etapas. Antes de entrar en cada una de ellas conviene decir que en cualquier caso se puede llevar a cabo una optimización de tipo uni- o multivariante. En el primero de los casos, en cada uno de los pasos se fijarán todas las variables excepto una, cuya influencia se estudie en dicha etapa de la optimización. El éxito de este tipo de optimización dependerá en gran medida de la profundidad y rigor con que se hayan realizado los estudios preliminares. En el segundo tipo de optimización, sin embargo, las etapas experimentales se verán bastante reducidas, ya que se habrán programado unas cuantas experiencias que nos permitan estudiar la influencia de varias variables simultáneamente.

En los métodos electroforéticos puestos a punto en esta tesis doctoral, se utilizó la optimización univariante en todos los casos, y se siguieron estas etapas [185,286,287,288]:

*Selección del capilar: dimensiones y acondicionamiento*

La selección de la longitud del capilar está relacionada con la resolución entre picos y el tiempo de separación, ya que, cuanto más largo sea un capilar, mayor es el tiempo de análisis y mayor la separación o resolución entre picos. Por otra parte, la sensibilidad de un método depende en gran medida del diámetro interno del capilar.

Un capilar de diámetro interno de 50-75 μm es muy aconsejable para llevar a cabo un gran número de separaciones evitando los problemas debidos a la excesiva generación de corriente. Para obtener la máxima sensibilidad posible se pueden utilizar capilares de 100 μm de diámetro interno, aunque en este caso se debe reducir el voltaje y/o la concentración de la disolución de separación ya que los problemas de calentamiento interno aumentan con el diámetro del capilar.

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[286] 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.

[287] V. M. Morris, C. Hargreaves, K. Overall, P. J. Marriott, J. G. Hughes. "Optimization of the capillary electrophoresis separation of ranitidine and related compounds". *J. Chromatogr. A* (1997) 245-254.

[288] V. Dolník. "Selectivity, differential mobility and resolution as parameters to optimize capillary electrophoretic separation". *J. Chromatogr. A* 744 (1996) 115-121.

Durante el desarrollo de un método es también importante optimizar el ciclo de lavado del capilar para obtener unos resultados reproducibles. Cada método necesita un tiempo de acondicionamiento del capilar en función de la disolución de separación que se esté empleando y la matriz analizada.

*Optimización de la temperatura*

La temperatura ejerce un papel importante en muchas separaciones ya que la movilidad de los analitos y el FEO están relacionados con este parámetro. La mayoría de los instrumentos comerciales de CE tienen el capilar termostatizado y el intervalo de temperatura permitido por los equipos está comprendido normalmente entre 10 y 50 ºC. Los cambios en la temperatura pueden incluso afectar al pH de la disolución reguladora.

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. Trabajar a temperaturas muy bajas no presenta casi ninguna ventaja en la mayoría de las aplicaciones electroforéticas, si bien es cierto que cuanto más baja sea la temperatura, más efectiva es la disipación del calor y, por tanto, se agranda la región de trabajo en la que se puede trabajar antes de que el efecto Joule se convierta en un problema.

*Optimización de la condiciones de detección*

Este apartado se particulariza según usemos como detector la espectrofotometría UV-Vis o la espectrometría de masas.

Si los analitos a separar absorben en la región del UV-Vis y el sistema de detección va a ser espectrofotométrico, se seleccionará la longitud de onda a la cual se produce la máxima absorción de la mayoría de los analitos objeto de estudio. En casi todos los programas de control disponibles es posible seleccionar varias longitudes de onda de forma simultánea si el detector es de diodos en fila, como ya se comentaba anteriormente. Con respecto a la disolución empleada para la separación, en este caso debe tener limitada absorción en el UV (190-220 nm).

Si el sistema de detección a utilizar va a ser la espectrometría de masas, habrá que optimizar tanto la composición del líquido adicional empleado en la interfase, como el resto de parámetros del electrospray (temperatura del gas de secado, flujo del gas de

secado, presión del gas nebulizador, estabilidad de compuestos y flujo del líquido adicional).

*Selección del voltaje y la corriente*

La fuerza del campo eléctrico aplicada al capilar es el motor de la separación. Tanto la velocidad electroforética como la electroosmótica son directamente proporcionales al campo eléctrico, por lo que un aumento del voltaje aplicado proporcionará mayor rapidez (tiempos de migración cortos). La conducción de la corriente eléctrica a través de una disolución genera calor debido a las colisiones entre iones. Si el calor no se disipa, al mismo tiempo que se produce, la temperatura dentro del capilar aumenta y podrían aparecer burbujas dentro del capilar que podrían interrumpir el paso de corriente. La producción de calor a altos voltajes, es la limitación más relevante a un aumento del potencial, ya que el efecto Joule contribuye significativamente a la dispersión de los analitos y, por tanto, al solapamiento de los picos.

*Elección de la disolución de separación*

   *pH*

Las características de la disolución de separación (pH, capacidad reguladora, fuerza iónica, aditivos, etc.) juegan un papel más decisivo en la separación electroforética que los parámetros instrumentales (voltaje, temperatura o tiempo/modo de inyección).

El pH es una variable de gran repercusión en la selectividad de los métodos electroforéticos pues influye en la carga neta de especies ácidas o básicas débiles que migran electroforéticamente (que es un factor clave), junto al tamaño, para su separación. La selección de una disolución de análisis depende del pH requerido para la separación de los analitos. Teóricamente a medida que aumenta el pH aumenta la movilidad electroosmótica. En primer lugar, porque elevados valores de pH producen más disociación de los grupos silanoles Si-OH a Si-O⁻ dentro de las paredes del capilar. Por otro lado, el potencial zeta es proporcional a la carga de la superficie de las paredes internas del capilar, por lo que a medida que aumenta el pH, aumenta el potencial zeta y consecuentemente la velocidad electroosmótica.

   *Capacidad reguladora y composición*

Además de la influencia del pH, otros aspectos de la disolución de separación empleada como la capacidad reguladora deben tenerse en cuenta. 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. Hay que tener en cuenta que esta capacidad reguladora disminuye 10 veces por cada unidad de pH que se desplace de su máximo (pH=pK$_a$). La capacidad reguladora de un anfolito en la zona de pH cercana a su pI depende de la magnitud de la diferencia pI-pK1, cuando esta diferencia es superior a 2.5 el anfolito no puede usarse como disolución reguladora.

*Fuerza iónica*

La fuerza iónica de la disolución de separación es una variable de importancia similar al pH si se tiene en cuenta su impacto en la eficacia, resolución y sensibilidad de la CE, ya que tiene una influencia decisiva en las movilidades electroforéticas y electroosmóticas. Normalmente, se puede afirmar que, al aumentar la concentración de la disolución de separación (es decir la fuerza iónica), se mejora la resolución de los analitos y su forma. Sin embargo se genera más calor dentro del capilar y es necesario un buen sistema para regular la temperatura. El uso de capilares de pequeño diámetro permite aumentar la fuerza iónica de la disolución reguladora.

*Presencia de aditivos*

En general, el empleo de ligandos como *ciclodextrinas* y *poliéteres macrocíclicos* como aditivos, permite un notable incremento de la selectividad debido a las diferencias que exhiben una serie de compuestos pese a su analogía química (por ejemplo, isómeros posicionales y enantiómeros) en sus constantes de formación de los complejos de inclusión. Aditivos tales como *sales neutras*, *anfolitos, alquilaminas* y *polímeros neutros* se pueden usar para reducir o controlar el FEO y la adsorción de proteínas a la pared del capilar.

La modificación de la polaridad y la viscosidad de la disolución de separación por adición de disolventes orgánicos miscibles (por ejemplo alcoholes, acetonitrilo, etc.) pueden aprovecharse también para potenciar las separaciones en CE, pues cambian las movilidades electroforéticas y electroosmóticas de los analitos. Además, pueden usarse para potenciar la solubilidad de analitos hidrofóbicos.

También pueden manipularse la viscosidad de la disolución reguladora y por tanto el FEO y la movilidad electroforética por adición no sólo de metanol y acetonitrilo sino de alcoholes de mayor peso molecular (ej. propanol, glicerina) que aumentan la viscosidad del medio de forma notable.

También pueden adicionarse *sustancias poliméricas solubles,* tales como, metilcelulosa e hidroxipropilmetilcelulosa para reducir el potencial zeta de la pared interna del capilar, y por tanto para minimizar el fenómeno de la electroósmosis y reducir significativamente los fenómenos indeseables de adsorción.

### *Electrolitos de separación y disolventes en CE-MS*

Dentro de este apartado de elección de la disolución de separación, parece pertinente recoger en un subapartado algunas particularidades a tener en cuenta cuando el sistema de detección empleado sea MS. Como ya se ha comentado, existen diferentes parámetros que influyen en la compatibilidad entre CE y ESI-MS, siendo la elección del medio de separación en CE y la composición del liquido adicional (en el caso de una interfase ESI con flujo adicional como la usada en el desarrollo de nuestro trabajo experimental) de gran importancia. Normalmente en electroforesis capilar con detección UV-Vis se utilizan mayoritariamente electrolitos del tipo borato, fosfato, y en algunos casos aditivos como detergentes, selectores quirales, polímeros, etc. Estos compuestos van a originar en los análisis realizados por CE-ESI-MS un aumento significativo del ruido, disminución de la señal de los analitos en estudio y en condiciones extremas, la obstrucción y/o contaminación del espectrómetro. Además, el uso de sales de metales alcalinos puede dar lugar a la formación de aductos con los analitos en la fuente de ionización, reduciéndose así la cantidad de moléculas que serán detectadas en su forma protonada, y por tanto la sensibilidad del método de análisis. De este modo, se han llevado a cabo numerosos estudios sobre la influencia de la naturaleza y concentración del electrolito de separación en la intensidad de la señal [289,290,291,292]. Se puede

[ 289 ] M.A. Moseley, J. Shabanowitz, D.F. Hunt, K.B. Tomer. "Optimization of capillary zone electrophoresis/electrospray ionization parameters for the mass spectrometry and tandem mass spectrometry analysis of peptides". *J. Am. Soc. Mass Spectrom.* 3 (1992) 289-300.

[ 290 ] J.H. Wahl, R.D. Smith. "Comparison of buffer systems and interface designs for capillary electrophoresis-mass spectrometry". *J. Capillary Electrophor.* 1 (1994) 62-71.

[291] C. Simó, A. Rizzi, C. Barbas, A. Cifuentes. "Chiral capillary electrophoresis-mass spectrometry of amino acids in foods". *Electrophoresis* 26(7-8) (2005) 1432-1441.

[ 292 ] M. Pelzing, C. Neususs. "Separation techniques hyphenated to electrospray-tandem mass spectrometry in proteomics: Capillary electrophoresis versus nanoliquid chromatography". *Electrophoresis* 26(14) (2005) 2717-2728

concluir, de forma muy general, que el uso de medios de separación volátiles con una fuerza iónica entre baja y media proporciona los mejores resultados en el acoplamiento CE-ESI-MS.

En la actualidad se pueden encontrar un gran número de aplicaciones que utilizan el acoplamiento CE-MS con medios de separación que contienen varios de los siguientes compuestos volátiles: agua, etanol, metanol, acetonitrilo, isopropanol, ácido acético, ácido fórmico, ácido aminocaproico, acetato amónico, formiato amónico, bicarbonato amónico, hidróxido amónico, trietanolamina, trietilamina, etc.. Estos mismos compuestos son la base para la preparación de las disoluciones utilizadas en la interfase ESI con flujo adicional.

*Tipo y tiempo de inyección*

La muestra se puede introducir en el capilar usando la modalidad hidrostática o hidrodinámica (gravedad, presión o vacío) y 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. En general, en esta modalidad, el vial de entrada debe contener los analitos y el de salida ser un vial vacío (desecho). A diferencia de en la primera modalidad, en la inyección electrocinética, el vial de salida debe ser un vial con disolución de separación para que se pueda establecer la corriente entre los dos extremos.

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. Si se analiza una mezcla muy compleja, es decir, con un elevado número de analitos, muy probablemente no se podrán emplear tiempos de inyección elevados ya que se pueden producir solapamientos entre picos.

# Cromatografía Líquida de Alta Resolución (HPLC)

## Introducción histórica y definición

Los inicios de la Cromatografía, que la evolución convirtió en la actual HPLC, se centraron en los trabajos que Tswett [293,294,295] y Day [296] realizaron en columnas abiertas con rellenos sólidos variados, predominantemente alúmina. Después de estos primeros hallazgos, pasaron unos veinte años, en los que la cromatografía no fue apenas objeto de estudio de interés; es al inicio de la década de los treinta cuando parece redescubrirse y ya no deja de desarrollarse de modo prácticamente continuo [297,298]. Se comprobó entonces, con diversos trabajos experimentales, que disminuyendo el tamaño medio de la partícula de relleno, se conseguía, en líneas generales, mejorar la calidad de las separaciones. Sin embargo, este hecho, condicionaba enormemente los tiempos de análisis, haciéndolos del todo impracticables. Esta cromatografía en columna abierta se conoce como cromatografía clásica o gravitatoria, ya que la fase portadora (móvil) recorre el relleno por la simple acción de la gravedad. Esta cromatografía clásica o a baja presión presentaba importantes inconvenientes desde un punto de vista práctico [299]:

-Era lenta.

-Era poco eficaz, tanto en la capacidad de discriminación entre solutos, como en el número de solutos que podían separarse.

-Era tediosa por la necesidad de la intervención casi constante del operador, salvo que se dispusiera de colectores de fracciones automáticos, y

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[293] M. Tswett. "On a new category of adsorption phenomena and their application to biochemical analysis". *Proc. Warsaw Soc. Nat. Sci., Biol.* Sect. XIV (6) (1903). Lecture presentada en la sección de Biología de una reunión científica llamada Warsaw Society of Natural Sciences.

[294] M. Tswett, "Zur Kenntnis der Phaeophyceenfarbstoffe". *Ber. dtsch. botan. Ges.* 24 (1906) 235–244.

[295] M. Tswett. "Adsorptions analyse und chromatographische Methode. Anwendungen auf die Chemie des Chlorophylls". *Ber. Deutsch. Bot. Ges.* 24 (1906) 384-385.

[296] Day. Proc. Am. Phil. Soc. 36 (1897) 112-116.

[297] R. Kuhn, E. Lederer. "Zerlegung des Carotins in seine Komponenten. (Über das Vitamin des Wachstums, I. Mitteil.)". Ber. dtsc. Chem. Ges. 641(1931) 349-1356.

[298] 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) Pp 399-498.

[299] M. Valcárcel Cases, A. Gómez Hens. "Cromatografía líquida en columna (I). Generalidades" en "Técnicas analíticas de separación". Ed. Reverté S. A. (1990) Pp 437-484.

-No proporcionaba directamente el cromatograma al tener que aplicar una detección discontinua (off-line) a cada fracción del eluído.

Estos inconvenientes fueron restrictivos en el desarrollo de la cromatografía de líquidos en columna; e hicieron que su evolución fuese algo más lenta que, por ejemplo, el espectacular desarrollo de la Cromatografía de Gases (GC).

Empezaba a parecer evidente que para que la cromatografía de líquidos en columna se convirtiese en una modalidad competitiva (respecto a GC, por ejemplo) era preciso trabajar a elevadas presiones en lugar de utilizar sólo la fuerza de la gravedad para hacer pasar la fase móvil líquida a través de la fase estacionaria [300, 301, 302, 303]. La presión elevada (entre 500 y 5000 psi) de la fase móvil líquida:

-Permitiría reducir el tamaño de partícula de la fase estacionaria, que aunque muy empaquetada, deja que la fase móvil la atraviese. De este modo se podría aumentar espectacularmente la eficacia separativa.

-Reduciría drásticamente la duración de una separación cromatográfica (de 5 a 50 veces) en relación con la modalidad a baja presión, y se haría equiparable en este aspecto a la GC.

-Permitiría una detección continua del eluido, por lo que un montaje de este tipo puede considerarse como un cromatógrafo de líquidos, es decir, un instrumento que separa y suministra información cuali- y cuantitativa.

Lógicamente, esta nueva configuración a presión elevada comportaba complicaciones técnicas en comparación con la modalidad clásica, y un notable aumento del coste de adquisición y mantenimiento del instrumento. Pero este aspecto quedaba minimizado cuando se observaba la enorme potencialidad que presentaba, ya que podía cubrir aspectos inabordables o poco recomendables en GC (compuestos iónicos, muy polares,

[300] J Calvin Giddings. "Dynamics of Chromatography. Principles and Theory". Ed. Marcel Dekker. (1965).
[301] L. R. Zinder. "Modern Practice of Liquid Chromatography. Before and after 1971". *J. Chem. Education* 74 (1997) 37-44. (Waters symposium: high-performance liquid chromatography).
[302] J. J. Kirkland. "Modern Practice of Liquid Chromatography". Ed. Wiley-Interscience, New York. (1971).
[303] T. Greibrokk. "The contribution of Csaba Horváth to liquid chromatography". *J. Sep. Sci*. 27 (2004) 1249-1254.

termolábiles, no volátiles, fases acuosas de muestra…). Su desarrollo comercial pleno empezó al comienzo de los años setenta.

En el siguiente cuadro o tabla, se muestra una comparación entre las características del modo convencional y de alta eficacia; además se incluyen otros modos que emplean columnas de diámetros internos menores y flujos de fase móvil muchos más pequeños (microclomunas, nanocolumnas…).

*Tabla*. Algunos parámetros característicos de las diferentes técnicas de la cromatografía liquida en columna.

| Parámetro | LC convencional | LC alta eficacia (HPLC) | Micro LC | LC Capilar | Nano LC |
|---|---|---|---|---|---|
| *Diámetro interno de las columnas* | 10-15 mm | 1.5-4.5 mm | 0.8 mm | 0.18-0.32 mm | 0.075-0.1 mm |
| *Longitud del lecho cromatográfico* | 50-200 cm | 3-30 cm | 5-25 cm | 5-25 cm | 15-25cm |
| *Diámetro medio de partículas* | > 150-200 μm | 3-40 μm | 3-5 μm | 3-5 μm | 5 μm |
| *Flujo de fase móvil* | 1-2 ml/min | 0.2-2.5 ml/min | 10-100 μl/min | 1-10 μl/min | 0.1-1 μl/min |

Después de esta breve descripción de cómo se llegó históricamente a lo que hoy conocemos como HPLC, explicaremos en qué consiste. Con el ánimo de aclarar la terminología, sería interesante comentar, a modo de curiosidad, que el término HPLC (High-Performance Liquid Chromatography) a veces se asocia a High-Pressure Liquid Chromatography (Cromatografía Líquida de Alta Presión). Incluso, en ocasiones, podemos verla nombrada como High-Efficiency Liquid Chromatography o High Speed Liquid Chromatography (tal vez, ésta última conlleve en sí alguna connotación particular).

La Cromatografía Líquida de Alta Resolución es una técnica de separación en la que una mezcla de compuestos se distribuye entre dos fases (una fase móvil y una estacionaria), teniendo lugar la separación en función de las distintas afinidades que presente cada compuesto de la mezcla por las diferentes fases. 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, auténtico "corazón" del cromatógrafo de líquidos. 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.

## Instrumentación en HPLC

Un sistema moderno de HPLC está formado por los siguientes componentes [304]:

- Bomba: Es la encargada de impulsar el/los disolventes al resto del sistema. Las bombas modernas tienen la capacidad para impulsar varios disolventes en proporciones variables y programables, y disponen de sistema de desgasificación de fase móvil (f.m.).

- Inyector: Permite la introducción de una cierta cantidad de muestra en el sistema.

- Columna cromatográfica: Contiene la fase estacionaria y en ella tiene lugar la separación de los analitos. 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.

- Horno termostatizado: Mantiene la temperatura de la columna constante, asegurando una mayor reproducibilidad en las separaciones (no siempre es necesario).

- Detector: Encargado de producir señales o respuestas analíticas ante la presencia de un determinado compuesto. Puede utilizarse más de uno simultáneamente, colocándolos en serie. (Podemos hacer extensivas en este punto las características de un detector ideal que apuntábamos en la sección de CE).

- Sistema de adquisición de datos: Actualmente los ordenadores, dotados con programas específicos, se encargan de ello.

En la siguiente figura, se presenta un equipo de HPLC de los utilizados en el desarrollo del trabajo experimental recogido en esta memoria, indicando cada uno de sus componentes. Se puede ver con claridad, que no fue necesario el uso de horno termostatizado en este caso. La presencia de la pre-columna se explica con el fin de

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[ 304 ] 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) Pp 19-51.

alargar la vida de la columna. En la figura se ve cómo pueden ser utilizados varios detectores en serie (UV-Vis y MS en este ejemplo).
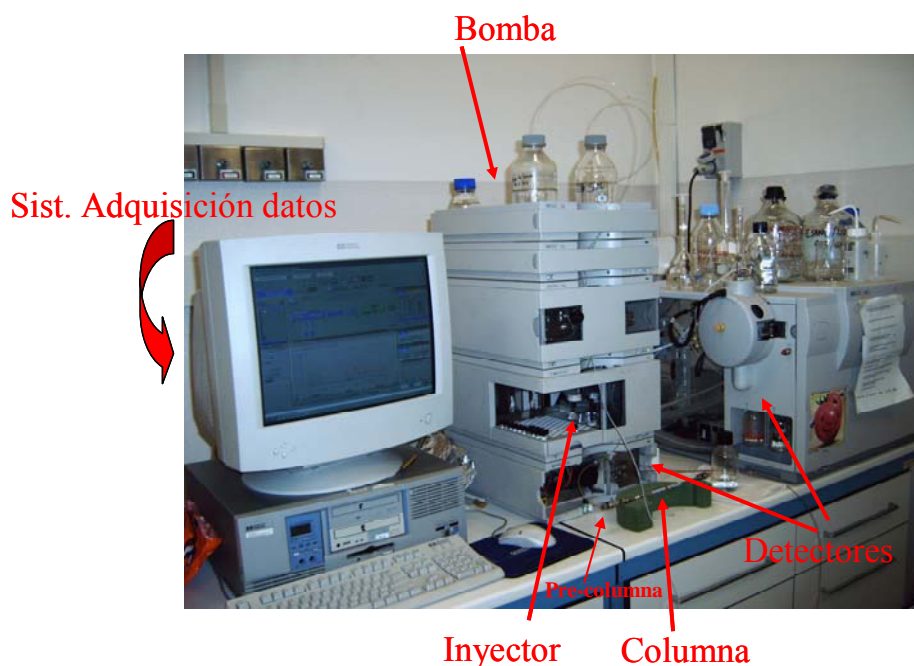


*Figura*. Equipo de HPLC empleado en el desarrollo experimental de varios de los capítulos presentados en esta tesis doctoral.

En una separación por HPLC, la fase móvil impulsada por la bomba transporta una banda de muestra a través de la columna cromatográfica. En ésta, 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. Una vez eluido cada compuesto debe ser detectado. Para ello, se colocan a la salida de la columna cromatográfica uno o varios detectores que proporcionarán una respuesta al paso de los analitos (absorbancia, emisión fluorescente, conductividad…). El procesado de esta señal produce el cromatograma, en el que representa la respuesta obtenida por el detector frente al tiempo. Cada analito, por tanto, estará representado por un pico que poseerá un determinado tiempo de retención ($t_R$). La intensidad de cada pico será directamente proporcional al factor de respuesta y la concentración del analito correspondiente en la muestra.

# Distintos tipos de Cromatografía

En HPLC encontramos una gran variedad de alternativas según la naturaleza de la fase estacionaria, como puede observarse en la siguiente tabla [305]:

*Tabla*. Tipos básicos de HPLC

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

*Muchos autores no la consideran un tipo de Cromatografía *per se*, sino más bien incluída en la cromatografía de partición o reparto [306,307].

En ella se observa que un sólido es la fase estacionaria activa más frecuente, además de actuar como soporte inerte en la cromatografía de partición. Este sólido tiene diferentes fundamentos de actuación [308]:

a) Físico en cromatografía de geles (exclusión). En este caso realiza la simple misión de un tamiz o filtro. La causa de la retención en el lecho cromatográfico es puramente física: el tamaño molecular.

b) Físico-químico en cromatografía de adsorción, que se basa en la actividad superficial del mismo que retiene con mayor o menor "fuerza" a los solutos. En la cromatografía de partición clásica (también conocida como cromatografía de reparto), la absorción es el fundamento de la retención de la fase estacionaria líquida.

c) Químico en cromatografía de cambio iónico, que se basa en el intercambio de especies cargadas (aniones y cationes)

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[305] La cromatografía líquida se puede clasificar atendiendo a varios criterios: a la forma física global de la fase estacionaria, a la dirección del flujo de la fase móvil, a la eficiencia de separación, a la interacción que se produce entre la fase estacionaria y el soluto…

[306] D. W. Armstrong. "Optical isomer separation by Liquid-Chromatography". *Anal. Chem.* 59(2) (1987) A84&.

[307] W. J. Lough. "Chiral Chromatography". Ed. Blackie. Glasgow. (1989).

[308] M. Valcárcel Cases, A. Gómez Hens. "Cromatografía líquida en columna (II). Técnicas de adsorción y partición" en "Técnicas analíticas de separación". Ed. Reverté S. A. (1990) Pp 485-531.

d) Bioquímico en la cromatografía de afinidad, que se basa en la retención reversible de solutos de naturaleza bioquímica basada en la interacciones bioespecíficas (acoplamiento llave-cerradura).

La distribución de un soluto entre las dos fases tiene lugar debido a la interacción en diferente proporción de las moléculas del mismo con las moléculas de cada fase. Esta interacción es debida a la existencia de tres tipos básicos de fuerzas intermoleculares: iónicas, polares y dispersivas. Además existen otras más puntuales: adsorciones bioespecíficas y exclusión por tamaños.

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).

La siguiente figura pone de manifiesto que los distintos modos que puede utilizar la cromatografía de líquidos tienden a ser complementarios en lo que a su campo de aplicación se refiere.
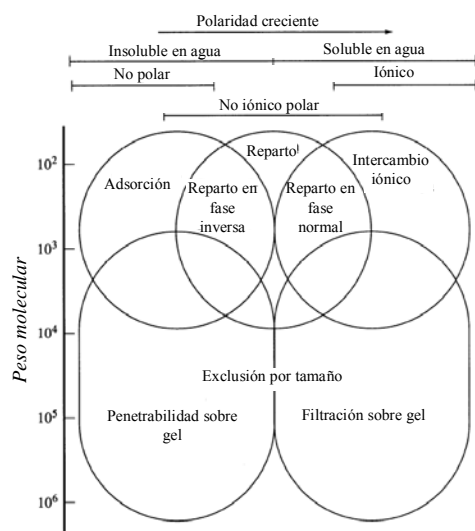


*Figura*. Campo de aplicación de los distintos modos que se pueden utilizar en HPLC.

## Cromatografía de partición o de reparto

Comentaremos de modo más particularizado este tipo de HPLC, por ser el tipo de cromatografía de líquidos más ampliamente utilizado y el que hemos utilizado en la parte experimental correspondiente a esta técnica.

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.

Deben establecerse dos distinciones básicas en este tipo de cromatografía. La primera se refiere a la naturaleza de las fases líquidas implicadas:

a) En "fase normal": la fase móvil es de naturaleza no polar (o poco polar) y la estacionaria es fuertemente polar (podemos decir que guarda cierta semejanza con la cromatografía de adsorción en este aspecto).

b) en "fase invertida" (o reversa), cuando la situación es la inversa: la fase estacionaria es no polar y la móvil, polar; se trata sin lugar a dudas del tipo de cromatografía estrella, ya que una gran mayoría de muestras de interés en diversos ámbitos tienen naturaleza hidrofílica.

Por otra parte, también se podría hacer otra distinción dentro de la cromatografía que nos ocupa atendiendo a la relación entre el sólido soporte inerte y la fase líquida estacionaria activa:

a) Fase estacionaria adsorbida: retenida por interacción físico-química del disolvente con los sitios activos del sólido soporte, y

b) Fase estacionaria ligada: cuando se establece un anclaje químico entre el sólido soporte y las moléculas de la fase estacionaria líquida. Las ventajas de esta posibilidad son más que notorias en términos de estabilidad, versatilidad, rapidez, etc.

Para hacernos una idea de la repercusión de cada una de esta variantes, cabe decir que la cromatografía de partición en fase ligada químicamente ha monopolizado la "casi" totalidad de aplicaciones prácticas. Además, el modo de trabajo en fase invertida, ha ocupado el 90% de las aplicaciones analíticas de interés.

### Relleno de las columnas

Una fase ligada químicamente aprovecha los grupos silanol libres de la superficie de las partículas de sílice haciéndolas reaccionar con alcóxidos o clorosilanos, de modo que finalmente los grupos silanol (que suelen ser indeseables en la mayoría de los casos) quedan bloqueados y, a la vez, la superficie del soporte silíceo queda recubierta de una capa firmemente unida al mismo cuya funcionalidad viene determinada por los reactivos de partida y, por tanto, puede variarse en un amplio intervalo.

En la superficie de la sílice se pueden producir reacciones con reactivos mono o polifuncionales. En teoría, la reacción con reactivos monofuncionales produce capas de "pelos" hidrocarbonatos sobre la superficie de la sílice; la longitud del grupo que se une a la sílice depende lógicamente del reactivo que se eligiese en la reacción (C18 (ODS), C8, fenil y un largo etc) [309]. Las columnas más empleadas son, sin lugar a dudas las C18; los porcentajes para cada tipo de fase estacionaria se muestran en la tabla [310].

*Tabla*. Uso relativo (%) de las fases estacionarias más usuales.

| Fase estacionaria | % Uso relativo |
|---|---|
| *C18* | 39 |
| *C8* | 26 |
| *Cianopropil\** | 14.5 |
| *Fenil* | 12 |
| *C4* | 3.7 |
| *Interacción hidrofóbica* | 1.8 |
| *C2* | 1.1 |
| *C1* | 0.8 |
| *Otros* | 0.8 |
| *Polímeros* | 0.8 |

\*En este caso, para calcular el % se incluye también el uso en fase normal

Por el contrario, en el caso de reactivos trifuncionales, las reacciones no sólo pueden tener lugar con los grupos silanol libres de la superficie del soporte, sino también con

[ 309 ] J. F. Rubinson, K. A. Rubinson. "Separaciones y cromatografía" en "Química Analítica contemporánea". Ed. Prentice Hall, México. (2000) Pp 404-457.
[310] R. E. Majors. "The cleaning and regeneration of Reversed-Phase HPLC Columns". *LG·GC Europe* Column Watch (2003) 2-6.

los propios grupos silanol que se forman en el curso de las reacciones. Esto conduce a un cierto grado de entrecuzamiento y se habla entonces de que el material resulta polimérico y ya no es una monocapa de pelos hidrocarbonatos. Los grupos silanol resultantes (igual que los no bloqueados en el caso de reactivos monofuncionales) se eliminan de manera casi completa mediante reacción con silanos monofuncionales de pequeño tamaño molecular (por ejemplo, TMCS, en un proceso que se conoce como *end capping*). En la siguiente reacción se muestra cómo se da el proceso explicado en los párrafos anteriores.
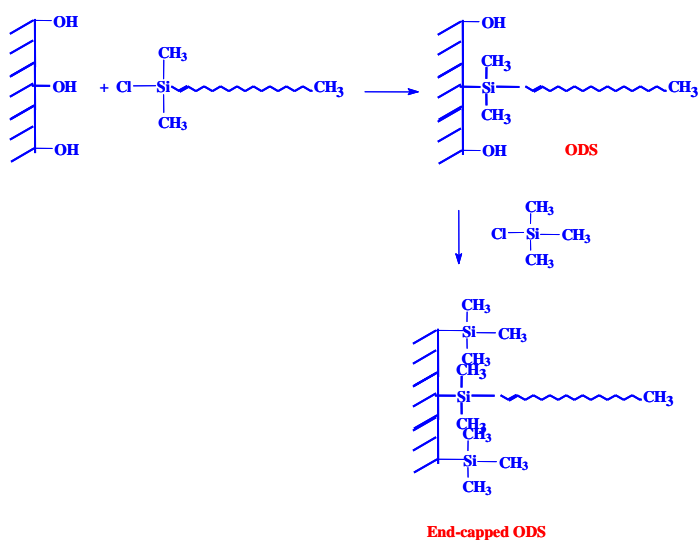


*Figura.* Reacciones que se suelen dar en un proceso de *end capping*.

Como se puede pensar de modo intuitivo, al aumentar la longitud de la cadena hidrocarbonada que se liga sobre la superficie de la sílice, ésta resultará cada vez menos polar.

En la figura mostrada a continuación se ve lo que sería el aspecto típico del interior de una columna C18, donde se ven las cadenas de 18 carbonos (grupos octadecilo) unidas a la sílice. Estos grupos orgánicos ligados, producen un efecto similar al que tendría una capa extremadamente fina de disolvente orgánico sobre la superficie de las partículas de sílice.
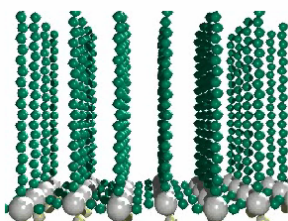


*Figura.* Interior de una columna C18.

Un segundo tipo de fase estacionaria usada en cromatografía en fase reversa está compuesto de partículas esféricas de polímero orgánico [310]. Un polímero típico es una resina compuesta de poliestireno y divinilbenceno. Los componentes del divinilbenceno forman enlaces entre las cadenas de poliestireno-enlaces cruzados. Los enlaces cruzados producen un reforzamiento físico del polímero, de modo que una mayor densidad de enlaces cruzados se asocia con un material rígido.

Otra fase estacionaria relativamente nueva es una variedad de grafito. El material se llama carbono granítico poroso (PGC), que es fundamentalmente carbono puro, y las superficies son molecularmente planas. El PGC es menos polar que los disolventes, por lo que, en general, se incluye en el tipo de rellenos para la cromatografía en fase reversa. Sin embargo, la propiedad más interesante del PGC es que la fuerza de interacción entre él y las moléculas del soluto depende casi exclusivamente del área molecular que contacta con las láminas de carbono [311,312].

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 [313].

Por el contrario, sí se ha podido trabajar con partículas de tamaños inferiores a 1 μm mediante la técnica de UHPLC recientemente patentada (Ultrahigh-pressure liquid chromatography), que es básicamente una adaptación de un equipo de HPLC para trabajar a presiones elevadas. Lo malo es que esta técnica aún no ha llegado a cuajar en el análisis rutinario.

[311] "Hypercarb™ HPLC Columns Technical Guide, the solution for problem separations" de Thermon Electron Corporation.
[312] K. A. Rubinson, J. F. Rubinson. "Cromatografía líquida" en "Análisis Instrumental". Ed. Pearson Education S. A. Madrid. (2000) Pp 636-676.
[313] 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 (17-18) (2004) 1431-1440.

La aparición de nuevas fases estacionarias denominadas monolíticas ha supuesto una gran innovación en lo relativo a los rellenos de las columnas de HPLC, superando el problema asociado a la baja permeabilidad de los empaquetamientos tradicionales [314,315]. Las columnas monolíticas son columnas cuya fase estacionaria, en lugar de ser un sólido particulado, está formada por un material sólido dispuesto en una sola pieza, con una estructura tridimensional compuesta por un esqueleto con poros de diferentes tamaños (macroporos y mesoporos).

Su elevada permeabilidad tiene dos ventajas fundamentales: por un lado permite aumentar considerablemente la longitud de la columna (aumentando, por tanto, el número de platos teóricos). Por otro lado, permite separaciones más rápidas gracias a la posibilidad de operar con flujos de fase móvil elevados sin pérdida de eficiencia.

## Detectores

El papel del detector es proporcionar información sobre la presencia de las especies separadas en la columna cromatográfica y, por tanto, debe elegirse éste en función de las características de las especies de interés. Las características de lo que sería un detector ideal se comentaron en la sección de CE, y evidentemente, son aplicables ahora. Muchos son los detectores que se han acoplado a la técnica separativa de HPLC (UV-Vis, índice de refracción, fluorescencia, electroquímicos, radioactivos, de dispersión de luz (ELS), espectrómetros de masas…).

En la presente memoria, cuando se llevaron a cabo separaciones cromatográficas se emplearon como sistemas de detección 2 tipos de detectores: espectrofotométricos UV-Vis y espectrómetros de masas. Ambos fueron ya comentados en la correspondiente sección de CE, aquí nos limitaremos a incluir algunas puntualizaciones pertinentes.

**Detector espectrofotométrico UV-Vis**

Es el más usual en los laboratorios analíticos, aunque en la mayor parte de los casos viene con otro detector *on-line* que complementa la información que el detector de

[ 314 ] Z. Lin, Z. H. Xie, X. H. Lu, X. C. Lin, X. P. Wu, G. N. " On-column coaxial flow chemiluminescence detection for underivatized amino acids by pressurized capillary electrochromatography using a monolithic column". *Anal. Chem*. 78(15) (2006) 5322-5328.
[315] N. Wu, J. Dempsey, E. M. Yehl, A. Dovletoglou, D. Ellison, J. Wyvratt. "Practical aspects of fast HPLC separations for pharmaceutical process development using monolithic columns". *Anal. Chim. Acta* 523 (2004) 149-156.

absorción UV-Vis nos aporta. Los hay de tres tipos como ya se comentó en la correspondiente sección de detectores para CE.

**Espectrometría de masas**

En la puesta a punto de métodos cromatográficos y en el desarrollo de sus aplicaciones se utilizaron dos tipos de sistemas de ionización (ESI, APCI) y tres tipos de analizadores de masas (IT, TOF y Q).

**APCI (Atmospheric Pressure Chemical Ionization)**

La ionización química a presión atmosférica (APCI) es un complemento para ESI que se emplea con mucha frecuencia. APCI no suele generar iones con cargas múltiples y opera a altas temperaturas. Normalmente, se emplea en el análisis de compuestos menos polares que en ESI, pequeños y que sean térmicamente estables.

La primera interfase APCI para HPLC-MS fue desarrollada por Horning y col [316] en los años 70. A partir de entonces se han sucedido diferentes diseños, algunos de los cuales han llegado a comercializarse.

Utilizando la sonda de APCI el líquido que procede del cromatógrafo es nebulizado y rápidamente evaporado por la acción de la temperatura elevada (300-500 ºC). A pesar de que a esta temperatura teóricamente ciertos analitos pueden ser degradados, los altos flujos de gas de nebulización y de $N_2$ coaxial (también llamado a veces gas de make-up), previenen la ruptura de las moléculas. De este modo los iones que están presentes en la disolución pueden pasar a fase vapor [317].

Para incrementar el proceso de ionización se suele aplicar una descarga en la corona, del orden de 2-6 kV, justo a la salida de la sonda de APCI en el spray. Esta descarga eléctrica no sólo ioniza a las moléculas de analito, sino también a las del disolvente de la fase móvil. Éstas, a su vez, pueden transferir su carga a la de los analitos en la fase gaseosa, de lo que resulta la ionización química de los mismos.

[316] E. C. Horning, D. I, Carroll, I. Dzidic, K. D. Haegele, M. G. Horning, R. N. Stillwell. "Liquid chromatograph-mass spectrometer-computer analytical systems. Continuos flow system based on atmospheric pressure ionization mass spectrometry". J. Chromatogr. 99 (1974) 13-21.
[317] O. Quintela, A. Cruz, M. Concheiro, A. De Castro, M. López-Rivadulla. "Metodología LC-MS. Aspectos generales de la técnica y sus aplicaciones en el campo de la toxicología". Rev. Toxicol. 22 (2005) 7-14.
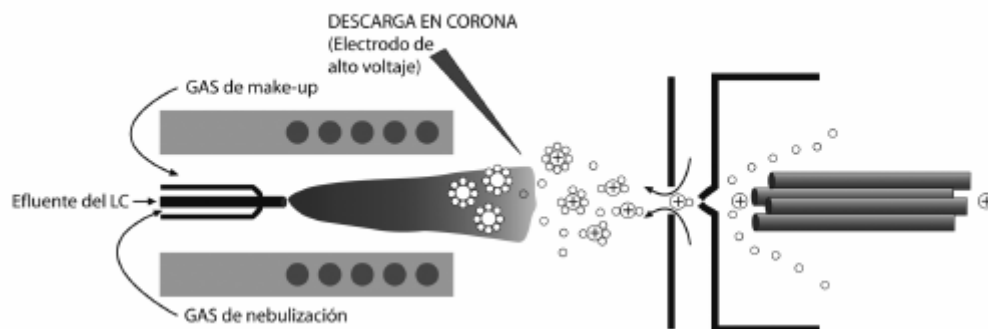
*Figura*. Fuente de APCI. La mezcla de líquido y vapor calientes se expande en la interfase a presión atmosférica, donde se produce una primera ionización mediante la descarga de la corona. Los iones formados a partir de las moléculas del solvente transfieren su carga a los analitos, produciéndose así su ionización química.

En pocas palabras, es similar a ESI; la diferencia estriba en que el líquido procedente del sistema cromatográfico pasa a través de un vaporizador calentado que facilita la rápida desolvatación/vaporización de las gotas. Las moléculas de analito y disolvente atraviesan una región donde mediante una corona de descarga se facilitan las reacciones ión-molécula entre el analito y los iones formados desde el disolvente en fase vapor a presión atmosférica, con un alto grado de eficiencia.

La transferencia protónica (protonación $[M+H]^+$) se produce en el modo de APCI positivo y la transferencia electronica $(M–H)^-$ en el modo de APCI negativo. Moléculas cargadas de manera múltiple, como se podían hallar en ESI, no se observan utilizando la interfase APCI.

En ciertas ocasiones, APCI puede producir mejores resultados que ESI. Podemos enumerar los siguientes casos en los que sería recomendable usar este tipo de interfase:

- Cuando la muestra tiene una mala respuesta en ESI.
- La muestra no contiene grupos ácidos ni básicos (como hidrocarburos, alcoholes, aldehídos, cetonas, ésteres…).
- La muestra es térmicamente estable y puede ser vaporizada.
- El flujo óptimo de trabajo, los disolventes o aditivos nos son compatibles con ESI.

**Cuadrupolo (Q)**

Este analizador es más barato y compacto que cualquier otro tipo de espectrómetro de masas. El corazón del cuadrupolo es un conjunto de cuatro barras cilíndricas paralelas que actúan como electrodos.
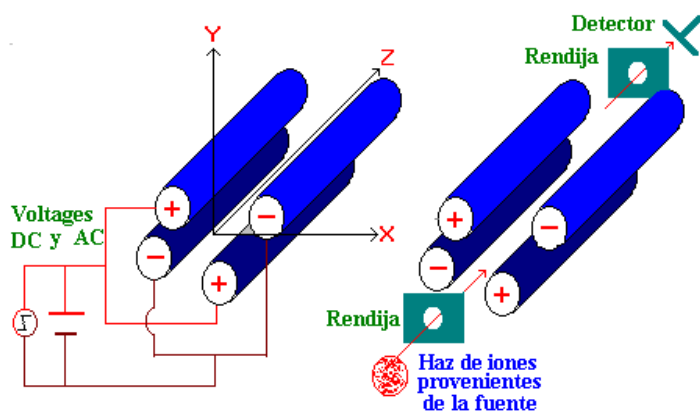


*Figura*. Barras cilíndricas paralelas que actúan como electrodos en el cuadrupolo.

 Las barras opuestas se conectan eléctricamente, un par está unido al polo positivo de una fuente variable de corriente continua y el otro par se une al terminal negativo. Además, se aplican a cada par de barras potenciales variables de corriente alterna de radiofrecuencia, que están desfasados 180 grados. Para obtener un espectro de masas con este dispositivo, los iones se aceleran en el espacio entre las barras mediante un potencial  de 5 a 10 V. Entre tanto las tensiones de corriente continua y de corriente alterna se incrementan simultáneamente mientras se mantiene constante su relación. En cualquier momento, todos los iones, excepto aquellos que tengan un determinado valor de m/z inciden en las barras y se convierten en moléculas neutras [318].

[318] D. A. Skoog, F. J. Holler, T. A. Nieman. "Principios de análisis instrumental". 5ª Edición. McGraw-Hill/Interamericana de España S. A. U. Madrid. (2001).
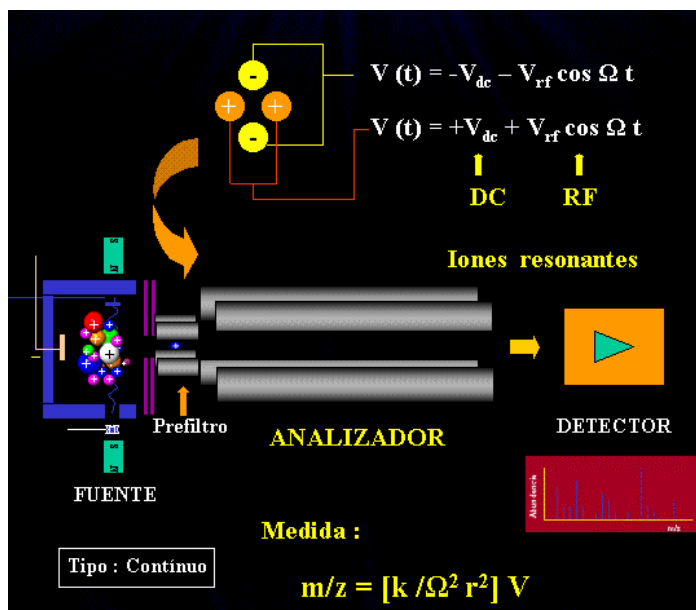
*Figura*. Resumen del fundamento del funcionamiento de un analizador de cuadrupolo, que muestra cuatro rodillos paralelos a los que se aplica una corriente continua (DC) sobre la que se superpone un potencial de radiofrecuencia (RF). El campo creado en los rodillos actúa a modo de filtro y determina que iones alcanzarán el detector. Los iones, en esta región de campo variable, oscilarán dependiendo del campo de radiofrecuencia aplicado, así como de su relación masa/carga, por lo que solo determinados iones alcanzaran el detector. De este modo, un espectro de masas se conseguirá barriendo (scanning ) el campo RF dentro de un rango de frecuencias.

El analizador de cuadrupolo es uno de los más extendidos hoy día, como ya adelantábamos en anteriores apartados de esta memoria. A su relativa sencillez se une una alta tolerancia a vacíos relativamente pobres, rango de masas de hasta 3000 Da que le hace muy adecuado para ser acoplado a interfases de cualquier tipo, incluida la ESI, y para el análisis de proteínas y biomoléculas. Además, otra cosa a su favor, es su relativo bajo costo. Su principal desventaja es la imposibilidad de realizar análisis de alta resolución, masas exactas, etc, así como su limitación en cuanto a rango de masas.
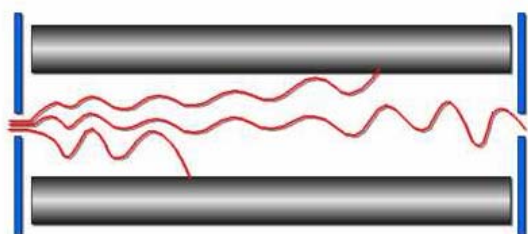


*Figura*. Representación de la trayectoria seguida por los iones en un analizador cuadrupolar; todos los iones, excepto aquellos que tengan un determinado valor de m/z inciden en las barras y se convierten en moléculas neutras.

Por tanto, sólo los iones cuyo valor de m/z esté dentro de un intervalo limitado, alcanzarán el detector.

# Desarrollo de un método en HPLC

Está claro que cuando se pone a punto un método cromatográfico, se persigue obtener una buena resolución, tiempo de análisis, precisión, altura de pico, trabajando a una presión adecuada y consumiendo el menor disolvente posible. Se recoge en distintos libros de texto sobre cromatografía o técnicas separativas en general [298,319], que un esquema o diagrama de flujo a seguir en el desarrollo de un procedimiento de cromatografía líquida en fase invertida podría ser:

```
┌─────────────────────┐   k grande    ┌─────────────────────┐
│ Elución con 100% ACN │─────────────▶│ Probar con HPLC en   │
└─────────────────────┘               │ fase normal          │
          │ k pequeña                 └─────────────────────┘
          ▼
┌─────────────────────┐   colas       ┌─────────────────────┐
│ Disminuir el % de ACN│─────────────▶│ Utilizar aditivos    │
│ hasta que 1<K        │               └─────────────────────┘
│ <20 para todos los   │
│ picos                │
└─────────────────────┘
          │
          ▼
┌─────────────────────┐
│ Ajustar el % de ACN  │
│ para regular la      │
│ selectividad (α)     │
└─────────────────────┘
          │
          ▼
┌─────────────────────┐
│ Cambiar a MeOH con   │
│ igual fuerza eluyente│
└─────────────────────┘
          │
          ▼
┌─────────────────────┐               ┌───────────────────────────┐
│ Cambiar a una mezcla │─────────────▶│ Probar mezclas ACN/MeOH si │
│ 50/50 MeOH/ACN       │               │ se aprecian cambios en el  │
└─────────────────────┘               │ espaciado de los picos     │
          │                           └───────────────────────────┘
          │              Cambios en el
          │              espaciado de las
          │              bandas
          ▼
┌─────────────────────┐               ┌───────────────────────────┐
│ Cambiar a THF con    │─────────────▶│ Probar mezcla 50/50 MeOH/  │
│ igual fuerza eluyente│               │ THF                        │
└─────────────────────┘               └───────────────────────────┘
          │
          ▼
┌─────────────────────┐
│ Probar una mezcla    │
│ 1:1:1 ACN/MeOH/THF   │
└─────────────────────┘
          │
          ▼
┌─────────────────────┐               ┌───────────────────────────┐
│ Ajustar las          │─────────────▶│ Recurrir a otra técnica    │
│ proporciones si los  │               │ cromatográfica             │
│ cromatogramas indican│               └───────────────────────────┘
│ que será posible     │
│ mejorar la           │
│ selectividad         │
└─────────────────────┘
```

*Figura*. Diagrama de flujo en el desarrollo de un procedimiento de cromatografía líquida en fase invertida. (ACN: acetonitrilo; MeOH: metanol; THF: tetrahidrofurano).

[319] L. R. Snyder, J. J. Kirkland, J. L. Glajch. "Systematic approach to the Reversed-Phase separation of regular samples" en "Practical HPLC method development". Ed. John Wiley & Sons, Inc. New York. (1997) Pp 403-437.

El esquema muestra el modo en el que se puede aproximar la optimización experimental de una separación usando este tipo de cromatografía líquida mediante un número limitado de experimentos.

Inicialmente, se puede trabajar con una fase móvil de elevada fuerza eluyente (acetonitrilo 100%) y un flujo de 1-1.5 ml/min para columnas de dimensiones usuales (10-25 cm de longitud por aprox 4.6 mm de diámetro interno), habiendo, lógicamente seleccionado unas condiciones de detección que permitan establecer la presencia de todos los compuestos de interés. Si la retención de los compuestos es muy baja, habremos elegido bien la cromatografía liquida en fase reversa (RP-HPLC). Habrá entonces que disminuir la fuerza eluyente de la fase móvil para lograr separaciones más prácticas; se persigue que todos los componentes de interés manifiesten factores de retención ($k$) comprendidos entre 1 y 20. En principio, al disminuir la proporción de modificador es de esperar una mayor retención, que no en todos los casos vendrá acompañado de una mejor separación.

Si los picos aparecen con colas, suele ser necesario adicionar a la fase móvil una pequeña cantidad de aditivo (TEA, trietilamina) para reducir o eliminar ese efecto.

Cuando tengamos detectado cuál es un valor (%) de acetonitrilo que logra una separación aceptable, será un buen momento para ver el efecto que produce un cambio en la naturaleza del modificador orgánico. Una sencilla prueba sería cambiar el ACN por metanol; aunque como la proporción de ACN seleccionada proporcionaba una retención aceptable, deberíamos utilizar una proporción de metanol en la fase móvil con igual o similar fuerza eluyente que la correspondiente a los mejores ensayos realizados hasta ese momento (por ejemplo, el equivalente isoelutrópico en metanol al 40% de ACN, es el 51%). Si el metanol no funcionase bien, podríamos probar nuevos cambios (tetrahidrofurano), o bien mezclas ternarias o incluso cuaternarias.

Este proceso puede ser más o menos largo según la dificultad de la separación, aunque, por lo general, no se suele partir de una ignorancia absoluta acerca del problema planteado, sino que se dispone de alguna información bibliográfica y ello puede ahorrar gran parte de este trabajo. Hoy en día, incluso se puede recurrir a sistemas de optimización asistida por ordenador.

**Elución isocrática y en gradiente y otras condiciones a optimizar**

En ciertas ocasiones, las muestras incluyen analitos de muy diversa naturaleza y con propiedades físico-químicas diferentes, pudiendo resultar algunas especies de interés de la muestra muy retenidas mientras que otras eluyen muy rápidamente. En este caso, los cambios de selectividad comentados antes, no surtirían efecto, ya que afectarían de modo distinto a unas y otras especies. Una solución a este tipo de problemas, consistiría en poder aprovechar las modificaciones tanto de la fuerza eluyente (retención), como de la naturaleza (selectividad) de la fase móvil de una forma dinámica, modificándolas en el tiempo durante la propia elución.

Podemos emplear una elución isocrática o una elución en gradiente. En las eluciones isocráticas se mantiene constante la fuerza del eluyente de la fase móvil. En las eluciones por gradiente, en cambio, es la fuerza eluyente de la fase móvil la que se modifica durante la elución y pueden diseñarse de modo que la selectividad se mantenga constante o no.

El saber cuándo debemos utilizar o no una elución en gradiente es una cuestión complicada, ya que no podemos estar seguros de que sea lo más conveniente simplemente porque una elución isocrática simple no pueda resolver el problema. Un criterio práctico bastante útil es el propuesto por Snyder [320]. Consiste en ejecutar un gradiente lineal entre extremos de porcentaje de modificador en un tiempo razonable, tratando de que los picos sean eluídos con factores de retención inferiores a 20. Se mide el intervalo de tiempo entre la aparición del primero y el último de los picos de la muestra y se divide por el tiempo de ejecución del gradiente. Si el cociente resulta ser superior a 0.25 será aconsejable trabajar usando un gradiente, puesto que ello significa que una elución isocrática que, con el mismo modificador, pudiera proporcionar factores de retención análogos produciría muy mala separación de los primeros picos, o bien, que para mantener dicha resolución en valores aceptables, el tiempo requerido para la elución isocrática correspondiente sería muy elevado.

Una vez demostrada la conveniencia o necesidad de trabajar en gradiente, habrá que opimizar otra serie de parámetros fundamentales que no quedaban recogidos en el esquema anterior que contenía un diagrama de flujo en el desarrollo de un procedimiento en RP-HPLC, como son:

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[320] L. R. Snyder, J. J. Kirkland, J. L. Glajch. "Non-ionic samples: Reversed- and Normal-Phase HPLC" en "Practical HPLC method development". Ed. John Wiley & Sons, Inc. New York. (1997) Pp 233-264.

- Temperatura

Será necesario comprobar si es mandatario o no controlar y mantener constante la temperatura en las separaciones, especialmente si el procedimiento de separación se va a utilizar en análisis de rutina.

- 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 serán 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. Aunque aquí, hemos de hacer notar que cuando la proporción de disolvente orgánico crece en la fase móvil, la determinación de la escala de pH se hace insegura y los valores de $pK_a$ de los solutos pueden resultar alterados, en ocasiones, significativamente.

# Semejanzas entre CE y HPLC

La electroforesis capilar y la cromatografía líquida de alta resolución pueden considerarse técnicas separativas complementarias que, al trabajar con muestras en estado líquido, presentan ciertas semejanzas.

• La primera semejanza entre la CE y la HPLC es que ambas pueden operar generalmente con detección on-line y utilizan sistemas de detección que recogen la señal respuesta frente al tiempo. La detección on-line otorga a estas técnicas la capacidad de hacer análisis cuali y cuantitativo. Los resultados de la CE o de la HPLC (electroferogramas o cromatogramas) muestran los analitos separados, y estos pueden ser caracterizados por su tiempo de retención. Además, ambas técnicas pueden ser acopladas a más de un sistema de detección. El más ampliamente utilizado es el UV/Vis, pero, cada vez más, la fluorescencia, la espectrometría de masas y la detección electroquímica (conductividad, amperometría y potenciometría) se están convirtiendo en sistemas de detección ampliamente utilizados. Hoy en día, hay disponibles comercialmente instrumentos de CE de tercera generación, que tienen incluso la opción de trabajar con más de dos detectores simultáneamente.

• Ambas pueden utilizarse empleando diferentes modos, y existe un paralelismo entre los modos electroforéticos que se pueden emplear y los modos en HPLC. Así: La electroforesis capilar en zona (CZE) o electroforesis capilar en zona libre y el isoelectroenfoque capilar (CIEF) son similares a la cromatografía de intercambio iónico, ya que las separaciones se basan en las diferencias de carga y tamaño entre los analitos; la electroforesis capilar en medios micelares (MECK) simula a la cromatografía en fase reversa; la cromatografía por exclusión de tamaño y la electroforesis capilar en gel separan atendiendo al tamaño molecular de las sustancias a separar, y las interacciones por afinidad usadas en cromatografía sirven también en CE.

• Tanto los sistemas de CE como los de HPLC pueden ser automatizados. Los automuestreadores y los sistemas automáticos y programas informáticos para controlar todos los pasos de un método separativo están presentes en prácticamente todos los instrumentos disponibles comercialmente.

• La automatización descrita en el punto anterior, permite incluso realizar la recolección de distintas fracciones de interés, pero el límite máximo de carga de muestra en CE ha ocasionado que la recolección o aislamiento de fracciones esté en el rango de nanogramos. Lógicamente las limitaciones en la cantidad de muestra que puede ser inyectada, también limitan la cantidad de muestra que puede ser recolectada. Como modificaciones y mejoras del proceso tecnológico se está persiguiendo un aumento en la cantidad de muestra a inyectar, ya que esto mejoraría proporcionalmente la capacidad de la CE micro-preparativa.

## Diferencias entre CE y HPLC

Aunque las semejanzas entre las dos técnicas son claras, también existen diferencias significativas que han conducido al desarrollo simultáneo de ambas.

• Una de las razones de más peso que han favorecido el desarrollo de la CE es su capacidad para facilitar la separación de macromoléculas en disolución. Los grandes biopolímeros son resistentes a las transferencias de masa y, por lo tanto, se separan más eficientemente mediante técnicas "non-partitioning" como la electroforesis, que mediante técnicas "partitioning" como la cromatografía.

Las moléculas pequeñas sí se ajustan bien a las capacidades de la cromatografía a causa de su buen coeficente de difusión y sus características en lo que a transporte de materia se refiere. Los grandes biopolímeros, como las proteínas, se ajustan mejor a las capacidades de las técnicas electroforéticas por la resistencia que ofrecen a la difusión y a la transferencia de masa. En teoría, las separaciones en CE pueden lograr eficacias cercanas a los millones de platos teóricos para moléculas grandes.

• Un factor adicional que lleva a la CE a proporcionar una mayor eficacia es el perfil del flujo. En HPLC, las fuerzas de fricción entre el sólido y el líquido dan lugar a perfiles de flujo laminares o parabólicos, existiendo un gradiente de velocidad importante que da lugar a un perfil de velocidades que es mayor en el centro y menor en las proximidades de las paredes. En CE, el flujo final tiene un perfil prácticamente plano que hace que todos los solutos, independientemente de su posición en la sección del

capilar, presenten la misma velocidad eluyendo como picos estrechos y, por tanto, con alta eficacia.

• Otra diferencia de estas dos técnicas es que no hay correlación entre los tiempos de retención en HPLC y los tiempos de migración en CE; el orden de elución no será el mismo para ambas técnicas.

• Una diferencia de las más notables se halla en el volumen requerido de muestra y disoluciones reguladoras o de separación empleadas. Mientras HPLC consume litros de fase móvil, la CE necesita sólo unos mililitros de disolución reguladora.

• Cuando la detección se realiza mediante técnicas ópticas, la longitud del paso de luz en la celda de detección en HPLC es normalmente de varios milímetros, mientras que en CE es del orden de micrómetros.

• La corrección del área en función del tiempo de migración es necesaria para llevar a cabo el análisis cuantitativo mediante la CE. Hay una tendencia generalizada que lleva a muchos analistas a cometer el error de integrar un electroferograma como si fuese un cromatograma. En HPLC, la separación de los analitos se realiza en la columna y la detección se hace una vez que estos han sido eluídos de la misma. El detector está localizado después de la columna separativa y la detección se lleva a cabo off-columna. La velocidad a la cual viajen los analitos a través del detector es independiente de la columna y de la separación; ésta sólo depende del flujo de la bomba de alta presión. Si el flujo es constante, como ocurre típicamente en HPLC, entonces todos los analitos se moverán a través del detector a una velocidad constante.

Contrastando con esto, la detección en CE es casi siempre on-columna. En este tipo de detección, el detector está localizado en algún punto a lo largo del capilar en el que la separación aún se está llevando a cabo. Como la separación se basa en que los analitos migran a diferentes ritmos, los analitos separados pasarán a través del detector a distintas velocidades. Estas diferencias de ritmo, para cada analito, deben ser consideradas cuando se desarrolle un trabajo cuantitativo.

# OBJETO Y JUSTIFICACIÓN

La relevancia de ese denominado "oro líquido" que es el aceite de oliva varía según el punto desde el que se enfoque y es diferente de un país a otro, pero es muy notable cuando se analizan las economías de ciertas regiones del mundo. 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. Nuestro país, en particular, se encuentra a la cabeza de la producción mundial y es, además el mayor país exportador del mundo. Dentro de España las zonas olivareras están bastante localizadas, de hecho, un 60% del olivar español está localizado en nuestra tierra, Andalucía, donde se produce el 75% del aceite de oliva español.

Dentro de la complejidad de la matriz aceite de oliva, los compuestos fenólicos son una familia muy importante que pertenece a la fracción insaponificable del mismo. Estos despiertan gran interés debido a su poder antioxidante, que les dota de un efecto quimio-protector en seres humanos y les hace tener gran influencia en la estabilidad a la oxidación que presenta el aceite de oliva. También influyen en las propiedades organolépticas de los aceites de oliva vírgenes. Además, su identificación y cuantificación pueden ser una buena vía para llevar a cabo la caracterización de aceites.

Por otra parte, uno de los problemas más importantes que se da hoy en día en la industria alimentaria es garantizar la seguridad y genuinidad de los alimentos a lo largo de la cadena alimentaria, mejorando la calidad de los mismos. En este sentido es necesaria la puesta a punto de herramientas de análisis más rápidas y fiables que permitan determinar y asegurar la calidad, autenticidad y trazabilidad de un alimento.

Dado lo anteriormente dicho y conocido, el interés de diferentes campos científicos en los compuestos fenólicos, y la necesidad de disponer de métodos analíticos los más rápidos, potentes, reproducibles, robustos y fiables posible, nos pareció adecuado orientar la presente tesis doctoral al estudio de los compuestos fenólicos en aceite de oliva mediante técnicas y metodologías separativas avanzadas, empleando varias de ellas para llevar a cabo del modo más satisfactorio y riguroso posible la determinación los componentes ya mencionados del aceite de oliva. Pensamos que sería útil utilizar no solo una técnica separativa, y por ello, se han usado tanto la electroforesis capilar, como

la cromatografía líquida de alta resolución. Asimismo, éstas se acoplaron a varios sistemas de detección (absorción UV-Vis y espectrometría de masas); la espectrometría de masas, podría complementar la información proporcionada por la detección óptica, dándonos información estructural muy valiosa. En lo que respecta al segundo sistema de detección emplado, se utilizaron analizadores de trampa de iones, y los modernos de tiempo de vuelo.

Con la idea de alcanzar el objetivo de caracterizar la fracción más polar del aceite de oliva, diseñamos un perfil de trabajo en el que se usaríamos varios métodos electroforéticos y cromatográficos, y se estudiarían distintos aspectos que caracterizan y afectan a la composición fenólica de los aceites, trabajando en todo momento según un protocolo científico correcto, preciso y minucioso.

De modo más pormenorizado los objetivos parciales planteados, fueron los siguientes:

- Mostrar la potencialidad de la CE (mediante el desarrollo de varios métodos electroforéticos) para la determinación y cuantificación de compuestos fenólicos en aceite de oliva.

- Ratificar la utilidad de HPLC para caracterizar y cuantificar los polifenoles presentes en el aceite de oliva.

- Comparar los resultados obtenidos mediante las dos técnicas separativas empleadas.

- Acoplar sistemas de detección modernos y potentes a CE y HPLC que nos permitan llevar a cabo le determinación estructural con errores mínimos, y detectar compuestos no descritos aún en aceite de oliva.

- Estudiar con los métodos desarrollados algunos parámetros agronómicos y tecnológicos importantes que pueden afectar a la composición del aceite de oliva en lo que a compuestos fenólicos se refiere (variedad de aceituna, procedencia geográfica, tratamiento térmico…)

- Determinar la capacidad antioxidante individual de los compuestos fenólicos mayoritarios del aceite de oliva.

- Seguir ahondando en la influencia que los compuestos fenólicos ejercen sobre las propiedades sensoriales del aceite.

# PARTE DE INVESTIGACIÓN DOCUMENTAL Y EXPERIMENTAL. DISCUSIÓN DE LOS RESULTADOS

En esta sección que lleva por título "PARTE DE INVESTIGACIÓN DOCUMENTAL Y EXPERIMENTAL. DISCUSIÓN DE LOS RESULTADOS" discutiremos los resultados obtenidos durante la realización de la presente tesis doctoral. La estructura que seguiremos para llevar a cabo dicho propósito consiste en incluir en cada uno de los capítulos una pequeña introducción en la que se explican las motivaciones que nos llevaron a la realización de ese trabajo experimental y los objetivos que se persiguieron. Tras eso, se adjunta el artículo que se publicó como consecuencia de la experimentación realizada, y en algunos casos, al trabajo, le sigue una información adicional o anexo que aporta algunas notas relevantes.

Así, el primer capítulo recopila un trabajo fruto del estudio de la bibliografía publicada hasta la fecha concerniente al análisis de polifenoles del aceite de oliva mediante distintas técnicas. Los tres capítulos siguientes se han dedicado al desarrollo de métodos electroforéticos para el análisis de ácidos fenólicos. El quinto, fue fundamental para el posterior desarrollo de otros capítulos, no sólo porque se centró en el estudio de la actividad antioxidante de los fenoles mayoritarios del aceite de oliva, sino porque nos dotó de "estándares coleccionados" mediante HPLC semi-preparativa que facilitaron el estudio de esta fracción de modo notable. A éste, le sigue un capítulo puramente electroforético en el que se desarrolla un método CE-UV que demuestra tener gran potencial y ser muy rápido, sencillo y fiable. Dicho método da lugar a los capítulos séptimo y octavo, que son aplicaciones del mismo al análisis de distintas muestras. En esos tres capítulos además de identificar nuevos fenoles mediante CE, cuantificar el contenido fenólico de los aceites (tanto de modo individual, como clasificando los fenoles en familias), y comparar dicho contenido en diversos aceites, se intentó dilucidar la relación existente entre estos compuestos y algunas características sensoriales del aceite de oliva. En el noveno capítulo, se evaluó la influencia de la oxidación térmica en la composición fenólica y en la actividad antioxidante de aceites de oliva virgen-extra empleando tanto HPLC-UV y HPLC-MS, como CE-UV. Los últimos dos capítulos combinan técnicas separativas con detección por espectrometría de masas; en el décimo, se describe la optimización y desarrollo de un método CE-ESI-IT MS que se aplica al análisis semi-cuantitativo de varias muestras de aceite de oliva español; tras éste, se cierra el trabajo experimental de la presente memoria (capítulo 11), poniendo a punto dos métodos, CE- y HPLC-ESI-TOF MS, que demuestran la

potencialidad de este nuevo detector y son capaces de determinar casi 50 compuestos en un solo análisis.

# Capítulo 1

## Metodologías analíticas para la extracción, separación y cuantificación de compuestos fenólicos en aceite de oliva

Uno de los pasos más relevantes cuando se afronta cualquier tema de investigación es la revisión bibliográfica, ya que nos debe dotar de una idea general acerca de *qué hay hecho, cómo se realizó experimentalmente y qué tendría interés hacer posteriormente*. Por eso se estimó oportuno la realización del review que compone este primer capítulo de la tesis. En él se recogen resumidamente los métodos analíticos para la determinación de compuestos fenólicos en aceite publicados hasta la fecha de su publicación utilizando distintas técnicas.

En líneas generales, un procedimiento para determinar los compuestos fenólicos del aceite de oliva lleva consigo tres pasos: la extracción de los compuestos de interés de la matriz de aceite, la separación analítica y la cuantificación de los mismos.

En lo que respecta al primer paso, la extracción, han sido descritos muchos procedimientos, en su mayoría utilizando extracción líquido-líquido y extracción sólido-líquido. Todos ellos se intentan resumir en el review, aunque también se dedican apartados a la hidrólisis (que en ocasiones es utilizada como paso que minimiza las interferencias y simplifica el ulterior análisis mediante una técnica separativa) y a la extracción con fluídos supercríticos.

Las técnicas analíticas que se recogen en esta revisión bibliográfica incluyen desde las primeras determinaciones espectrofotométricas hasta las más modernas técnicas separativas acopladas a distintos sistemas de detección. En muchos de esos métodos se logra llevar a cabo una cuantificación exitosa, aunque la comparación directa entre las concentraciones de compuestos fenólicos en aceite de oliva recogidas en bibliografía es realmente difícil de hacer, ya que no es raro encontrar que los datos difieren, incluso, en órdenes de magnitud. Estas discrepancias se pueden explicar atendiendo a que son numerosos los factores que afectan la composición polifenólica de un aceite (características genéticas de la variedad de aceituna cultivada, modificaciones tecnológicas durante el procesamiento de las aceitunas…) y a que cada método analítico expresa los resultados en un formato distinto (cuantifica todos los compuestos fenólicos respecto a un estándar (que puede ser distinto al elegido por otro autor), agrupa los compuestos por familias y las cuantifica respecto a otro patrón comercial…) debido a la ausencia de patrones comerciales para la mayoría de los compuestos fenólicos presentes en el aceite.

En nuestra opinión, el review que se adjunta a continuación, y que constituye el capítulo primero de la presente memoria, recopila de manera rigurosa, sistemática y clara, las publicaciones más relevantes con relación a los tres, antes citados, pasos esenciales en una determinación analítica de compuestos fenólicos en aceite de oliva de modo satisfactorio.

**Alegria Carrasco-Pancorbo**[1]
**Lorenzo Cerretani**[2]
**Alessandra Bendini**[2]
**Antonio Segura-Carretero**[1]
**Tullia Gallina-Toschi**[2]
**Alberto Fernández-Gutiérrez**[1]

[1]Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain
[2]Department of Food Science, University of Bologna, P.zza Goidanich 60, 47023 Cesena (FC), Italy

# Analytical determination of polyphenols in olive oils

The increasing popularity of olive oil is mainly attributed to its high content of oleic acid, which may affect the plasma lipid/lipoprotein profiles, and its richness in phenolic compounds, which act as natural antioxidants and may contribute to the prevention of human disease. An overview of analytical methods for the measurement of polyphenols in olive oil is presented. In principle, the analytical procedure for the determination of individual phenolic compounds in virgin olive oil involves three basic steps: extraction from the oil sample, analytical separation, and quantification. A great number of procedures for the isolation of the polar phenolic fraction of virgin olive oil, utilizing two basic extraction techniques, LLE or SPE, have been included. The reviewed techniques are those based on spectrophotometric methods, as well as analytical separation (gas chromatography (GC), high-performance liquid chromatography (HPLC), and capillary electrophoresis (CE)). Many reports in the literature determine the total amount of phenolic compounds in olive oils by spectrophometric analysis and characterize their phenolic patterns by capillary gas chromatography (CGC) and, mainly, by reverse phase high-performance liquid chromatography (RP-HPLC); however, CE has recently been applied to the analysis of phenolic compound of olive oil and has opened up great expectations, especially because of the higher resolution, reduced sample volume, and analysis duration. CE might represent a good compromise between analysis time and satisfactory characterization for some classes of phenolic compounds of virgin olive oils.

## 1 Introduction

### 1.1 Importance of olive oil and its differences from other vegetable oils

Olive oil has been produced for about 6000 years, but in the last thirty years there has been a growing interest in the use of olive oil in cooking because of a greater knowledge of Mediterranean food and an awareness of the healthy virtues of a Mediterranean diet, and particularly olive oil [1, 2].

Among the different vegetable oils, virgin olive oil (VOO) is unique because it is obtained from the olive fruit (*Olea europaea L.*) solely by mechanical means, without further treatment other than washing, filtration, decantation, or centrifugation [3].

Olive oil can be consumed in the natural unrefined state or as a refined product. The refined product is made either from virgin olive oil and called refined virgin oil (RVO) or from solvent-extracted oil [4] and called refined husk oil (RHO).

Its chemical composition consists of major and minor components. The major components, that include glycerols, represent more than 98% of the total weight. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1 *n*-9) ranges from 56 to 84% of total fatty acids [5], while linoleic acid (18:2 *n*-6), the major essential fatty acid and the most abundant polyunsaturate in our diet, is present in concentrations between 3 and 21% [6, 7].

Minor components, amounting to about 2% of the total oil weight, include more than 230 chemical compounds, e.g., aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and antioxidants [8]. The main antioxidants of VOO are carotenes and phenolic compounds, including lipophilic and hydrophilic phenols [9]. While the lipophilic phenols, among which are tocopherols, can be found in other vegetables oils, some hydrophilic phenols of VOO are not generally present in other oils and fats [9, 10].

**Correspondence:** A. Segura Carretero or A. Fernández Gutiérrez, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain. Phone: +34 958 243296/7 Fax: +34 958 249510.
E-mail: ansegura@ugr.es or albertof@ugr.es

**Review**

## 1.2 Phenolic compounds in VOO

Polyphenols is a broad term used in the natural products literature to define substances that possess a benzene ring bearing one or more hydroxy groups, including functional derivatives [11].

According to Harborne et al. [11] phenolic compounds are grouped into the following categories: 1. phenols, phenolic acids, phenylacetic acids; 2. cinnamic acids, coumarins, isocoumarins and chromones; 3. lignans; 4. ten group of flavonoids; 5. lignins; 6. tannins; 7. benzophenones, xanthones, and stilbenes; 8. quinones; 9. betacyanins. Most phenolic compounds are found in nature in a conjugated form, mainly with a sugar molecule.

In the case of virgin olive oils, "polyphenols" mostly refers to hydrolysis products of oleuropein and ligstroside, aglycones, and related compounds.

The phenolic fraction of VOO consists of an heterogeneous mixture of compounds, each of which varies in chemical properties and impacts on the quality of VOO [12]. The occurrence of hydrophilic phenols in VOO was observed more than 40 years ago by Cantarelli and Montedoro [13, 14]. They established a set of research priorities related to polyphenols which remain practically unchanged to this day:

– Development of an analytical procedure to quantify phenolic compounds in oils.
– Estimation of the levels of phenolic compounds in vegetables oils.
– Possible relationship between these compounds and the characteristics of the olive fruit (variety, degree of ripeness).
– Effect of extraction technology and refining process on the level of polyphenols.
– Importance of phenolic compounds as natural antioxidants.
– Possible role of polyphenols in justifying why olive oils with high peroxide values have considerable stability.

It has not been easy to satisfy these points and many researchers are still working on them. However, very interesting systematic studies of the individual classes of hydrophilic phenols in VOO have been developed recently, and it is possible to say that the composition of VOO is today largely elucidated.

VOO contains different classes of phenolic compounds such as phenolic acids, phenolic alcohols, flavonoids, hydroxy-isocromans, secoiridoids, and lignans as reported in **Table 1**.

**Phenolic acids** with the basic chemical structure of C6-C1 (benzoic acids) and C6-C3 (cinnamic acids), such as caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic, and *p*-hydroxybenzoic acid, were the

first group of phenols observed in VOO [15, 16]. Several authors confirmed the occurrence of phenolic acids as minor components in VOO [17–23].

The prevalent phenols of VOO, however, are the **secoiridoids**, that are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure [24]. These compounds, e.g., oleuropein, demethyloleuropein, and ligstroside, are derivatives of the secoiridoid glucosides of olive fruits. Breakdown products of two major phenolic constituents of the olive fruit, oleuropein and ligstroside, form the majority of the phenolic fraction.

The most abundant secoiridoids of VOO are the dialdehydic form of elenolic acid linked to hydroxytyrosol = (3,4-dihydroxyphenyl)-ethanol or tyrosol = (*p*-hydroxyphenyl)-ethanol (3,4-DHPEA-EDA or *p*-HPEA-EDA) and an isomer of the oleuropein aglycone (3,4-DHPEA-EA). For the first time, these compounds were found by Montedoro et al. [20,25] who also assigned their chemical structure [26]. Later these structures were confirmed by other authors [27]. Recently, oleuropein and ligstroside aglycone were also detected as minor phenolic components in VOO [28, 29].

Hydroxytyrosol and tyrosol are the main **phenolic alcohols** in VOO. It is also possible to find in VOO hydroxytyrosol acetate [30], tyrosol acetate [31], and a glucosidic form of hydroxytyrosol [32].

Several authors have reported that **flavonoids** such as luteolin and apigenin were also phenolic components of VOO [33, 34]. (+)-Taxifolin, a flavanonol, has recently been found in Spanish virgin olive oil [23].

The last group of phenols found in VOO are the **lignans**; Owen et al. [28,35] and Brenes et al. [36] have recently isolated and characterized (+)-1-acetoxypinoresinol, (+)-pinoresinol, and (+)-1-hydroxypinoresinol as the most frequent lignans in VOO. Lignans are also found as prevalent phenolic compounds in VOO.

A new class of phenolic compounds, **hydroxy-isochromans**, was found in different samples of extra-VOO. In particular, the presence of 1-phenyl-6,7-dihydroxy-isochroman and 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman has been demonstrated [37].

## 1.3 The family of polyphenols in the minority compounds and their antioxidant, health, and sensory properties

The antioxidant potential of phenolic compounds in olive oil has also been a subject of considerable interest, both because of its chemoprotective effect in human beings [38–43] and because it is a major factor in the high stability (shelf-life) of olive oils [42–46]. The antioxidant activity of VOO components has been related to the pro-

**Table 1**. Phenolic compounds in virgin olive oil and their chemical general structure.



| Phenolic compounds in virgin olive oil | |
|---|---|
| **Phenolic acids** **Benzoic acid derivatives** Gallic acid Gentisic acid Benzoic acid Vanillic acid Protocatecuic acid *p*-Hydroxybenzoic acid Syringic acid **Cinnamic acid derivatives** Caffeic acid *p*-Coumaric acid *o*-Coumaric acid Ferulic acid Cinnamic acid Sinapinic acid **Other phenolic acids and derivatives** 4-(Acetoxyethyl)-1,2-dihydroxybenzene | **Hydroxy-isocromans** 1-phenyl-6,7-dihydroxy-isochroman 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman |

Dopac (3,4-dihydroxyphenylacetic acid)
4-hydroxyphenylacetic acid

| | |
|---|---|
| **Phenolic alcohols** (3,4-Dihydroxyphenyl)ethanol (3,4-DHPEA) *p*-Hydroxyphenyl)ethanol (*p*-HPEA) (3,4-Dihydroxyphenyl)ethanol-glucoside 2-(4-hydroxyphenyl)ethyl acetate | **Flavonoids** **Flavones** Apigenin Luteolin **Flavanonol** (+)-Taxifolin |
| **Secoiridoids** Oleuropein Dialdehydic form of oleuropein aglycon Dialdehydic form of ligstroside aglycon Ligstroside aglycon Oleuropein aglycon (3,4-DHPEA-EA) Dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA (3,4-DHPEA-EDA) Dialdehydic form of decarboxymethyl elenolic acid linked to *p*-HPEA (*p*-HPEA -EDA) | **Lignans** (+)-1-Acetoxypinoresinol (+)-Pinoresinol (+)-1-Hydroxypinoresinol |

Dialdehydic forms of secoiridoids derivatives

tection against important chronic and degenerative diseases such as coronary hearth diseases (CHD), ageing neuro-degenerative diseases, and tumours of different localizations [47−49]. Among these protective effects, it possible to highlight, for example, the protection of low density lipoprotein (LDL) oxidation [50]; the reduced oxidative damage of the human erythrocytes by 3,4-DHPEA [51] and the reduction of free radical production in the faecal matrix [52]. Moreover, in several studies, it was affirmed that the phenolic substances isolated and purified from olive oil were much more potent antioxidants than the classical in-vivo and in-vitro free radical scavengers, vitamin E and dimethyl sulfoxide [28, 52−54].

Polyphenols also contribute to the organoleptic properties of VOOs and are commonly described as bitter and astringent [44, 55−57] and responsible for organoleptic characteristics in general [58]. Less commonly, polyphenols are associated with pungency, that is, peppery, burning, or hot sensations [8, 44,59]. However, the relationships between individual hydrophilic phenols of VOO and its sensory characteristics are not totally defined. For instance, several authors associated the off-flavour note of "atrojado" with the presence of phenolic acids in VOO [60], but other studies did not show any relation between bitter sensory note and phenolic acid content in a VOO [61]. The relations between the secoiridoid derivatives and the bitterness of VOO have also been studied; first, interest was focused on two derivatives of oleuropein and demethyloleuropein, such as 3,4-DHPEA-EDA and *p*-HPEA-EA [62, 63]. In this case, García et al. [63] studied the reduction of oil bitterness by heating of olive fruits, and a good correlation between oil bitterness and content of hydroxytyrosol secoiridoid derivatives was found. In later studies it was observed that a relation exists between the bitter and pungent sensory properties and ligstroside derivative content [64] the content of the aldehydic form of oleuropein aglycone [65].

## 1.4 Importance and difficulties of quantification of phenolic compounds in virgin olive oils

The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by the agronomic and technological conditions of its production.

Several agronomic parameters can modify the phenolic concentration of VOO. The most widely studied aspects include cultivar, fruit ripening, pedoclimatic conditions, and some agronomic techniques such as irrigation [66–68]. As reported by different authors the phenolic composition of fruits is affected qualitatively by the cultivar.

Since the occurrence of hydrophilic phenols in VOO is strictly related to the activities of various endogenous enzymes of olive fruits, their concentration in the oil is strongly affected by the extraction conditions. Crushing and malaxation are the most important critical technological points of the mechanical oil extraction process [69–71].

For all these reasons, the identification and quantification of the individual components of VOO are of great interest.

Many analytical procedures directed towards the determination of the complete phenolic profile have been proposed (spectrophotometric methods; biosensors; paper chromatography, TLC, GC with different detectors, and HPLC coupling with several detection systems; NMR and IR techniques for the characterization and identification of these compounds; capillary electrophoresis (CE)); however, the variety of extraction techniques, chromatographic conditions, and methods of quantification have contributed to differences in reported levels of virgin olive oil phenolics.

Direct comparison between the concentrations of olive oil phenols reported in the literature is difficult, as the reported concentrations often differ greatly (sometimes even in orders of magnitude). Several authors have explained this by the fact that there are numerous factors which affect phenolic compounds of VOO, such as various genetic characteristics of the olive cultivar [44] or technological modifications during processing the olives [72]. These reasons may partly, but not completely, explain these discrepancies. Pirisi and co-workers raised the question whether the discrepancies may be caused by the various analytical methods used and/or the expression of the results in various formats [73].

In fact, individual phenolic compounds give different responses during UV detection after their HPLC separation [31]. Another cause of confusion is the use of different standard equivalent units in the case of the Folin-Ciocalteu colorimetric assay for total phenolics, depending on the chosen calibration curve (e.g., caffeic acid, gallic acid, syringic acid, tyrosol, oleuropein equivalents).

It would be really interesting, as Tsimidou proposed [44], to perform a possible collaborative study using the same analytical method to ensure that the differences in magnitude of the phenol content depend mainly on the variety. Two years later, Pirisi et al. [73] stated that before such a study can be undertaken, it is necessary to study, in detail, the influence of differences in milling conditions on the polyphenol content of oils.

Despite a general recognition of the problems associated with the analysis and quantification of phenolic compounds in olive oil, only recently has a paper been published with the objective of highlighting the differences between the various units used for expressing levels of "olive phenolics" [74].

In general, an analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, analytical separation, and quantification. These steps will be explained in later sections of this article.

## 2 Sample preparation

### 2.1 Hydrolysis

In a number of instances, an hydrolysis step has been included to minimize interferences in the subsequent chromatography and to simplify chromatographic data, particularly in instances where appropriate standards are commercially unavailable.

Acid hydrolysis has been the traditional approach to measurement of aglycones and phenolic acids from flavonoid glycosides and phenolic acid esters, respectively.

However, two forces have driven the use of alkaline hydrolysis. First, commercial processing of many plant-derived foods now involves alkali-treatment and the stability of plant phenols under these conditions becomes of interest. For instance, the major characteristic phenols of olive are secoiridoids and their reactivity in alkali has been examined [75].

Several examples showing the use of alkaline or acid hydrolysis of the polar fraction of olive oil are mentioned in this review [18, 20, 97].

### 2.2 Extraction of phenolic compounds from virgin olive oils

Isolation of phenolic compounds from the sample matrix is generally a prerequisite for any comprehensive analysis scheme although enhanced selectivity in the subsequent quantification step may reduce the need for sample manipulation. The ultimate goal is the preparation of a sample extract uniformly enriched in all compounds of interest and free from interfering matrix components [76].

A great number of procedures for the isolation of the polar phenolic fraction of VOO utilizing two basic extraction techniques, LLE or SPE, have been published in the literature. Various isolation systems have been proposed by different authors, depending, among other aspects, on the aim of the particular study. The systems do not vary only in solvents and/or solid-phase cartridges used, but

also in the amount of the sample taken for analysis, volumes of solvents, and further details [74].

In addition, the phenolic fraction of VOO consists of an heterogeneous mixture of compounds, which are, in most of cases, not commercially available. This represent a problem in calculating the recovery of an isolation technique. Therefore, other similar phenolic compounds have been frequently used in the past. In recent studies, to overcome this obstacle, a refined phenolics-free olive oil is spiked with an exactly specified dose of a phenolic extract that is prepared by the extraction of VOO.

### 2.2.1 Liquid-liquid extraction

Traditionally, the phenolic fraction of olive oil has been isolated by extraction of an oil solution in a lipophilic solvent with several portions of methanol [28, 35] or methanol/water (with different levels of water that range between 0 and 40% [16, 18–21, 77]), followed by solvent evaporation of the aqueous extract and a cleanup of the residue by solvent partition [20, 23, 30, 78–83]. The most commonly used solvent has been hexane, but petroleum ether and chloroform have also been proposed; however, the addition of hexane or other organic solvents to the oil before extraction did not yield significant differences in the phenol recovery efficiency [20].

Tensioactive substances (e. g., Tween 20 (2% *v/w*)) have repeatedly been used to liberate the phenolic compounds of the lipoprotein membranes [20,84].

Extraction with tetrahydrofuran/water followed by centrifugation has also been performed [85], as well as extraction with *N,N*-dimethylformamide [86].

Montedoro et al. [20] examined the extractive methods for simple and hydrolyzable phenolic compounds in VOO, studying different schemes where olive oil was dissolved in various solvents, and the volume and the percentage of these solvents were changed. The best results were obtained using methanol/water (80:20, *v/v*) in agreement with data reported in the literature [84, 87] and optimum extraction was achieved on extracting 100 g of olive oil with two 20-mL volumes of solvent. However, several years later Angerosa et al. [88] reported results that were in contrast with these; the incomplete recovery of some components and the considerable emulsion formation between the oil and the methanol/water layer prompted them to choose chose neat methanol as the extraction solvent.

Cortesi et al. [85] examined the extraction of the polar fraction of olive oil with tetrahydrofuran/water (80:20, *v/v*) followed by centrifugation, and they concluded that the recoveries were 5 times higher with this method in terms of hydroxytyrosol and 2 times for tyrosol than with methanol/water (60:40, *v/v*).

The used of *N,N*-dimethylformamide seemed to show interesting results in terms of recovery efficiency and sample manipulation [86].

After the liquid-liquid extraction process in order to isolate the desired analytes from unsaturated, interfering species, residual oil is removed by overnight storage at sub-ambient temperature [88], by centrifugation or by further extraction with hexane, although Sephadex column [20, 25] and Policlar AT: Celite 560 (1:2) [17] chromatography have also been used to effect further clean-up.

### 2.2.2 Solid-phase extraction

The versatility of SPE has been exploited for the recovery of phenolic compounds from olive oil and various systems employing SPE, either as isolation or clean-up step, have been reported in the literature.

Some of the suitable sorbents are alkylsilicas, such as $C_8$ [73, 89] or $C_{18}$ [22, 90](but incomplete extraction of the phenolic fraction [91] and partial oil separation [73] have been reported). Despite the common assumption that $C_{18}$ phase is less suitable for the isolation of polar components from a non-polar matrix than normal-phase SPE, $C_{18}$-cartridges have often been tested for isolation of phenols from VOO [92–94]. Anionic exchange cartridges have been also used to isolate the phenolic fraction from various seed oils, but recoveries were low (53–62%) for some components [95]. Promising results were obtained by Mateos et al. [31], who worked with amino-phase cartridges and diol-bond phase SPE cartridges and found for the latter high recovery (>90%) of all major olive phenolic compounds.

In doped refined olive oil samples the recoveries were studied using a $C_{18}$-cartridge with total supression of residual silanol groups ($C_{18 EC}$, end capped) [96]. In this study the discrimination between $C_{18}$ and $C_{18 EC}$ was not so vast as expected since the two stationary phases differ only in the presence of free silanol groups; however, their presence ($C_{18}$) seems to improve the release mechanism, increasing the recovery.

Anyway, in all the cases, one of two experimental approaches was used. In one procedure, a solution of the oil in hexane was applied [22] to a pre-conditioned (typically reversed-phase) cartridge which was washed with hexane-ethoxyethane or hexane-cyclohexane [89] mixtures to remove the non-polar lipid fraction. Phenols were then eluted with acetonitrile or methanol. Alternatively, the polar fraction of olive oil has been partitioned into aqueous methanol from a hexane solution [97] and fractionated into two parts (A and B) by SPE. Analysis of the two fractions show that Part A (eluted from Sep Pak $C_{18}$ with methanol/water (50:50 *v/v*)) contains only simple phenols and phenolic acids, while Part B (eluted with mixtures of methanol/chloroform) has a complex nature.

It is possible to find some papers where the authors use a combination of LLE and SPE in the same sample preparation protocol. For instance, Buiarelli and co-workers [98] extracted phenolic acids of olive oil with borate buffer at pH 9.2. Then the extraction buffer solution was loaded on a phenyl cartridge, and the eluent of this step was acidified to pH 2.6 and loaded on a $C_{18}$ cartridge. Plant phenols are ionizable with typical $pK_a$ values ranging from 8 to 12 and oil/water partition coefficients ranging from $6 \times 10^{-4}$ to 1.5. Thus, they exhibit considerable diversity in terms of acidity as well as polarity, ranging from hydrophobic to hydrophilic in character. The range of physicochemical behaviour must be considered when determining sample handling strategies such as, for example, in pH control to ensure favourable partitioning behaviour during extraction [99]. Another example of a similar process has been reported [100]. In a recently published paper, two different methods were necessary for the complete quantification of phenolic compounds, a methanolic extraction and an extraction which included a SPE cleaning step [101].

Summarizing all the points raised so far in this section, we can say that a reasonable performance of $C_{18}$-phase could generally be demonstrated for simple phenols; however, poor recovery has been found for secoiridoid derivatives, especially dialdehydic forms [22, 86, 92, 93]. In a recently published study [102], a group of 15 phenolic compounds (one of which occurs naturally in VOO) were extracted in order to study the recoveries of each analyte, and this work showed that LLE gave the best results in terms of recovery of the phenolic standard mixture added to the refined peanut oil. In the same study, diol-SPE showed higher recoveries of total phenols, o-diphenols, tyrosol, hydroxytyrosol, and secoiridoids than the other extraction procedures ($C_8$-SPE; $C_{8\,mod.}$-SPE; and $C_{18}$). This is in accordance with another published work [74], where the experimental work demonstrates that the application of LLE led to significantly recovery of total phenolic compounds (93%) than SPE-diol (68%) and SPE-$C_{18}$ (38%). These total averages were calculated by spiking a refined phenolics-free olive oil with an exactly specified dose of a phenolic extract that was prepared by the extraction of VOO.

### 2.2.4 Supercritical fluid extraction

Supercritical fluid extraction (SFE) was developed in the 1960s and, in recent years, has acquired some relevance for the extraction of polyphenols from plant sources. The main advantage of SFE is that it combines the characteristics of gases and liquids for extraction. The low viscosity of the supercritical fluids confers a high capacity for diffusion and improves access to phenolic compounds bound to the cell wall [103]. Moreover, their relatively high density confers a high solvation power, which greatly facilitates the extraction process. Furthermore, it minimizes

any possible degradation processes [104], such as oxidations or isomerizations, that may occur with other more conventional extraction techniques, because it reduces extraction time and because the process can be carried out in the absence of light and air.

The extraction behaviour of phenolic compounds covering a range of polarities has been modelled using supercritical carbon dioxide and an inert support as a sample matrix [105]. Extraction and collection variables were optimized and revealed that the use of methanol as modifier was mandatory. Dynamic SFE produces clean extracts with higher recoveries of total phenols from dried olive leaf [106] than sonication in liquid solvents such as n-hexane, ethoxyethane, and ethyl acetate. However, the extraction yield obtained was only 45% of that obtained with liquid methanol.

The use of this extraction system is still rare for the extraction of phenolic compounds of olive oil; perhaps future studies will make this extraction system more common.

## 3 Spectrophotometric determination: advantages and disadvantages

One method that is widely used for the quantitative determination of total phenols in VOO is colorimetric assay, based on the reaction of Folin-Ciocalteu reagent with the functional hydroxy groups of phenolic compounds [107, 108]. The method consists of calibration with a pure phenolic compound, extraction of phenols from the sample, and the measurement of absorbance after the color reaction. The popularity of this assay can mainly be attributed to its simplicity and speed of analysis [109].

The major disadvantage of the colorimetric assay is its low specificity, as the color reaction can occur with any oxidizable phenolic hydroxy group. Recently an interesting approach to the content of total extractable phenolic compounds in different food samples involving comparison of chromatographic and spectrophotometric methods has been reported, accounting for the possible influence of other substances as interfering compounds [110].

Furthermore, the method does not distinguish between individual compounds differing in molar mass (ranging from 138 to 416 g/mol in the case of the major olive oil phenols) and structure (i. e. the number of active hydroxy groups) [74].

Thus, samples with comparable total phenolic content, but widely varying phenolic composition, will give a different response in the colorimetric method.

However, Singleton et al. [111] have shown that the molar absorptivity per reactive hydroxy group is comparable in compounds with otherwise similar structure. The molar absorbance of an olive phenol therefore primarily depends on the number of hydroxy groups, with mono-

phenolic compounds leading to half of the color complex formation as compared to diphenolic compounds and therefore displaying approximately half of the molar absorbance. That means that the values obtained by the colorimetric assay can provide direct information on the amount of antioxidant phenolic groups in olive oil extracts. They are consequently related to the oxidative stability of VOO [109].

### 3.1 From Folin-Denis to Folin-Ciocalteu reagent

The original paper by Folin and Denis [112] described the significant parts of this procedure with the use of Folin-Denis reagent. Several features could cause inconvenience or possible error. Saturated sodium carbonate varies in composition with temperature, and equilibrium is not reached rapidly from the super-saturated condition. Beer's law might not be obeyed over a sufficiently wide range, and, in particular, abnormally low results were obtained if samples of too high absorbance were diluted with a blank solution instead of repeating the entire assay procedure after further dilution. If samples were inadequately mixed between additions or if reactants were added in the wrong sequence, results were erratic. Depending upon temperature and other factors, the blue color might be formed or disappear at an appreciable rate after 30 minutes and produce erratic results, or a required rigid time schedule might be difficult to maintain with a large group of samples. And, most serious of all, a precipitate might form slowly and will affect results unless noticed and removed.

For all these reasons, these studies were followed by a long series of papers by Folin and his co-workers and by others noting problems and suggesting improvements in the original methods. Modifications to the phenol reagent itself culminated in the report by Folin and Ciocalteu [113]. The modifications to the procedure consisted mainly in a longer heating period, the presence of hydrochloric acid, and the addition of lithium sulfate. The Folin-Denis reagent contains two classes of substances, one more sensitive than the other to reduction, whereas the Folin-Ciocalteu reagent has a greater incorporation of molybdate into the complex, giving the form highly sensitive to reduction [114]. Folin-Ciocalteu phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids, in which the molybdenum and tungsten are in the $6^+$ state. On reduction with certain reducing agents, so-called molybdenum blue and tungsten blue are formed, in which the mean oxidation state of the metals is between 5 and 6. As the reduced Folin-Ciocalteu reagent is blue, it is possible to carry out the detection with an spectrophotometer in the range of 500 to 750 nm.

Several studies confirm that the Folin-Ciocalteu reagent is preferable to the Folin-Denis reagent for several reasons; the former is expected to give a better estimate of total phenolic content. This method is considered the method of choice for estimating total phenol content in complex plant products.

### 3.2 Protocol of spectrophotometric determination of total phenolic content

The determination of total phenolic content is based on the previously mentioned procedure of Folin-Ciocalteu [108]. For example, a typical protocol using this method could be as follow [115]: An aliquot of the aqueous-methanolic solution of phenolic compounds extracted from a VOO (from $\approx 2$ g of olive oil) is diluted in 6 mL of water, followed by the addition of 0.5 mL of Folin-Ciocalteu reagent. After 1 min, 2 mL of sodium carbonate solution (15% *w/v*) is added to the reaction mixture, which is finally mixed and diluted with water to 10 mL. The absorbance of the solution is measured after 2 hours against a blank sample (the same protocol but using 0.1 mL of methanol/water (50:50 *v/v*)) at a wavelength of 750 nm. In this case the calibration curve is constructed using standard solutions of gallic acid within the range of 0.01 to 1 mg/mL.

Several modifications of this method have been made by different authors [107, 116, 117].

### 3.3 Spectrophotometric determination of *o*-diphenols

In 1973, Vázquez Roncero et al. [83] emphasized the great variation in the levels of polyphenols in Spanish olive oil and pointed out that there was a good correlation between polyphenols and the oxidative stability of the oil. Several years later, Gutfinger et al. [107] also found a good relationship between olive oil stability and the content of total phenols or total *o*-diphenols.

After this, many authors studied both the spectrophotometric determination of total phenols and the spectrophotometric determination of *o*-diphenols. A typical protocol could be [31]: the methanolic extract obtained from olive oil after the use of the extraction system (using $\approx 2.5$ g of olive oil) is evaporated, the residue dissolved in 10 mL methanol/water (50:50 *v/v*), and the solution filtered. A mixture of 4 mL of the solution with 1 mL of a 5% solution of sodium molybdate dihydrate in ethanol/water (50:50 *v/v*) is shaken vigorously. After 15 min, the absorbance at 370 nm is measured. A blank is obtained by measuring a mixture of 4 mL of phenolic solution with 1 mL of ethanol/water (50:50 *v/v*) [118].

Several modifications of this method have been made by different authors [115, 119].

# 4 Chromatographic determination of the phenolic profile of virgin olive oils

The need for profiling and identifying individual phenolic compounds necessitates the replacement of traditional methods by high-performance chromatographic analyses. Separation is commonly achieved by HPLC, although GC is used in some instances. The most common mode of separation exploits reversed-phase systems typically with $C_{18}$ columns and various mobile phases. Detection is routinely achieved by ultraviolet absorption often involving a photodiode array detector although the versatility of the latter often appears to have been neglected.

Coupled techniques, particularly various mass spectral methods, are being used increasingly for routine work although analyte collection using preparative-scale HPLC and off-line identification are often still needed for non-routine samples.

## 4.1 Thin-layer chromatography

Paper and thin-layer chromatography (TLC) methods have been devised and exploited for the preliminary separation/clean-up of phenolic extracts of VOO.

In the past, paper chromatography had limited application to the separation of phenolic compounds of VOO [120]. However, the separation of approximately 20 phenolic compounds or several group of phenols from olive oil using TLC on silica, cellulose, and polyamide has been described. Elution of the compounds of the polar extract of olive oil has been done by dimensional-TLC using different systems:

n-Butanol/acetic acid/water (4:1:5) (System A) and water (System B); toluene/ethyl formate/formic acid (5:4:1) (System A) and acetic acid (2%) (System B); benzene/methanol/water (80:1:1) (System A) and benzene/methanol/water (45:8:4) (System B) [15, 84].

Ragazzi et al. [121] used TLC with cellulose and polyamide as stationary phases. In the first case, the mobile phase was as follows: chloroform/acetic acid/water (8:2:1) or n-butanol/acetic acid/water (6:2:1), while in the second case, they used methanol/acetone/water (3:1:1) or methanol/acetone/water (6:1:1).

## 4.2 GC and GC-MS analysis of phenolic compounds from olive oil

The qualitative and quantitative determination of phenolic compounds from olive oil can be accomplished by capillary gas chromatography (GC) of these compounds or their derivatives. GC in general assumes that the compounds injected are volatile at the temperature of analysis and that they do not decompose at either the temperature of injection or that of analysis. In standardized analytical methods, flame ionization detection (FID) is most widely used. Mass spectrometry (MS) allows acquisition of molecular mass data and structural information, and identification of compounds.

The first paper about the separation of phenolic compounds of olive oil by GC was published by Janer del Valle et al. [122] 25 years ago. At the same time, this technique was also used by Solinas et al. [77,123], who used GC for identification of mixtures between virgin olive oils and refined oils. In 1987, Forcadell et al. [124] developed a protocol for the preparation of trimethylsilyl (TMS) derivatives. In the same year, Solinas [19] published a paper on development of a GC method for the qualitative/quantitative evaluation of phenolic compounds in VOOs from different cultivars at diverse degrees of ripeness. In this work, some phenolic compounds were found in all cases although their amounts varied in each variety. Ratios between some of these phenolic compounds seemed to be constant during olive maturation, so that their levels could be used as varietal markers. The methodology involved liquid-liquid extraction of the phenolic compounds, clean-up of the methanolic extract followed by an azeotropic distillation to remove the solvents, low-pressure column chromatography to clean-up the extracts, and finally capillary GC analysis of the trimethylsilyl derivatives. Although the method permitted characterization of the simplest compounds, other linked phenols occurring in large quantities were not identified.

Improvement in the identification of compounds was obtained with sophisticated analytical techniques such as GC-MS [79, 125] and GC-MS/MS [126]. For instance, Angerosa et al. [88] used a modified extraction procedure followed by capillary GC-MS to identify the simple and the linked phenols present in VOO.

Compared with direct inlet mass spectra, the GC-MS data, in general, exhibit the same typical fragmentation patterns but with slight differences in intensities. In instances where the classical mass spectrometric gas phase ionization techniques such as EI and CI are unsuitable (e.g., with polar, non-volatile, and thermolabile phenols), chemical derivatization usually involving silylation may overcome these limitations but can introduce further difficulties by increasing the molecular mass of the analyte possibly beyond the range of the mass analyzer [104]. Derivatization also often produces mixtures of partially derivatized compounds [27] from a single analyte.

Nevertheless, in the last years, the qualitative/quantitative analysis of the phenolic profile of VOO by GC has been used both in analytical [96] and in applied [79,126–128] work; however, the other chromatographic methods are more often employed because they avoid the use of derivatizing reagents. Another problem of this technique, as

**Table 2**. GC conditions for determination of phenolic compounds in olive oil.

| Temperature range | Column characteristics | Analysis time | Derivatization | Detector system | Observations | References |
|---|---|---|---|---|---|---|
| Progam 1: 160–250°C Program 2: 130–250°C | Capillary column SE-30, 3% on Chromosorb W HP 2 m, 4.0 mm ID 80–100 mesh | 120 min | Trimethylsilylation (Use of 3 different compounds: TRI-SIL (HMDS/TMCS in Pyridine (2 : 1 : 10); TRI-SIL/BSA P (BSA and Pyridine); TRI-SIL Z(TMSI in dry Pyridine)) | FID | TY, HYTY and phenolic acids | Janer del Valle et al. [122] |
| 140–260°C | OV 17, OV 101 and SE 30 (1 : 1 : 1) 3% on Chromosorb W HP; 100–200 mesh | 120 min | Silylation | FID | Phenolic acid and simple phenols. TY, HYTY and 4-hydroxyphenylacetic acid represented about 50% of total phenol content. HPLC analysis too | Solinas et al. [77, 123] |
| 60–270°C | Capillary column 25 m, 0.3 mm ID | 45 min | Silylation | FID; MS | Use of TLC for separating polar fraction of olive oil | Cortesi et al. [18] |
| 70–275°C | Capillary column SE 52 15 m, 0.15 μm ID | 107.5 min | Trimethylsilylation | FID | Study of relation between oxidation products and negative organoleptic note | Solinas et al. [19] |
| 70–275°C | Capillary column SE 54 25 m, 0.32 μm ID 0.10 μm film thickness | 108 min | Trimethylsilylation | FID; MS; NMR | The presence of a ligstroside aglycon containing no carbomethoxy group and oleuropein aglycone derivatives was evidenced | Angerosa et al. [88] |
| 40–320°C | DB5 MS Capillary column 30 m, 0.25 μm ID 0.25 μm film thickness | ≈60 min | Trimethylsilylation (bis(trimethylsilyl)trifluoroacetamide (BSTFA)) | MS (CI); NMR | Phenolic and secoiridoids aglycones | Angerosa et al. [27] |
| 70–280 °C | Supelco silica capillary column SE-54; 30 m, 0.25 μm ID 0.25 μm film thickness | 50 min | Trimethylsilylation (Derivatives were produced with BSTFA) | MS | Study of the evolution of phenolic compounds in VOO during storage. HPLC analysis too. | Cinquanta et al. [66] |
| 70–270 °C | SPB-5 fused-silica capillary column; 30 m, 0.32 μm ID 0.10 μm film thickness | 110 min | Trimethylsilylation (bis(trimethylsilyl)trifluoroacetamide) | FID; MS | Comparative study of SPE using C18 and C18 EC. TY, HYTY, phenolic acids and oleuropein aglycone and ligstroside aglycone. | Liberatore et al. [96] |
| 70–300°C | HP1 capillary column (Hewlett-Packard) of 30 m × 0.32 mm ID 0.10 μm film thickness | ≈95 min | Trimethylsilylation (trimethylsilyl ethers were obtained with a silylation mixture made up of pyridine, hexamethyldisilazane and trimethylchlorosilane (2 : 1 : 1)) | FID; MS (ES) | HYTY, TY, oleuropein, oleuropein aglycones and oleoside-11-methyl ester in olive fruits | Marsilio et al. [129] |
| 60–275 °C | J&W DB-5MS column, 30 m × 0.25 mm ID 0.25 μm film thickness | ≈30 min | Derivatization with BSTFA : trimethylchlorosilane (TMCS) (99 : 1) | MS; MS/MS | Phenolic compounds in Sicilian olive oils. LLE (methanol/water (80 : 20 v/v). TY, HYTY, decarbomethoxy ligstroside and oleuropein aglycones in the dialdehydic forms were the most abundant compounds | Saitta et al. [130] |

mentioned before, is that the use of high temperature could damage the analytes. **Table 2** shows some GC temperature ranges used, type of derivatization, characteristics of the column employed, and several observations about several published works, as well as the time of analysis in each case. We have included all the GC references mentioned during this review and two new references [129, 130]. **Figure 1** depicts an example of a chromatogram of phenolic extracts of a VOO, showing the presence of interferences represented by the TMS derivatives of some fatty acids.

### 4.3 HPLC and LC-MS analyses

The limited volatility of many phenols has restricted the use of GC for their separation, so HPLC currently represents the most popular and reliable technique for analysis of phenols.

HPLC is normally used for separating non-volatile, high-molecular-mass constituents, in either adsorption or partition mode. Adsorption chromatography, namely normal phase chromatography, is widely used to separate classes of constituents according to the nature and number of polar functional groups [131]. In normal phase HPLC the absorbent is silica gel and the eluent is a nonpolar solvent. Initial experiments were done using this mode [132], but in the same period the best results, in terms of reproducibility of retention time and separation of the most polar compounds, were obtained using reverse-phase HPLC [60, 123, 133, 134].
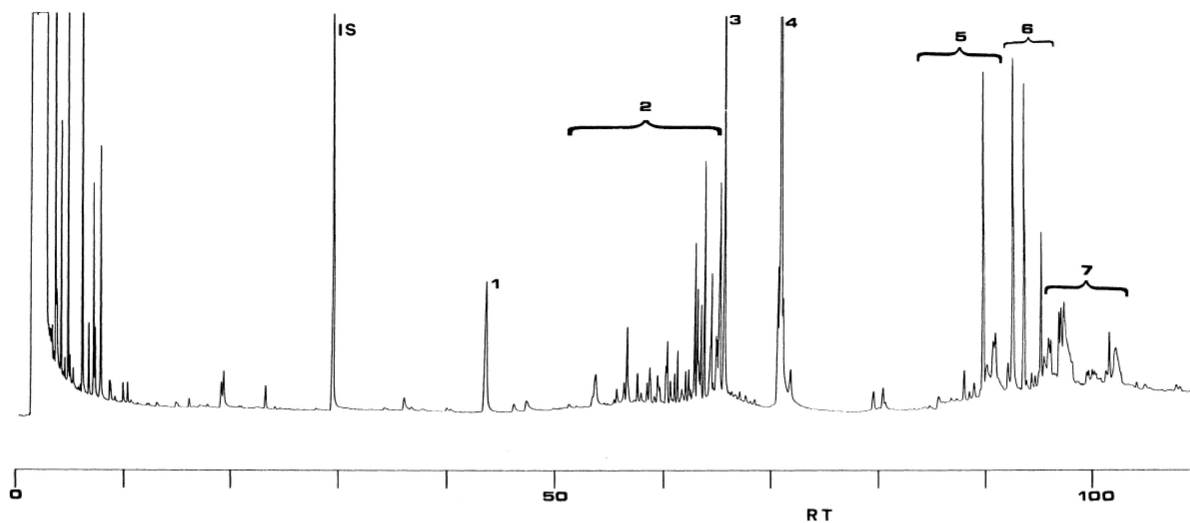
**Figure 1.** Example of a GC chromatogram of a phenolic extract of virgin olive oil. Key: I.S. resorcinol (internal standard); **1**, tyrosol; **2**, hydroxytyrosol and phenolic acids zone; **3**, $C_{16:0}$ fatty acid TMS derivative; **4**, $C_{18:0}$, $C_{18:1}$, and $C_{18:2}$ fatty acids TMS derivatives; **5**, ligstroside aglycone zone; **6**, monoglyceryl TMS derivatives zone; **7**, oleuropein aglycone zone. From reference [96] with permission of the authors.

Reverse-phase HPLC, which is based on partition chromatography, is used to separate individual components that belong to one constituent class [131]. In this case, the stationary phase usually consists of a non-polar octadecylsilane ($C_{18}$) bonded phase, while the mobile phase is a polar solvent.

Columns are commonly 100 to 300 mm in length with 10 $\mu$m or 5 $\mu$m packings, shorter columns being given preference.

In some instances, isocratic elution has provided adequate resolution due to the selectivity effects of one or more components (e. g., acetonitrile) of the mobile phase, although gradient elution has usually been mandatory in recognition of the complexity of the phenolic profile of most samples. Numerous mobile phases have been employed, but binary systems comprising an aqueous component and a less polar organic solvent such as acetonitrile or methanol remain common. Acid (acetic, formic, or phosphoric acid) is usually added to both components to maintain a constant acid concentration during gradient runs. The decrease of pH helps to avoid the dissociation of phenolic compounds, improving also the asymmetry of the peak and reducing the peak-tailing [135].

For example, a method available for analyzing the phenolic fraction is reversed-phase HPLC using isocratic elution with an aqueous solution of sulfuric acid-acetonitrile [89] or with methanol-aqueous acetic acid [136]. The technique, as stated before, has also been applied using gradient elution with methanol-aqueous acetic acid [80] or acetonitrile-aqueous acetic acid [137].

Routine detection in HPLC is typically based on measurement of UV-Vis absorption at 225, 240, or 280 nm. Because some phenolic compounds show several absorption maxima, the use of simultaneous multiple UV (photodiode array) is recommended for identification purposes [20, 67, 89]. Indeed, there are significant differences in absorption maxima and molar absorptivities [80] of even the major phenols identified in a single olive fruit. This creates problems in quantification as discussed by Tsimidou et al. [80] who classified the various phenols into four groups and used a single calibration standard for the members of each group. The most commonly used wavelength for routine detection has been 280 nm which represents a suitable compromise, although detection at other wavelengths and dual wavelength [20] detection have been applied. The advantages of low wavelength detection at 225 nm have been demonstrated [89] but problems associated with high background absorption of typical mobile phases in RPLC have limited its use.

Identification of the eluted phenols in HPLC (and in GC) is usually based on correspondence of the retention data with an appropriate standard. The limited availability of suitable standards for quantification is a problem that can be overcome, in part, by synthesis of the relevant compounds. Alternatively, the relevant compounds isolated by preparative scale chromatography can serve as reference standards [31, 35, 59].

In many instances, quantification is carried out by reference to one or more appropriately selected reference compounds.

However, all phenols possess a strong chromophore system. Their UV spectra are particularly informative, providing considerable structural information that can distinguish the type of phenol and the oxidation pattern. Furthermore, spectra of eluting peaks obtained at, for example, the apex and the two inflection points of the peak can be compared and used as indicators of purity.

The complementary nature of fluorescence detection has been demonstrated. In fact, the lignans show a good response to fluorescence excitation and this technique has been studied by Brenes et al. [138] to analyze these phenolic compounds in olive oils. Although fluorescence detection offers some advantages over UV detection in terms of enhanced selectivity and sensitivity, not all the phenolic compounds found in food fluoresce [139]. The analysis of phenolic compounds in olive fruit using fluorescence detection has also been reported [140], but there is only one paper reporting the analysis of some cinnamic acids in olive oils.

Coulometric electrode array detection coupled to HPLC was also used for qualitative and quantitative determination of phenolic compounds in VOO [86, 138]. The advantage of this method, besides easy sample preparation, is the possibility of separating and quantifying co-eluted substances with different potentials [141, 142]. The detector measures the different potentials at which the phenolic compounds are oxidized [143]. In addition, an amperometric detector was also used to quantify phenolic compounds in olive oils, showing great sensitivity and stability although the contamination of the electrode with the oxidation products is an important disadvantage [144].

Despite the obvious successes of GC-MS, as was commented before, it is the hyphenation of liquid chromatography with MS that has revolutionized the analysis of non-volatile species, as evidenced by the number of papers.

LC-MS interfacing has been achieved in a number of ways; however, it was with the advent of API techniques that LC-MS came of age. API is a soft ionization source for the analysis of polar, non-volatile, thermolabile, and high molecular mass molecules such as plant phenols (e.g., phenolic compounds of olive oil [145] and olive leaf [146]). Although API has revolutionized the application of LC-MS, some problems remain, the major limitation being the strong dependency of the response on the nature of the analyte plus the mobile phase [104]. Thus, generation of mass spectral libraries is difficult. Moreover, it is difficult to optimize conditions for a typical extract containing a broad range of analytes although many instruments now have provision for programmed operation of spectral conditions.

API mass spectra typically comprise protonated molecular ions, [M+H]+ or sodium adduct ions [M+Na]+ in positive ion mode, or deprotonated molecular ions, [M−H]− in negative ion mode with few fragment ions and thus have a low structure information content. On rare occasions, LC-MS can provide data sufficient for full structure analysis but more generally it is used to determine molecular mass and to establish the distribution of substituents on the phenolic ring(s).

HPLC with tandem mass spectrometry (LC-MS-MS) and negative APCI was used for the analysis of phenolic acids, tyrosol and oleuropein derivatives [147].

Negative ESI was more sensitive for the majority of phenols. Thus, molecular masses of the separated phenolic compounds were obtained through prominent [M−H]− ions for most of the compounds.

There are several examples of papers where MS or MS/MS is used for the analysis of phenolic compounds in olive oils and olives (see **Table 3**).

In instances where mass spectral data are insufficient to establish a definitive structure, NMR spectrometry is a powerful complementary technique for structural assignment. NMR spectra of phenolic compounds are frequently complex and identification of the isolated compounds is complicated in the absence of suitable reference standards which requires time-consuming syntheses of the relevant materials. Although 2D NMR spectrometry can be used for structural analysis without a reference compounds, the technique requires relatively large amounts of the compounds. Limited sensitivity and the need to isolate relatively large quantities of sample are currently the greatest limitations of NMR spectrometry.

In many instances, the combination of UV, MS, and $^1$H NMR will provide adequate information for structural elucidation [28, 31, 35, 74]. In other cases, information on the $^{13}$C NMR signals is necessary plus 2D correlations experiments such as COSY or $^1$H-$^{13}$C correlation experiments such as HMBC or HSQC.

Several authors have analyzed phenolic compounds in olive oil, olive oil waste water, and olive fruits using different methods of HPLC, with different extraction systems and coupling diverse detector systems. All this information is summarized in Table 3, where the time of analysis, the type of elution employed, the mobile phases, the stationary phase, the extraction system, the detection system used, and several pertinent observations are given. We have included all the HPLC references mentioned during this review and several new references [148−157]. Obviously, it is impossible to list all papers in this table. We have therefore chosen several of the most representative papers.

For all the reasons explained before, HPLC is the technique most commonly employed for analysis of the polar fraction of olive oil. However, GC and HPLC could be

**Table 3**. Separation of phenolic compounds of polar fraction of olive oil using HPLC methods

| Time of analysis | Mobile phases | Stationary phase | Type of elution | Extraction system | Detection system | Observations | References |
|---|---|---|---|---|---|---|---|
| 45 – 60 min | MeOH:H$_2$O (50:50 $v/v$) + CH$_3$COOH 0.2% | μBondapak C$_{18}$, 30 cm × 3.9 mm ID 10 μm | Isocratic | LLE with methanol/ water (60:40 $v/v$) (30 g of olive oil) | UV (280 nm) | Study of different Spanish virgin olive oils obtained from olives at different degree of ripeness | Graciani-Costante et al. [60,133] |
| 60 min | **A**: H$_2$O + CH$_3$COOH 2% **B**: MeOH | Hibar RP$_{18}$ | Gradient | LLE with methanol/ water (60:40 $v/v$) | UV | Analysis of virgin, refined and solvent extracted olive oils | Cortesi et al. [134] |
| 60 min | **A**: H$_2$O + CH$_3$COOH 0.2% **B**: MeCN | μBondapak C$_{18}$ | Gradient | LLE with methanol | UV | Tentative identification of mixtures between virgin olive oils and refined oils | Solinas et al. [123] |
| 70 min | **A**: H$_2$O + CH$_3$COOH 2% **B**: MeCN:MeOH (50:50 $v/v$) | Hibar RP$_{18}$, 25 cm × 4.6 mm ID 7 μm or Spherisorb ODS C$_{18}$, 25 cm × 4.6 mm ID 5 μm | Gradient | LLE | UV | Use of alkaline or acid hydrolysis of the polar fraction of olive oil | Cortesi et al. [18] |
| 70 min | A: H$_2$O B: MeOH | Ultrasphere ODS C$_{18}$, 25 cm × 2.0 mm ID 5 μm | Gradient | SPE (C$_{18}$) | UV (225 nm) | Correlation of bitter taste of VOO and instrumental HPLC analysis | Gutiérrez et al. [90] |
| 45 min | **A**: H$_2$O + CH$_3$COOH 2% (pH 3.1) **B**: MeOH | Erbasil C$_{18}$, 15 cm × 4.6 mm ID | Gradient | LLE with methanol/ water in different proportions | UV | Use of TLC. Elenolic acid and 4 unknown compounds (239 nm). Phenolic acids and secoiridoids. | Montedoro et al. [20] |
| | HPLC method of Montedoro et al. [20] | | Gradient | LLE with methanol/ water (80:20 $v/v$) evaporation to syrup concentration; addition of acetonitrile; washing with hexane. | UV | Characterization of oleuropein aglycone and 3 hydrolyzable phenols. Analysis of phenolic acids and secoiridoids. Use of hydrolysis of phenolic extracts, TLC and HPLC for doing standard compounds | Montedoro et al. [25] |
| | HPLC method of Montedoro et al. [20] | | Gradient | LLE with methanol/ water | UV; NMR and IR | Spectroscopic characterization of secoiridoids derivatives | Montedoro et al. [26] |
| 60 min | **A**: H$_2$O + CH$_3$COOH 3% **B**: MeOH | Spherisorb ODS 2, 25 cm × 4.6 mm ID 5 μm | Gradient | Aqueous ethanol extraction LLE | UV | Calculation of molar adorptivities of individual compounds. Calibration with 4 standard curves in groups (130 min → segments of mobile phase) | Tsimidou et al. [80] |
| 50 min | **A**: H$_2$O + CH$_3$COOH pH 3.2 **B**: MeOH | Erbasil C$_{18}$, 25 cm × 4.6 mm ID 10 μm | Gradient | LLE with methanol (30 g of olive oil) | GC-MS; MNR analysis | Development of HRGC method of phenol dosage. Characterization of several complex molecules by means of GC-MS | Angerosa et al. [88] |
| 130 min | **A**: H$_2$O + CH$_3$COOH 2% **B**: MeOH | HPLC-DAD: Lichrospher 100 RP$_{18}$, 25 cm × 4.0 mm ID 5 μm | Gradient | LLE as Vázquez Roncero et al. [34] (50 g olive oil) | Variable wavelenght UV detectors (280 and 285 nm); electrochemical | Variable wavelength UV detectors were found to be more suitable than DAD for quantitative information | Tsimidou et al. [21] |
| 40 min | 10$^{-3}$ M H$_2$SO$_4$ and CH$_3$CN | ODS-1, ODS-2, C$_8$, C$_1$, CN and Phanyl analytical columns (Spherisorb, 25 cm × 4.6 mm ID 3 μm) | Isocratic | SPE (C$_8$) | DAD ($\lambda$ = 225 nm) | Low quantities of olive oil used in the extraction system (1 g). Simple and complex phenols on different columns. | Pirisi et al. [89] |
| 120 min | **A**: H$_2$O + CH$_3$COOH 3% **B**: MeOH | Spherisorb ODS, 25 cm × 4.6 mm ID 10 μm | Gradient | LLE and SPE; acid and basic hydrolysis | Colorimetry | Two part of polar fraction= A: simple phenols and phenolic acids; B. complex nature | Litridou et al. [97] |
| 93 min | **A**: H$_2$O + CH$_3$COOH 0.5% **B**: MeCN | Spherisorb ODS 2, 25 cm × 4.6 mm ID | Gradient | LLE with methanol/ water (80:20 $v/v$) | UV; MS (ESI) in positive ion mode | Flavonoids such as luteolin and apigenin were detected as phenolic components of VOO | Rovellini et al. [33] |

**Table 3**. Continued.

| Time of analysis | Mobile phases | Stationary phase | Type of elution | Extraction system | Detection system | Observations | References |
|---|---|---|---|---|---|---|---|
| 50 min | **A**: $H_2O$ + $CH_3COOH$ 2%<br>**B**: MeOH | μBondapak $C_{18}$, 15 cm × 4.6 mm ID | Gradient | Angerosa et al. [88] | Photodiode array ($\lambda$ = 280 nm) | Study of the evolution of phenolic compounds in VOO during storage. GC-MS analysis too | Cinquanta et al. [66] |
| 25 min | **A**: $H_2O$ (adjusted pH 2.5 by $H_3PO_4$)<br>**B**: MeCN | Supelcosil ABZ + Plus, 25 cm × 4.0 mm ID 5 μm | Gradient | 100 g of frozen pulp were extracted as Amiot et al. [157] | Photodiode array | Demethyloleuropein as possible varietal marker. Elenolic acid glucoside and HYTY as indicators of maturation for olives. | Esti et al. [67] |
| 100 min | **A**: $H_2O$ (adjusted pH 3.2 by $H_3PO_4$)<br>**B**: MeCN | Lichrosorb $RP_{18}$, 25 cm × 4.6 mm ID 5 μm | Gradient | Aqueous ethanol extraction with bisulfite; hexane patitioning and SPE. (LSE procedure with Extrelut cartridge) | HPLC-MS; HPLC-DAD | Olive fruit. Verbascoside, anthocyanic compounds adn oleuropein derivatives. HPTLC | Romani et al. [148] |
| 60 min | **A**: $H_2O$ + $CH_3COOH$ 0.2%<br>**B**: MeOH | Spherisorb ODS 2, 25 cm × 4.6 mm ID | Gradient | Same as Montedoro et al. [20] | Photodiode array; MS; NMR | Simple phenols, flavonoids, secoiridoids | Brenes et al. [30] |
| HPLC method of Montedoro et al. [20] | | | | Various, e.g., aqueous methanol extraction containing diethyldithio-carbamate followed by SPE | HPLC-DAD | Olive fruit, virgin olive oil, vegetation waters, and pomace | Servilli et al. [93] |
| 70 min | **A**: $H_2O$ + $CH_3COOH$ 2%<br>**B**: MeOH + $CH_3COOH$ 2% | Column RP18 Pecosphere; 8.3 cm × 4.6 mm ID 3 μm | Gradient | LLE with aqueous methanol | DAD | Bitterness index K225 and autoxidation stability | Beltrán et al. [82] |
| HPLC method of Montedoro et al. [20] | | Column RP18 Latex; 25 cm × 4.0 mm ID 5 μm | Gradient | LLE with methanol (500 g of olive oil) | UV; MS (ESI) in negative and positive ion mode; NMR | Identification of lignans as major components in polar fraction of olive oil. Preparative thin-layer chromatography (PLC). | Owen et al. [35] |
| HPLC method of Montedoro et al. [20,25,26] | | Column RP18 Latex; 25 cm × 4.0 mm ID 5 μm | Gradient | LLE with absolute methanol and methanol/water (80:20 *v/v*) | UV; MS (ESI) in negative and positive ion mode; NMR | Use of TLC, GC, GC-MS Study of antioxidant/anticancer capacity (Owen et al. [27]) (using the same method) | Owen et al. [28] |
| 65 min | Method UV: HPLC method of Brenes et al. [30]<br>Method EC: **A**: 30 mM $LiClO_4$ solution (pH 3.1 with $HClO_4$)<br>**B**: MeOH containing 30 mM $LiClO_4$ solution | Spherisorb ODS 2, 25 cm × 4.6 mm ID 5 μm | Gradient | LLE: Montedoro et al. [20];<br>SPE: Favati et al. [92];<br>New extraction method: *N,N*-dimethylformamide (DMF) | UV; Electrochemical detector (EC) | Treatment of extracted oil with 2 N HCl to check the effectiveness of the extraction methods. | Brenes et al. [86] |
| 25 min | $H_2O$:$CH_3CN$ (82:18 *v/v*) + $CH_3COOH$ 0.02% | Nucleosil ODS, 25 cm × 2.1 mm or 25 cm × 1.1 mm ID 5 μm | Isocratic | LLE with buffer; SPE with phenyl cartridges (acidification) | UV, Spectrofluorimetric, MS, MS/MS HPLC-APCI (negative ion mode) | Phenolic acids | Cartoni et al. [100] |
| Method 1: 40 min<br>Method 2: 30 min | Method 1: HPLC method of Pirisi et al. [89]<br>Method 2: **A**: HPLC method of Montedoro et al. [20] | Spherisorb ODS 2, 25 cm × 4.6 mm ID 3 μm | Gradient | LLE and SPE systems | UV; DAD | Simple and complex phenols | Pirisi et al. [73] |
| HPLC method and conditions of Cortesi et al. [85] | | $C_{18}$ column (RP) Alltech 25 cm × 4.6 mm ID | Gradient | LLE: Montedoro et al. [20] using butylated hydroxytoluene (BHT) | MS; MS/MS | Analysis of oleuropein aglycone by APCI-MS. Phenolic compound profile | Caruso et al. [149] |
| HPLC method and conditions of Brenes et al. [30] | | | | | Photodiode array | Enzymes during malaxation | García et al. [81] |
| 50 min | **A**: $H_2O$ + $CH_3COOH$ 1%<br>**B**: MeOH/ MeCN/ $CH_3COOH$ (95:5:1 *v/v/v*) | $C_{18}$ column (RP) 15 cm × 4.6 mm ID 5 μm | Gradient | Aqueous methanol extraction | UV; APCI and ESI (negative and positive ion mode) detection | Olive fruit Semiprepartive scale HPLC too; antioxidant activity studies | McDonald et al. [117] |
| HPLC method of Romani et al. [148] | | Lichrosorb $RP_{18}$, 25 cm × 4.6 mm ID 5 μm | Gradient | LLE with EtOH/water (70:30 *v/v*), the water was acidified with formic acid (pH 2.5) | DAD; MSD | HPLC analysis of phenolic acids, secoiridoids and flavonoids | Romani et al. [127] |

**Table 3**. Continued.

| Time of analysis | Mobile phases | Stationary phase | Type of elution | Extraction system | Detection system | Observations | References |
|---|---|---|---|---|---|---|---|
| ≈ 50 min | **A**: $H_2O$ + $CH_3COOH$ 2% **B**: MeOH | Ultrasphere ODS $C_{18}$, 25 cm × 4.6 mm ID 5 μm | Gradient | According to the method described by Caponio et al. [42] | UV (278 nm) | Influence of the degree of olive ripeness on organoleptic characteristic and shelf-life (VOO from Coratina and Ogliarola salentina cultivars) | Caponio et al. [43] |
| 50 min | **A**: $H_2O$ + $CH_3COOH$ 0.01% **B**: MeCN | Column RP18 Symetry 10 cm × 4.6 mm ID 3.5 μm | Gradient | Olive oil residues adjusted to pH 3 with HCl and extracted with ethyl acetate. | UV/Vis | Olive oil residues. Simple phenolic compounds | Lesage-Meesen et al. [150] |
| 100 min | **A**: $H_2O$ (adjusted pH 3.2 by $H_3PO_4$) **B**: MeCN | Lichrosorb $RP_{18}$, 25 cm × 4.6 mm ID 5 μm | Gradient | Acidification of waste waters and LSE (Extrelut cartridge). Elution steps: 1) hexane; 2) ethyl acetate; 3) acid methanol Extraction of olive pulp (as Romani et al. [148]) | DAD; MS | Olive oil waste water and related olive samples | Mulinacci et al. [151] |
| 50 min | **A**: $H_2O$ + $CH_3COOH$ 3% **B**: MeCN:MeOH (50:50 v/v) | Lichrospher 100 $RP_{18}$, 25 cm × 4.0 mm ID 5 μm | Gradient | Comparative studies of LLE and SPE using diol-phase cartridges; unwanted substances washed out with hexane and hexane/ethyl acetate (90:10, v/v) | UV; DAD NMR (for ligstroside aglycone) | Phenols, flavones and lignans. Colorimetric determination of o-Diphenols. GC-MS | Mateos et al. [31] |
| 70 min | Elution solvents used: **A**: $H_2O$ + $CH_3COOH$ 1% **B**: MeOH **C**: Acetonitrile **D**: Isopropanol | Apex octadecyl 104 $C_{18}$; 25 cm × 0.4 mm ID 5 μm | Gradient | LLE with methanol and isopropanol/methanol mixture | UV-Vis | Phenolic compounds and tocopherols (280 nm) (simple and complex phenols and α-tocopherol) | Tasioula-Margari et al. [152] |
| 60 min | **A**: $H_2O$ + $CH_3COOH$ 2mM **B**: MeOH + $CH_3COOH$ 2mM | Nucleosil ODS, 25 cm × 2.1 mm ID 5 μm | Gradient | LLE with methanol/water (80:20 v/v), acidification and passed through a $C_{18}$ cartridge | MS and MS/MS (API/MS in negative ion mode) | Identification of a new class of phenolic compounds in olive oils: hydroxy-isochromans | Bianco et al. [37] |
| | Method UV: HPLC method of Brenes et al. [30] | | Gradient | LLE with N,N-dimethylformamide (DMF) | DAD; fluorescence | Phenolic compounds in Picual variety | García et al. [153] |
| HPLC method and conditions of Brenes et al. [30] | | | | | UV; electrochemical, fluorescence, MS. | Use of a lignan (1-acetoxypinoresinol) to authenticate Picual olive oils. Use of GC too. | Brenes et al. [138] |
| 65 min | **A**: $H_2O$ + $CH_3COOH$ 5% **B**: MeOH **C**: MeCN | Spherisorb S3 ODS 2, 25 cm × 4.6 mm ID 5 μm | Gradient | Same as Mateos et al. [31] | DAD (240, 280, 335 nm) | Phenolic acids, secoiridoids, lignans in Cornicabra virgin olive oils (50 min of separation + 15 min to clean the column) | Gómez-Alonso et al. [154] |
| 50 min or 70 min | **A**: $H_2O$ + Phosphoric acid 0.5% **B**: MeOH/ MeCN (50:50 v/v) | Lichrospher 100 $RP_{18}$, 25 cm × 4.0 mm ID 5 μm | Gradient | SPE (diol-bound phase) | UV, HPLC-MS in ESI(positive ion mode) | Dialdehydic and aldehydic forms of oleuropein aglycone and ligstroside aglycone | Gutiérrez-Rosales et al. [56] |
| 65 min | **A**: $H_2O$ + $CH_3COOH$ 2% **B**: MeOH/ MeCN (50:50 v/v) | $C_{18}$ Luna column, 25 cm × 3.0 mm ID 5 μm | Gradient | Comparative study of 5 extraction methods (LLE and SPE) | UV, DAD; MS | HPLC method and capillary electrophoresis method. (HYTY, TY, oleuropein and ligstroside aglycone, and decarboxymethyl oleuropein aglycone) | Bendini et al. [102] |
| 65 min | **A**: $H_2O$ + $CH_3COOH$ 2% **B**: EtOH | Phenomenex Luna (phenyl-hexyl)phase; 25 cm × 4.6 mm ID 5 μm | Isocratic | LLE with methanol/water (80:20 v/v) Montedoro et al. [20] | UV; MS (ESI in negative ion mode) | Isolation of individual polyphenols to study sensory properties | Andrewes et al. [59] |
| | Method UV: HPLC method of Brenes et al. [30] | | Gradient | LLE with N,N-dimethylformamide (DMF) | DAD; fluorescence | Phenolic composition of commercial virgin olive oils | García et al. [155] |
| 60 min | **A**: $H_2O$ + $CH_3COOH$ 0.2% (pH 3.1) **B**: MeOH | Inertsil ODS-3; 15 cm × 4.6 mm ID 5 μm | Gradient | LLE with methanol/water (80:20 v/v) | Photodiode array (280 nm and 339 nm) | Total phenol content after storage period (secoiridoid derivatives and 3, 4 – DHPEA-AC) | Morelló et al. [156] |

**Table 3**. Continued.

| Time of analysis | Mobile phases | Stationary phase | Type of elution | Extraction system | Detection system | Observations | References |
|---|---|---|---|---|---|---|---|
| 75 min | **A**: $H_2O$ + $CH_3COOH$ 0.5% **B**: MeCN | $C_{18}$ Luna column, 25 cm × 3.0 mm ID 5 µm | Gradient | LLE with methanol/ water (60:40 *v/v*) | DAD; MS (ESI in negative ion mode) | Effect of olive ripening degree on the oxidative stablility and organoleptic properties of olive oil | Rotondi et al. [119] |
| HPLC method of Rotondi et al. [119] | | | Gradient | LLE with methanol/ water from olive oil. SLE from olive fruits. | DAD; MS (ESI in positive and negative ion mode) | HPLC and CE analysis. 3 simple phenols, a secoiridoid derivative and 2 lignans | Bonoli et al. [176] |
| 60 min | **A**: $H_2O$ + $CH_3COOH$ 0.5% **B**: MeOH/ MeCN (50:50 *v/v*) | Lichrospher 100 $RP_{18}$, 25 cm × 4.0 mm ID 5 µm | Gradient | Comparative study of LLE and SPE (diol and $C_{18}$-phase) | Photodiode array detector; MS, NMR. | Simple phenols, secoiridoids and lignans | Hrncirik et al. [74] |
| 60 min | **A**: $H_2O$ + $CH_3COOH$ **B**: MeOH | Spherisorb ODS 2, 25 cm × 4.6 mm ID 5 µm | Gradient | Methanolic extraction and extraction which included SPE cleaning step. | DAD; DAD/ ESI-MS/MS | Olive fruits. Simple phenols, secoiridois, flavonoids and anthocyanins. | Vinha et al. [101] |
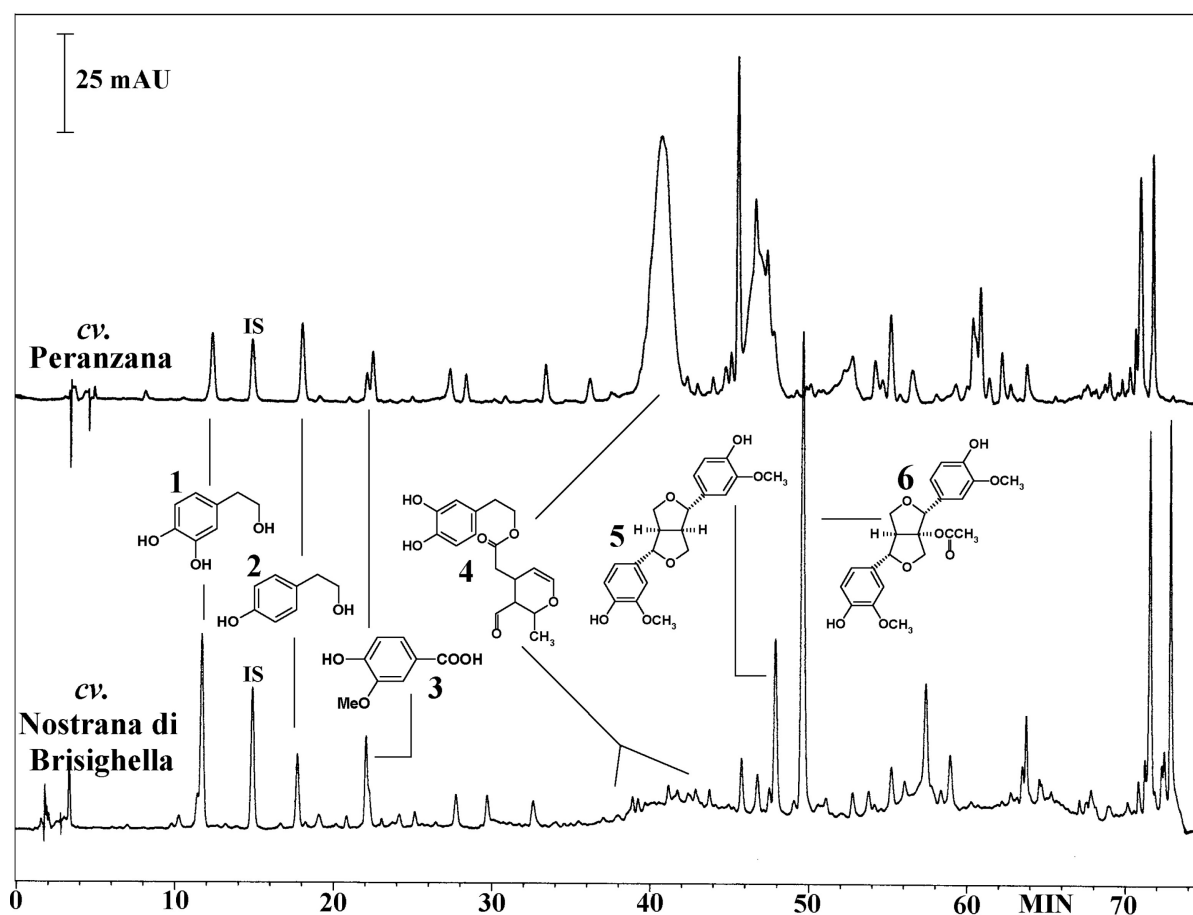


**Figure 2**. HPLC chromatogram at 280 nm of the extracts from Peranzana and Nostrana di Brisighella *cv*. olive oils. Peak identification: **1**, hydroxytyrosol; **2**, tyrosol; **3**, vanillic acid; **4**, deacetoxyoleuropein aglycone zone; **5**, (+)-pinoresinol; **6**, (+)-1-acetoxypinoresinol; I.S, internal standard (3,4-dihydroxyphenylacetic acid). From reference [157]. Mobile phase A, water/formic acid (99.5/0.5 *v/v*); mobile phase B, acetonitrile. Column: $C_{18}$ Luna™ column 5 µm, 25 cm × 3.0 mm ID (Phenomenex). (Other conditions in [157].)

complementary techniques; in fact, in several recent studies the isolation and characterization of VOO phenolic compounds have been done using HPLC and GC simultaneously [79, 127, 158].

In **Figure 2**, it is possible to see the typical HPLC-phenolic profile of two extracts of VOO [159]. These extracts were obtained from extra-VOO obtained from Peranzana and Nostrana di Brisighella olive *cvv*. In both samples, tyrosol,

hydroxytyrosol, and vanillic acid were detected; moreover, the extract from Peranzana oil showed a high concentration of deacetoxyoleuropein aglycone (DAOA), while the Nostrana di Brisighella sample showed two lignans; (+)-pinoresinol and (+)-1-acetoxypinoresinol.

Compound elution is typical of reversed-phase HPLC, i. e. polar compounds (e. g., simple phenols and phenolic acids) elute first, followed by those of decreasing polarity.

## 5 Capillary electrophoresis: A useful technique for the analysis of olive oils

Many works in the literature, as has been said before, determine the total phenolic amount in olive oils by spectrophotometric analysis and characterize their phenolic patterns by capillary gas chromatography (CGC) and, mainly, by reverse phase high performance liquid chromatography (RP-HPLC). Even if the characterization and quantification of phenolic compounds from olive oils have been successfully carried out by HPLC, this analytical technique needs accurate sample preparation and, generally, is time-consuming due to the complexity of the matrix. Actually, in the worst case the whole HPLC analysis of olive oil's phenols requires more than 90 min.

Therefore, the use of faster analytical techniques and screening tools, allowing a rapid screening of phenolic compounds of olive oils, is strongly recommended. CE can represent a good compromise between analysis time and satisfactory characterization for some classes of phenolic compounds in VOO.

CE has become an alternative or complementary to the HPLC technique. The speed, resolution, and simplicity of CE, combined with low operating costs, make the technique an attractive option for the development of improved methods of food analysis for the new millennium [160–166]. CE offers the analyst a number of key advantages

for the analysis of the components of food. Indeed, in recent years, it has proved to be a high-resolution technique and has been applied successfully to the analysis of phenolic compounds of a large variety of samples (honey, plant extracts, wine, beer, tea, fruits, vegetables, juices ) requiring only small amounts of sample and buffer and a short analysis time [167–172]. Unfortunately, few data are available on the phenolic content in products that are waste from the olive oil industry (olive mill wastewater [173] and alperujo [174]) and in olive oil [23,98, 102,175, 176] obtained directly with this technique.

This review is presented as a summary of the most important publications in which a study on the polyphenolic compounds in olive oil is carried out. For this reason, we only summarize the information of the last mentioned references (see **Table 4** and **Table 5**).

All the gathered methods use simple CZE methodologies based on a borate run buffer at alkaline pH. In the literature, the most efficient operative mode to separate phenolic compounds has been found to be borate-based CZE, but borate-phosphate-based micellar electrokinetic chromatography (MECK) methods with sodium dodecylsulfate (SDS) as micellar agent have been also used [177–179].

Between all these methods, differences can be found regarding applied voltage, internal diameter of the capillary, time of injection (in all the cases was hydrodynamic injection), effective length of capillary, and buffer concentration. However, as previously stated, both type of buffer and pH are practically the same in all these methods. An increase in pH values causes higher migration times; in fact, higher pH values lead to a higher ionization state of the species and at the selected basic pH, the phenolic compounds are negatively charged and would migrate towards the anode, i.e. away from the detector. Nevertheless, due to the large electroosmotic flow (EOF) in the sys-

**Table 4.** Summary of optimized conditions of capillary electrophoresis methods where olive oil samples are analyzed. $\lambda_d$, wavelength of detection; $V$, voltage; $T$, temperature, ID, internal diameter of capillary; $L_{ef}$, effective length of capillary; [Buffer]; buffer concentration.

| References | Instrumental variables | | | | | | Experimental variables | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\lambda_d$ [nm] | $V$ [kV] | T [°C] | ID [µm] | $L_{ef}$ [cm] | $t_{inj}$ [s] | Type of buffer | [Buffer] [mM] | pH |
| Bendini et al. [102] | 200 | 27 | 30 | 50 | 40 | 3 s (0.5 p.s.i) | Sodium tetraborate | 45 | 9.6 |
| Bonoli et al. [175] | CZE method of Bendini et al. [102] | | | | | | | | |
| Bonoli et al. [176] | CZE method of Bendini et al. [102] | | | | | | | | |
| Carrasco Pancorbo et al. [23] | 210 | 25 | 25 | 75 | 50 | 8 s (0.5 p.s.i) | Sodium tetraborate | 25 | 9.6 |
| Buiarelli et al. [98] | 200 | 18 | 25 | 50 | 36 | 2 s (1.5 p.s.i) | Sodium tetraborate | 40 | 9.2 |

**Table 5.** Summary of extraction systems used and detected compounds in olive oil samples with the application of each method. HYTY, hydroxytyrosol; TY, tyrosol; DHPE, 2,3-dihydroxyphenylethanol; VA, vanillic acid; DAOA, deacetoxy oleuropein aglycone; Ac Pin, (+)-1-acetoxypinoresinol.

| References | Extraction system | Initial quantity of oil → Final quantity of solvent (MeOH/H$_2$O (50 : 50 *v/v*) ) in the extraction process | Detected compounds in olive oil |
|---|---|---|---|
| Bendini et al. [102] | LLE (Pirisi et al. [73]) | 2 g → 1 mL | HYTY, TY, unidentified secoiridoids compounds |
| Bonoli et al. [175] | LLE (Pirisi et al. [73]) | 2 g → 1 mL | HYTY, TY, DHPE, unidentified oleuropein aglycone derivatives |
| Bonoli et al. [176] | LLE (Pirisi et al. [73], modified by Rotondi et al. [119]) | 2 g → 0.5 mL | HYTY, TY, VA, DAOA, Ac Pin |
| Carrasco Pancorbo et al. [23] | LLE [23] | 60 g → 0.5 mL | 14 phenolic acids |
| Buiarelli et al.[98] | Combination of LLE-SPE [98] | 10 g → non specified | 5 phenolic acids |

tem, polyphenols are propelled together with the bulk solution towards the cathode, but at a much lower rate. Therefore, phenols and polyphenols are less dissociated and those with a bigger molecular mass are first detected since they are less able to migrate upstream [168]. Moreover, the lower analyte velocities observed at higher pH values could be explained by the increase in ionic strength of the running buffer, which leads to lower electroosmotic flow [180].

For all these reasons, the best compromise in terms of resolution of the phenolic compounds and total analysis time was obtained, in all cases, at about a pH 9.5 buffer.

The differences in internal diameter of the capillary and time of injection, as well as the extraction system, the quantity of olive oil used in the extraction protocol, and the volume of solvent, MeOH/H$_2$O (50 : 50 *v/v*), for redissolving the phenolic compounds extracted, cause the differences in sensitivity between these methods.

In three of these papers [102, 175, 176] the aim of the authors is the study of compounds of all the most representative phenolic compounds in extra virgin olive oil, such as simple phenols (hydroxytyrosol and tyrosol and some phenolic acids), secoiridoids (oleuropein aglycone derivatives), and lignans ((+)-1-acetoxypinoresinol). The objective for the authors of the other two papers [23, 98] was the characterization of a specific family among the phenolic compounds, the phenolic acids. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables (e.g., olive oil), against oxidative damage diseases (coronary heart disease, stroke, and cancers) [181].

**Figure 3** shows one of the mentioned examples where the aim of the authors is the study of all the phenolic fraction of olive oil [159]. The figure shows the CE-profile of two extracts from extra-virgin olive oils obtained from Per-
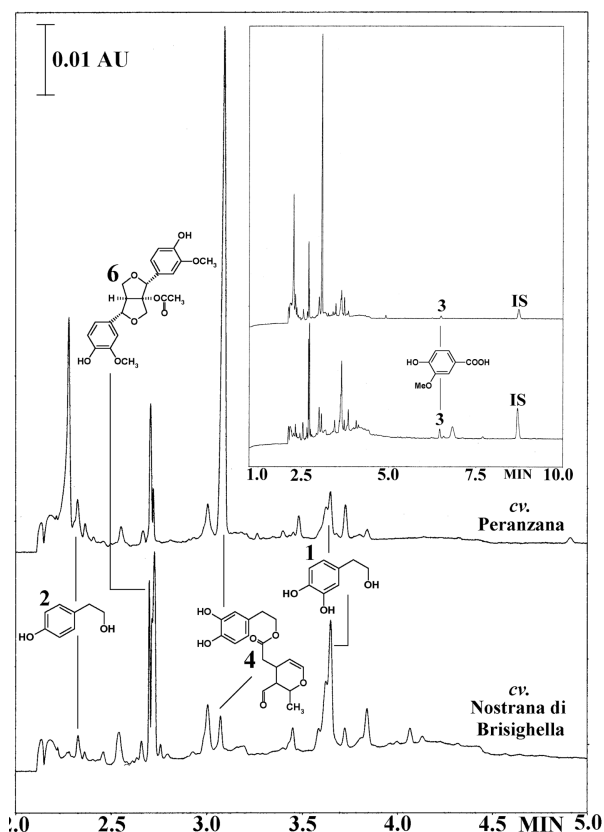


**Figure 3**. CZE electropherograms at 200 nm of the extracts from Peranzana and Nostrana di Brisighella *cvv.* olive oils. See Figure 2 for analyte identification numbers. Separation conditions: capillary, 47 cm × 50 μm; applied voltage, 27 kV; applied temperature, 30°C; buffer, 45 mM Sodium borate (pH 9.60); hydrodynamic injection, 0.5 p.s.i. for 3 s. From reference [157].

anzana and Nostrana di Brisighella olives *cvv.* (they are the same samples as those shown in the HPLC graphics; so it is possible to compare the two techniques). In this
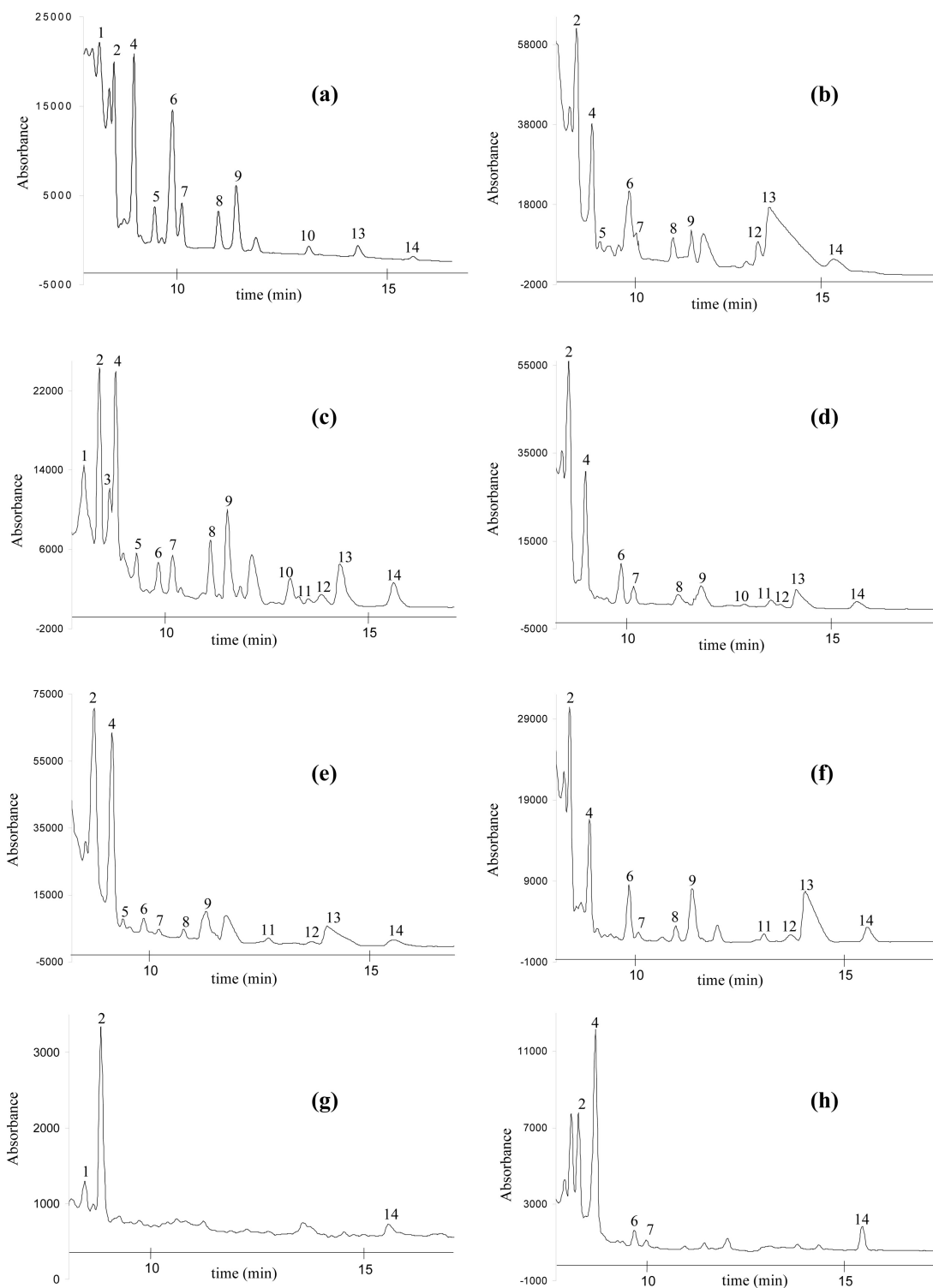
**Figure 4.** CZE electropherogram of phenolic fraction extracted from real olive oil sample by LLE. (a) Arbequina, (b) Lechín of Sevilla, (c) Picual, (d) Hojiblanca, (e) Lechín of Granada, (f) Cornicabra, (g) refined olive oil and (h) mixture of refined and virgin olive oil. From reference [51]. Separation conditions: capillary, 57 cm × 75 µm; applied voltage, 25 kV; applied temperature, 25°C; buffer, 25 mM Sodium borate (pH 9.60); hydrodynamic injection, 0.5 p.s.i. for 8 s. Detection was performed at 210 nm. Peak identification numbers: **1**, *trans*-cinnamic acid; **2**, 4-hydroxyphenylacetic acid; **3**, sinapinic acid; **4**, gentisic acid; **5**, (+)-taxifolin; **6**, ferulic acid; **7**, *o*-coumaric acid; **8**, *p*-coumaric acid; **9**, vanillic acid; **10**, caffeic acid; **11**, 4-hydroxybenzoic acid; **12**, dopac; **13**, gallic acid and **14**, protocatechuic acid.

case, the elution order, according to the different principle of separation, was different from the HPLC one. Tyrosol and hydroxytyrosol were recognized in both oils. Similarly to that detected by HPLC, deacetoxy oleuropein aglycone (DAOA) by CZE was the highest peak of the Peranzana extract. Furthermore, the CZE-DAOA peak was particularly sharp and well resolved (at the baseline) from the other peaks, as well in the Nostrana di Brisighella extract.

This work reports a qualitative comparison between HPLC and CE separations of extra-VOO phenolic compounds. The main advantage of the CE separation reported was the short analysis time, with respect to the HPLC method. Actually, tyrosol, hydroxytyrosol, (+)-1-acetoxypinoresinol and DAOA were analyzed within 10 min by CZE, while HPLC separation required up to 75 min. A preliminary quantification of phenols realized by the two techniques showed good agreement, confirming the correct assignment of the CZE peaks.

**Figure 4** shows one of the examples mentioned where the aim of the authors is the study of a specific family in the phenolic fraction of olive oil [23]. A sensitive, rapid, efficient, and reliable method for the separation and determination of phenolic acids by CZE was developed and applied to 6 monovarietal extra-virgin olive oil samples, different refined olive oils, and commercial mixtures of refined and virgin olive oils in order to compare the amounts of phenolic acids. The differences in the phenolic acid profiles shown in the electropherograms of the different varieties of olive oil extracts are very clear. It is an important factor to bear in mind, in order to compare the analysis of the samples correctly, that the absorbance scales of the different electropherograms for each variety of olive oil are different. When refined olive oil and mixtures of refined and virgin olive oil were analyzed, the amounts of phenolic compounds obtained were smaller than for the other types of olive oils.

Compounds such as *trans*-cinnamic acid, sinapinic acid, (+)-taxifolin, caffeic acid, and dopac, which only appeared in several olive oils, could be considered as potential markers for geographical origin or olive fruit varieties in the future.

In this work [23] the potential of the CE technique with UV detection for fast and sensitive simultaneous determination of 14 compounds of the same family in extra-virgin olive oils obtained from different varieties has been demonstrated.

## 6 Conclusions

Polyphenols are significantly related to the quality of virgin olive oil and their contribution to the oxidative stability of the oil is widely accepted. The qualitative and quantitative composition of VOO hydrophilic phenols is strongly affected by the agronomic and technological conditions of its production. For these reasons, the identification and quantification of the individual components of VOO are of great interest. Many analytical procedures directed towards the determination of the complete phenolic profile have been proposed; in general, an analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, analytical separation, and quantification. The variety of extraction techniques, chromatographic conditions, and methods of quantification have contributed to the differences in reported levels of virgin olive oil phenolic compounds.

The use of capillary electromigration methods to analyze phenolic compounds of olive oil is nowadays increasing, although the usual procedure now encompasses a high-performance separation technique in combination with diode array detection or mass spectrometry.

The high resolution, efficiency, and analysis speed provided by CE together with the minimum sample and reagents consumption have promoted the use of this technique. Also the suitability of CE for coupling to different types of detectors may make CE a powerful tool for the characterization of the phenolic fraction of olive oil.

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# Capítulo 2

## Determinación de ácidos fenólicos en aceite de oliva virgen-extra mediante CZE

El trabajo experimental de la presente memoria se comenzó intentando ahondar en una de las familias que forman parte de la fracción más polar del aceite de oliva: los ácidos fenólicos. Estos, en general, son compuestos fenólicos que poseen un grupo carboxilo y pueden ser divididos en dos grupos: ácidos hidroxicinámicos e hidroxibenzoicos. Aunque el esqueleto básico es siempre el mismo, el número y la posición de los grupos hidroxilo en el anillo aromático son los responsables de la gran variedad de ácidos fenólicos presentes en la naturaleza en general y en el aceite de oliva en particular.

Los ácidos fenólicos han despertado gran interés por las funciones biológicas que pueden desempeñar como metabolitos secundarios y por el papel que juegan en la calidad de los alimentos y en sus propiedades organolépticas. Además, muy recientemente han tomado mayor protagonismo a causa de la potencial protección que pueden ejercer contra las enfermedades provocadas por daños oxidativos (riesgo cardiovascular, cáncer, deterioro cognitivo…) [321,322,323,324].

Por todos estos motivos, se consideró interesante poner a punto un método para la determinación de esta familia en aceite de oliva, realizando antes una serie de estudios preliminares que nos permitieran confirmar la viabilidad del desarrollo de este método, que incluyeron:

- Estudio de la potencialidad de la técnica de CE para determinar estos compuestos.
- Optimización de un sistema de extracción adecuado para los ácidos fenólicos puesto que están a baja concentración en una matriz muy apolar como es el aceite.
- Selección lo más acertada posible de los analitos a estudiar, de modo que dispusiéramos de patrones comerciales de los mismos con objeto de facilitar su

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identificación y cuantificación, y que los elegidos estuviesen realmente en el aceite.

Hasta el momento en el que se desarrolló este método, no existía ninguna alternativa a HPLC que ofreciese resultados fiables y reproducibles, con tiempos de análisis cortos, y que lograse detectar cantidades tan pequeñas de ácidos fenólicos en aceite.

# Sensitive Determination of Phenolic Acids in Extra-Virgin Olive Oil by Capillary Zone Electrophoresis

ALEGRÍA CARRASCO PANCORBO, CARMEN CRUCES-BLANCO,
ANTONIO SEGURA CARRETERO,* AND ALBERTO FERNÁNDEZ GUTIÉRREZ

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n,
E-18071 Granada, Spain

A sensitive, rapid, efficient, and reliable method for the separation and determination of phenolic acids by capillary zone electrophoresis has been carried out. A detailed method optimization was carried out to separate 14 different compounds by studying parameters such as pH, type and concentration of buffer, applied voltage, and injection time. The separation was performed within 16 min, using a 25 mM sodium borate buffer (pH 9.6) at 25 kV with 8 s of hydrodynamic injection. With this method and using a liquid−liquid extraction system, with recovery values around 95%, it has been possible to detect small quantities of phenolic acids in olive oil samples. This is apparently the first paper showing the quantification of this specific family of phenolic compounds in virgin olive oil samples.

## INTRODUCTION

In recent years, interest in natural antioxidants from vegetable substances has been related to their therapeutic properties (*1*). Among the different vegetable oils, extra-virgin olive oil is unique because it is obtained from the olive fruit (*Olea europaea* L.) solely by mechanical means, without further treatment other than washing, filtration, decantation, or centrifugation. This process retains minor compounds originally present in the olive fruit, which are usually removed from other vegetable oils during various stages of refining.

The cultivars of olive fruit vary considerably in size, shape, oil content, and flavor; for this reason several commercially important olive oil varieties are grown in Spain (*2*).

The increasing popularity of olive oil is mainly attributed to its high content of oleic acid, which may affect the plasma lipid/lipoprotein profiles (*3*), and its richness in phenolic compounds, which act as natural antioxidants (*4, 5*), and may contribute to the prevention of human disease.

Among the various components of the unsaponifiable fraction of olive oil, phenolic compounds are one of the most important because they are natural antioxidants and a factor to be considered in the evaluation of the quality of an extra-virgin olive oil due to their role in its autoxidation stability, nutritional value, and flavor [bitterness (*6*) and astringency], and organoleptic characteristics in general (*7*).

In discussions of plant metabolites, the term "phenolic acids" refers to a distinct group of organic acids. Phenolic acids are the monomeric phenols in a large class of aromatic plant metabolites named simply phenolics, which exhibit certain sensorial and physiological properties of great interest (*8*).

In recent years, capillary electrophoresis (CE) has proven to be a high-resolution technique and has been applied successfully in the analysis of phenolic compounds of a large variety of samples [honey (*9*), plant extracts (*10*), wine (*11*), oils (*12, 13*), and olive mill wastewater (*14*)], requiring only small amounts of sample and buffer and short times of analysis. CE offers the analyst a number of key advantages for the analysis of the components of food (*15, 16*).

Despite these characteristics, the quantitative determination of the special family among the phenolic compounds, the phenolic acids, in olive oil samples has never been done before by capillary zone electrophoresis (CZE) with diode array detection.

For this reason, the aim of the present work has been to demonstrate the potential of the CE technique with ultraviolet (UV) detection for the fast and sensitive simultaneous determination of 14 compounds in extra-virgin olive oils from different varieties.

## MATERIALS AND METHODS

**Apparatus.** Experiments were performed with a Beckman P/ACE System MDQ CE instrument comprising a 0−30 kV high-voltage built-in power supply, a diode array detector, and the Gold software for system control and data handling. All capillary tubing (bare fused silica) was from Beckman Instruments (Fullerton, CA). The temperature was controlled by use of fluorocarbon-based cooling fluid.

**Reagents and Stock Solutions.** The phenolic acids 4-hydroxyphenylacetic acid, gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihy-

* Address correspondence to this author at Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain (e-mail ansegura@ugr.es; fax +34958249510).
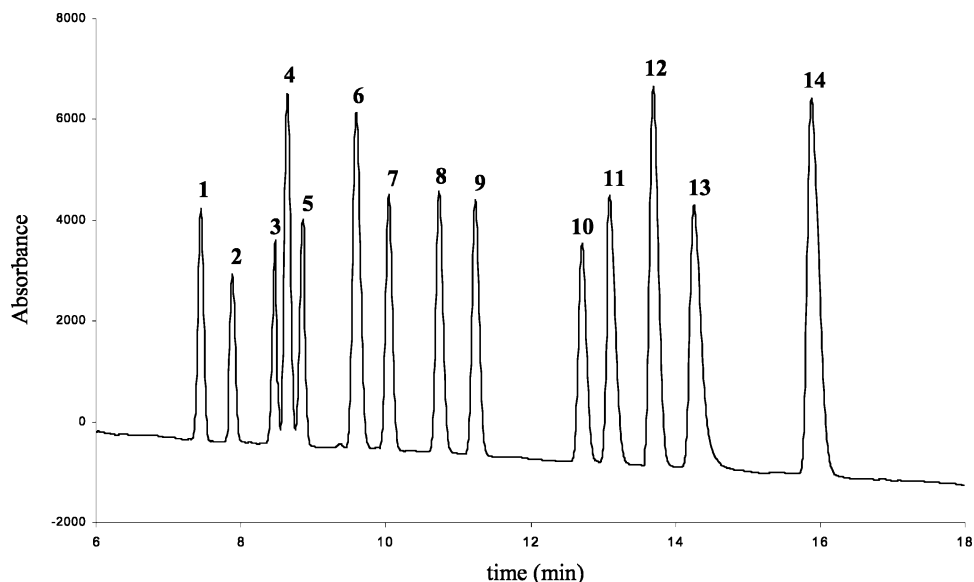
**Figure 1.** CZE separation of a standard mixture composed of 14 phenolic compounds under optimized conditions. Separation conditions: capillary, 57 cm × 75 μm; applied voltage, 25 kV; applied temperature, 25 °C; buffer, 25 mM sodium borate (pH 9.60); hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 210 nm. Peaks: 1, *trans*-cinnamic acid; 2, 4-hydroxyphenylacetic acid; 3, sinapinic acid; 4, gentisic acid; 5, (+)-taxifolin; 6, ferulic acid; 7, *o*-coumaric acid; 8, *p*-coumaric acid; 9, vanillic acid; 10, caffeic acid; 11, 4-hydroxybenzoic acid; 12, dopac; 13, gallic acid; 14, protocatechuic acid.

droxybenzoic acid), caffeic acid (3,4-dihydroxycinnamic acid), dopac (3,4-dihydroxyphenylacetic acid), *p*-coumaric acid (4-hydroxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), *trans*-cinnamic acid, *o*-coumaric acid (2-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and gentisic acid (2,5-dihydroxybenzoic acid) were obtained from Sigma-Aldrich (St. Louis, MO). 4-Hydroxybenzoic acid was acquired from Fluka. The flavanonol (+)-taxifolin was obtained from Extrasynthèse (Genay, France), and all of the analytes were used as received.

The stock solution containing all 14 compounds was prepared in methanol/water (50:50, v/v) at a concentration of 500 μg/mL for each analyte.

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium borate (borax), glycine (aminoacetic acid), and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) were obtained from Sigma and sodium carbonate anhydrous and ammonium chloride from Panreac (Barcelona, Spain), which were all used as running buffers at different concentrations and pH values. Methanol and *n*-hexane were acquired from Panreac and were of HPLC grade.

Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA).

**Electrophoretic Procedure.** CE separation was performed on a fused silica capillary (75 μm i.d., 375 μm o.d., total length = 57 cm, effective length = 50 cm). Good repeatability was assured by rinsing the capillary with 0.1 M sodium hydroxide for 5 min followed by 2 min with Milli-Q water at the beginning of each experimental session. The capillary was equilibrated with the running buffer (25 mM sodium borate adjusted to pH 9.6) for 5 min before each sample injection. The optimized running buffer was prepared by dissolving an appropiate amount of solid salt in Milli-Q water and adding a proper amount of 1.0 M NaOH.

Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 psi) for 8 s (1 psi = 6895 Pa),

Each electrophoretic run was carried out at 25 kV with the capillary temperature maintained at 25 °C, resulting in a current of ∼95 μA. After each electrophoretic run, the capillary was flushed with Milli-Q water for 5 min. All samples, buffers, and solutions were filtered through a 0.20 μm syringe filter. The running buffer was changed after five runs.

UV detection was performed at 210 and 275 nm simultaneously. Diode array detection was used over the range of 190−600 nm to achieve spectral data. Peak identification was done by comparing both

migration time and spectral data obtained from real samples and standards and also with spiked real samples at different concentration levels. Peak areas were used for quantification of the analytes.

Before the first use of a new capillary, it was preconditioned by rinsing with 0.5 M NaOH for 10 min, followed by a 5 min rinse with Milli-Q water.

**Analysis of Extra-Virgin Olive Oils.** Extra-virgin olive oil samples were obtained using dual phase decanter centrifugation from six Spanish monovarietal olive oils named Picual, Hojiblanca, Cornicabra, Lechín de Granada, Lechín de Sevilla, and Arbequina.

Different refined olive oils and commercial mixtures of refined and virgin olive oils were used to compare the amounts of phenolic acids.

**Liquid−Liquid Extraction (LLE) of Phenolic Acids from Real Samples.** The extraction conditions and amounts of the extraction system were optimized and, finally, phenolic acids were extracted from extra-virgin olive oils as follows: Oil (60 ± 0.001 g) was dissolved in 60 mL of hexane, and the solution was extracted successively with four 20 mL portions of 60% aqueous methanol. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure and a temperature of 40 °C, and the residue was redissolved in 500 μL of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter before the CE analysis.

## RESULTS AND DISCUSSION

**Selection of Analytes.** To obtain a representative phenolic acid mixture to propose a potent analytical method for the analysis of these compounds in any kind of olive oil, all of the varieties we used had been previously studied in depth to check what compounds were present. With this information and all of the data of compounds previously reported from virgin olive oil, we made a family of phenolic acids and another compound, (+)-taxifolin (flavanonol), very appropriate for real samples. (+)-Taxifolin was included in the family because it was extracted with the LLE system used and appeared in the zone of phenolic acids in the electropherograms of extra-virgin olive oil samples.

Optimization of the separation conditions was achieved through testing of the migration behavior of a standard mixture containing the 14 compounds under study.
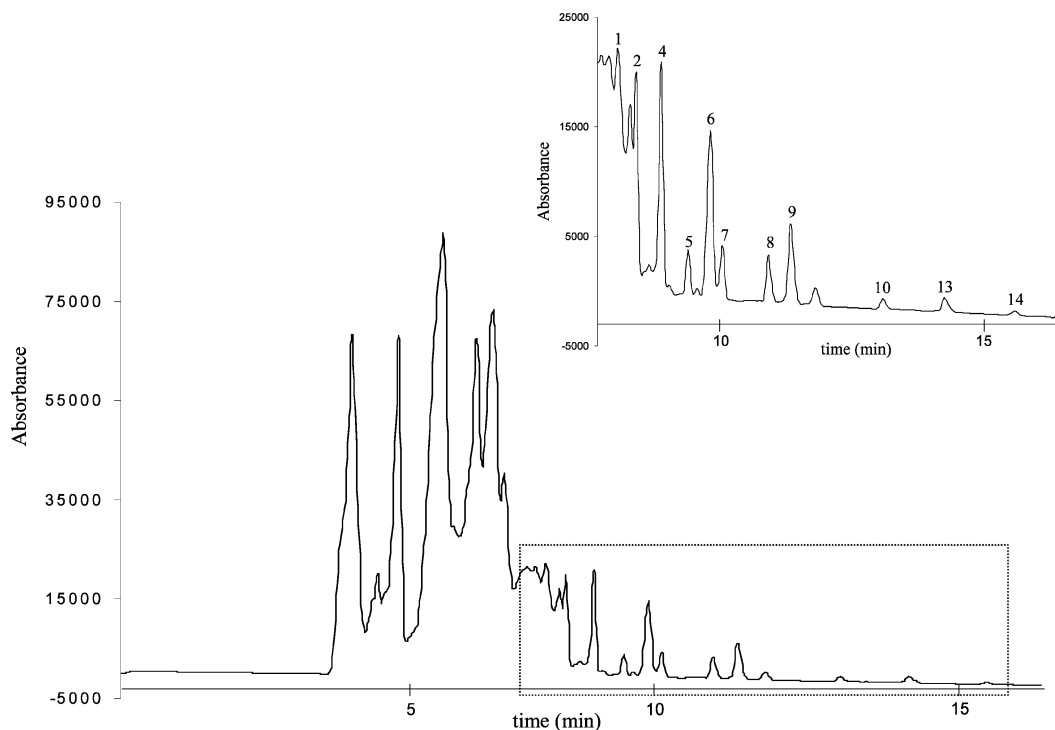
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**Figure 2.** CZE electropherogram of phenolic fraction extracted from extra-virgin olive oil Arbequina by LLE. In the square at the right of the total electropherogram are shown the last ≫8 min of a run. See **Figure 1** for analyte identification numbers. Detection was performed at 210 nm (other conditions as **Figure 1**).

**Effect of Experimental Variables.** The effect of pH on the peak resolution of the 14 standard compounds was first studied. This effect was studied by adjusting the buffer pH to 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, or 10.5 by adding proper amounts of 1.0 M NaOH or 1.0 M HCl.

It was observed that at high pH values, the separation achieved was better than at lower pH values because the phenolic acids have a double-negative charge and present migration times longer than those of other compounds that can also be extracted with the LLE method employed. A more detailed study between pH 9 and 10 was carried out to obtain the optimum pH value for the correct resolution of the analytes under study by adjusting the buffer pH to 9.2, 9.4, 9.6, or 9.8. A value of pH 9.6 was selected for the rest of the experimental work as providing reasonable analysis times and very good resolution between peaks.

Five different aqueous buffers were tested: glycine, CAPSO, ammonium chloride, sodium carbonate, and sodium borate, all adjusted to range in pH between 9 and 10 (9.2, 9.4, 9.6, and 9.8).

Among the different buffers tested, sodium borate gave the best separation plus a satisfactory analysis time. The influence of sodium borate concentration on migration times and resolution was investigated using concentrations between 5 and 75 mM. It was concluded that migration and resolution increased when the concentration of sodium borate also increased. However, at concentrations >40 mM an unstable baseline and badly shaped peaks began to appear in the electropherograms of the mixture compounds. For this reason, a 25 mM concentration of sodium borate buffer (pH 9.6) was selected for the rest of the experimental work in order to reduce the analysis time and maintain good resolution.

**Effect of Instrumental Variables.** The effect of the applied voltage on the resolution of the 14 compounds was studied using the optimized buffer composition. When the applied voltage was increased from 10 to 30 kV, shorter analysis times and

**Table 1.** Recoveries Calculated by Analyzing, Three Times, Physically and Chemically Refined and Purified Oil Spiked with a Mixture of 14 Standard Phenolic Compounds

| | phenolic compounds recovery (%) ($n = 3$) | | | | | |
| | 20 μg/mL | | 50 μg/mL | | 200 μg/mL | |
| analyte | av | SD[a] | av | SD | av | SD |
|---|---|---|---|---|---|---|
| *trans*-cinnamic acid | 99.2 | 5.53 | 98.9 | 6.32 | 98.20 | 4.25 |
| 4-hydroxyphenyl-acetic acid | 125.11 | 4.28 | 113.10 | 4.78 | 102.35 | 5.78 |
| sinapinic acid | 91.83 | 6.23 | 91.74 | 5.89 | 89.10 | 6.23 |
| gentisic acid | 92.32 | 7.54 | 91.98 | 7.98 | 89.74 | 6.18 |
| (+)-taxifolin | 82.10 | 5.29 | 80.10 | 6.29 | 78.74 | 7.03 |
| ferulic acid | 91.31 | 6.44 | 89.31 | 5.99 | 86.10 | 6.12 |
| *o*-coumaric acid | 90.03 | 9.05 | 91.03 | 8.53 | 89.98 | 9.54 |
| *p*-coumaric acid | 89.95 | 10.01 | 90.10 | 9.03 | 88.73 | 10.25 |
| vanillic acid | 95.47 | 9.98 | 95.30 | 10.02 | 94.31 | 9.80 |
| caffeic acid | 92.26 | 8.23 | 92.01 | 7.63 | 87.05 | 9.78 |
| 4-hydroxybenzoic acid | 95.17 | 7.57 | 97.12 | 7.98 | 96.12 | 5.25 |
| dopac | 98.97 | 6.75 | 97.97 | 8.51 | 95.03 | 4.78 |
| gallic acid | 94.20 | 8.25 | 92.10 | 4.28 | 89.16 | 6.82 |
| protocatechuic acid | 98.80 | 5.15 | 97.90 | 5.12 | 94.02 | 7.25 |

[a] Standard deviation.

higher separation efficiencies were obtained. As the voltage is increased, there is more joule heat generated in the capillary, which can be detrimental to the separation because it causes broader peaks, the possibility of sample decomposition, or the formation of bubbles in the capillary. The better the heat is dissipated by the equipment, the higher the voltage that can be used. In the present work, it was possible to work at 25 kV without losing resolution and reducing the duration of the run analysis.

Among the different injection modes, hydrodynamic injection was selected. It is observed that as the sample plug length is increased, efficiency decreases and peaks broaden. An injection time of 8 s was finally selected.
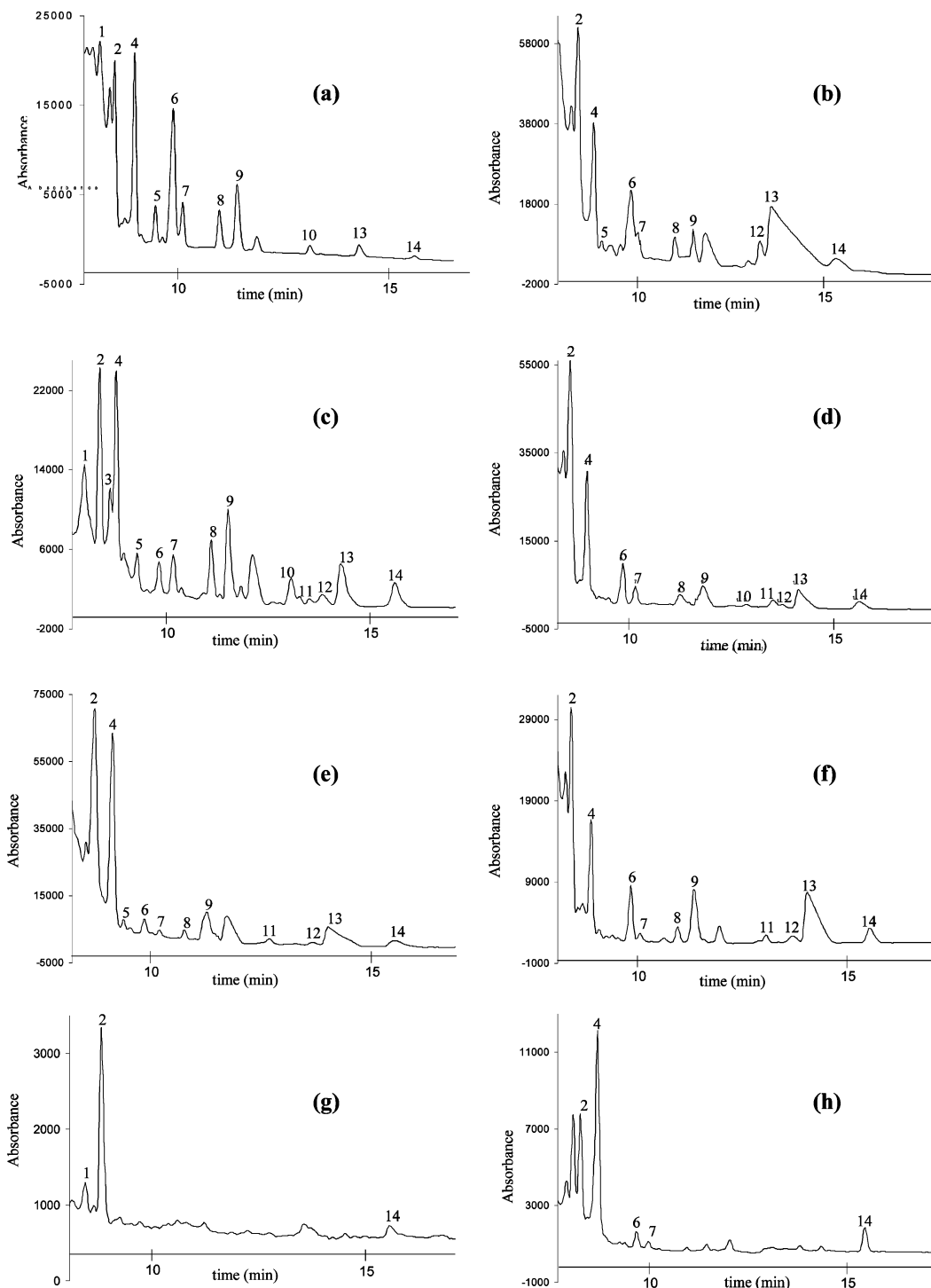
**Figure 3.** CZE electropherogram of phenolic fraction extracted from real olive oil sample by LLE: (**a**) Arbequina; (**b**) Lechín de Sevilla; (**c**) Picual; (**d**) Hojiblanca; (**e**) Lechín de Granada; (**f**) Cornicabra; (**g**) refined olive oil; (**h**) mixture of refined and virgin olive oils. For identification of the compounds see **Figure 1**. Detection was performed at 210 nm (instrumental and experimental parameters as **Figure 1**).

At the optimized conditions, the optimum electropherogram of the mixture of the 13 phenolic acids and the flavanonol (+)-taxifolin is presented in **Figure 1**, where the elution order observed is as follows: *trans*-cinnamic acid, 4-hydroxyphenyl-acetic acid, sinapinic acid, gentisic acid, (+)-taxifolin, ferulic acid, *o*-coumaric acid, *p*-coumaric acid, vanillic acid, caffeic acid, 4-hydroxybenzoic acid, dopac, gallic acid, and protocat-echuic acid. As can be seen, the peaks were completely separated in only 16 min.

**Repeatability Study.** Repeatability was assessed for each standard compound at two concentration levels (20 and 100 μg/

mL). The two solutions containing all of the analytes were prepared and analyzed on the same day (intraday precision, *n* = 12) and on three consecutive days (interday precision, *n* = 36). The relative standard deviations (RSDs) of peak areas and the RSDs of migration times were determined for each analyte in the two standard mixtures.

The intraday repeatabilities on the migration time (expressed as RSD) were 0.45 and 0.89% for the 20 and 100 μg/mL concentrations, respectively, whereas the interday repeatabilities on the migration time were within 0.51 and 1.41% for the 20 and 100 μg/mL concentrations, respectively.

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**Table 2.** Results of the Analysis of Real Samples (*n* = 7)[a]

| | μg/L = μg of analyte/L of olive oil = ppb | | | | | | | |
| analyte | Arbequina | Picual | Hojiblanca | Lechín de Sevilla | Lechín de Granada | Cornicabra | refined oil | mixture of oils |
|---|---|---|---|---|---|---|---|---|
| *trans*-cinnamic acid | nq | 620.80 ± 30.01 | nd | nd | nd | nd | nd | nq |
| 4-hydroxyphenyl-acetic acid | 849.71 ± 45.48 | 1069.75 ± 53.45 | 3563.34 ± 178.15 | 2648.35 ± 165.36 | 3493.21 ± 132.31 | 1333.58 ± 66.31 | 132.42 ± 3.99 | 202.25 ± 10.10 |
| sinapinic acid | nd | 387.23 ± 17.21 | nd | nd | nd | nd | nd | nd |
| gentisic acid | 423.73 ± 19.31 | 499.38 ± 20.20 | 581.29 ± 27.03 | 545.32 ± 26.05 | 1486.34 ± 64.28 | 327.66 ± 15.25 | nd | 272.06 ± 13.62 |
| (+)-taxifolin | 129.42 ± 5.47 | 107.69 ± 4.89 | nd | nq | nq | nd | nd | nd |
| ferulic acid | 412.61 ± 17.63 | 116.45 ± 5.32 | 337.50 ± 16.88 | 351.30 ± 16.25 | 100.21 ± 4.28 | 174.31 ± 7.11 | nd | nd |
| *o*-coumaric acid | 103.85 ± 3.89 | 186.70 ± 7.25 | 137.73 ± 7.02 | 155.69 ± 7.75 | nq | nq | nd | nd |
| *p*-coumaric acid | 125.25 ± 5.05 | 429.99 ± 18.75 | 142.67 ± 7.15 | 136.56 ± 6.28 | 114.97 ± 5.53 | nq | nd | nd |
| vanillic acid | 156.24 ± 5.98 | 347.06 ± 16.99 | 236.90 ± 12.10 | 213.07 ± 10.25 | 318.21 ± 14.91 | 235.94 ± 9.87 | nd | nd |
| caffeic acid | nq | 145.06 ± 5.28 | nq | nd | nq | nq | nd | nd |
| 4-hydroxybenzoic acid | nd | nq | nq | nd | nd | nd | nd | nd |
| dopac | nd | nq | nd | 72.58 ± 3.61 | nd | nd | nd | nd |
| gallic acid | 124.91 ± 5.12 | 181.71 ± 6.87 | 251.42 ± 11.23 | 1376.83 ± 65.01 | 411.06 ± 25.55 | 382.61 ± 17.25 | nd | nd |
| protocatechuic acid | 37.02 ± 1.63 | 110.24 ± 5.11 | 56.76 ± 2.63 | 107.29 ± 4.98 | 80.47 ± 3.59 | 56.26 ± 2.16 | nd | 33.91 ± 1.28 |

[a] Values are given as *X* ± SD. nd, nondetectable. nq, nonquantified.

**Table 3.** Analytical Parameters of Proposed Method

| analyte | linearity (%) | RSD (%) (mean value) | detection limit (mg/mL) | quantification limit (mg/mL) | calibration range (mg/mL) | calibration eqs | $r^2$ |
|---|---|---|---|---|---|---|---|
| *trans*-cinnamic acid | 98.56 | 3.86 | 3.22 | 10.75 | 5–200 | $y = 1720.3x - 1802.5$ | 0.998 |
| 4-hydroxyphenylacetic acid | 98.59 | 1.61 | 1.32 | 4.40 | 5–500 | $y = 893.6x + 2069.3$ | 0.997 |
| sinapinic acid | 96.79 | 6.51 | 5.35 | 17.83 | 5–200 | $y = 1232.3x - 2820.2$ | 0.994 |
| gentisic acid | 98.79 | 3.62 | 2.99 | 9.78 | 5–300 | $y = 2427.6x - 3854.6$ | 0.997 |
| (+)-taxifolin | 98.68 | 2.63 | 2.16 | 7.22 | 5–100 | $y = 1573.8x + 2200.8$ | 0.996 |
| ferulic acid | 97.67 | 5.28 | 3.05 | 10.51 | 5–200 | $y = 2407.2x - 2052.6$ | 0.996 |
| *o*-coumaric acid | 98.74 | 3.15 | 3.04 | 10.16 | 5–100 | $y = 1672.5x - 4413.0$ | 0.996 |
| *p*-coumaric acid | 97.52 | 11.10 | 3.50 | 12.25 | 5–200 | $y = 1638.2x - 5950.3$ | 0.995 |
| vanillic acid | 98.94 | 3.15 | 2.86 | 9.21 | 5–300 | $y = 2180.5x - 3266.5$ | 0.995 |
| caffeic acid | 96.98 | 5.25 | 3.02 | 11.15 | 5–100 | $y = 1923.2x - 4475.2$ | 0.997 |
| 4-hydroxybenzoic acid | 97.17 | 3.09 | 2.53 | 8.01 | 5–100 | $y = 2559.9x - 1432.2$ | 0.997 |
| dopac | 97.47 | 2.99 | 2.37 | 7.90 | 5–100 | $y = 3309.0x + 1129.6$ | 0.997 |
| gallic acid | 98.31 | 2.00 | 1.58 | 5.28 | 5–300 | $y = 2995.6x - 3840.8$ | 0.998 |
| protocatechuic acid | 98.97 | 1.22 | 0.96 | 3.21 | 5–200 | $y = 4323.4x - 7998.5$ | 0.998 |

The intraday repeatabilities on the total peak area (expressed as RSD) were 1.3 and 2.5% for the 20 and 100 μg/mL concentrations, respectively, whereas the interday repeatabilities on total peak area were 2.9 and 5.6% for the 20 and 100 μg/mL concentrations, respectively. For (+)-taxifolin only, the precision obtained (expressed as RSD) was ~9%, which could be due to the closeness to the gentisic acid peak.

Despite the intraday precision being higher than the interday precision, as could be expected, good overall repeatability has been obtained.

**Identification and Quantification of Extra-Virgin Olive Oil Components.** After the optimization of the conditions of the CZE for the separation of the mixture of 14 standard compounds, its usefulness was further checked by analyzing real samples.

The present method has been applied to extra-virgin olive oil samples of different geographical origins and different varieties of one type of olive fruit named Picual, Hojiblanca, Cornicabra, Lechín de Granada, Lechín de Sevilla, and Arbequina. Also, refined olive oils and commercial mixtures of refined and virgin olive oils were analyzed to compare the amounts of phenolic acids present. All samples were injected in the CE instrument seven times (*n* = 7).

In **Figure 2** is shown a typical electropherogram of a virgin olive oil extract. The compounds were identified by comparing

UV spectra of unknown peaks with those of a standard, by comparing migration times, and by spiking the samples with standard compounds at several concentration levels to clarify their identifications.

To determine the real amount of olive oil phenolic acids, it is very important to completely extract this fraction from the extra-virgin olive oil. In the literature it is often possible to find references disagreeing about effectiveness of extraction methods based on solid-phase extraction (SPE) or LLE (*12, 18−24*). The effectiveness of different extraction methods of phenolic acids was compared by evaluating the percentage recoveries for each analyte under study and the number of analytes that were extracted.

The results obtained indicated that the LLE procedure permitted the extraction of a higher number of phenolic acids, which give a greater potential to the present methodology for the analysis of these compounds in any kind of olive oil. The SPE procedures do not allow a sufficient amount of oil to be passed across the cartridge to produce more diluted extracts and, consequently, it is more difficult to detect compounds at sub-parts per million levels with diode array detection.

To study the recoveries of each extraction, a phenolic standard mixture was added to a physically and chemically refined and purified oil after it was checked that this olive oil did not contain

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any amount of the compounds under study; only a small amount of 4-hydroxyphenylacetic acid was detected.

All of the olive oil extracts were analyzed by CE, and the amount of each standard was compared to that of the standard mixture not subjected to any extraction procedure. Tests were performed in triplicate ($n = 3$) and for three different concentration levels (20, 50, and 200 $\mu$g/mL) to simulate the extraction system possibilities at real concentration levels found in olive oils.

All of the recoveries of the standard mixtures of the 14 compounds at the three concentrations levels shown in **Table 1** were ~95% for all of the analytes except for (+)-taxifolin. For 4-hydroxyphenylacetic acid, recoveries of >100% were obtained due to the small contribution of the physically and chemically refined and purified oil used for this study. If this contribution is eliminated, the real recovery for this analyte (at 50 $\mu$g/mL) was 95.35%. Naturally, the recoveries were worse when the concentration of spiked refined oil was higher, because the 500 mL methanol/water mixture (50:50, v/v) was not sufficient to carry away and redissolve all of the phenols in the flask after evaporation in the rotary evaporator.

The differences in the phenolic acid profiles shown in the electropherograms of the different varieties of olive oil extracts are very clear (see **Figure 3**). The differences observed in these electropherograms are completely logical because the phenolic content of an olive oil is the result of a complex interaction among several factors, including cultivar, location, type of crushing machine, degree of ripeness, and storage conditions of olive oil fruits as well as the type of oil extraction procedure used and olive oil storage conditions (*25*, *26*).

It is an important factor to bear in mind, in order to compare the analyses of the samples correctly, that the absorbance scales of the different electropherograms for each variety of olive oil are different. When refined olive oil and mixtures of refined and virgin olive oils were analysed, the amounts of phenolic compounds obtained were smaller than for the other types of olive oils.

The differences among the varieties of olive oils were greater when the relative quantities of these compounds were determined (see **Table 2**) than when the profiles of the electropherograms were studied by checking what analytes were in each olive oil.

Nevertheless, there are compounds such as *trans*-cinnamic acid, sinapinic acid, (+)-taxifolin, caffeic acid, and dopac that appeared in only several olive oils; therefore, these compounds could be considered as potential markers for the geographical origin or the olive fruit variety in the future.

Finally, the detection limit (DL), quantification limit (QL), and precision (as RSD of the intermediate concentration value of the linear range) were calculated for the studied analytes using the method proposed by Curie (*27*). Three replicates of each analyte at different concentrations were made to set up the calibration.

All of the features of the proposed method are summarized in **Table 3**.

**Conclusions.** A CZE method for the quantification of phenolic acids in extra-virgin olive oil extracts at sub-parts per million levels has been developed and optimized. Separation and identification of 14 compounds in olive oils of different geographical origins and different varieties of olive fruit were performed after LLE with good repeatability and short analysis times.

The results obtained are very promising, but more detailed studies are necessary to confirm the phenolic acid pattern that

is typical for each type of extra-virgin olive oil and its relationship with the different factors that affect these profiles.

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Como complemento a la que se describe en el artículo anterior, creemos interesante indicar que en la aplicación del método a muestras reales tras su extracción previa en las condiciones óptimas elegidas tras los estudios preliminares, se observó que los electroferogramas de dichos extractos se podían dividir en dos partes claramente diferenciadas.
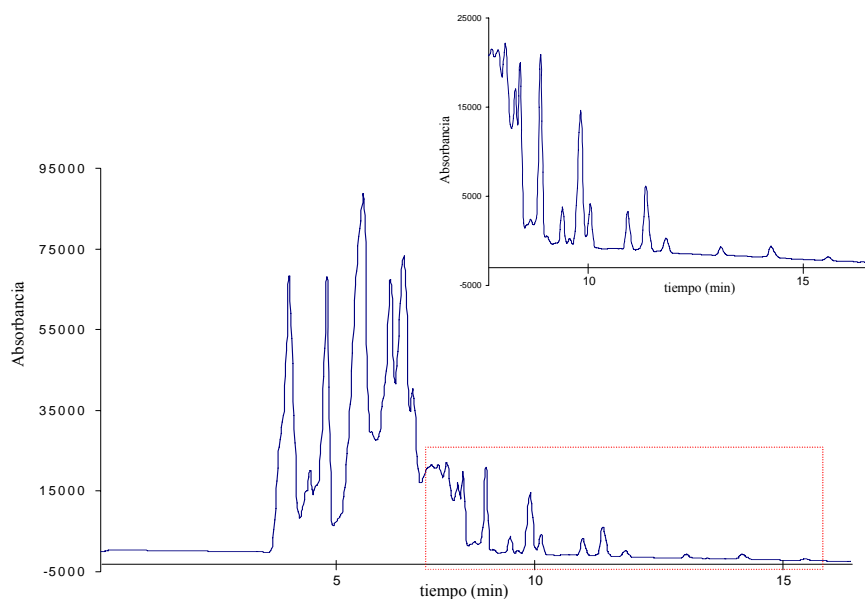


*Figura*. Electroferograma de un extracto en metanol/agua (60/40 v/v) de una muestra de aceite de oliva virgen extra de la variedad Arbequina. La zona que se amplía a la derecha, sería la correspondiente a los ácidos fenólicos.

El primer grupo de analitos contiene *compuestos polihidroxilados*, que están en gran cantidad en el aceite de oliva, por lo que salen picos muy intensos y bastante solapados; para poder resolverlos el tiempo de análisis sería demasiado grande. Además la mayoría de los compuestos que forman parte de ese grupo no pueden ser encontrados comercialmente como patrones, lo que dificulta su identificación y cuantificación.

El segundo grupo de analitos es el de los *ácidos fenólicos* (zona marcada con puntos en la anterior figura y ampliada a la derecha), fueron los elegidos como objeto de estudio para este método, ya que son compuestos que se podrían resolver bien con las condiciones adecuadas y de los que se comercializaban estándares.

Sin lugar a dudas, este método, que fue cronológicamente el primero que se puso a punto en esta tesis, nos amplió el conocimiento de la fracción fenólica del aceite de oliva y nos indicó la línea a seguir en nuestra investigación. En posteriores

metodologías puestas a punto en el trabajo experimental que se incluye en esta memoria, se consiguió llevar a cabo la identificación y cuantificación de muchos de los componentes *polihidroxilados* que en este artículo que compone el primer capítulo de esta tesis, quedaban como "incógnitas".

# Capítulo 3

## Determinación de ácidos fenólicos en aceite de oliva virgen-extra usando modificadores de flujo electroosmótico y polaridad invertida

La idea de desarrollar este nuevo método para analizar la misma familia de compuestos que ya se había estudiado en el capítulo anterior, fue además de comparar los resultados y comprobar la fiabilidad de los mismos mediante el empleo de otro método, utilizar una metodología electroforética diferente y algo más innovadora en la que se invierte la polaridad de los electrodos en el equipo y con ello, a priori, se podrían reducir los tiempos de análisis. Usar "polaridad invertida" quiere decir que la inyección se lleva a cabo en el extremo negativo (cátodo), mientras que el detector se coloca en el extremo positivo (ánodo). Con la inversión de la polaridad, se consigue que los analitos con carga negativa (como es el caso de los ácidos fenólicos al pH de trabajo) migren hacia el electrodo positivo, con lo que la atracción hacia el mismo podría hacer que el análisis requiriese menos tiempo que en el caso del anterior trabajo.

Además el orden de elución de los compuestos presentes en los extractos de aceite sería aproximadamente el inverso, y así, todos los picos intensos que en el método expuesto en el capítulo 2 salían al inicio del electroferograma, saldrían al final con estas condiciones y no interferirían tanto en la determinación de los ácidos fenólicos.

Sin embargo, hay que tener en cuenta que no sólo con invertir la polaridad sería viable este método, ya que el FEO tendería hacia el sentido opuesto al que nos interesa (donde se sitúa el detector UV-Vis) e impediría que los analitos atravesasen el capilar. Por ello, hubo modificar la superficie del capilar para hacer factible el desarrollo de el método aquí descrito.

De manera general, podemos decir que el control del FEO tiene una gran relevancia en las separaciones en CE. Para controlarlo, se han utilizado muchas estrategias, como son: cambios en las propiedades físico-químicas de la disolución reguladora, modificaciones de la superficie del capilar, o aplicación de un voltaje radial externo al capilar [325]. Centrándonos en los cambios en la superficie del capilar, se han utilizado recubrimientos tanto permanentes [326] como dinámicos [327]. Cuando el capilar no está recubierto permanentemente y la polaridad está invertida, se debe de añadir un surfactante para disminuir, suprimir o incluso invertir el FEO. Los surfactantes catiónicos han sido ampliamente utilizados (CTAB, CTAH, TTAB); aunque también

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otros surfactantes de diferente naturaleza, como el HDB (también recogido a veces en bibliografía como HDM) [328]. Incluso se pueden encontrar artículos donde se explica cómo se llevó a cabo la separación electroforética añadiendo al electrolito se separación alquilaminas, EDTA o mezclas de varios surfactantes [328].

De todas las estrategias mencionadas, nos decantamos por modificar la superficie del capilar mediante varios recubrimientos dinámicos observando cuál daba mejor resultado.

Las variedades de aceite de oliva virgen-extra que se estudiaron fueron las mismas que las empleadas en el capítulo 2. También se utilizó el mismo sistema de extracción, puesto que la familia de interés era la misma y era necesario pre-concentrar para poder detectar todos los analitos.

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**Alegría Carrasco Pancorbo**
**Antonio Segura Carretero**
**Alberto Fernández Gutiérrez**

Department of Analytical
Chemistry, Faculty of Sciences,
University of Granada,
C/Fuentenueva s/n,
E-18071 Granada, Spain

# Co-electroosmotic capillary electrophoresis determination of phenolic acids in commercial olive oil

Fourteen phenolic acids have been selectively determined in olive-oil samples using the co-electrosmotic capillary electrophoresis mode with UV detection after the LLE extraction system. A polycationic surfactant (hexadimetrine bromide, HDB), which dynamically coats the inner surface of the capillary and causes a fast anodic electroosmotic flow, was added to the electrolyte. The main factors affecting co-electroosmotic flow (EOF) such as type of modifier, concentration, and influence of organic solvents have been studied. Other parameters such as pH, type, and concentration of buffer, applied voltage, and injection time were also optimised using hydrodynamic injection for 8 s and UV detection at 210 nm. The composition optimum of the running buffer used was a 20% 2-propanol, 0.001% HDB, and 50 mM sodium borate at a pH value of 9.6. The method has been applied to determination and quantification of fourteen phenolic acids at ppb levels in olive oil samples after a liquid-liquid extraction.

## 1 Introduction

The incidence of arteriosclerosis is low in populations using olive oil as their primary source of fat [1–3]. Triacylglycerols comprise about 98% of the oil and oleic acid is its main fatty acid but virgin olive oil, obtained from the first cold pressing, also contains a range of antioxidants and flavour constituents [4, 5].

Phenolic compounds are some of the antioxidants that constitute the polar fraction of virgin olive oils, and there is evidence that the stability of the oils to autoxidation is partly due to their high content of these compounds [6, 7]. Phenolic compounds also contribute to the pungent astringency and bitter taste of virgin olive oil [8] and have also been used in chemometric studies aimed at the characterization of the geographical origin of virgin olive oils [9, 10], although the phenolic composition of oils depends to a large extent on olive variety, pedoclimatic conditions, maturation degree, and oil extraction procedure [11, 12].

Dietary phenolic compounds have generally been considered as non-nutrients, and their possible benefit to human health has only recently been considered. The capacity to be active as antioxidants of the simple phenolic acids

usually found in olive fruit and virgin olive oil has been demonstrated [13–15] but there are few methods that permit their selective quantification in olive oil.

In recent years, capillary electrophoresis has developed into a tool suitable for the analysis of small molecules and ions [16, 17]. Many examples of the application of CE to the analysis of phenolic compounds can be found in the recent literature [18–20], the majority of which use simple CZE or MECK methods based on a borate run buffer at alkaline pH. However, few data are available on the phenolic acid content in products from the olive oil industry as determined by this technique [21–23].

The application of an electric field when using capillary electrophoresis induces a flow of the bulk solution towards the cathode called the electrosmotic flow (EOF). Control of the EOF is of major importance for the optimization of separations. Different strategies have been adopted to control the EOF, including changes in the physicochemical buffer properties, modifications of the capillary surface, or application of an external radial voltage to the capillary [24–26].

For the second approach, either dynamic [27–30] or permanent [28, 30, 31] wall coating has been reported. Among the compounds mentioned for dynamic coatings, the use of cationic surfactants is of great interest in the design of anion separations. CE with reversed EOF has already been applied for the separations of several

**Correspondence:** Antonio Segura Carretero, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain. Fax: +34 958 249510.
E-mail: ansegura@ugr.es.

families of phenolic compounds [32–36] in different kinds of samples.

The aim of this paper is to selectively analyse the phenolic acids in extra-virgin olive oils of several varieties by co-electroosmotic capillary electrophoresis with UV detection.

## 2 Experimental

### 2.1 Reagents and stock solutions

The phenolic acids 4-hydroxyphenylacetic acid, gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), caffeic acid (3,4-dihydroxy-cinnamic acid), dopac (3,4-dihydroxyphenilacetic acid), *p*-coumaric acid (4-hydroxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), *trans*-cinnamic acid, *o*-coumaric acid (2-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), and gentisic acid (2,5-dihydroxybenzoic acid) standard were obtained from Sigma-Aldrich (St. Louis, MO). 4-Hydroxybenzoic acid was acquired from Fluka. The flavanonol, (+)-taxifolin (3,3′,4′,5,7-pentahydroxyflavanone), was obtained from Extrasynthèse (Genay, France) and all the analytes were used as received. All common names and structures are summarized in **Table 1**.

The stock solutions containing all the 14 compounds were prepared in methanol/water (50 : 50 *v/v*) at a concentration of 500 mg L$^{-1}$ for each analyte. Individual stock solutions of each analyte were prepared at the appropriate concentration for the calibration process.

Sodium hydroxide was purchased from Merck (Darmstadt, Germany), sodium borate (borax), sodium phosphate, and CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) were obtained from Sigma, and anhydrous sodium carbonate and ammonium chloride were from Panreac (Montcada I Reixac, Barcelona, Spain); these were all used as running buffers at different concentrations and pHs.

Cetyltrimethylammonium bromide (CTAB), tetradecyltrimethylammonium bromide (TTAB), and hexadimetrine bromide (HDB) were obtained from Sigma. Stock aqueous solutions of CTAB, TTAB, and HDB were prepared weekly at 10 mM for CTAB and TTAB and 0.5% (*w/v*) for HDB.

2-Propanol was obtained from Merck. Methanol and *n*-hexane were acquired from Panreac (Montcada I Reixac, Barcelona, Spain) and were of HPLC grade.

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

**Table 1.** Name and molecular structures for the 14 analytes under study.

| Name | Structure |
| --- | --- |
| Protocatechuic acid |  |
| Dopac |  |
| 4-Hydroxybenzoic acid |  |
| Gallic acid |  |
| Caffeic acid |  |
| Vanillic acid |  |
| *p*-Coumaric acid |  |
| *o*-Coumaric acid |  |
| Ferulic acid |  |
| (+)-Taxifolin |  |
| Gentisic acid |  |
| 4-Hydroxyphenylacetic acid |  |
| Sinapinic acid |  |
| *trans*-Cinnamic acid |  |

## 2.2 Extra-virgin olive oils

Six monovariety extra-virgin olive-oil samples were obtained from varieties of olives called Picual, Hojiblanca, Cornicabra, Lechín de Granada, Lechín de Sevilla, and Arbequina. As the concentration of polyphenols in oils decreases during storage, all the olive oil samples used in this study were obtained at the same time of year (December 2003). Also, refined olive oils without phenolic acids were used as blanks.

## 2.3 Liquid-liquid extraction of phenolic acids

The phenolic acids were extracted from extra-virgin olive oils as follows: $60 \pm 0.001$ g of oil was dissolved in 60 mL hexane and the solution was extracted successively with three 20 mL portions of 60% aqueous methanol. The combined extracts of the hydrophilic layer were dried in a rotary evaporator under vacuum and at a temperature of $40°C$ and the residue was redissolved in 500 $\mu$L of methanol/water (50 : 50 *v/v*) and filtered through a 0.20 $\mu$m filter prior to capillary electrophoresis analysis.

## 2.4 Electrophoretic procedure

CE determination was performed with a Beckman P/ACE™ System MDQ capillary electrophoresis instrument. A diode-array detector was used to detect the individual compounds at their optimum wavelengths and to identify them by comparing their UV spectra with those of the reference compounds. Data acquisition and processing were carried out with GOLD software installed on a personal computer. The system incorporates a $0-30$ kV high-voltage built in power supply. All capillaries (fused silica) used were obtained from Beckman instrument, Inc. (Fullerton, CA, USA) and had an inner diameter of 75 $\mu$m, a total length of 57 cm, and an effective separation length of 50 cm. The temperature was controlled using a fluorocarbon based cooling fluid.

Good repeatability was assured by rinsing the capillary with 0.5 M sodium hydroxide for 5 min followed by rinsing for 2 min with Milli-Q water at the beginning of each experimental session. The capillary was equilibrated with the running buffer (50 mM sodium borate adjusted to pH 9.6, 0.001% HDB and 20% of 2-propanol) for 5 min before each sample injection.

Samples were injected hydrodynamically at the cathodic end in low-pressure mode (0.5 psi) for 8 s. (1 psi = 6895 Pa).

Each electrophoretic run was carried out at $-25$ kV maintaining the capillary temperature at $25°C$, resulting in a current of approximately $-80$ $\mu$A.

After each electrophoretic run, rinsing was performed for 5 min with the run buffer by applying both high voltage with normal polarity of the electrodes (+25 kV) and pres-

sure (20 psi), which resulted in separations with higher repeatability than obtained after solely purging the capillary with electrolyte. The effect of purging and high voltage can be explained by a faster regeneration of the inner capillary surface and, consequently, a stable EOF. Then, the capillary was replenished with fresh electrolyte solution (5 min flush). Pre-conditioning of the capillary and the purging sequence between two runs were essential to obtain reproducible results. All washing steps were performed at $25°C$.

All samples, buffers, and solutions were filtered through a 0.20 $\mu$m syringe filter. As the filtration is commonly a problematic step, we checked the absence of analyte retention in the filter. The running buffer was changed after 5 runs.

UV detection was performed at 210 nm and 275 nm simultaneously. Diode-array detection was used over the range of 190 to 600 nm to obtain spectral data. Peak identification was accomplished by comparing both migration time and spectral data obtained from real samples and standards and also with spiked real samples at different concentration levels. Peak areas were used for quantification of the analytes.

# 3 Results and discussion

## 3.1 Selection of analytes

With the information and all the data of compounds previously reported from virgin olive oil, we set up a group of phenolic acids and a flavonol compound, (+)-taxifolin, very common in olive oil samples and that elutes in the same window time of phenolic acids. The phenolic compounds selected were: protocatechuic acid, dopac, 4-hydroxybenzoic acid, gallic acid, caffeic acid, vanillic acid, *p*-coumaric acid, *o*-coumaric acid, ferulic acid, gentisic acid, 4-hydroxyphenylacetic acid, sinapinic acid, and *trans*-cinnamic acid.

The (+)-taxifolin was included in this group of compounds under study because it was extracted with the LLE system used and appeared in the zone of phenolic acids in the electropherograms of extra-virgin olive-oil samples [37].

## 3.2 Effect of surfactant concentration and chain length on resolution

Long-chain alkylammonium salts (cetyltrimethylammonium and tetradecyltrimethylammonium bromide (CTAB and TTAB)) as well as polycations (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (HDB)) are suitable for use as electroosmotic flow modifiers. Their behavior, especially the reversal of the electroosmotic mobility, can be explained by assuming the formation of hemimicelles on the capillary wall. That is, cationic surfactants are first adsorbed individually by electrostatic interactions

**Figure 1.** CZE electropherograms with co-electroosmotic flow of a standard mixture composed by 14 phenolic compounds under study using different surfactant. (a) 0.7 mM CTAB, (b) 0.001% HDB, (c) 2.5 mM TTAB. Detection was performed at 210 nm.

and then begin to associate into hemi-micelles by Van der Waals attraction. The formation of hemi-micelles changes the surface charge of the capillary wall from negative to positive and causes the reversal of the electroosmotic mobility. Consequently, the polarity of the electrodes has to be reversed and the sample is injected at the cathodic end and detection is performed at the anodic end.

Optimisation of the separation conditions was achieved through testing the migration behavior of a standard mixture containing the 14 compounds under study. The concentration ranges studied were from 0.03–1.9 mM for CTAB, from 0.05–5 mM for TTAB, and 0.0001–0.02% for HDB. This study was carried out varying the modifier concentrations under conditions of constant pH, organic sol-

vent percentage, and ionic strength. Within the concentration range evaluated, all three surfactants produce flow reversal in a single step [38, 39]. An increase in surfactant concentration in the bulk has no effect on the extent of adsorption at the surface and the flow magnitude remains practically constant.

As can be seen in **Figure 1**, the best resolution was obtained when HDB was used at a concentration of 0.001%.

### 3.3 Effect of pH and buffer

The effect of pH was studied between 7.5 and 10.5 and we observed that the migration times of the analytes decreased with increasing pH values. High pH values cause the silanol groups of the interior of the capillary walls to become negatively charged and the interaction with the positive charges of the polycationic surfactant (HDB) to become stronger.

To ensure a high inherent electrophoretic mobility of the phenols, an alkaline buffer with a pH value above the respective $pK_a$ values of the analytes must be used. For this reason a more detailed study between pH 9 and 10 was carried out to obtain the optimum pH value for the total resolution of the analytes under study by adjusting the buffer pH to 9.2, 9.4, 9.6, and 9.8.

Five different aqueous buffers were tested: sodium phosphate, CAPSO, ammonium chloride, sodium carbonate, and sodium borate, all adjusted to the pH range between 9 and 10 (9.2, 9.4, 9.6, and 9.8). Sodium borate gave the best separation plus a satisfactory analysis time; no complexation was observed and a value of pH 9.6 was selected for the rest of the experimental work (see **Figure 2**). The separation achieved was good because the −OH groups of phenolic acids are de-ionized and the present migration times are shorter than those of other compounds of the polar fraction of olive oil which are extracted with 60% aqueous methanol.

The EOF on a bare capillary decreases with increasing ionic strength. Changes in the mobility of the analytes and in the EOF were observed when the ionic strength of a borate buffer is increased by raising the buffer concentration between 10 to 80 mM. The concentration selected for the rest of the experimental work was 50 mM.

### 3.4 Influence of organic solvent

The use of organic solvents as electrolyte additives favors the resolution of the phenolic acid but the separation time is often increased when high solvent concentrations are used, due to a higher viscosity and reduction of the zeta potential. Different organic solvents such as methanol, acetonitrile, 1-propanol, and 2-propanol were tested, the best results being obtained with 2-propanol. We have

studied contents of 2-propanol between 5 and 30% and found the best resolution to occur at 20% (see **Figure 3**).

### 3.5 Effect of instrumental variables

The effect of the applied voltage on the resolution of the 14 compounds was studied using the optimized buffer composition. When the applied voltage increased from −10 to −30 kV, shorter analysis times were obtained, albeit at the expense of a deteriorating efficiency of separation. Thus, a voltage of −25 kV was selected.

Among the different injection modes, hydrodynamic injection was selected. We observed that as the sample plug length is increased, the efficiency decreases and peaks become broader. An injection time of 8 s was finally selected.

The temperature during washing and analysis was 25°C.

The optimum electropherogram obtained from a standard mixture of 14 analytes can be seen in **Figure 4**.

### 3.6 Repeatability study

Repeatability was assessed for each standard compound at two concentration levels (20 and 100 mg L$^{-1}$). The two solutions containing all the analytes were prepared and analyzed on the same day (intraday precision, $n = 12$) and on 3 consecutive days (interday precision, $n = 36$). The relative standard deviations of peak areas and the relative standard deviations of migration times were determined for each analyte in the two standard mixtures.

The intraday repeatability of the migration time (expressed as relative standard deviation) was 0.89 and 1.25% for the 20 mg L$^{-1}$ and 100 mg L$^{-1}$ concentrations, respectively, whereas the interday repeatability of the migration time was within 0.98 and 1.65% for the 20 mg L$^{-1}$ and 100 mg L$^{-1}$ concentrations, respectively.

The intraday repeatability of the total peak area (expressed as relative standard deviation) was 2.5 and 4.9% for the 20 mg L$^{-1}$ and 100 mg L$^{-1}$ concentrations, respectively, while the interday repeatability of the total peak area was 3.5 and 6.1% for the 20 mg L$^{-1}$ and 100 mg L$^{-1}$ concentrations, respectively.

Although the interday precision was worse than the intraday precision, good overall repeatability was still obtained.

### 3.7 Analytical parameters

The detection limits and quantitation limits of the method were tested for the studied analytes [40]. The results obtained for the phenolic acids are summarized in **Table 2**. Standard calibration graphs were prepared for each analyte. All calibration curves showed good linearity between different concentrations depending on the analytes studied. Each point of the calibration plot was
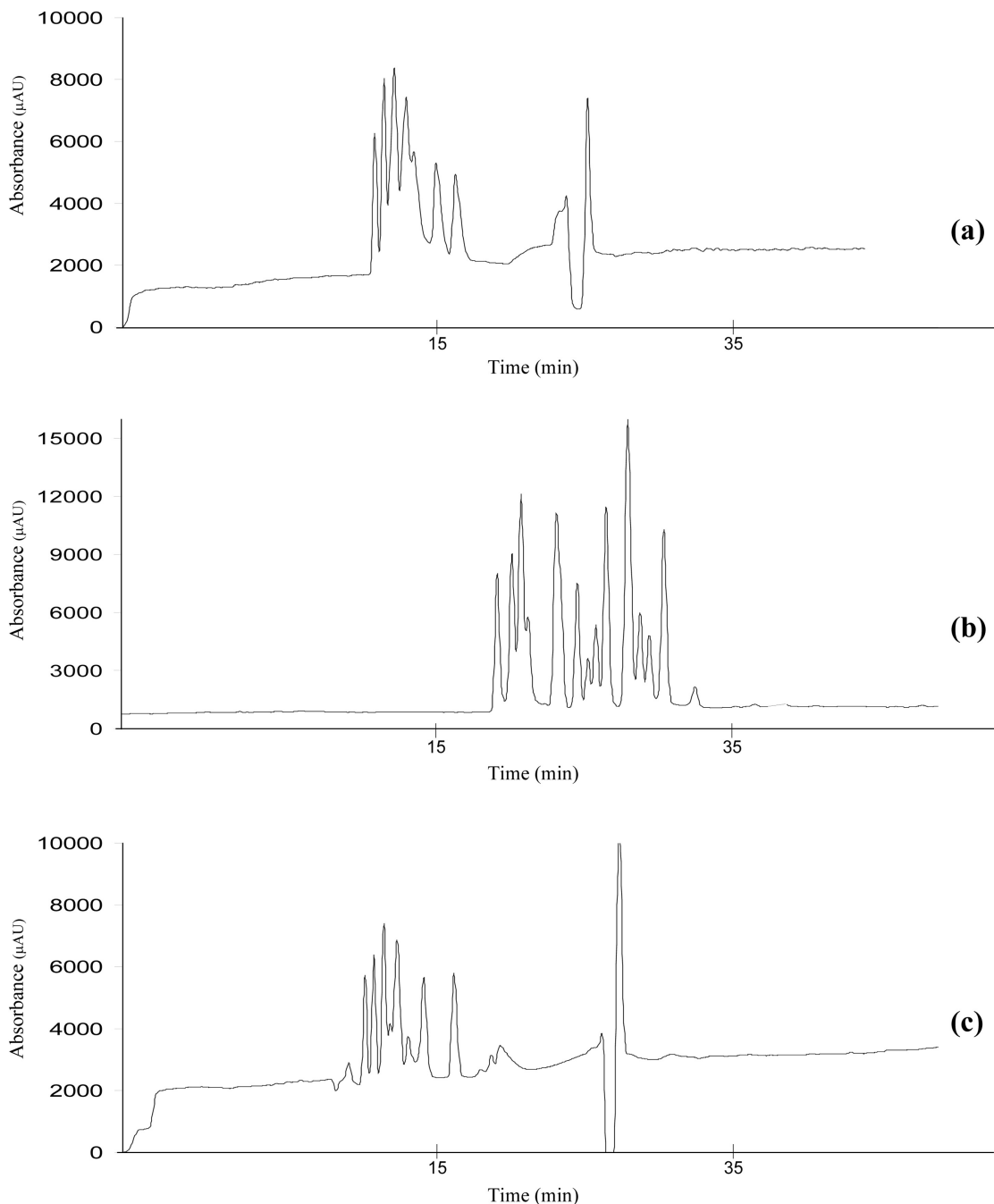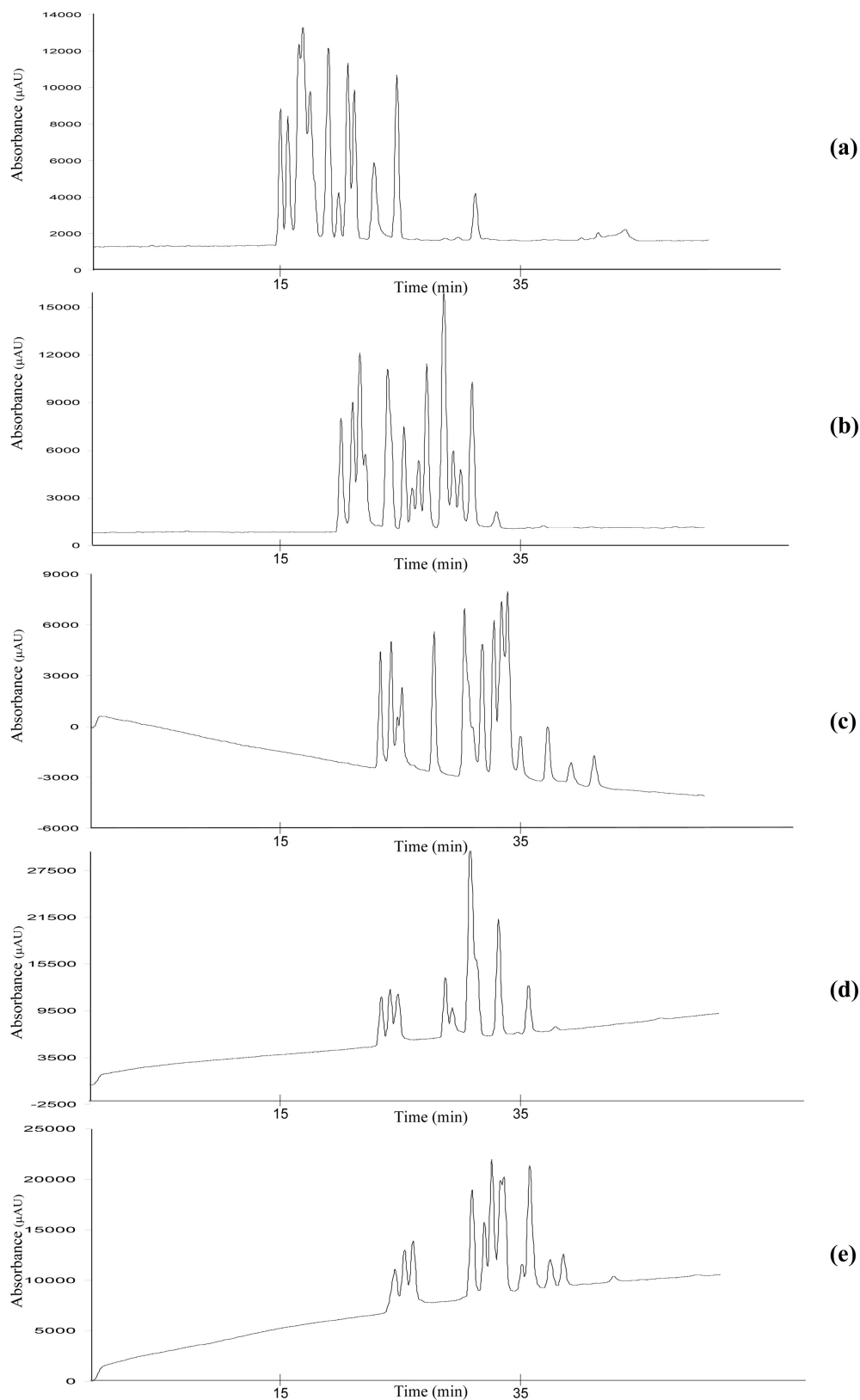
**Figure 2.** CZE electropherograms with co-electroosmotic flow of a standard mixture composed by 14 phenolic compounds at different values of pH. (a) pH 10, (b) pH 9.6, (c) pH 9, (d) pH 8.5, (e) pH 8. Detection was performed at 210 nm.

**Table 2.** Analytical parameters of proposed method.

| Analyte | Detection limit [mg L$^{-1}$] | Quantification limit [mg L$^{-1}$] | Calibration range[a] [mg L$^{-1}$] | Calibration equations | $r^2$ |
|---|---|---|---|---|---|
| Protocatechuic acid | 1.69 | 5.63 | 200 | $y = 3140.9x - 781.6$ | 0.996 |
| Dopac | 2.18 | 7.27 | 100 | $y = 2428.8x + 8282.0$ | 0.997 |
| 4-Hydroxybenzoic acid | 1.30 | 4.33 | 100 | $y = 4074.5x - 23918.0$ | 0.999 |
| Gallic acid | 1.73 | 5.77 | 500 | $y = 3065.7x - 15180.0$ | 0.998 |
| Caffeic acid | 2.77 | 9.23 | 100 | $y = 1912.3x + 10646.0$ | 0.997 |
| Vanillic acid | 1.76 | 5.87 | 200 | $y = 3012.4x + 18129.0$ | 0.996 |
| *p*-Coumaric acid | 2.40 | 8.00 | 200 | $y = 2210.9x - 23128.0$ | 0.996 |
| *o*-Coumaric acid | 1.90 | 6.33 | 100 | $y = 2791.3x + 42655.0$ | 0.995 |
| Ferulic acid | 2.20 | 7.33 | 100 | $y = 2402.8x - 19635.0$ | 0.996 |
| (+)-Taxifolin | 1.46 | 4.87 | 100 | $y = 3622.0x + 112225.0$ | 0.999 |
| Gentisic acid | 3.40 | 11.33 | 1500 | $y = 1556.0x - 1300.2$ | 0.995 |
| 4-Hydroxyphenylacetic acid | 0.53 | 1.77 | 1000 | $y = 10027.0x - 26044.0$ | 0.997 |
| Sinapinic acid | 2.63 | 8.77 | 500 | $y = 2014.0x - 25536.0$ | 0.998 |
| *trans*-Cinnamic acid | 1.17 | 3.9 | 1000 | $y = 4517.2x + 20462.0$ | 0.997 |

[a]  The calibration ranges were from LOQ to the value indicate in the table.
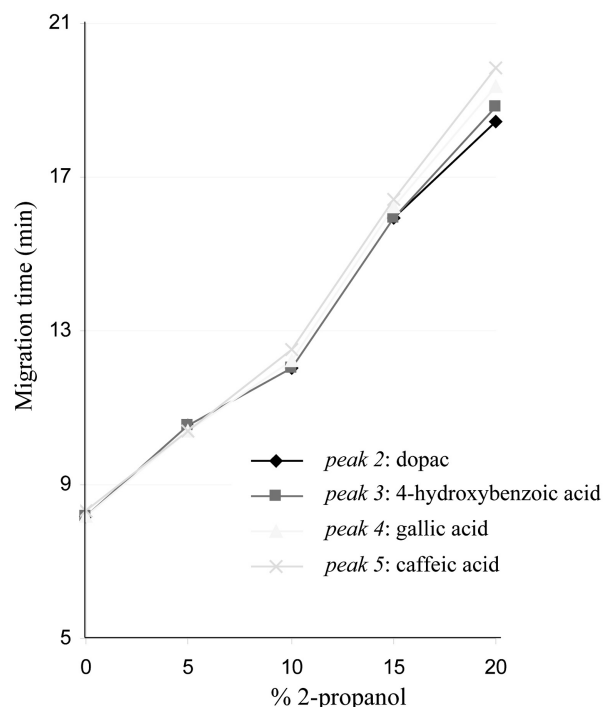


**Figure 3.** Effect of % of 2-propanol in the migration time of peaks: 2, dopac; 3, 4-hydroxybenzoic acid; 4, gallic acid; and 5, caffeic acid.

repeated three times in the same way. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.997 for all the compounds quantified.

### 3.8  Analysis of olive oil samples

After optimization of the conditions for the separation of the 14 standard compound mixture, the method was checked by analyzing extracts of olive-oil samples.

The main olive-oil producing countries have been particularly active in recent years in studying the chemical composition of olive oil varieties or oil produced in a specific area and how this relates to oil quality. For this reason the method has been applied to six monovariety extra-virgin olive-oil samples of six types of olive fruit called: Picual, Hojiblanca, Cornicabra, Lechín of Granada, Lechín of Sevilla, and Arbequina.

All the samples were subjected to replicate capillary electrophoresis seven times ($n = 7$).

The compounds were identified by comparing the UV spectra of unknown peaks with those of standard peaks, comparing migration times, and by spiking the samples with standard compounds at several concentration levels to confirm their identification.

The differences in the phenolic acid profiles are shown in **Figure 5**.

The absorbance scales in the six electropherograms are different, due to considerable concentration differences between the compounds under study in each variety. The quantitative results are presented in **Table 3**. It is important to bear in mind that the units in this table are in µg analyte/L olive oil because we have considered a preconcentration factor of ≈120 times for the liquid-liquid extraction
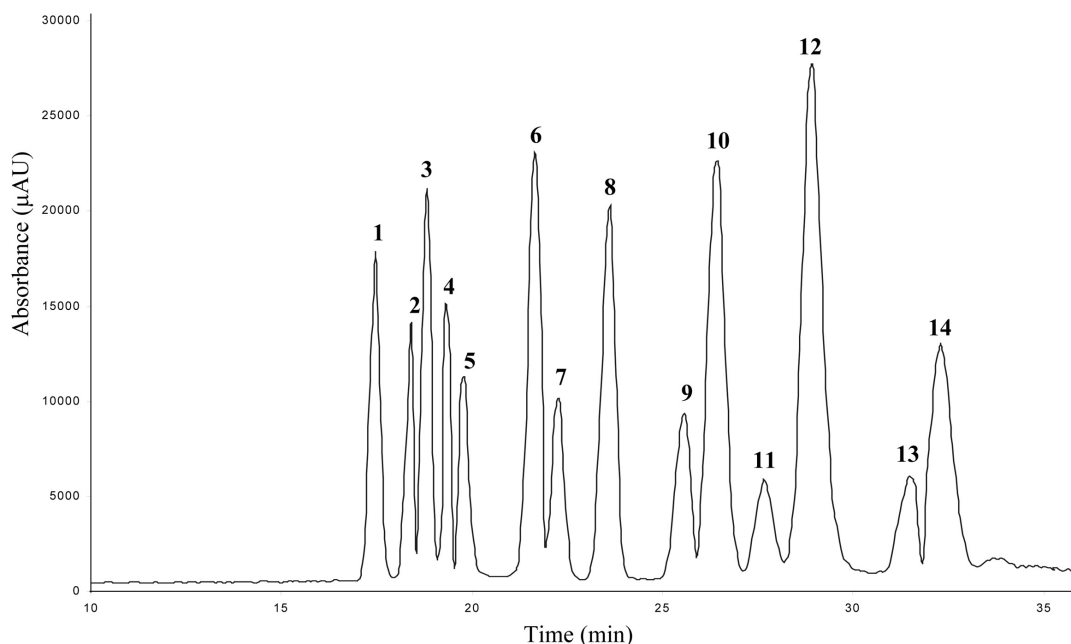
**Figure 4.** CZE with co-electroosmotic flow of a standard mixture composed of 14 phenolic compounds under optimized conditions. Separation conditions: capillary, 57 cm × 75 μm; applied voltage, −25 kV; applied temperature, 25°C; buffer, 50 mM sodium borate (pH 9.60) with 20% 2-propanol; hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 210 nm. Peak identification numbers: 1, protocatechuic acid; 2, dopac; 3, 4-hydroxybenzoic acid; 4, gallic acid; 5, caffeic acid; 6, vanillic acid; 7, *p*-coumaric acid; 8, *o*-coumaric acid; 9, ferulic acid; 10, (+)-taxifolin; 11, gentisic acid; 12, 4-hydroxyphenylacetic acid; 13, sinapinic acid; and 14, *trans*-cinnamic acid. All the analytes were at 100 mg L$^{-1}$.

**Table 3.** Results of the analysis of real samples ($n$ = 7) (Value = $X \pm$ SD).

| Concentration [μg L$^{-1}$ = μg analyte/L olive oil] | | | | | | |
|---|---|---|---|---|---|---|
| Analyte | Arbequina | Picual | Hojiblanca | Lechín de Sevilla | Lechín de Granada | Cornicabra |
| Protocatechuic acid | 75.8 ± 3.8 | 32.7 ± 1.7 | 18.0 ± 1.5 | 58.0 ± 1.5 | n.d. | n.d. |
| Dopac | 109.5 ± 7.7 | n.d. | n.d. | 54.2 ± 2.1 | n.d. | n.d. |
| 4-Hydroxybenzoic acid | 25.5 ± 3.6 | 70.4 ± 2.9 | n.d. | n.d. | n.d. | n.d. |
| Gallic acid | 113.9 ± 6.8 | 240.1 ± 9.6 | 450.2 ± 9.3 | 2270.5 ± 68.9 | 294.2 ± 23.5 | 662.2 ± 38.5 |
| Caffeic acid | 24.5 ± 0.7 | 80.7 ± 4.6 | n.q. | 184.6 ± 6.5 | n.q. | 93.2 ± 5.2 |
| Vanillic acid | 201.4 ± 13.1 | 295.6 ± 12.6 | 384.4 ± 29.2 | 397.8 ± 47.8 | 524.7 ± 25.6 | 191.7 ± 7.3 |
| *p*-Coumaric acid | 217.8 ± 9.8 | 561.7 ± 12.9 | 145.4 ± 17.8 | 201.9 ± 54.2 | 272.6 ± 14.2 | 547.5 ± 23.3 |
| *o*-Coumaric acid | 32.4 ± 2.9 | 140.1 ± 3.5 | 99.1 ± 3.4 | 138.7 ± 9.6 | n.q. | n.q. |
| Ferulic acid | 237.2 ± 4.1 | 258.8 ± 10.7 | 199.2 ± 6.2 | 214.5 ± 6.4 | 159.1 ± 4.8 | 254.5 ± 7.6 |
| (+)-Taxifolin | 51.3 ± 2.6 | 23.1 ± 1.6 | n.q. | n.q. | n.d. | n.d. |
| Gentisic acid | 459.7 ± 41.1 | 250.8 ± 15.9 | 1029.9 ± 64.6 | 823.8 ± 41.4 | 1280.4 ± 75.5 | 668.6 ± 45.8 |
| 4-Hydroxyphenylacetic acid | 435.3 ± 19.6 | 884.9 ± 39.8 | 3542.7 ± 159.4 | 2157.5 ± 97.1 | 2819.9 ± 124.3 | 2545.1 ± 114.5 |
| Sinapinic acid | n.d. | 1217.1 ± 25.3 | n.d. | n.d. | n.d. | n.d. |
| trans-Cinnamic acid | 745.5 ± 22.6 | 4476.9 ± 203.8 | n.d. | n.d. | n.d. | n.d. |

n.d.: non detectable
n.q.: non quantified
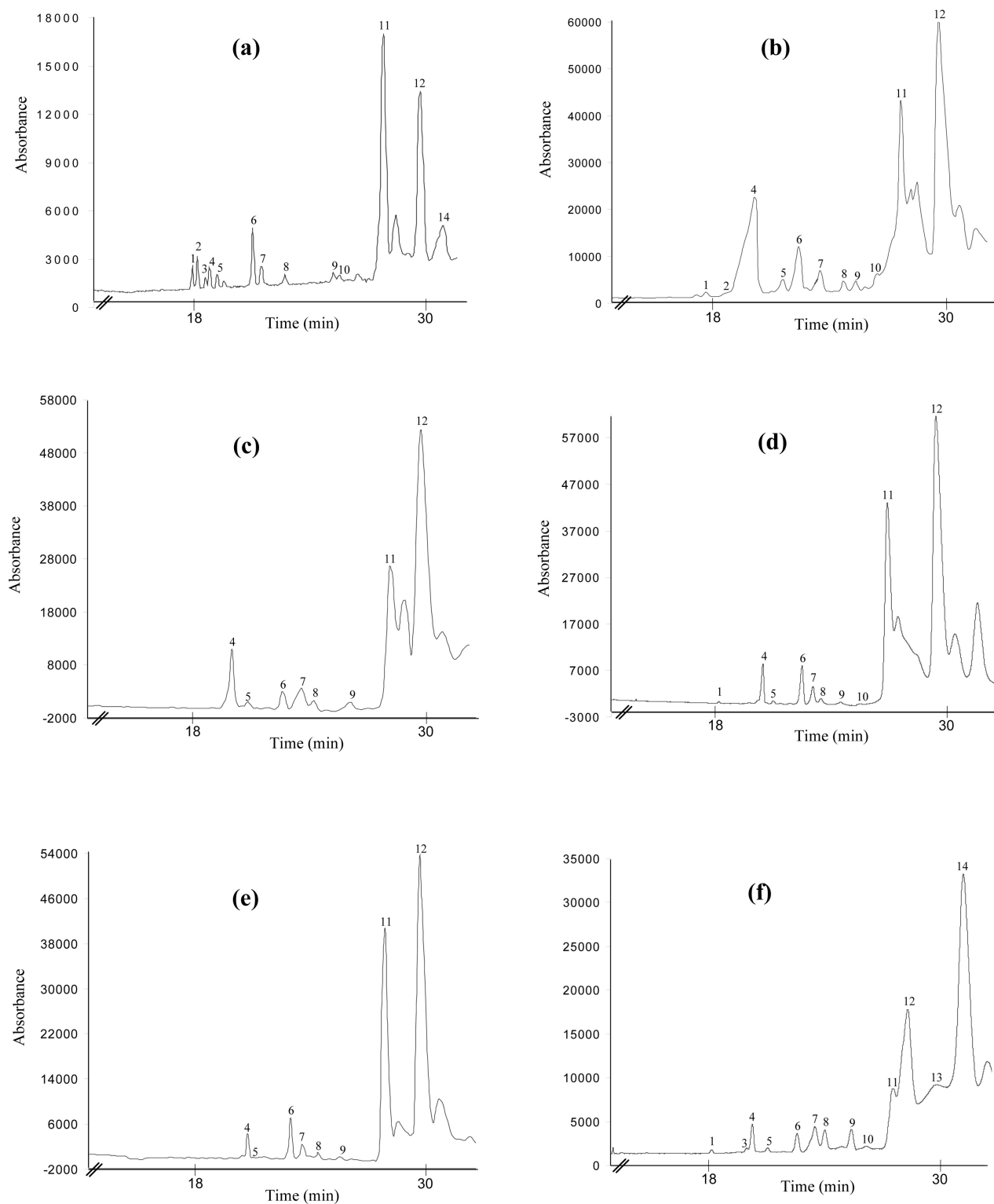
**Figure 5.** CZE electropherogram with co-electroosmotic flow in optimum conditions of phenolic fraction extracted from real olive oil sample by LLE. (a) Arbequina, (b) Lechín de Sevilla, (c) Cornicabra, (d) Hojiblanca, (e) Lechín de Granada, and (f) Picual. For identification of the compounds see Figure 4. Detection was performed at 210 nm. The absorbance scales (µAU) in the six electropherograms are different because there are considerable concentration differences between the compounds under study in each variety. (instrumental and experimental parameters as Figure 4).

system used (obviously depending on the % recovery of each analyte).

## 4 Conclusions

The price of virgin olive oil depends on olive variety and quality, and analytical tools are necessary to authenticate monovarietal oils and to determine their quality. Polyphenols are significantly related to the quality of virgin olive oil, but this relation is always established for the major components of the methanol-water extract of the virgin olive oil. We have demonstrated that it should be possible to find a relation between the quality or variety of a virgin olive oil and a group of minority components such as phenolic acids. Separation and identification of 14 compounds of the hydrophilic fraction of olive oil were accomplished. The amounts of these substances were determined in several extracts obtained from different olive oil varieties. In fact, the distribution of the phenolic compounds shows quantitative differences, which are related to the variety of the olive fruit. The method described provides good repeatability and satisfactory analysis times.

## Acknowledgements

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Como se puede deducir, los resultados obtenidos en el capítulo 2 y en el 3 están muy de acuerdo en lo referido al orden de magnitud; las diferencias apreciadas son debidas a que los aceites no eran exactamente del mismo período de la campaña oleícola y, como se ha mencionado ya en varias partes de esta memoria, son muchos los factores agronómicos que afectan a la composición fenólica del aceite de oliva.

En relación al tiempo de análisis no pudimos cumplir unos de los objetivos que nos motivó para afrontar el desarrollo de este método, ya que finalmente eran necesarios más de 30 minutos para determinar los 14 analitos, mientras que en el método descrito en el capítulo 2 bastaban algo más de 15 minutos. Esta demora en la migración de los compuestos, tiene su explicación en que fue bastante difícil lograr la resolución adecuada entre dopac, ácido 4-hidroxibenzoico, ácido gálico y ácido cafeico; no siendo posible separarlos sin la adición de un 20% de 2-propanol.

En la figura que se muestra a continuación, es interesante comparar los perfiles obtenidos para la variedad Lechín de Sevilla por el método en zona con polaridad normal (capítulo 2) y éste con polaridad invertida y modificador del FEO. El orden de elución no fue exactamente el inverso, pero sí estuvo cercano a serlo.
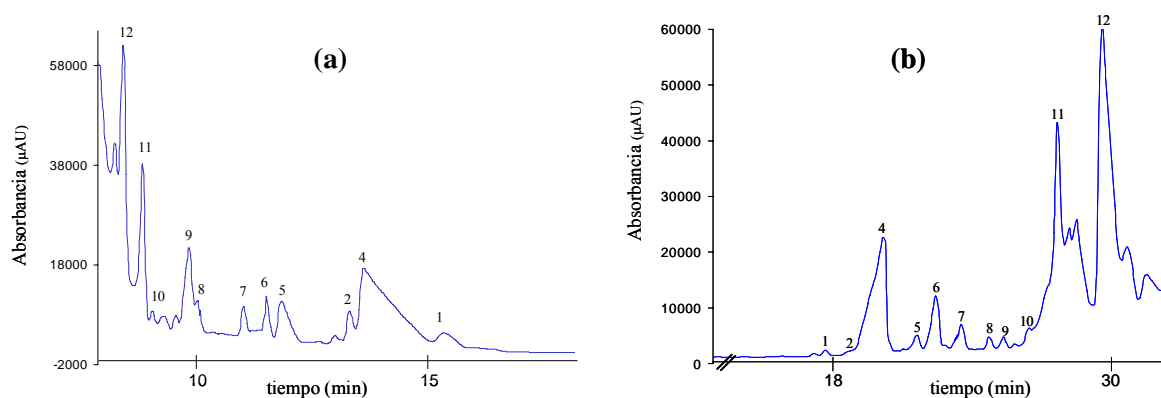


(a) Electroferograma obtenido con las condiciones óptimas del método desarrollado en el capítulo 2 usando una metodología en zona con polaridad normal. (b) Electroferograma obtenido con las condiciones óptimas del método desarrollado en el capítulo 2 usando una metodología en zona con polaridad invertida y un modificador del FEO.
Identificación de los picos: (1) ácido protocatecuico; (2) dopac; (3) 3,4-hidroxibenzoico; (4) ácido gálico; (5) ácido cafeico; (6) ácido vanílico; (7) ácido p-coumárico; (8) ácido o-coumárico; (9) ácido ferúlico; (10) (+)-taxifolin; (11) ácido gentísico; (12) ácido 4-hidroxifenilacético; (13) ácido sinapínico; (14) ácido trans-cinámico.

De cualquier modo, aunque el tiempo de análisis fue peor que en el caso del método en zona con polaridad normal, logramos poner a punto un método fiable, que nos permitía determinar de modo satisfactorio todos los componentes de la familia de ácidos fenólicos, y que introducía algunas innovaciones metodológicas para el análisis de estos analitos extraídos de la matriz del aceite de oliva.

# Capítulo 4

# Determinación de ácidos fenólicos usando capilares recubiertos de EpyM-DMA y CE con flujo invertido

Como ya se adelantaba en el capítulo anterior, una de las estrategias que se puede adoptar para controlar el FEO o evitar interacciones indeseables entre los analitos y las paredes del capilar, es llevar a cabo un recubrimiento de la pared interna del capilar, ya sea dinámico o estático (permanente o semi-permamente). La modificación de las paredes de un capilar puede ser clasificada en tres categorías [329]:

(a) recubrimientos dinámicos añadiendo a la disolución de separación un modificador catiónico o neutro [330],

(b) provocar la adsorción de un modificador catiónico a la pared del capilar de modo permanente mediante una adsorción física [331], o

(c) fijar una capa hidrofílica permanente mediante un enlace covalente y/o un entrecruzamiento [332].

Un recubrimiento de capilar ideal es aquel que es estable durante las condiciones que se requieren para llevar a cabo la separación, y que además sigue siendo estable en un rango amplio de valores de pH. Hoy en día, otro factor que determina la idealidad de un recubrimiento es que éste sea compatible con CE-MS.

Como se comentó antes, para obtener un recubrimiento dinámico, es necesario añadir determinadas sustancias a la disolución reguladora que interaccionen con la pared interna del capilar; este tipo de recubrimientos conllevan un grave problema cuando el detector que está acoplado al equipo de CE es MS, puesto que existe el riesgo de contaminación de la interfase y disminución de la señal obtenida.

Por otra parte, se ha demostrado que el empleo de recubrimientos físicos o covalentes del capilar, puede ser una buena alternativa para suprimir las interacciones de los analitos con las paredes del mismo y, a su vez, evitar la contaminación del espectrómetro de masas. En general, estos recubrimientos suelen tener naturaleza polimérica.

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Algunos de los recubrimientos covalentes o entrecruzados requieren de procedimientos bastante complicados para fijar el propio recubrimiento a la pared del capilar, aunque ofrecen la gran ventaja de que son estables durante mucho tiempo. Por otra parte, los recubrimientos físicos tienen un tiempo de vida algo menor, pero ofrecen buena reproducibilidad y su uso es más sencillo.

Estos recubrimientos físicos se han utilizado en algunas ocasiones [331, 333] para controlar el FEO y reducir los fenómenos de adsorción, pero como la fijación al capilar se basa en fenómenos de adsorción, en los casos mencionados siempre era necesario añadir un poco de agente para el revestimiento del capilar en el tampón de separación. Este hecho, lógicamente representa un problema en CE-MS.

Recientemente, González y col [334] sintetizaron un copolímero (2-etil-(2-pirrolidina) metacrilato de N,N-dimetilacrilamida (EpyM-DMA) que daba la posibilidad de obtener un recubrimiento físico simple, rápido y reproducible, que se podía aplicar al análisis de proteínas básicas y ácidas mediante CE-MS. Su gran ventaja es que no era necesario añadir cantidades de esta sustancia en el tampón de separación, bastaba simplemente con lavar el capilar durante dos minutos con una disolución diluida del polímero justo antes de equilibrar el capilar con la disolución de separación.

El objetivo que nos planteamos al realizar el presente capítulo fue demostrar la utilidad del recubrimiento polimérico descrito para llevar a cabo el análisis de dos familias de compuestos que tienen gran relevancia: los ácidos fenólicos y los aminoácidos.

El interés que despiertan los primeros ha sido puesto ya de manifiesto en la presente memoria; respecto a los segundos, cabe decir que son muy importantes en multitud de campos. El análisis de la composición de proteínas y péptidos es esencial para estudiar la estructura primaria en Bioquímica. Además la determinación de aminoácidos en fluidos biológicos como la orina y sangre, puede ayudar en el diagnóstico y tratamiento de diversas enfermedades. En el ámbito alimentario, los aminoácidos se miden para establecer posibles tendencias en el flavor, controlar el proceso de fermentación y asegurar la calidad del producto final.

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En el caso de los ácidos fenólicos, el detector utilizado fue UV-Vis, y para los aminoácidos (derivatizados con fluoresceína isotiocianato (FITC)), la fluorescencia inducida por láser (FIL).Así demostramos que el rango de posible uso del EpyM-DMA es muy amplio, ya que se puede utilizar para el análisis de estas dos familias teniendo acoplados a la CE diferentes detectores. Además, para lograr la separación completa de los analitos (en ambos métodos) hubo que añadir modificadores orgánicos al medio de separación, cosa que nos permite dar fe de su repetibilidad y reproducibilidad incluso con el uso de estos modificadores.

Los aminoácidos se salen un poco del hilo conductor de la presente tesis, pero se incluyeron en este estudio porque el objetivo de este trabajo no fue analizar las dos familias de compuestos mencionadas en una matriz en particular, sino poner a punto dos métodos potencialmente útiles para un amplio abanico de muestras.

Si comparamos los resultados obtenidos en el caso de los ácidos fenólicos con los dos capítulos anteriores, vemos que son claramente mejores que aquellos obtenidos usando el método del capítulo 3, y que en cuestión de tiempo, son muy similares al método de zona con polaridad normal descrito en el capítulo 2. La única diferencia es que en la familia aquí analizada no se incluyó el (+)-taxifolin al no ser un ácido fenólico.

**ELSEVIER**

# Coelectroosmotic capillary electrophoresis of phenolic acids and derivatized amino acids using *N,N*-dimethylacrylamide-ethylpyrrolidine methacrylate physically coated capillaries

Alegría Carrasco-Pancorbo [a], Alejandro Cifuentes [b], Sonia Cortacero-Ramírez [a], Antonio Segura-Carretero [a,*], Alberto Fernández-Gutiérrez [a]

[a] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, 18071 Granada, Spain*
[b] *Institute of Industrial Fermentations, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

## Abstract

Two different families of compounds, i.e., phenolic and amino acids have been separated by capillary electrophoresis using a physically adsorbed polymer as capillary coating. The polymer used was *N,N*-dimethylacrylamide-ethylpyrrolidine methacrylate (DMA-EpyM) and it provided an stable coating by only flushing the capillary with a DMA-EpyM aqueous solution for 2 min between runs. The usefulness of this procedure has been demonstrated through the fast analysis of different families of solutes. Two different detection systems, diode-array detector and laser-induced fluorescence, have been used to determine phenolic acids and derivatized amino acids with fluorescein isothiocyanate, respectively. The main factors affecting reversal of electroosmotic flow (EOF) such as pH, type and concentration of buffer, and concentration and influence of organic solvents, as well as all the instrumental conditions were studied and optimized for both families of compounds.
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## 1. Introduction

Capillary electrophoresis (CE) is a rapidly growing, powerful separation technique that is being used for the separation and characterization of a wide variety of analytes. Because of a good suitability, CE methods have been often used for the separation of small molecules and ions [1–4]. The application of an electric field when using capillary electrophoresis induces a flow of the bulk solution towards the cathode called electroosmotic flow (EOF). Control of the EOF is of major importance for the optimization of separations [5,6]. Different strategies have been adopted to control the EOF including changes in the physicochemical buffer properties, modifications of the capillary surface or application of an external radial voltage to the capillary [7–11].

Concerning to the second approach, there are other reasons for chemical modification of the capillary wall in electrophoresis. Goals may also include reduction or elimination of analyte–wall interactions, improved reproducibility, or resolution of particularly difficult separation problems [12]. The general modification of the capillary wall can be classified into the following categories [13]: to perform dynamic coating by adding the cationic or neutral modifier to the electrolytes [14–16], to adsorb the cationic modifier to the capillary wall permanently by physical adsorption [17,18], and to fix the hydrophilic layer permanently by covalent bonding and/or cross-linking [19,20]. An ideal capillary wall coating is stable under conditions required for separation, preferably over a broad range of buffer pH. Nowadays, another factor to bear in mind to evaluate the suitability of a coating is if its use is compatible with CE–MS applications.

The dynamic coating can cause severe problems when CE is combined with MS because the presence of the non-volatile buffer constituents may deteriorate the ionization of the analytes. Hence, permanent modification would be preferable. Some of the covalent bondings and/or cross-linkings require a relatively

complicated coating procedure, although their advantage is that they have a long lifetime [13]. On the other hand, the physical adsorption has a shorter lifetime, but it has a simple coating procedure and good reproducibility [21].

Phenolic acids and amino acids are two families of compound very important for health. One vast area of interest has been in food quality [22–24]. Phenolic acids have been associated with color, sensory qualities, and nutritional and antioxidant properties of foods [25]. One impetus for analytical investigations has been the role these phenolics have in organoleptic properties (flavor, astringency, and hardness) of foods [26,27]. Additionally, the food industry has investigated the content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives [28]. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables, against oxidative damage diseases (coronary heart disease, stroke, and cancers) [29–31].

Amino acids are also very important in many fields [32]. Their compositional analysis in proteins and peptides is essential for the study of the primary structure in biochemistry [33]. Determination of amino acids in body fluids such as urine and blood can help in the diagnosis and treatment of diseases [33]. While in food science, amino acids are measured to correlate flavor trends, monitor fermentation and assess the quality of the final product [34–37].

Different CE methods with reversal EOF have already been applied for the separations of phenolic compounds [38–43] and amino acids [44,45] using different non-permanent coatings. The *N,N*-dimethylacrylamide-ethylpyrrolidine methacrylate (DMA-EpyM) proposed in this work is a simple and reproducible physically adsorbed coating that was synthesized by reaction of *N*-(2-hydoxyethyl)-2-pyrrolidine with methacryloyl chloride and purified by a column chromatography [46]. The use of other physically adsorbed polymers has been proved as an interesting strategy to reduce the adsorption phenomena in several previous papers [18,47,48]; however, because the attachment of the coating to the wall is based on adsorption, a small amount of coating agent is frequently added to the separation medium to keep the coating on the capillary wall surface, fact that as we have commented before could be a problem in CE–MS.

Using DMA-EpyM, adding coating agent in the buffer is not necessary, its regeneration is achieved just only flushing the capillary with a polymer solution between injections and for this reason its use is compatible with CE–MS methods. The usefulness of this polymer coating has been demonstrate in the analysis of proteins in food [46,49] and, recently in the analysis of amino acids by chiral capillary electrophoresis-mass spectrometry [50].

The main goal of this research is the development of new CE procedures based on coelectroosmotic capillary electrophoresis using the mentioned DMA-EpyM copolymer as coating for demonstrating that it is useful for the analysis of two families of compounds (phenolic acids and amino acids, using DAD-detector and LIF, respectively), which are very interesting from a medical and industrial point of view. We have also proved that its use permits the addition of organic modifiers to the buffer without losing repeatability or reproducibility.

## 2. Materials and methods

### 2.1. Instrumentation

CE determination of phenolic acids was performed with a Beckman P/ACE[TM] MDQ capillary electrophoresis instrument. A diode-array detector was used to detect the individual compounds at their optimum wavelengths and to identify them by comparing their UV spectra with those of the reference compounds, comparing migration times, and by spiking the mixture of all the phenolic acids with standard compounds. UV detection was performed at 210 and 275 nm simultaneously in all the analyses. Data acquisition and processing were carried out with GOLD software installed in a personal computer.

The detection of amino acids was carried out using a Beckman 5500 capillary electrophoresis equipped with a laser-induced fluorescence detector, using an argon ion laser as excitation source (488 nm) and the electropherograms were recorded by monitoring the emission intensity at 520 nm.

All capillaries (fused silica) used were obtained from Beckman instrument, Inc. (Fullerton, CA, USA) and had an inner diameter of 75 μm, a total length of 57 cm and an effective separation length of 50 cm. The temperature was controlled in both instruments using a fluorocarbon based cooling fluid.

### 2.2. Chemicals

The phenolic acids: 4-hydroxyphenylacetic acid, gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxy-benzoic acid), caffeic acid (3,4-dihydroxycinnamic acid), dopac (3,4-dihydroxyphenylacetic acid), *p*-coumaric acid (4-hydroxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), *trans*-cinnamic acid, *o*-coumaric acid (2-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid) and gentisic acid (2,5-dihydroxybenzoic acid) standard were obtained from Sigma–Aldrich (St. Louis, MO). 4-Hydroxybenzoic acid was acquired from Fluka. The stock solutions containing all the 13 compounds were prepared in methanol/water (50:50, v/v) at a concentration of 200 mg L$^{-1}$ for each analyte.

The family of amino acids was made with three non-essential (glutamic acid, aspartic acid and glycine) and two essential amino acids (threonine and tryptophan), all of them were obtained from Sigma (St. Louis, MO, USA). Stock standard solutions of a mixture of these five compounds were prepared in doubly deionized water containing different concentrations of the analytes under study (5 mg L$^{-1}$ for glycine and threonine; 10 mg L$^{-1}$ in the case of aspartic acid and glutamic acid; and 15 mg L$^{-1}$ for tryptophan). For the first four mentioned compounds we used these concentrations to obtain the same signal height (approx.); in the case of tryptophan the detection and quantification limits were lower, for this reason we used a bigger concentration of this compound in the mixture of five amino acids.

The solutions used for the derivatization procedure of the amino acids were: FITC ($1.0 \times 10^{-2}$ M) in acetone (obtained

from Scharlau), and a 0.2 M sodium carbonate/bicarbonate buffer (pH 9).

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) and di-sodium hydrogen phosphate were obtained from Sigma, and sodium acetate and potassium hydrogen phthalate from Panreac (Montcada I Reixac; Barcelona (Spain)), which were all used as running buffers at different concentrations and pHs. Methanol was acquired from Panreac (Montcada I Reixac, Barcelona, Spain) and 2-propanol was obtained from Merck (Germany); they were HPLC grade.

### 2.3. Coating procedure

The synthesis of the DMA-EpyM polymer used as coating has been described elsewhere [46]. A very simple coating strategy was used flushing the capillary prior to each run with a diluted polymer solution (0.1 mg mL$^{-1}$ in water) for 2 min and then flushing the capillary with the separation buffer for 2 min.

### 2.4. CE conditions for phenolic acids

Separation of phenolic acids was performed in a coated fused silica capillary with 75 μm internal diameter (i.d.) and a total length of 57 cm (50 cm to the detector).

A buffer solution of 50 mM Na$_2$HPO$_4$ at pH 6 with 10% of 2-propanol (v/v) was selected as optimum for CE separations after appropriate optimization (see Section 3.1). In these conditions a mixture of 13 phenolic acids was injected hydrodynamically in the cathodic end at 3450 Pa (0.0345 bar or 0.5 psi) for 8 s. Each electrophoretic run was carried out at −25 kV (resulting in a current of −125 μA approx.) maintaining the capillary temperature at 22 °C. UV detection was performed at 210 and 275 nm simultaneously. Diode-array detection was used over the range of 190–600 nm to achieve spectral data.

All solutions were filtered through a 0.45 μm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary.

Every time a new capillary was used it was preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5 min rinse with Milli-Q water. Between injections, capillaries were rinsed with water for 1 min, 2 min of polymer solution (0.1 mg mL$^{-1}$) and 2 min of buffer solution.

The running buffer was changed after four runs.

### 2.5. Derivatization of amino acids and CE conditions

The derivatization reaction for the amino acids consisted in 495 μL of 0.2 M carbonate buffer (pH 9), 1000 μL of FITC solution (1.0 × 10$^{-2}$ M) and 1000 μL of acetone mixed with 600 μL of a mixture of amino acids at different concentrations, adding doubly deionized water to a final volume of 5 mL in a test tube, using very similar conditions to those described elsewhere [51]. This solution was introduced in a thermostatic bath for 2 h at 50 °C, and 2 mL of the resulting solution were diluted to a final volume of 10 mL with doubly deionized water before the analysis.

This solution was injected in the CE–LIF for the analysis.

Separation of the derivatized amino acids was performed in a coated fused silica capillary with 75 μm internal diameter (i.d.) and a total length of 57 cm (50 cm to the detector).

As above, the CE buffer for amino acids analysis was carefully selected obtaining the best results using a solution of 25 mM Na$_2$HPO$_4$ at pH 6 and 20% propanol (v/v).

The derivatized mixture was injected hydrodynamically at 0.5 psi for 12 s and detection was carried out by on-column measurement of 488 nm wavelength excitation and 520 nm emission. The voltage applied was −25 kV and the current achieved was −59 μA maintaining the capillary temperature at 22 °C.

All solutions were filtered through a 0.20 μm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary.

The capillary conditioning was the same that the protocol described in Section 2.4. The running buffer was changed after four runs.

## 3. Results and discussion

### 3.1. Separation of phenolic acids using DMA-EPyM coated capillaries

This family of phenolic acids was made to obtain a representative phenolic acid mixture to propose a potent analytical method for the analysis of these compounds in a wide variety of samples. Caffeic, *p*-coumaric, vanillic, ferulic and protocatechuic are acids present in nearly all plants [52]. Furthermore, several of the compounds we have included in the standard mixture are present in grape and berry extracts, orange, apple and other juices, bread, carrots, dried lentils, white, red and rose whines, olive fruits, olive oils, beer, etc.

The instrumental and experimental conditions were carefully studied to obtain the best sensitivity and resolution among the analytes under study. The effect of pH was studied between 5.0 and 9.5 using different buffers depending on the range of pH. The DMA-EPyM coated capillaries show an anodal EOF at low pH values, a nearly zero EOF at pHs around 6 and a low cathodal EOF at pHs higher than 8 [46].

After the preliminary studies, we decided to compare the different buffer solutions at a concentration of 50 mM. Sodium acetate/acetic acid was used in the range of pH from 4.5 to 5.5, observing that the migration times of the analytes decreased with an increase of the pH value. Potassium hydrogen phthalate/sodium hydroxide was used between 4.5 and 6, but using this buffer the current was not very stable. Best results were obtained with di-sodium hydrogen phosphate/monobasic sodium phosphate buffer; it was checked in the range of pH 6–8. Finally, to study higher pH values, sodium tetraborate with HCl or NaOH was used (between 8 and 10). When sodium tetraborate was used, the time of analysis in all the range of pH was longer than that obtained with other buffers; this fact could be explained for the cathodal EOF generated, and also obviously for the different separation power of each buffer. In Fig. 1, the behaviour of three of the used buffers is shown. A more detailed study between pH 6 and 8 was carried out (in steps of 0.2) after choosing di-sodium
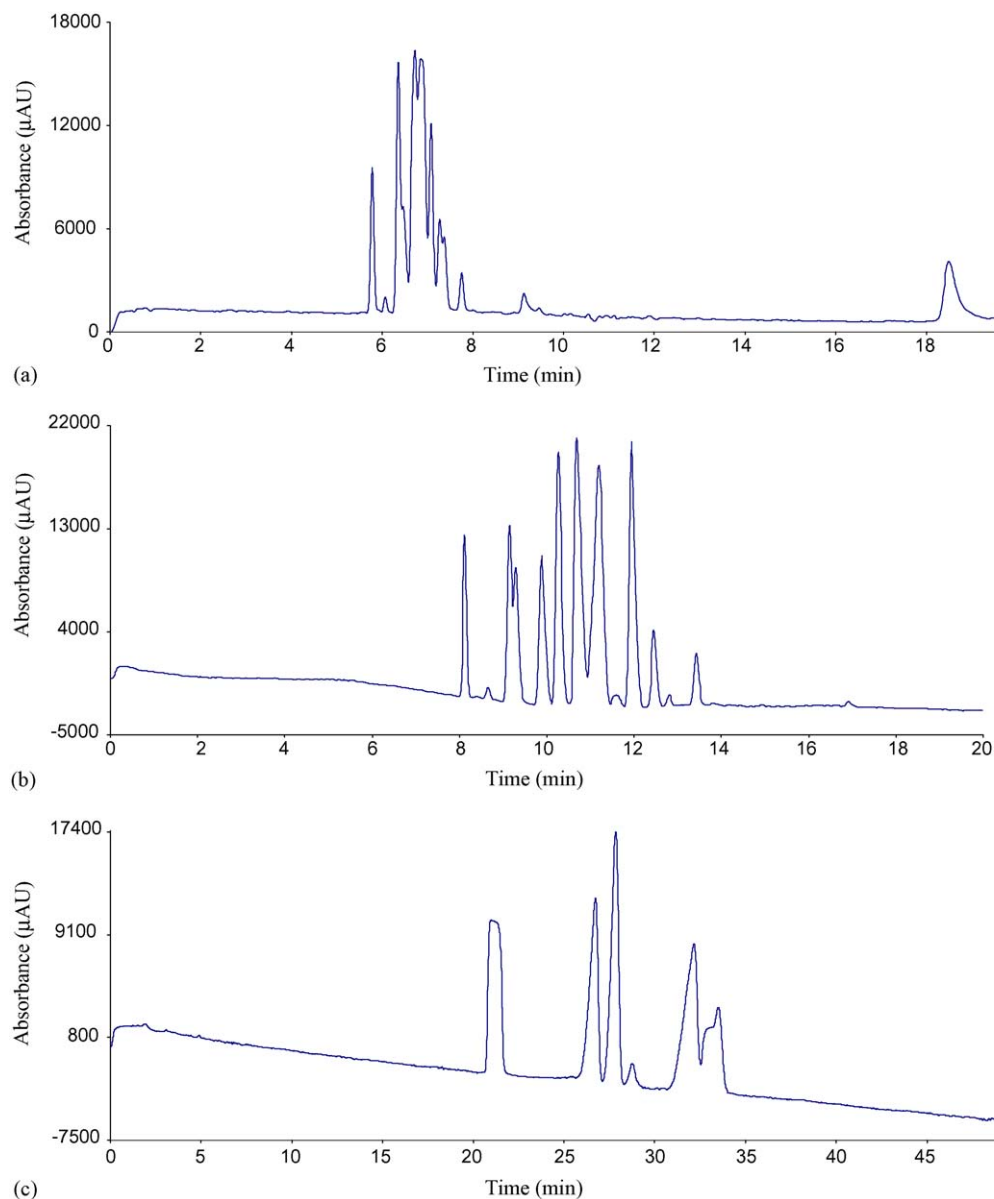
Fig. 1. Electropherograms of a sample containing the 13 phenolic acids under study (in methanol/water (50:50, v/v) at a concentration of $40\,mg\,L^{-1}$ of each compound) using different buffer systems: (a) sodium acetate/acetic acid at a pH value of 5.5; (b) di-sodium hydrogen phosphate/monobasic sodium phosphate buffer at a pH value of 6; and (c) sodium tetraborate at a pH of 9.6. Other separation conditions: capillary, $57\,cm \times 75\,\mu m$ (effective separation length of $50\,cm$); applied voltage, $-25\,kV$; buffer concentration, $50\,mM$; hydrodynamic injection, $0.5\,psi$ for $8\,s$. Detection was performed at $210\,nm$.

hydrogen phosphate solution as buffer and the optimum value was pH 6.

The buffer concentration was investigated in a range between 25 and 75 mM (in steps of 5). We found that 50 mM of di-sodium hydrogen phosphate buffer at pH 6 represented a good compromise for the resolution of the compounds studied and a reasonable analysis time, since higher buffer concentrations improved the separation but provided longer analysis time.

In order to achieve the total separation of phenolic acids the addition of different organic solvents to the separation buffer was studied. Namely methanol, acetonitrile, 1-propanol and 2-propanol were tested, obtaining the best results with 2-propanol. It could be observed that the resolution of all the analytes after

study was sufficient with 10% of 2-propanol (v/v); with higher percentages of 2-propanol, the resolution was not improved, but rather it was even worse (see Fig. 2). The percentage of 2-propanol was studied between 0 and 30% (v/v). The addition of organic modifiers to the buffer could also permit us to prove the repeatability and reproducibility of DMA-EPyM in presence of organic solvents.

The effect of the applied voltage on resolution of the 13 compounds was studied using the previously mentioned buffer composition, and the optimum value was −25 kV. As we were working with compounds which have negative charge at the optimum pH of the buffer, changing the polarity of the electrodes (reversed) makes sense to reduce the analysis time. The
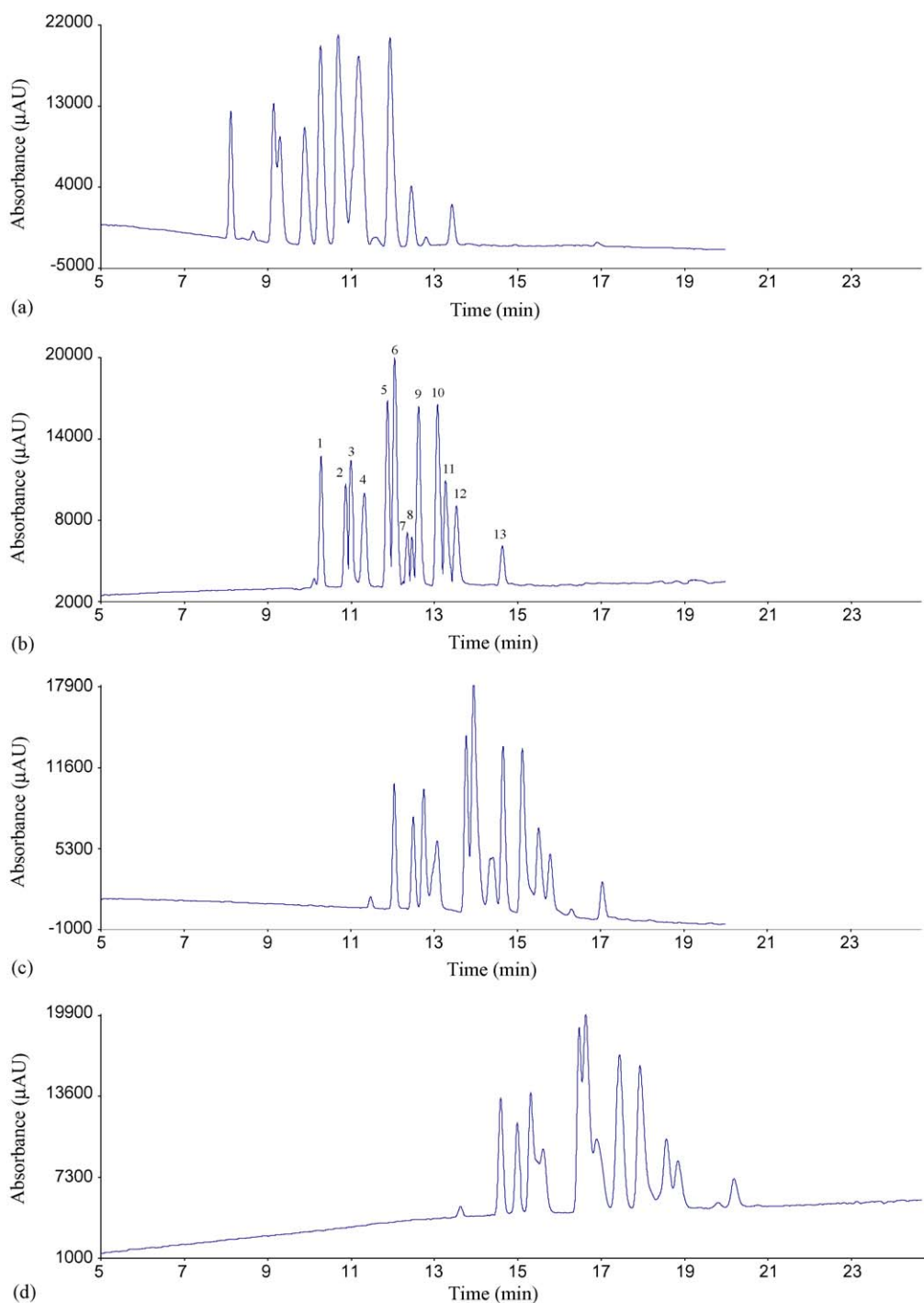
Fig. 2. Study of the influence of the addition of organic modifier (2-propanol) at different concentrations to the buffer solution: (a) 0% of 2-propanol; (b) 10% of 2-propanol; (c) 15% of 2-propanol; and (d) 20% of 2-propanol. Other separation conditions: capillary, 57 cm × 75 μm (effective separation length of 50 cm); applied voltage, −25 kV; di-sodium hydrogen phosphate/monobasic sodium phosphate buffer at a pH value of 6 and 50mM; hydrodynamic injection, 0.5 psi for 8 s. Sample containing all the 13 phenolic acids at 40 mg L$^{-1}$. Detection was performed at 210 nm. Peak identification numbers: 1, 4-hydroxybenzoic acid; 2, gentisic acid; 3, protocatechuic acid; 4, vanillic acid; 5, gallic acid; 6, *trans*-cinnamic acid; 7, 4-hydroxyphenylacetic acid; 8, *o*-coumaric acid; 9, *p*-coumaric acid; 10, dopac; 11, caffeic acid; 12, ferulic acid; and 13, sinapinic acid.

samples were injected at the negative end (hydrodynamic injection during 8 s) and detection was performed at the positive end.

The temperature during washing and analysis was 22 °C.

The optimum electropherogram obtained from a standard mixture of these 13 phenolic acids can be seen in Fig. 2b.

### 3.2. Separation of amino acids using DMA-EPyM coated capillaries

Glutamic acid, aspartic acid and glycine (non-essential), and threonine and tryptophan (essential) were selected for their separation using coelectroosmotic capillary electrophoresis using

DMA-EPyM. We chose these compounds because of their importance from a medical and nutritional point of view, and their presence in a lot of foods (fish, meat, beans, dairy products, cottage cheese, bananas, etc.). Furthermore, if we consider their side chains, working with these five compounds, we analyze the simplest amino acid (glycine) since it has the smallest possible side chain (–H), one which contains an aromatic side chain (tryptophan), one amino acid that contains an extra hydroxyl group (threonine), and two with acidic side chains. This family permitted to demonstrate that the separation of these compounds can be carried out using LIF as detection system and adding organic modifiers in the separation buffer using DMA-EpyM as stationary polymer in the capillary.

For the development of this method the steps carried out in the optimization were the same than those described in the Section 3.1.

In this case, three different salts were tested as buffers (di-sodium hydrogen phosphate, sodium borate and sodium acetate), and again, di-sodium hydrogen phosphate provided the best electropherograms. The effect of pH was studied between 5.7 and 8.0 using 25 mM di-sodium hydrogen phosphate buffer solution (concentration value selected in the preliminary studies) and a value of pH 6 gave rise to the best resolution plus a satisfactory analysis time.

The effect of ionic strength on resolution for the mixture of five amino acids was also checked varying the concentration of di-sodium hydrogen phosphate between 20 and 50 mM at optimum pH value, obtaining the best resolution with a concentration of 25 mM.

Then, the percentage of 2-propanol was studied between 0 and 25% (v/v) (using a 25 mM di-sodium hydrogen phosphate at pH 6). The increase of this percentage improved the resolution between peaks, and finally we decided to select 20% as optimum value.

The instrumental variables were carefully studied as well. The optimum voltage value was −25 kV and hydrodynamic injection time (0.5 psi) was used selecting as optimum time 12 s.

Fig. 3 shows the electropherogram of the separation of these five derivatized amino acids in the optima conditions. The elution order is easily understood observing the structure of the molecules under study: firstly, aspartic and glutamic acids which contain in their chemical structure two –COOH groups. These two compounds only differ in one –CH$_2$– group in their side chains. The other three compounds have the same charge at the optimum pH, so the elution order will depend on the size of the molecules and will be glycine, threonine and tryptophan.
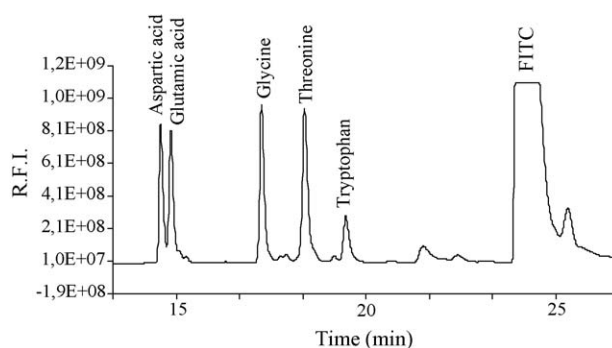


Fig. 3. Electropherogram obtained for a sample of five amino acids under optimized conditions using laser-induced fluorescence as detector. Optima separation conditions: capillary, 57 cm × 75 μm (effective separation length of 50 cm); applied voltage, −25 kV; buffer, 25 mM Na$_2$HPO$_4$ at pH 6 and 20% propanol; hydrodynamic injection, 0.5 psi for 12 s. Conditions of detection: 488 nm wavelength excitation and 520 nm emission. Sample containing the five analytes under study in these concentrations: 120 μg L$^{-1}$ for glycine and threonine; 240 μg L$^{-1}$ in the case of aspartic acid and glutamic acid; and 360 μg L$^{-1}$ for tryptophan.

### 3.3. Repeatability and reproducibility study

#### 3.3.1. Study of the repeatability and reproducibility of the CE–UV method for phenolic acids

Repeatability was studied by performing series of separations using the optimized method on one of the samples containing all the standards (40 mg L$^{-1}$ of each phenolic acid) on the same day (intraday precision, $n = 8$) and on three consecutive days (interday precision, $n = 12$). The relative standard deviations (R.S.D.s) of peak areas and migration times were determined considering five of the phenolic compounds under study (4-hydroxybenzoic acid, protocatechuic acid, *o*-coumaric acid, caffeic acid and sinapinic acid). We chose these compounds to check the repeatability and reproducibility in all the zones of the electropherogram, with analytes with short, medium and long migration time.

The intraday repeatabilities on the migration time for these five analytes (expressed as R.S.D.) were found inside the range between 0.49 and 0.94%, whereas the interday repeatabilities on the migration time were between 0.89 and 1.64%.

The intraday repeatabilities on the total peak area (expressed as R.S.D.) were between 1.24 and 2.32%, whereas the interday repeatabilities on total peak area were found in the range from 2.14 to 3.22%. These data are summarized in Table 1.

In practice, batch-to-batch reproducibility is a key issue. The reproducibility was studied by performing eight separations

Table 1
R.S.D. values of the peak area and migration time obtained in the study of repeatability of the CE–UV method for the phenolic acids

| Compound | Intraday ($n = 8$) | | Interday ($n = 12$) | |
|---|---|---|---|---|
| | Peak area | Migration time | Peak area | Migration time |
| 4-Hydroxybenzoic acid | 1.24 | 0.54 | 2.14 | 0.89 |
| Protocatechuic acid | 2.32 | 0.49 | 2.35 | 1.23 |
| *o*-Coumaric acid | 1.76 | 0.67 | 2.16 | 0.91 |
| Caffeic acid | 1.87 | 0.89 | 3.22 | 0.98 |
| Sinapinic acid | 2.01 | 0.94 | 2.28 | 1.64 |

Table 2
R.S.D. values of the peak area and migration time obtained with different columns, different sample and different operator of the CE–UV method for the phenolic acids

| Compound | Reproducibility ($n = 8$) | |
|---|---|---|
| | Peak area | Migration time |
| 4-Hydroxybenzoic acid | 2.32 | 1.23 |
| Protocatechuic acid | 3.01 | 1.54 |
| *o*-Coumaric acid | 2.89 | 2.54 |
| Caffeic acid | 2.03 | 2.67 |
| Sinapinic acid | 2.77 | 2.33 |

of other sample containing all the standards of phenolic acids (40 mg L$^{-1}$ of each phenolic acid under study) 24 h later than the analysis of the first, under the same conditions but different operator and different capillary. The results obtained, as shown in Table 2, were between 1.23 and 2.67% for the migration times and between 2.03 and 3.01% for total peak area (expressed as R.S.D.) considering the five analytes previously mentioned.

### 3.3.2. Repeatability and reproducibility study of the CE–LIF method for amino acids

This study was carried out performing series of separations using the optimized method, as we have described in the previous section. The repeatability of the DMA-EpyM coating was evaluated by measuring the relative standard deviations (R.S.D.s) of migration times of all the five amino acids under study (glutamic acid, aspartic acid, glycine, threonine and tryptophan). Eight replicates of migration time measurement for the five amino acids at the same day were used to examine the short-term stability of the coated capillary. These data can be seen in Fig. 4, the migration time data indicate that the short-term stability of the coated capillary is reasonably good (with R.S.D.s in the range of 0.39–0.65%). Similarly, eight replicates of migration time measurement for the five amino acids on different days in one week were used to test the long-term stability of the stationary polymer coated capillary. As shown in Fig. 5, the migration time data indicate that the long-stability of the coated capillary is also reasonably good (with R.S.D.s in the range of 0.33–1.03%).
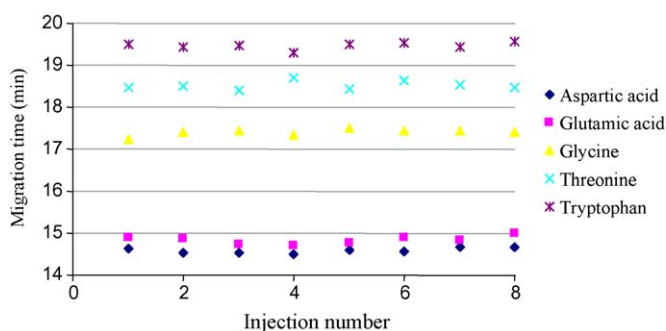


Fig. 4. Study of intraday repeatability for the CE–LIF method. Eight replicates of migration time measurement for the five amino acids at the same day were used to examine the short-term stability of the coated capillary. Sample containing the five analytes under study in these concentrations: 120 µg L$^{-1}$ for glycine and threonine; 240 µg L$^{-1}$ in the case of aspartic acid and glutamic acid; and 360 µg L$^{-1}$ for tryptophan.
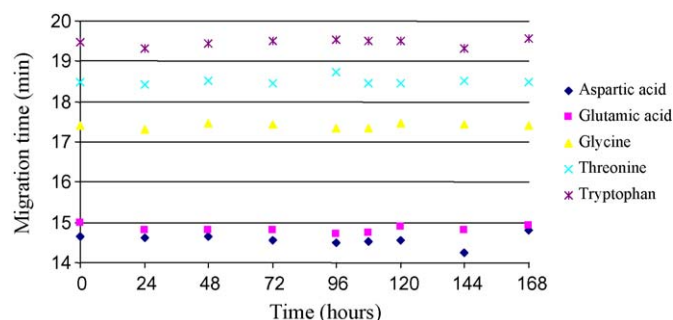


Fig. 5. Study of interday repeatability for the CE–LIF method. Eight replicates of migration time measurement for the five amino acids on different days in one week were also used to examinate the long-term stability of the stationary polymer coated capillary. Sample containing the five analytes under study in these concentrations: 120 µg L$^{-1}$ for glycine and threonine; 240 µg L$^{-1}$ in the case of aspartic acid and glutamic acid; and 360 µg L$^{-1}$ for tryptophan.

As commented before, reproducibility is a very important issue. The reproducibility was studied by performing 24 separations of other sample containing all the standards of amino acids 24 h later than the analysis of the first, under the same conditions but different operator and different capillary. The results obtained were between 1.02 and 2.98% for the migration times (expressed as R.S.D.) considering the five analytes previously mentioned (2.98% for glutamic acid, 2.32% for aspartic acid, 1.54% for glycine, 1.23% for threonine and 1.02% for tryptophan), suggesting that this physically adsorbed copolymer gives a good batch-to-batch reproducibility using this CE–LIF method for amino acids.

### 3.4. Calibration, linearity and detection limits

#### 3.4.1. Calibration curves, linearity and detection limits for phenolic acids

The detection limits (LOD = $3\sigma_b/b$) and quantitation limits (LOQ = $10\sigma_b/b$) of the method were tested for the studied analytes using the IUPAC method, where $\sigma_b$ is the white standard deviation and $b$ the slope of the calibration curves [53]. Standard calibration graphs were prepared for each analyte and all calibration curves showed good linearity between LOQ depending on the analytes studied and 200 mg L$^{-1}$. Each point of the calibration plot was repeated three times in the same way. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.996 for all the compounds quantified. The results obtained for the phenolic acids are summarized in Table 3.

#### 3.4.2. Calibration curves, linearity and detection limits for amino acids

Standard calibration graphs were prepared for each analyte in the range of LOQ–1200 µg L$^{-1}$ for glycine and threonine, from LOQ to 2400 µg L$^{-1}$ in the case of aspartic and glutamic acid, and from LOQ to 3600 µg L$^{-1}$ for tryptophan and three replicates of each point of the calibration curve were made to set up the calibration. The detection (LOD) and quantification limit (LOQ) were calculated for the studied analytes using the method proposed by Curie [53]. The calibration plots indicate

Table 3
Limit of detection (LOD) and quantification (LOQ) for each phenolic acid under study

| Compound | LOD (mg L$^{-1}$) | LOQ (mg L$^{-1}$) |
|---|---|---|
| 4-Hydroxybenzoic acid | 1.23 | 4.10 |
| Gentisic acid | 1.87 | 6.23 |
| Protocatechuic acid | 1.34 | 4.47 |
| Vanillic acid | 1.92 | 6.40 |
| Gallic acid | 0.98 | 3.28 |
| *Trans*-cinnamic acid | 0.46 | 1.53 |
| 4-Hydroxyphenylacetic acid | 3.23 | 10.77 |
| *o*-Coumaric acid | 3.47 | 11.57 |
| *p*-Coumaric acid | 0.96 | 3.20 |
| Dopac | 1.11 | 3.70 |
| Caffeic acid | 1.46 | 4.87 |
| Ferulic acid | 1.59 | 5.30 |
| Sinapinic acid | 1.79 | 5.98 |

Table 4
Limit of detection (LOD) and quantification (LOQ) for each amino acid under study

| Compound | LOD (µg L$^{-1}$) | LOQ (µg L$^{-1}$) |
|---|---|---|
| Aspartic acid | 45 | 150 |
| Glutamic acid | 51 | 170 |
| Glycine | 18 | 61 |
| Threonine | 14 | 46 |
| Tryptophan | 134 | 446 |

good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.995 for all the compounds quantified. The results obtained for the amino acids are summarized in Table 4.

## 4. Conclusions

The aim of this study has been to demonstrate that coelectroosmotic capillary electrophoresis using DMA-EpyM as stationary polymer can be used for the analysis of phenolic acids and amino acids. Two methods have been developed; in the case of phenolic acids, we used a CE–UV, and for the amino acids, a CE–LIF method was chosen. Both of them could be used for the analysis of the extracts of a plenty of products which contain these compounds of great interest.

The relative standard deviations (R.S.D.) obtained in the study of repeatability were lower than 0.94% (intraday results) and 1.64% (interday) for the migration times in the case of CE–UV for phenolic acids, and lower than 0.65% (intraday) and 1.03% (interday) for the CE–LIF method to study amino acids.

Detection limits in the CE–UV method for the phenolic acids were between 0.46 mg L$^{-1}$ for *trans*-cinnamic acid and 3.47 mg L$^{-1}$ for *o*-coumaric acid, and in the CE–LIF method were between 14 µg L$^{-1}$ for threonine and 134 µg L$^{-1}$ for tryptophan.

It is important to highlight that these values demonstrate that DMA-EpyM provides a stable coating even when the separation buffer contains organic modifiers. All these facts make broader the usefulness of this coating.

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Nos parece útil, recoger en este anexo alguna información tanto estructural como sobre el comportamiento del polímero empleado en este capítulo como recubrimiento del capilar para ayudar a entender *cómo funciona* el mismo.

La estructura del polímero es:
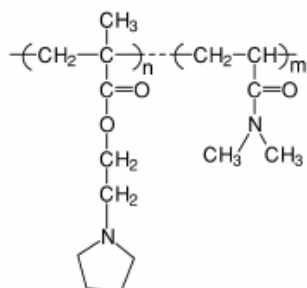


*Figura*. Estructura del 2-etil-(2-pirrolidina) metacrilato de N,N-dimetilacrilamida.

En la siguiente figura se representan los valores del FEO en función del pH del electrolito de separación de un capilar sin recubrimiento interno y de un capilar con el recubrimiento polimérico EpyM-DMA.
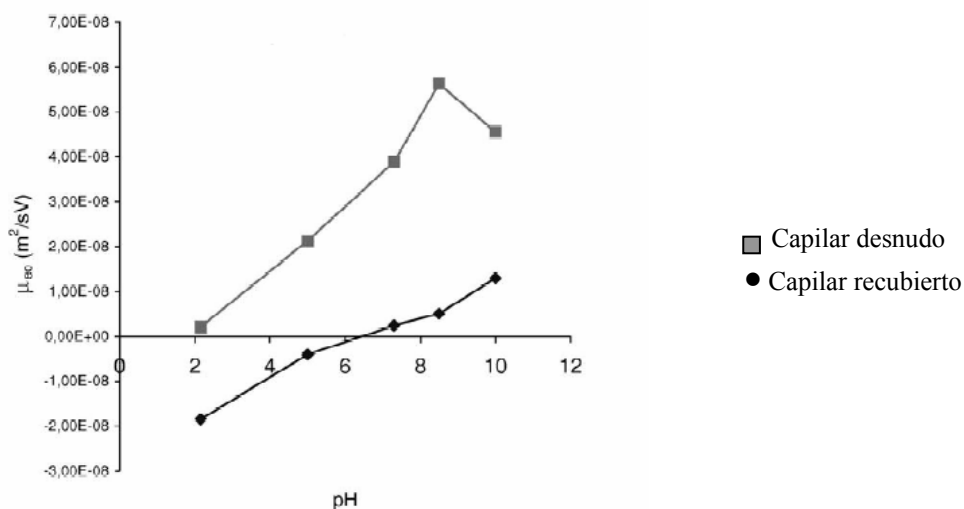


*Figura*. Valores del FEO según el pH del tampón de separación en un capilar sin recubrimiento interno y en otro con el recubrimiento polimérico EpyM-DMA.

Utilizando el recubrimiento polimérico, a un pH de separación próximo a 6, el FEO es prácticamente cero. Por debajo de este valor, el FEO es anódico, y por encima de pH 6, es catódico. Este efecto se puede explicar teniendo en cuenta que la carga neta de la pared interna del capilar es el resultado de las cargas positivas de los grupo amino procedentes del polímero, y de las cargas negativas de los grupos siloxano que no han interaccionado con el polímero. De este modo, a pHs ácidos, los grupos amino (con carga positiva) son los grupos mayoritarios, lo que da como resultado un FEO anódico.

Por otro lado, a pHs básicos, el número de cargas positivas disminuye, y comienzan a ser predominantes los grupos con carga negativa de la sílice, lo que se traduce en un FEO catódico.

Conviene tener en cuenta que incluso a pHs muy básicos el FEO obtenido en el capilar con recubrimiento polimérico es unas 10 veces menor que el obtenido en el capilar "desnudo".

# Capítulo 5

# Evaluación de la capacidad antioxidante individual de compuestos fenólicos presentes en el aceite de oliva

Antes de ahondar en el argumento tratado en este capítulo, parece necesario hacer una pequeña aclaración. Tal vez, lo más adecuado sería colocar este capítulo agrupando así aquellos capítulos en los que se trabaja con HPLC, pero considero que parte del trabajo experimental aquí expuesto ha sido fundamental para poder desarrollar algunos de los capítulos en los que sólo se utiliza la técnica de CE, ya que se coleccionaron estándares de compuestos fenólicos no disponibles comercialmente que nos permitieron llevar a cabo la identificación de los mismos en los perfiles obtenidos mediante CE.

Cronológicamente hablando, también es más adecuado este orden, ya que tanto el trabajo experimental que aquí se recoge, como el que se describe en el siguiente capítulo 6, se desarrollaron durante una estancia de investigación en la Universidad de Bolonia (Dpto. Ciencia de los Alimentos) que tuvo lugar antes de realizar el trabajo experimental que se resume en siguientes capítulos.

Dicho esto y ya en relación con este capítulo 5, es interesante fijar la atención en el hecho de que 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 [335].

El aceite de oliva se considera resistente a la oxidación debido a su bajo contenido en ácido grasos poliinsaturados y a la presencia de antioxidantes naturales tales como los compuestos fenólicos o los tocoferoles.

Antioxidantes son aquellas moléculas que, aunque estén presentes en una baja concentración, son capaces de prevenir la oxidación [336]. Una clasificación de los antioxidantes que se basa en su mecanismo de acción es [337]: antioxidantes primarios, antioxidantes con efecto sinérgico y antioxidantes secundarios. Los polifenoles son uno

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de los grupos principales de compuestos fenólicos que actúan como antioxidantes primarios al inhibir la oxidación en el aceite de oliva.

Los antioxidantes pertenecientes al primer grupo (AH), atrapan los radicales libres (LOO·, LO·) interrumpiendo el paso de propagación formando un radical antioxidante (A·) de reactividad tan baja que impide la reacción posterior con los lípidos [338].

$$LOO· + AH \rightarrow LOOH + A·$$
$$LO· + AH \rightarrow LOH + A·$$
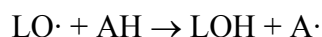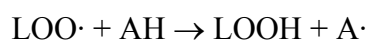
Los secuestradores de radicales frecuentemente donan un electrón al electrón desapareado del radical libre, reduciéndolo. Los polifenoles son muy activos en este sentido.

Los extractos más hidrofílicos del aceite de oliva contienen una gran cantidad de compuestos fenólicos: fenoles simples, lignanos, secoiridoides…Para poder evaluar de manera individual la capacidad antioxidante de cada compuesto, hay que sintetizarlos o aislarlos.

Nosotros optamos por la segunda opción; se analizaron aceites comerciales mediante HPLC-UV y HPLC-MS y, una vez que estaba caracterizada la fracción de interés, se procedió al coleccionamiento de varios de los compuestos individuales (de los cuales no existen patrones comerciales) utilizando una columna semi-preparativa. Tras comprobar la pureza de los "estándares" aislados, se realizaron medidas de la capacidad antioxidante de los mismos mediante tres tests basados en técnicas totalmente diferentes, cosa que permitiría comparar los resultados y ver si todas las determinaciones estaban de acuerdo en cuanto a la capacidad de cada compuesto. Los tres tests fueron: Un estudio de "radical scavenging" empleando el conocido DPPH, una oxidación acelerada en sistema lipídico modelo (OSI, que es equiparable al sistema Rancimat), y un novedoso método electroquímico.

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# Evaluation of the Antioxidant Capacity of Individual Phenolic Compounds in Virgin Olive Oil

A. Carrasco-Pancorbo,[†] L. Cerretani,*,[‡] A. Bendini,[‡]
A. Segura-Carretero,[†] M. Del Carlo,[§] T. Gallina-Toschi,[‡] G. Lercker,[‡]
D. Compagnone,[§] and A. Fernández-Gutiérrez[†]

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n,
E-18071 Granada, Spain, Department of Food Science, University of Bologna, P.zza Goidanich 60,
47023 Cesena (FC), Italy, and Department of Food Science, University of Teramo,
Via C. Lerici 1, Mosciano Stazione (TE) 64023, Italy

Virgin olive oil has a high resistance to oxidative deterioration due to its tryacylglycerol composition low in polyunsaturated fatty acids and due to the presence of a group of phenolic antioxidants composed mainly of polyphenols and tocopherols. We isolated several phenolic compounds of extra virgin olive oil (phenyl-ethyl alcohols, lignans, and secoiridoids) by semipreparative high-performance liquid chromatography (HPLC) and identified them using ultraviolet, atmospheric pressure chemical ionization, and electrospray ionization MS detection. The purity of these extracts was confirmed by analytical HPLC using two different gradients. Finally, the antioxidant capacity of the isolated compounds was evaluated by measuring the radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl radical, by accelerated oxidation in a lipid model system (OSI, oxidative stability instrument), and by an electrochemical method.

KEYWORDS:  Polyphenols; olive oil; HPLC; antioxidant activity; antioxidant power; DPPH; OSI

## INTRODUCTION

Oxidative stability is a central parameter in the estimation of extra virgin olive oil (VOO) quality, as it gives a reliable evaluation of the susceptibility of the oil to oxidative degeneration, which is the main cause of its damage (*1*, *2*). Although inevitable, the oxidation process can be delayed by antioxidants that enhance the oxidative stability by preventing the propagation of lipid peroxidation or removing free radicals. Antioxidants are reported as molecules, which, when present even at low concentrations, significantly prevent oxidation (*3*).

Olives and olive-derived products are an important part of the Mediterranean diet and are recognized as a valuable source of natural phenolic antioxidants (*4*). VOO, one of the few oils consumed without any chemical treatment, has a high resistance to oxidative deterioration mainly due to two reasons:  First, its fatty acid composition is characterized by a high monounsaturated-to-polyunsaturated fatty acid ratio, and second, it contains many minor compounds with powerful antioxidant activity among which polyphenols stand out (*5*, *6*). As a consequence of their fundamental chemical and antioxidant properties, the phenolic compounds continue to attract considerable research efforts.

A classification of the antioxidants based on their mechanism of action can be established as follows:  primary antioxidants, synergistic, and secondary antioxidants (*7*). Polyphenols are one of the main groups of phenolic compounds acting as primary antioxidants (AH) to inhibit oxidation in VOO. They mainly act as chain breakers by donating a radical hydrogen to alkylperoxyl radicals (ROO•) formed during the initiation step of lipid oxidation and subsequently forming a stable radical (A•) through the reaction (*8*):

$$ROO^\bullet + AH \rightarrow ROOH + A^\bullet$$
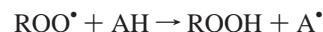
Olive oil hydrophilic extracts contain a large number of phenolic compounds including simple phenols, lignans, and secoiridoids (*9*), which exhibit antioxidant properties. To evaluate the antioxidant contribution that these individual phenolic compounds have in olive oil, it is important to either synthesize or isolate individual polyphenols for their analysis. Synthesis is currently not practical, so isolation procedures must be used.

Much literature is available on the development of methods for the analysis, isolation, and identification of polyphenols in olives and olive oils (*4*, *9*−*20*). Typically, these methods have been applied to monitoring differences between olive varieties and changes during ripening and oil processing. The isolation of polyphenols has been performed to assess the properties of the two main parts of the polar fraction of VOO as antioxidants after separation by solid phase extraction (*21*), but only a few

* To whom correspondence should be addressed. Tel:  +390547338121. Fax:  +390547382348. E-mail:  lorenzo.cerretani2@unibo.it.
    † University of Granada.
    ‡ University of Bologna.
    § University of Teramo.
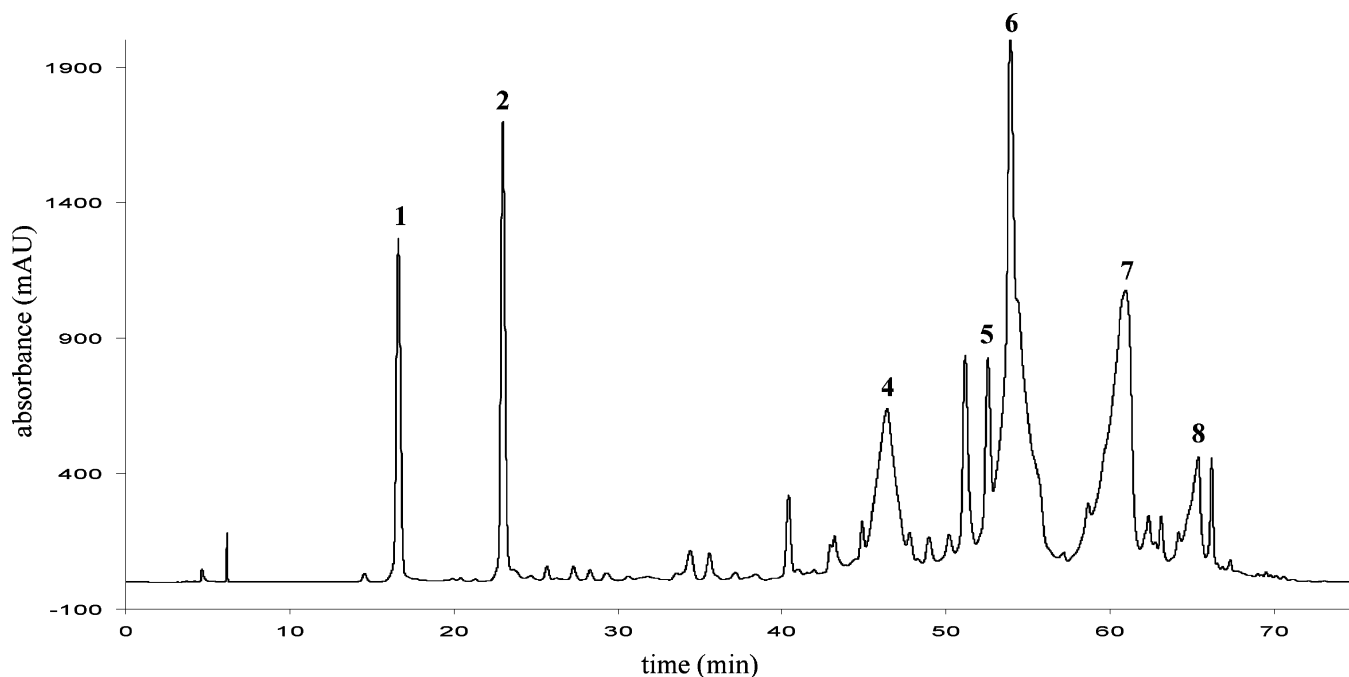
**Figure 1.** Chromatogram of extra VOO sample under optimized conditions using semipreparative HPLC. Detection was performed at 280 nm. Peak identification numbers: 1, hydroxytyrosol; 2, tyrosol; 3, elenolic acid (was detected at 240 nm); 4, deacetoxy oleuropein aglycon; 5, (+)-pinoresinol; 6, 1-(+)-acetoxypinoresinol; 7, oleuropein aglycon; 8, ligstroside aglycon.

data are available to the date to assess the individual antioxidant properties of these compounds directly isolated from extra VOO.

The aim of this work was to evaluate the antioxidant activity of different single phenolic compounds of VOO by chemical methods (1,1-diphenyl-2-picrylhydrazyl, DPPH), by accelerated oxidation in a lipid model system (OSI, oxidative stability instrument), and by an electrochemical method [flow injection analysis (FIA)−amperometry and cyclic voltammetry] and to compare the information to obtain insight of the chemical groups involved in the antioxidant mechanisms.

## MATERIALS AND METHODS

**Apparatus.** High-performance liquid chromatography (HPLC) analyses were performed with a HP 1100 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump delivery system, degasser, autosampler, diode array UV−vis detector, and a mass spectrometer detector. The semipreparative HPLC column used was a Phenomenex Luna ($C_{18}$) column, 10 $\mu$m i.d., 25 cm × 10 mm, and the flow rate was 3 mL/min. The analytical HPLC column used was a $C_{18}$ Luna column, 5 $\mu$m i.d., 25 cm × 3.0 mm (Phenomenex, Torrance, CA), with a $C_{18}$ precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min.

The electrochemical behavior of the compounds studied was evaluated using hydrodynamic and cyclic voltammetry. The hydrodynamic voltammetry was performed in FIA, and cyclic voltammetry was performed using stop flow voltammetry. The apparatus consisted of a Minipuls II peristaltic pump (Gilson, France), a high-pressure injection valve model 7125 (Rheodyne, Rohnert Park, CA) equipped with a 20 $\mu$L loop, an electrochemical cell model UniJet (BAS, West Lafayette, IN) using a glassy carbon working electrode, and Autolab potentiostat (Ecochemie, Amsterdam, Netherlands).

**Reagents, Stock Solutions, and Reference Compounds.** Dopac (3,4-dihydroxyphenylacetic acid) was acquired from Sigma Aldrich (St. Louis, MO), and oleuropein (oleuropein glucoside) was obtained from Extrasynthèse (Genay, France). The stock solution containing these two analytes was prepared in methanol/water (50:50, v/v) and in methanol at a concentration of 500 $\mu$g/mL for each one. These compounds were used to do the calibration curves. DPPH and Trolox were acquired from Sigma Chemicals Co. (St. Louis, MO). Methanol and *n*-hexane (HPLC grade) were from Merck (Darmstadt, Germany). Doubly deionized

water with a conductivity of 18.2 M$\Omega$ was obtained by using a Milli-Q system (Millipore, Bedford, MA).

**Samples.** Commercial extra VOO samples were used for this study (from Bertolli, Unilever Bestfoods Italia S.p.A., Inveruno-MI, Italy). Triolein was used as glyceridic matrix without phenolic compounds for OSI studies and was obtained from Fluka (Buchs, Switzerland).

**Liquid−Liquid Extraction (LLE) of Phenolic Compounds from Olive Oils.** A LLE system was used to extract the phenolic compounds present in VOO. According to Carrasco-Pancorbo et al. (*22*), 60 g of oil (±0.001 g) was dissolved in 60 mL of hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v) solution. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure and a temperature of 40 °C. Finally, the residue was redissolved in 0.5 mL of methanol/water (50:50, v/v) and filtered through a 0.20 $\mu$m filter.

**Isolation of Polyphenols Using Semipreparative HPLC.** In the semipreparative HPLC analysis for the isolation of the single phenolic compounds, the mobile phases were water with acetic acid (0.5%) (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.

The injection volume for the isolation of the reference compounds was 100 $\mu$L of extracts obtained from extra VOO in methanol/water 50:50 (v/v) using the LLE system. The analyses were carried out at room temperature. The wavelengths were set at 240, 280, and 330 nm. In this way, we have used semipreparative reverse phase HPLC to isolate the following polyphenols from VOOs: hydroxytyrosol (CAS no. 10597-60-1), tyrosol (CAS no. 501-94-0), elenolic acid, deacetoxy oleuropein aglycon (named decarboxymethyl oleuropein aglycon also), (+)-pinoresinol (CAS no. 487-36-5), (+)-1-acetoxypinoresinol, oleuropein aglycon, and ligstroside aglycon. The analysis of these compounds was then done with an analytical column to check the purity of the isolated compounds and to confirm their identity, using the same gradient used in the semipreparative HPLC method plus another gradient. A chromatogram obtained using semipreparative HPLC is shown in **Figure 1**.

**Analysis of the Isolated Compounds Using Analytical HPLC.** After isolation, the analysis of these compounds was done with the

analytical HPLC column to check the purity of the isolated compounds and to confirm their identity, using the same gradient used in the semipreparative HPLC method. The injection volume was 10 $\mu$L. The wavelengths were set at 240, 280, and 330 nm. The detection was made using MS as well, and the analyses were carried out using an electrospray (ESI) interface operating in positive mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psi; drying gas temperature, 350 °C; capillary voltage, 3000 V; and fragmentor voltage, 60 V; and also using atmospheric pressure chemical ionization (APCI) interface operating in negative mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 30 psi; drying gas temperature, 350 °C; vaporizer temperature, 450 °C; capillary voltage, 3000 V; and fragmentor voltage, 60 V. The polarity of ESI and APCI and all of the parameters of MS detector were optimized using the height of the MS signal for the phenolic compounds isolated previously from the methanol−water extracts of extra VOO as an analytical parameter.

Using the same mobile phases, another gradient was used to confirm the purity of the isolated compounds keeping in mind the fact that they only produced one peak in the chromatograms. The solvent gradient changed according to the following conditions: from 0 to 10 min, 90% A:10% B to 70% A:30% B; from 10 to 20 min, 70% A:30% B to 65% A:35% B; from 20 to 30 min, 65% A:35% B to 55% A:45% B; from 30 to 40 min, 55% A:45% B to 50% A:50% B; from 40 to 45 min, 50% A:50% B to 5% A:95% B; from 45 to 48 min, 5% A:95% B to 95% A:5% B.

**Quantification of Isolated Compounds.** Three standard calibration curves were done to quantify the isolated phenolic compounds using two commercial reference compounds, DOPAC (3,4-dihydroxyphenyl-acetic acid) and oleuropein (oleuropein glucoside). Tyrosol and hydroxytyrosol were quantified using the calibration curve of DOPAC at 280 nm; (+)-pinoresinol, (+)-1-acetoxypinoresinol, deacetoxy oleuropein aglycon, oleuropein aglycon, and ligstroside aglycon were quantified with the calibration curve of oleuropein obtained at 280 nm. However, the curve of oleuropein at 240 nm was used for the quantification of elenolic acid. The analyses were carried out using the analytical HPLC column and the gradient of 75 min.

**Radical Scavenging Activity of Isolated Phenolic Compounds.** The DPPH radical scavenging activity of the isolated compounds was evaluated following the Parejo et al. (*23*) and Brand-Williams et al. (*24*) analytical protocols, modified according to that of Rotondi et al. (*25*). Briefly, 2.9 mL of DPPH (100 $\mu$M) in methanol/water (80:20, v/v) solution was added to 0.1 mL of the solution of the isolated phenolic compound to be tested. After 30 min at 23 °C, absorbance was measured at 515 nm and compared to a control sample prepared without adding phenolic compounds [with 0.1 mL of methanol/water (50:50, v/v) solution]. The radical scavenging activity was expressed as TEAC (Trolox equivalent antioxidant capacity) based on a calibration curve ($r^2 = 0.9971$).

**Oxidative Stability of Oil Samples.** The oxidative stability of the phenolic compounds was also evaluated by the OSI, using an eight-channel OSI (Omnion, Decatur, IL). The instrumental conditions were set following the analytical protocol described by Jebe et al. (*26*); to obtain the OSI, a stream of purified air (120 mL min$^{-1}$ air flow rate) was passed through the sample of oil-free phenolic compounds (with 50 or 218 mg/kg of each compound individually), and the effluent air for the oil sample was then bubbled through a vessel containing deionized water. The effluent air contained volatile organic acids swept from the oxidizing oil, which increased the conductivity of the water. The temperature to carry out this test was 110 °C. The OSI index (or OSI time) was expressed in hours ($n = 4$).

As we have commented before, triolein was used as a glyceridic matrix without phenolic compounds for OSI studies. We prepared triolein spiked with the individual phenolic compounds as follows: 0.25 or 1.09 mg of each isolated compound dissolved in methanol was added to 5 g of triolein for doing the experiments in the OSI. We added these quantities to check the results at two different concentrations of each analyte (50 or 218 mg/kg in each case). After homogenization by intensive shaking, the solvent was evaporated using nitrogen. We decided to add these quantities of the isolated compounds because the

compounds under study were found in extra VOO samples at similar concentrations (or in the same order of magnitude).

**Electrochemical Study of these Single Phenolic Compounds.** The electrochemical behavior of the studied phenols was measured [in equimolar solution (10 $\mu$M; approximately between 1.38 and 4.16 ppm depending on the molecular weight of the compound)] using hydro-dynamic voltammetry performed in FIA. Three replicate injections for each potential step were performed in the potential interval 0−300 mV vs Ag/AgCl; the increasing potential step was 25 mV, and the flow rate was 150 $\mu$L/min. The buffer was phosphate in a concentration of 50 mM at a pH of 7.4 with 50 mM KCl. Twenty microliters of sample was injected in the flowing buffer, and the current produced in the electrochemical oxidation was recorded.

The cyclic voltammograms of the single phenolic compounds were performed using a stop flow approach. This procedure allowed the use of smaller amounts of sample (a fact that is interesting because of the limited avaibility of HPLC fractions). The voltammetric scans were performed in the range −100 to +700 mV vs Ag/AgCl with a scan rate of 50 mV/s. The anodic peaks offered an insight into the electrochemistry of the single phenolic compounds.

A classification of the antioxidant power (AOP) of the evaluated phenols was proposed considering as a major feature the oxidation plateau regions of the hydrodynamic voltammograms; when a comparable oxidation potential was recorded, the higher current produced at that potential was considered as an index of energetically favored oxidation (higher AOP).

## RESULTS AND DISCUSSION

**Confirmation of the Purity of the Isolated Phenolic Compounds in VOO.** With the use of two different gradients in the analytical scale column and performing the detection at three wavelengths and also using MS (ESI interface operating in positive mode and APCI interface operating in negative mode), we were able to confirm the purity of these isolated compounds. **Table 1** shows the retention time, absorption maxima, and the fragmentation patterns using the two interfaces for all of these described compounds, and in **Figure 2**, the chromatographic profiles of an extra VOO and individual isolated compounds obtained using an analytical column are shown. For the isolation of elenolic acid, we worked at 240 nm, but it is also added in the figure in order to show the analysis of all of the isolated compounds using the gradient of 75 min.

**Calibration Curves.** Standard calibration graphs were prepared for two reference compounds: DOPAC and oleuropein (oleuropein glucoside) at two wavelengths. It was impossible to use the standards (isolated phenolic compounds) obtained with semipreparative HPLC to investigate method linearity because the quantity obtained of each compound using HPLC was not enough to be weighed and to determine the exact weight of each one; this fact caused all of the compounds quantified in this work to be expressed in terms of two commercial standards (DOPAC and oleuropein glucoside).

The detection limit (DL), quantification limit (QL), and precision [as relative standard deviation (RSD) of the intermediate concentration value of the linear range] of this method were calculated for the studied analytes using the method proposed by Curie (*27*).

All calibration curves showed good linearity between different concentrations depending on the analytes studied. Each point of the calibration plot was repeated three times in the same way. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.990 for oleuropein and DOPAC at the two wavelengths. All of the features of the proposed method are summarized in **Table 2**.

**Radical Scavenging Activity (DPPH Test).** The H-transfer reactions are monitored by UV−vis spectroscopy recording the

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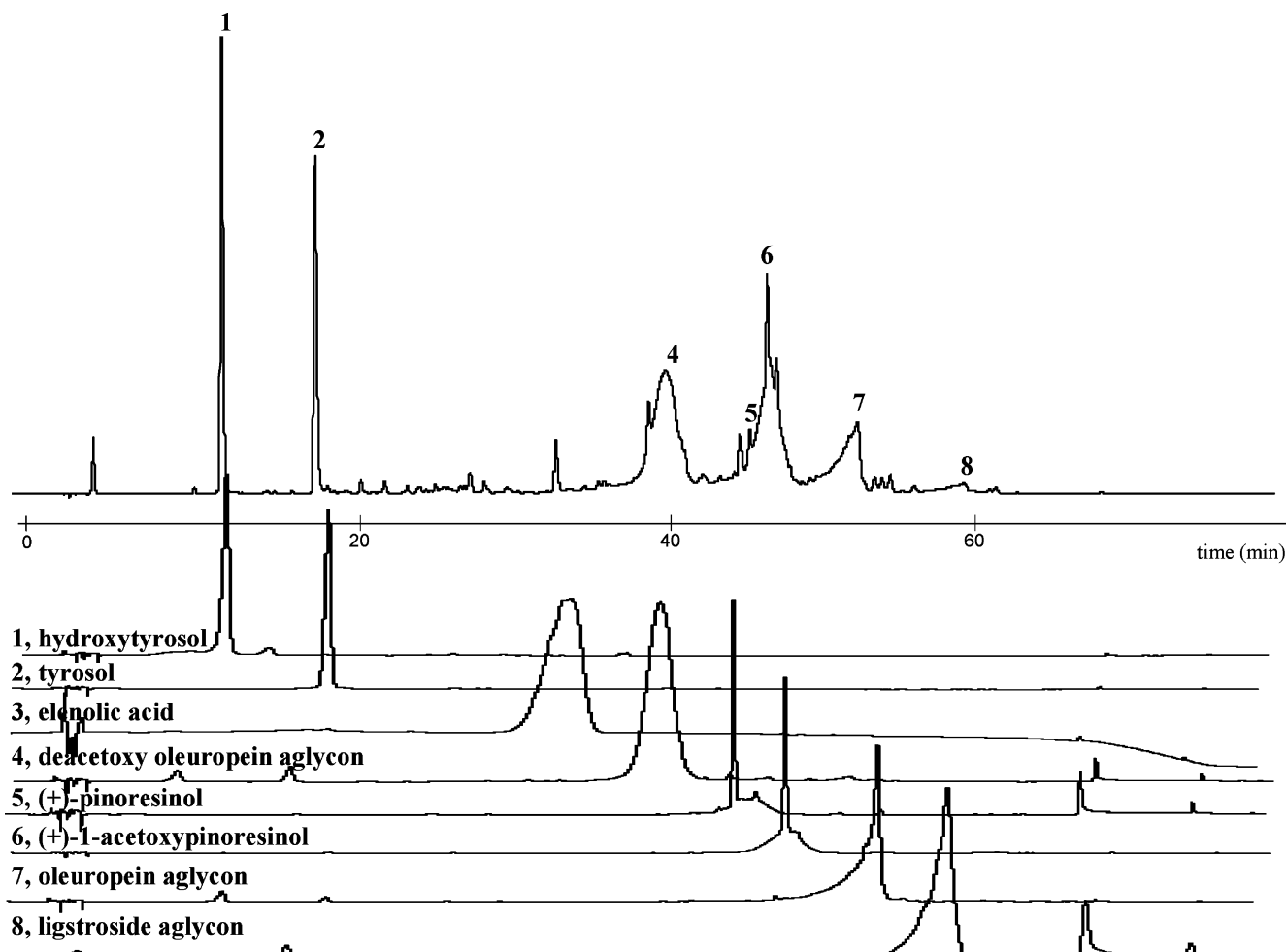


**Figure 2.** Chromatograms of extra VOO sample and the isolated phenolic compounds under optimized conditions using analytical HPLC. Detection was performed at 280 nm (only in the analysis of elenolic acid, number **3**, the detection was performed at 240 nm).

**Table 1.** Retention Times, Absorption Maxima, and the Fragmentation Patterns Using the Two Interfaces for All of the Compounds under Study[a]

| | | | | major fragments APCI negative | | | | major fragments ESI positive | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| analyte | $t_r$ (min) | $\lambda_{max}$ (nm) | molecular mass | $[M - H]^-$ | $[2M - H]^-$ | $[M - H_2O]^-$ | other fragments | $[M + H]^+$ | $[2M + H]^+$ | $[M + Na]^+$ | $[2M + Na]^+$ | $[M - H_2O + H]^+$ | other fragments |
| HYTY | 11.64 | 232/280 | 154 | 153.1 | | 137.1 | 225.1 | | | | | 137.1 | |
| TY | 17.74 | 230/276 | 138 | | nondetectable in APCI | | | | | | | 121.1 | 183.1 |
| EA | 35.75 | 240 | 242 | 241.1 | | | | 243.1 | 485.1 | 265.1 | 507.2 | 225.1 | |
| DAOA | 40.25 | 234/282 | 320 | 319.2 | 639.1 | 301 | 195 163.1 | | | 343.1 | | | 137.1 |
| PIN | 45.41 | 234/280 | 358 | 357.2 | | | | 359.1 | | 381.2 | 739.0 | 341.1 | 398.7 403.1 |
| ACPIN | 46.57 | 234/280 | 416 | 415.1 | 831.1 | | | 417.1 | | | | 398.4 | 385.1 485 |
| OA | 53.11 | 236/282 | 378 | 377.1 | 755.1 | | | 379.1 | | 401.1 | | 417.1 137.1 225.1 | |
| LA | 59.65 | 230/276 | 362 | 361.2 | | 344 | | 363.1 | | 385.1 | | 121.1 225.1 | |

[a] HYTY, hydroxytyrosol; TY, tyrosol; EA, elenolic acid; DAOA, deacetoxy oleuropein aglycon; PIN, (+)-pinoresinol; ACPIN, (+)-1-acetoxypinoresinol; OA, oleuropein aglycon; LA, ligstroside aglycon.

decay of the DPPH visible absorption band ($\lambda_{max} = 515$ nm in MeOH) that reflects the conversion of the DPPH radical into the corresponding colorless hydrazyne (DPPH-H) by the antioxidant. The antioxidant potential of the isolated phenolic compounds is shown in **Table 3**; the test was done at two different concentrations for each compound (50 and 218 ppm).

A number of structural features seem to be important in determining the antioxidant trend. The foremost consideration

is the extent and nature of the hydroxylation pattern of the aromatic rings. We can establish a classification of these compounds according to their AOP. In decreasing order, with 218 ppm of each compound, we have hydroxytyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon > (+)-pinoresinol > ligstroside aglycon > tyrosol > elenolic acid > (+)-1-acetoxypinoresinol. Doing the same test with 50 ppm of each compound, the order was practically the same: hydroxy-

**Table 2.** Analytical Parameters of the Proposed Method

| analyte | RSD (%) (intermediate value) | DL ($\mu$g/mL) | QL ($\mu$g/mL) | calibration range ($\mu$g/mL)[a] | calibration equations | $r^2$ |
|---|---|---|---|---|---|---|
| 3,4-dihydroxyphenylacetic acid | 3.65 | 0.22 | 0.73 | 2000 | $y = 21.30x - 20.91$ | 0.9988 |
| oleuropein glucoside, $\lambda = 280$ nm | 3.37 | 0.99 | 3.30 | 4000 | $y = 4.65x - 4.02$ | 0.9967 |
| oleuropein glucoside, $\lambda = 240$ nm | 4.93 | 0.34 | 1.11 | 4000 | $y = 13.96x + 2464.60$ | 0.9901 |

[a] From QL to the value in the table.

**Table 3.** Values of DPPH Test for the Isolated Phenolic Compounds at Two Different Concentrations and Order of Classification in Terms of Antiradical Power [Mean Values ± SD ($n = 6$)][a]

| | ARP (218 ppm) | classification | ARP (50 ppm) | classification |
|---|---|---|---|---|
| hydroxytyrosol | 26.23 ± 0.56 | 1 | 5.03 ± 0.10 | 1 |
| tyrosol | 0.76 ± 0.05 | 6 | 0.18 ± 0.01 | 8 |
| elenolic acid | 0.69 ± 0.04 | 7 | 0.29 ± 0.01 | 7 |
| deacetoxy oleuropein aglycon | 7.61 ± 0.29 | 2 | 2.42 ± 0.14 | 2 |
| (+)-pinoresinol | 1.35 ± 0.08 | 4 | 1.08 ± 0.03 | 4 |
| (+)-1-acetoxy-pinoresinol | 0.60 ± 0.03 | 8 | 0.30 ± 0.01 | 6 |
| oleuropein aglycon | 6.11 ± 0.12 | 3 | 1.54 ± 0.03 | 3 |
| ligstroside aglycon | 0.79 ± 0.04 | 5 | 0.38 ± 0.03 | 5 |

[a] ARP, antiradical power (in $\mu$mol/g).

tyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon > (+)-pinoresinol > ligstroside aglycon > (+)-1-acetoxy-pinoresinol > elenolic acid > tyrosol.

Phenolic compounds can be active as antioxidants for a number of potential factors. The most important is likely to be by free radical scavenging in which the phenol can break the free radical chain reaction. The presence of different substituents in the phenol backbone structures modulates their antioxidant properties, in particular their hydrogen-donating capacities.

We have proved that molecules with *o*-dihydroxyl functionalities are characterized by high antioxidant activity because of the formation of intramolecular hydrogen bonds during the reaction with the free radicals, but it is necessary to underline that the electronic and steric effects of substituents near the phenolic hydroxyl groups may be of importance in governing the hydrogen-donating capacity of monohydroxyl phenols. Electron-donating substituents in the ortho position tend to weaken the O—H bond of phenol and provide extra stability to the phenoxyl radical.

Hydroxytyrosol was the analyte that was found to exert the strongest antioxidant activity. Between deacetoxy oleuropein aglycon and oleuropein aglycon, it was logical that the first was more efficient as an antioxidant than oleuropein aglycon; the group —COOCH$_3$ in oleuropein aglycon caused a decrease in the antioxidant capacity because it is not an electron donor group. This fact could be observed when we studied the different antioxidant capacities of (+)-pinoresinol and (+)-1-acetoxy-pinoresinol where —COOCH$_3$ is also present. We also observed in these compounds that the *o*-methylation (—OCH$_3$) of the hydroxyl group of the benzoic rings caused a decrease in the antioxidant capacity, because this group was not able to form a hydrogen bond, in comparison with molecules having a catecholic group in their structure. Tyrosol and ligstroside aglycon are monosubstituted phenols and, as was expected, exhibited a very poor radical scavenging activity.

Elenolic acid is not a phenol, but undoubtedly, it is contained in the polar extract of VOO. Its presence in olive oil in a free form is due to the hydrolysis of oleuropein, ligstroside, and related compounds. At the two different concentrations that we studied, elenolic acid is one of the compounds that presents a weaker antioxidant activity; this result suggests that radical hydrogen donor ability is not easily attributable to compounds containing the closed ring of elenolic acid. Also, Briante et al. (*14*) showed in a previous paper that elenolic acid was a very poor antioxidant as compared to hydroxytyrosol.

**OSI Results.** For many years, *o*-diphenols are reported as being the highest contributors to oxidative stability in VOOs (*5*, *28*). Most of the papers studying the influence of different variables on changes in polyphenols also determined the stability measured by Rancimat (a method that works according to the same principle as OSI), and good correlation coefficients between the two parameters are found in all the studies (*1*, *28*– *30*). Studies of individual compounds added to refined oils or oils stripped of antioxidants demonstrate the important contribution of hydroxytyrosol in the effective inhibition of oxidation (*30*–*33*). Servili et al. (*33*) found that the oleosidic forms of hydroxytyrosol have the same antioxidant efficiency as hydroxytyrosol. However, in this study, the OSI test is applied to a glyceridic matrix-free phenolic compounds spiked with eight singular phenolic compounds.

To estimate the stability or susceptibily of a fat to oxidation, the sample can be subjected to an accelerated oxidation test under standardized conditions and a suitable end point is chosen to determine signs of oxidative deterioration. Several parameters (such as temperature, metal catalysts, oxygen pressure, and shaking) are manipulated to accelerate oxidation and the development of rancidity in oils and emulsions. The induction period (IP) is measured as the time required to reach an end point of oxidation corresponding to either a level of detectable rancidity or a sudden change in the rate of oxidation. Measurements of IP under standard conditions are generally used as an index of antioxidant effectiveness.

In our case, as commented before, to obtain the OSI time, we used as instrumental conditions a temperature of 110 °C and an air air flow rate of 120 mL min$^{-1}$. When the results of this test are observed (in **Table 4**), it can be seen that three of these compounds have antioxidant activity, and the others have pro-oxidant effects. The OSI value for the triolein without any phenolic compound was 9.08 h ($n = 8$); this time was longer when hydroxytyrosol, deacetoxy oleuropein aglycon, and oleuropein aglycon were added to the ≈5 g of triolein; however, the OSI time was shorter when triolein was spiked with elenolic acid, (+)-1-acetoxypinoresinol, ligstroside aglycon, tyrosol, and (+)-pinoresinol.

Among the isolated phenolic compounds with antioxidant activity, the classification in terms of AOP was, in decreasing order: hydroxytyrosol > deacetoxy oleuropein aglycon > oleuropein aglycon. The pro-oxidant compounds can be ranked according with the OSI index: (+)-pinoresinol > tyrosol > ligstroside aglycon > (+)-1-acetoxypinoresinol > elenolic acid. These classifications are for the compounds added in a

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**Table 4.** OSI Results for the Eight Isolated Phenolic Compounds at Two Different Concentrations and Their Classification in Terms of Resistance Time to Oxidation [Mean Values ± SD ($n = 4$)]

| | OSI (218 ppm) | classification | OSI (50 ppm) | classification |
|---|---|---|---|---|
| hydroxytyrosol | 16.25 ± 0.61 | 1 | 11.40 ± 0.46 | 1 |
| tyrosol | 3.20 ± 0.12 | 7 | 5.15 ± 0.21 | 7 |
| elenolic acid | 7.98 ± 0.29 | 4 | 8.55 ± 0.28 | 5 |
| deacetoxy oleuropein aglycon | 11.25 ± 0.54 | 2 | 9.15 ± 0.23 | 3 |
| (+)-pinoresinol | 3.12 ± 0.09 | 8 | 4.86 ± 0.23 | 8 |
| (+)-1-acetoxy-pinoresinol | 7.58 ± 0.20 | 5 | 8.71 ± 0.43 | 4 |
| oleuropein aglycon | 9.76 ± 0.39 | 3 | 9.53 ± 0.41 | 2 |
| ligstroside aglycon | 3.50 ± 0.13 | 6 | 7.11 ± 0.21 | 6 |

concentration of 50 mg/kg. For 218 mg/kg, the order for the antioxidants was the same, but for the pro-oxidants, the order was (+)-pinoresinol > tyrosol > ligstroside aglycon > elenolic acid > (+)-1-acetoxypinoresinol.

Nevertheless, it is important to highlight that high temperature stability tests have several limitations. First, because reactions are very rapid and the concentration of hydroperoxide (ROOH) decreases rapidly with increasing temperature, it is obvious that phenolic antioxidants will become less effective with increasing temperature. Second, because ROOH are the products of the reaction of alkylperoxy radicals with the antioxidant, their immediate decomposition could even lead to chain propagation rather than chain breaking under these conditions. Finally, side reactions of oxidation or thermolability of phenolic compounds also have to be considered. However, the result of the OSI test may help to understand how phenolic compounds act as antioxidants and how their chemical structure contributes.

**Electrochemical Results for the Single Phenolic Compounds Studied.** In a previous work, the correlation between the oxidation potential measured in cyclic voltammetry and the antioxidant activity was described (*34*). Our group proposed a FIA−amperometry method to evaluate the radical scavenging activity of phenolic extract of VOO (*2*). The electrochemical study of these phenolic compounds using a glassy carbon working electrode offers the possibility of investigating the functional (antioxidant) characteristics of the isolated phenols without the use of a reactive compound.

Both cyclic voltammetry and hydrodynamic voltammetry were performed on the single phenolic compounds obtained by semipreparative HPLC, and a classification of the AOP of the evaluated phenols was proposed on the basis of the electrochemical results.

Electron-donating substituents in the ortho position of phenolic molecules tend to weaken the O−H bond of phenol and provide extra stability to the phenoxyl radical. It is important to stress that the electrochemical oxidation of the phenolic moiety goes through the formation of a phenoxyl radical. Therefore, it can be assumed that the AOP, measured on the basis of electrochemical features (oxidation potential and current) of the phenolic molecules, may better describe the radical scavenging activity as previously hypothesized and experimentally shown (*2, 35*).

Considering the results obtained in cyclic voltammetry experiments, it is logical to deduce that between deacetoxy oleuropein aglycon and oleuropein aglycon, the first has an higher AOP (lower oxidation potential) than the oleuropein aglycon. A similar electrochemical behavior was found when we studied the different AOPs of (+)-pinoresinol and (+)-1-acetoxypinoresinol where there is the −COOCH$_3$ too. We also
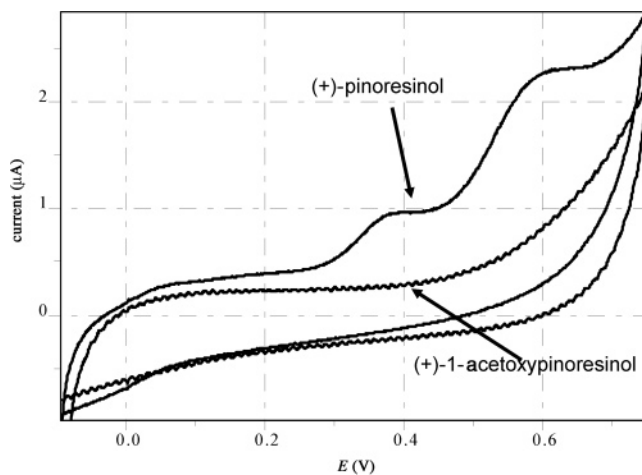


**Figure 3.** Cyclic voltammograms of (+)-pinoresinol and 1-(+)-acetoxypinoresinol.
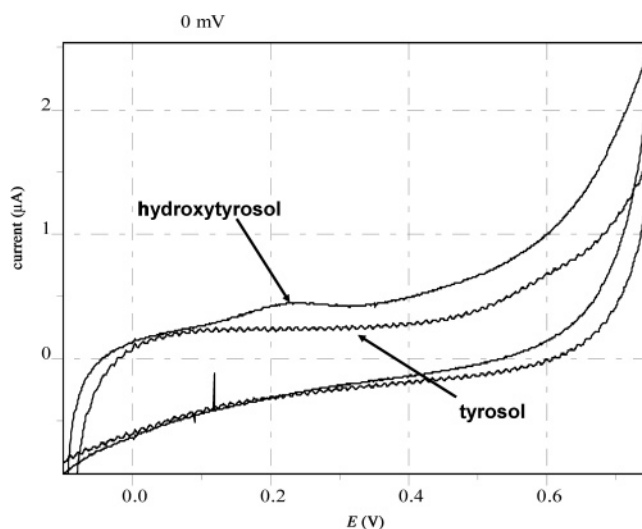


**Figure 4.** Cyclic voltammograms of hydroxytyrosol and tyrosol.

observed that the *o*-methylation (−OCH3) of the hydroxyl group of the benzoic rings caused a higher oxidation potential and a lower anodic current (see **Figure 3**). In these voltammograms, it is evident that the substitution of a hydrogen atom with an acetoxy group completely changes the electrochemical behavior of the molecule eliminating the occurrence of anodic peaks. The importance of an electron donating group was also evidenced by the cyclic voltammograms of tyrosol and hydroxytyrosol where the presence of the −OH group determines the appearance of an anodic peak at +250 mV (**Figure 4**). Finally, ligstroside aglycon is a monosubstituted phenol and, as was expected, exhibited a very poor electrochemical activity, and elenolic acid, which is not a phenol, did not show electrochemical activity.

Considering the results obtained in hydrodynamic voltammetry experiments (data reported in **Table 5**), it is possible to see some plateau regions for each molecule. The plateau regions correspond to the oxidation potential of a molecule moiety, thus indicating the energy required for the oxidation; this value is inversely proportional to the AOP of the molecule (*2, 35*). On the basis of the lower oxidation potential, we have proposed an AOP scale as follows: hydroxytyrosol > oleuropein aglycon > (+)-pinoresinol > deacetoxy oleuropein aglycon > (+)-1-acetoxypinoresinol > tyrosol > ligstroside aglycon = elenolic acid. This simple method, based on the electron-donating capability of each molecule to the electrode, may contribute to

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**Table 5.** Plateau Potential Regions ($E_{ox}$) Evaluated by Hydrodynamic Voltammetry (HV)[a]

| | $E_{ox}$ (HV) mV vs Ag/AgCl | AOP classification |
|---|---|---|
| hydroxytyrosol | 0.12–0.17 | 1 |
| tyrosol | 0.35 | 6 |
| elenolic acid | inactive | 7 |
| deacetoxy oleuropein aglycon | 0.15–0.30 | 4 |
| (+)-pinoresinol | 0.15–0.22–0.30 | 3 |
| (+)-1-acetoxypinoresinol | 0.22 | 5 |
| oleuropein aglycon | 0.15–0.20–0.30–0.40 | 2 |
| ligstroside aglycon | inactive | 7 |

[a] The AOP classification is based on the lower oxidation potential.

the understanding of the structure−function relationship of radical scavengers.

We should underline the differences among the results of the three tests. The reason for these discrepancies may be that while the DPPH test values the capacity of a group to block a radical (antiradical activity), the other test (OSI) allows the evaluation of the behavior of these groups also in the presence of oxygen (antioxigen and antiradical activity). To show the different behavior, we can use as an example the obtained values for lignans. The result of the chemical method (DPPH) showed that both lignans [(+)-pinoresinol and (+)-1-acetoxypinoresinol] are antioxidants and (+)-pinoresinol exerted a stronger antioxidant activity than (+)-1-acetoxypinoresinol; on the other hand, the result of the OSI test showed that both lignans have a pro-oxidant effect, but this pro-oxidant action is remarkable in the case of (+)-pinoresinol. To justify this result, it is important to consider their chemical structures, which contain one atom of oxigen in each central ring; this atom, in conditions of thermal stress, could cause the opening of the ring; therefore, the lignans could act as pro-oxidants. The −COOCH$_3$ group in the (+)-1-acetoxypinoresinol hinders the opening of the ring and causes a weaker pro-oxidant effect.

The electrochemical study of these phenolic compounds using a glassy carbon working electrode offers the possibility of investigating the functional (antioxidant) characteristics of the isolated phenols without the use of a reactive compound; their resulst are very similar to those obtained using the DPPH test.

In conclusion, in this work, the antioxidant capacity of simple phenols, lignans, and secoiridoids has been investigated. The antioxidant activity was evaluated by DPPH, OSI, and an electrochemical method and was closely related to the chemical structure of the isolated phenolic compounds.

The ability to act as a hydrogen donor and the inhibition of oxidation are enhanced by increasing the number of hydroxyl group in the phenols. We verified that, as is generally assumed, the presence of a single hydroxyl group confers a limited amount of antioxidant activity. On the other hand, the presence of an *o*-diphenol enhances the ability of the phenolic compounds to act as antioxidants.

The results obtained for the three tests show that hydroxytyrosol, deacetoxy oleuropein aglycon, and oleuropein aglycon are the strongest in the classification in terms of AOP. It is very interesting to observe the results obtained for the OSI test, where several of the compounds under study showed antioxidant activity, and the others showed pro-oxidant effects.

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En este Anexo del capítulo 5 nos parece conveniente adjuntar alguna información adicional acerca de cada uno de los tests que se utilizaron para medir la capacidad antioxidante de los compuestos aislados.

1) El primer test puede encuadrarse dentro de los "métodos de captación de radicales". La captación de radicales es el principal mecanismo de acción de los antioxidantes en los alimentos. Se han desarrollado muchos métodos en los que se mide la capacidad antioxidante a través de la captación de radicales libres sintéticos en disolventes orgánicos polares a temperatura ambiente. Los radicales más usados son del tipo 2,2-difenil-1-picrilhidrazilo (DPPH) y 2,2`-azinobis(3-etilbenzotiazolina-ácido sulfónico) (ABTS).
En el método DPPH se mide la captación de este radical a través de la disminución de la absorbancia, medida a 515 nm, que se produce por reducción de un antioxidante (AH) o por reacción con especies radicales (R·).

$$DPPH· + AH \rightarrow DPPH\text{-}H + A·$$
$$DPPH· + R· \rightarrow DPPH\text{-}R$$
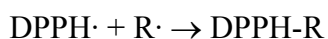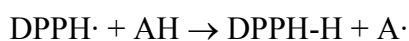
En la mayor parte de los casos el método DPPH se ha usado para medir la captación de radicales después de 15 ò 30 minutos de iniciada la reacción. El dato se puede expresar como valor $EC_{50}$, que expresa la concentración de antioxidante necesaria para captar el 50% de los radicales DPPH en un período de tiempo determinado; o bien, como valor TEAC (capacidad antioxidante equivalente Trolox).

2) El segundo test, OSI, se puede incluir entre los métodos predictivos que miden el índice de estabilidad del aceite (IEA). Estos métodos son una automatización del método del oxígeno activo (MOA). Los instrumentos para la determinación del IEA se denominan Rancimat$^{TM}$, fabricado por Metrohm (Basel, Suiza), y Oxidative Stability Instrument$^{TM}$, fabricado por Omnion (Rockland, EEUU). Estos aparatos miden el aumento de la conductividad eléctrica cuando se hace pasar el efluente de un aceite oxidado a través del agua. Los ácidos carboxílicos volátiles que se generan durante la oxidación del aceite incrementan la conductividad eléctrica del agua. Las muestras, cuantificadas por los métodos IEA, se mantienen a 100, 110, 120, 130 y 140ºC. la temperatura se suele ajustar hasta que el tiempo de oxidación esté incluido dentro del

rango aprox de 4-25 horas. El tamaño de la muestra es de 2.5 a 5 g, dependiendo del instrumento usado.



Fotografía del instrumento OSI empleado en el desarrollo experimental de este capítulo.

3) Sin duda, el tercer método fue el más novedoso. Este método puede determinar el "poder antioxidante" sin necesidad de usar ningún reactivo. El comportamiento electroquímico de cada uno de los compuestos se midió usando una voltametría hidrodinámica en un análisis por inyección en flujo (FIA). El sistema estaba formado por una bomba peristáltica (Wilson, Francia), una válvula de alta presión modelo 7125 (Rheodyne, Rohnert Park, CA), un loop de 20 µl, una celda electroquímica modelo UniJet (BAS, West Lafayette, IN) que usaba un electrodo vítreo de carbono, y un detector amperométrico AMEL 559 HPLC (AMEL, Milán, Italia) unido a un registrador de señal RC 102 (Pharmacia, Suecia).

La voltametría es una técnica electroanalítica en las que se aplica un determinado potencial eléctrico a un electrodo (denominado electrodo de trabajo) sumergido en una disolución que contiene una especie electroactiva y se mide la intensidad eléctrica que circula por este electrodo. La intensidad medida es función del potencial aplicado y de la concentración de la especie electroactiva presente.

En el estudio que compone este capítulo, utilizamos voltametría tanto hidrodinámica, como cíclica. En lo que respecta a la primera de ellas, podemos decir que está siendo ampliamente utilizada para la detección y determinación de compuestos oxidables y reducibles o de iones que han sido previamente separados por HPLC o por métodos de inyección en flujo. En estas celdas, el electrodo de trabajo está normalmente empotrado a la pared de un bloque aislante que está separado del electrodo auxiliar por un espaciador delgado. El volumen de una celda de este tipo suele ser 0.1-1 µl. el potencial

correspondiente a la región de corriente límite de los analitos se aplica entre un electrodo de trabajo de carbono vítreo (hay también otras opciones, pera ésta es la que nosotros usamos) y un electrodo de referencia de plata/cloruro de plata que está situado a la salida del flujo del detector. Lógicamente, esta tipo de voltametría es más sensible que la cíclica, que pasamos a comentar a continuación.

La voltametría cíclica se caracteriza porque la señal que se aplica al electrodo de trabajo, cuyas condiciones son estacionarias y sin agitación de la disolución, es una señal de potencial triangular.
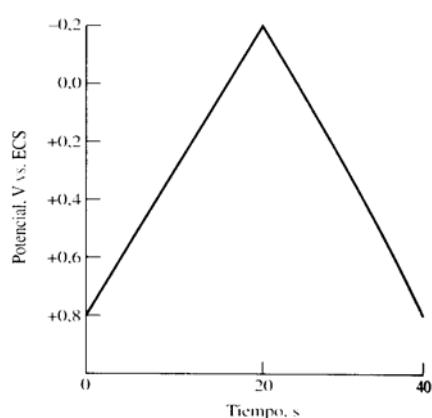


Figura. Ejemplo de una señal de excitación (de +0.8 V a -0.15 V en este caso) en voltamperometría cíclica que podría ser utilizada para obtener un voltamperograma.

Como puede verse en el dibujo anterior, el potencial aplicado va desde +0,8V a –0,15V. Y cuando se alcanza este valor de –0,15V se produce una inversión en el sentido del barrido y vuelve a su valor original de +0,8V. Este ciclo suele darse varias veces, y los potenciales en los que se da esta inversión de la dirección reciben el nombre de *potenciales de inversión*. El intervalo de potencial de inversión impuesto en cada experimento es aquel en el que tiene lugar la oxidación o la reducción controladas por difusión de uno o varios analitos, y la dirección en la que se produzca el barrido va a depender de la composición que presente la muestra.

Esta técnica voltamperométrica es usada, principalmente, como herramienta para análisis cualitativo, y los parámetros principales que permiten la identificación del analito son: el potencial de pico catódico, el potencial de pico anódico, la corriente de pico catódica y la corriente de pico anódico.

Esta técnica es importante para el estudio de los mecanismos y de las velocidades de los procesos de oxidación/reducción. A menudo, los voltamperogramas cíclicos revelan la presencia de intermedios en las reacciones de oxidación/reducción.
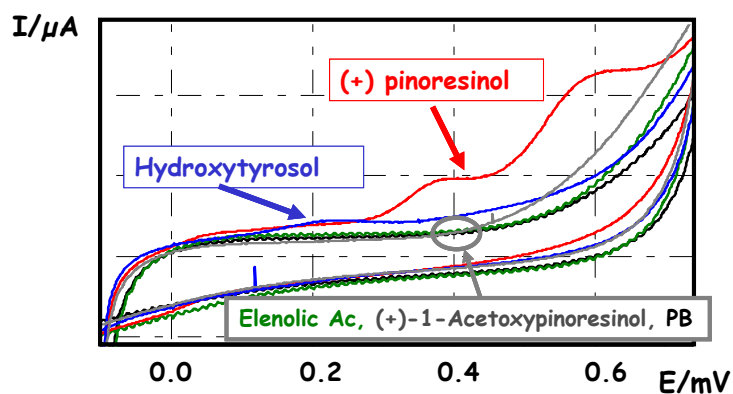


*Figura*. Ejemplo que recoge algunos de los datos obtenidos y recogidos en el artículo que compone este capítulo utilizando voltametría cíclica para mostrar cómo son los datos ofrecidos por esta técnica.

El sistema que fue utilizado en este estudio en su conjunto y una imagen de la celda electroquímica más detallada se muestran a continuación:
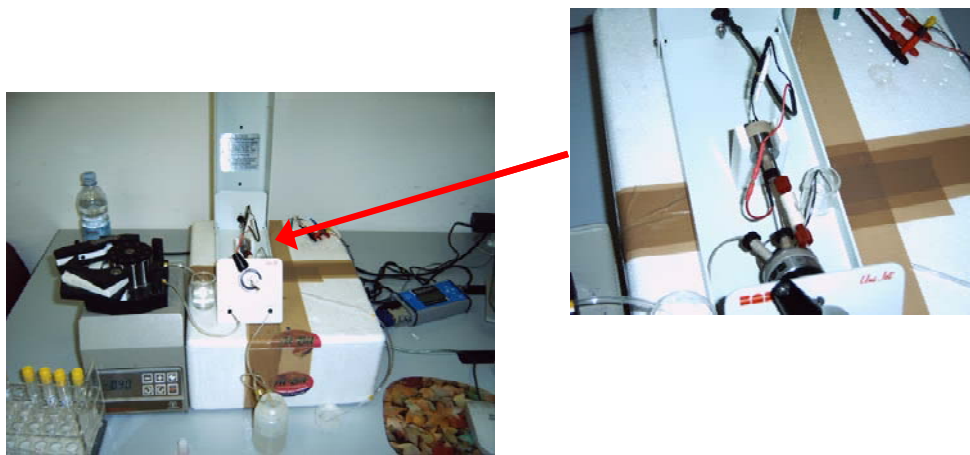


*Figura*. Fotografía del sistema electroquímico empleado y detalle de la celda electroquímica.

Observando los datos finales, se encontró una gran correlación entre el test DPPH y el test electroquímico. En el test OSI fue sorprendente que algunos compuestos mostraron actividad antioxidante y otros prooxidante, cosa que se explica por las condiciones tan drásticas de temperatura (110º C) a las que se realiza el mismo. Para entender

totalmente los resultados en este caso es muy importante conocer y tener presente la estructura de todos los compuestos empleados en este estudio.

Concluido el trabajo experimental comentado en este anexo, se habían conseguido dos objetivos muy interesantes de manera simultánea:

-Se había logrado caracterizar varios de los compuestos fenólicos (8 en particular: hidroxitirosol, tirosol, ácido elenólico, deacetoxi-oleupeína aglicona (también denominada decarboximetil-oleupeína aglicona), (+)-pinoresinol, (+)-1-acetoxipinoresinol, oleupeína aglicona y ligustrósido aglicona) atendiendo a su capacidad antioxidante medida mediante técnicas basadas en principios totalmente diferentes.

-Se habían recogido estándares de los 8 compuestos anteriormente mencionados, que tenían un gran valor, ya que en ese momento, de esos 8 compuestos sólo 1 estaba disponible comercialmente (tirosol).

Este segundo logro, nos permitió plantearnos la puesta a punto de un método electroforético para caracterizar estos compuestos mayoritarios en la fracción más polar del aceite de oliva (capítulo 6), cosa que aún no había podido ser lograda mediante CE.

# Capítulo 6

**Puesta a punto de un método electroforético sencillo y rápido para caracterizar fenoles simples, lignanos, fenoles complejos, ácidos fenólicos y flavonoides en aceite de oliva virgen-extra**

Como se puso de manifiesto en el capítulo 1, si comparamos los resultados que hay hasta el momento obtenidos por electroforesis capilar y aquellos obtenidos mediante otras técnicas (especialmente HPLC), la balanza está más que descompensada a favor de estas últimas.

Considerando los resultados que, hasta el momento en el que se inició el presente capítulo, habían sido logrados con el uso de CE para analizar compuestos fenólicos del aceite de oliva, pudimos ser conscientes de que había grandes carencias a las que se les debía intentar dar respuesta.

En la siguiente tabla, se muestra un resumen de los métodos electroforéticos publicados para analizar polifenoles en aceite de oliva antes del desarrollo del método que aquí se describe (Tabla 5 del capítulo 1 ampliada).

*Tabla*. Resumen de los sistemas de extracción y de los compuestos que eran estudiados aplicando cada uno de los métodos.

HYTY, hidroxitirosol; TY, tirosol; DHPE, 2,3-dihidroxifeniletanol; VA, ácido vaníllico; DAOA, deacetoxi-oleuopeína aglicona (o decarboximetil-oleuopeína aglicona); Ac Pin, (+)-1-acetoxipinoresinol; Pin, (+)-pinoresinol; Lig Agl, ligustrósido aglicona; Ol Agl, oleuropeína aglicona; EA, ácido elenólico; TY-Ac, tirosol acetato; 10-H-Ol Agl: 10-hidroxi-oleuropeína aglicona.

| Autores y referencia | Sistema de extracción | Cantidad inicial de aceite→Cantidad final de disolvente (MeOH/H$_2$O (50:50 v/v) ) en el proceso de extracción | Compuestos detectados (y cuantificados)** |
|---|---|---|---|
| Bendini y col [205] | LLE (Pirisi y col [339]) | 2 g → 1 ml | HYTY, TY, varios compuestos relacionados con los secoiridoides no identificados |
| Bonoli y col [340] | LLE (Pirisi y col [339]) | 2 g → 1 ml | HYTY, TY, DHPE, derivados de la oleuropeína sin identificar |
| Bonoli y col [341] | LLE (Pirisi y col [339], modificado por Rotondi y col [342]) | 2 g → 0.5 ml | HYTY, TY, VA, DAOA, Ac Pin |
| Carrasco Pancorbo y col [100] | LLE [100] | 60 g → 0.5 ml | 14 ácidos fenólicos |
| Buiarelli y col [343] | Combinación of LLE-SPE [343] | 10 g → no especificada | 5 ácidos fenólicos |
| Carrasco Pancorbo y col [213] | LLE [100] | 60 g → 0.5 ml | 14 ácidos fenólicos |
| Gómez Caravaca y col [206] | SPE (Cartuchos Diol) [206] | 60 g → 2 ml | TY, Pin, Ac Pin, DAOA, Lig Agl, HYTY, Ol Agl, EA |
| Carrasco Pancorbo y col [344]* | SPE (Cartuchos Diol) [206] | 60 g → 2 ml | TY, HYTY, TY-Ac, lignanos, Lig Agl (y sus formas isoméricas), Ol Agl (y sus formas isoméricas), 10-H-Ol Agl, EA |

*Los detalles de este método (CE-ESI-Trampa de Iones) se comentarán en próximos capítulos

** No en todos los casos se lleva a cabo la cuantificación. En ciertos casos, se cuantifican todos los fenoles conjuntamente

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[343] F. Buiarelli, S. Di Berardino, F. Coccioli, R. Jasionowska, M. V. Russo. "Determination of phenolic acids in olive oil by capillary electrophoresis". *Annali di Chimica* 94 (2004) 699-705.

[344] A. Carrasco Pancorbo, D. Arráez Román, A. Segura Carretero, A. Fernández Gutiérrez. "Capillary electrophoresis-electrospray ionization-mass spectrometry method to determine the phenolic fraction of extra-virgin olive oil". *Electrophoresis* 27 (2006) 2182-2196.

Como se puede deducir del estudio de la tabla, los ácidos fenólicos fueron objeto de estudio en tres de los métodos mencionados [100,213,343], mientras que en los otros, el objetivo de los autores fue estudiar la fracción completa de compuestos fenólicos que se halla en el aceite de oliva, intentando determinar varias familias distintas de manera simultánea [205,206,340,341,344].

Bendini y col [205,340,341] lograron identificar varios componentes muy importantes de la fracción polifenólica del aceite de oliva (TY, HYTY, lignanos y la zona de la oleuropeína aglicona decarboxilada (DAOA)) usando un método electroforético bastante rápido. Después, en un artículo de publicado por nuestro grupo de investigación [206], el número de compuestos identificados en un perfil electroforético de un aceite de oliva virgen-extra pudo verse aumentado mediante el uso de estándares coleccionados con HPLC semi-preparativa. Sin embargo, en este último caso, el tiempo de análisis era demasiado largo.

A pesar de todos estos logros y mejoras conseguidos por anteriores métodos, quedaban dos familias muy importantes dentro de la fracción fenólica del aceite de oliva aún por caracterizar: los **secoiridoides** y los **flavonoides**.

Los secoiridoides son una de las familas mayoritarias en la fracción polar del aceite [345]. Son derivados de las formas glucosídicas de los secoiridoides de la aceituna, y se caracterizan por la presencia en su estructura del ácido elenólico o algún derivado suyo. Son importantes desde un punto de vista médico y farmacológico por las propiedades que poseen [346], también por su poder antioxidante [347,348], y por la influencia que ejercen sobre percepciones sensoriales [146,147].

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Del mismo modo, los flavonoides han despertado gran interés en la comunidad científica por el amplio rango de propiedades biológicas y fisiológicas [349] que pueden llegar a poseer y porque pueden servir como marcadores quimiotaxonómicos [350].

Los flavonoides (luteolina y apegina) fueron encontrados en aceite de oliva por primera vez por Rovellini y col [109], y hasta la fecha sólo han sido identificados y cuantificados en esta matriz mediante HPLC.

Así pues, nos propusimos la mejora de los métodos electroréticos mencionados anteriormente para obtener un método simple y rápido que permitiese la separación de los componentes más importantes de la fracción fenólica extraída (SPE-Diol) [351] del aceite de oliva. Quisimos identificar el mayor número de compuestos presentes en esta fracción posible usando los estándares coleccionados mediante HPLC (capítulo 5), usando la información obtenida mediante HPLC-MS y teniendo en cuenta toda la información espectral.

Si se lograban estos objetivos, se alcanzaba incluso otro objetivo "colateral", como es el demostrar que la CE es capaz de proporcionar resultados que han sido obtenidos tradicionalmente mediante HPLC. Se dotaría al analista de una metodología alternativa para caracterizar los compuestos fenólicos del aceite de oliva, y se demostraría que en casos en los que HPLC no ofrece suficiente resolución, CE con la flexibilidad de condiciones experimentales que presenta, podría ser una técnica más que complementaria.

Del trabajo experimental desarrollado que se adjunta a continuación como una publicación en el J. Sep. Sci., podemos avanzarr que se llevó a cabo una concienzuda optimización de:

> -*longitud y diámetro del capilar*
>
> -*temperatura*
>
> -*pH y fuerza iónica del tampón de separación*
>
> -*voltaje de separación*

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[351] En este capítulo se cambió el sistema de extracción, ya que en un artículo de nuestro grupo de investigación (Gómez Caravaca y col [206]) se había demostrado que el sistema de extracción sólido-líquido con cartuchos Diol podía ser más conveniente y permitir una mayor automatización del proceso con la estación de vacío.

*-tipo y tiempo de inyección*

Y se llegó a la conclusión de que los parámetros óptimos eran: 40 cm de longitud efectiva del capilar x 50 µm de diámetro interno, 22ºC, 45 mM de tetraborato sódico a un pH de 9.3, 28 kV e inyección hidrodinámica durante 8 segundos.

Además, para poder identificar todos estos compuestos empleamos tres vías:

*a) uso de los estándares disponibles comercialmente*
*b) uso de los estándares coleccionados mediante HPLC*
*c) uso de 4 longitudes de onda para llevar a cabo la detección en cada análisis*

Aunque hay que reconocer que las condiciones electroféticas no distan mucho de las de otros métodos mencionados con anterioridad, la gran innovación y ventaja de este método radica en la identificación de un gran número de compuestos en el perfil electroforético de un extracto de aceite de oliva (hasta 26 analitos en 10 minutos).

**Alegria Carrasco-Pancorbo[1]**
**Ana Maria Gómez-Caravaca[1]**
**Lorenzo Cerretani[2]**
**Alessandra Bendini[2]**
**Antonio Segura-Carretero[1]**
**Alberto Fernández-Gutiérrez[1]**

[1]Department of Analytical
 Chemistry, Faculty of Sciences,
 University of Granada, Granada,
 Spain
[2]Department of Food Science,
 University of Bologna, Cesena
 (FC), Italy

## Original Paper

# A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra-virgin olive oil

We have devised a simple and rapid capillary electrophoretic method which provides the analyst with a useful tool for the characterization of the polyphenolic fraction of extra-virgin olive oil. This method that uses a capillary with 50 µm id and a total length of 47 cm (40 cm to the detector) with a detection window of $100 \times 200$ µm, and a buffer solution containing 45 mM of sodium tetraborate pH 9.3 offers valuable information about all the families of compounds present in the polar fraction of the olive oil. The detection was carried out by UV absorption at 200, 240, 280, and 330 nm in order to facilitate the identification of the compounds. Concretely, the method permits the identification of simple phenols, lignans, complex phenols (isomeric forms of secoiridoids), phenolic acids, and flavonoids in the SPE-Diol extracts from extra-virgin olive oil in a short time (less than 10 min) and provides a satisfactory resolution. Peak identification was done by comparing both migration time and spectral data obtained from olive oil samples and standards (commercial or isolated (by HPLC-MS) standards), with spiked methanol-water extracts of olive oil with HPLC-collected compounds and commercially available standards at several concentration levels, studying the information of the electropherograms obtained at several wavelengths and also using the information previously reported.

## 1 Introduction

In recent years, the number of reports describing the beneficial properties of olive oil has dramatically increased. Recent data have suggested that the components in olive oil may have more health benefits than previously thought, and consequently there have been numerous experiments which have investigated the metabolic fate of the constituents in olive oil [1, 2].

**Correspondence:** Dr. A. Segura-Carretero, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.
**E-mail:** ansegura@ugr.es.
**Fax:** +34-958249510.

**Abbreviations: Ac Pin,** (+)-1-acetoxypinoresinol; **DOA,** decarboxylated oleuropein aglycon; **EA,** elenolic acid; **EK,** electrokinetic; **HD,** hydrodynamic; **HYTY,** hydroxytyrosol; **Lig Agl,** ligstroside aglycon; **Ol Agl,** oleuropein aglycon; **Pin,** (+)-pinoresinol; **TY,** tyrosol

The composition of olive oil is primarily triacylglycerols and ∼0.5–1.0% nonglyceridic constituents [3]. Among other minor constituents, olive oil contains phenolic and polyphenolic compounds, many of which influence the sensorial properties of olive fruits and virgin olive oils [4, 5] and are important markers for studying fruit characteristics of different cultivars and for controlling oil production processes [6]. Their antioxidant effects are also correlated to the high stability of virgin olive oil compared to thermoxidation and autoxidation processes over time [7, 8].

The phenolic compounds identified and quantified in olive oil belong to phenylethylalcohols (as hydroxytyrosol (HYTY) and tyrosol (TY)), phenolic acids (as *p*-coumaric acid, vanillic acid, *etc.*), lignans ((+)-pinoresinol and (+)-1-acetoxypinoresinol), secoiridoids (several aglycone derivatives of oleuropein and ligstroside), and flavonoids (luteolin and apigenin) [9].

Separation and determination of individual phenolic compounds in the extracts obtained from olive oil by

liquid-liquid extraction (LLE) or SPE use HPLC analysis coupled mostly with UV detection [4, 5, 10–14], although electrochemical [15, 16], fluorescence [17, 18], and MS [5, 6, 19] detection systems have also been proposed. GC analysis is less common because the derivatization step is essential [20–23].

There has been increasing interest in CE due to the high separation efficiency which can be obtained, the short analysis times that can result, the relatively low cost of the instrumentation, and the suitability of CE for many types of compounds. In particular, this technique has recently been used for the analysis of phenolic compounds of extra-virgin olive oil [24–28], olive mill wastewater [29], and alperujo [30]. In previous papers [24–26], it was possible to identify several important compounds of the polyphenolic fraction in virgin olive oil samples (TY, HYTY, lignans, and decarboxylated oleuropein zone) by using a fast electrophoretic method. In a recently published paper of our group [31], however, the identification of several compounds (never seen before using CE) of the polyphenolic fraction in seven Spanish varieties of extra-virgin olive oil using standards obtained by semi-preparative HPLC, and their quantification using two reference compounds at two different wavelengths has been done using CE.

In spite of the effort of several research groups to develop rapid and reliable CE methods for the analysis of the complete polyphenolic fraction of virgin olive oil, there are two important families, present in this polar fraction, which are not well characterized by CE: secoiridoids and flavonoids.

The secoiridoids belong to a majority family of phenolic compounds of virgin olive oil [23, 32]. These secoiridoids are derived 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 (EA) or their EA derivatives in their molecular structure [33]. The prevalent secoiridoid compounds in the olive oil are the dialdehydic form of EA linked to HYTY or TY (3,4-DHPEA-EDA or *p*-HPEA-EDA, respectively) and isomers of oleuropein aglycon (Ol Agl) (3,4-DHPEA-EA) or ligstroside aglycon (Lig Agl) (*p*-HPEA-EDA) [14, 34–36].

It has been demonstrated that HYTY and the secoiridoids containing multiple hydroxyl groups are the natural antioxidants of virgin olive oil exhibiting the highest antioxidant power [7, 37, 38]. Furthermore, several healthy and pharmacological properties of the secoiridoid derivatives of virgin olive oil have also been studied [39, 40].

The relations between the secoiridoid derivatives and the bitterness of VOO have also been studied; first, interest was focused on two derivatives of oleuropein and demethyloleuropein, such as 3,4-DHPEA-EDA and *p*-DHPEA-EA [41, 42]. In recent studies, it was observed that a relationship between bitter and pungent sensory properties and ligstroside derivative content [43] or the amount of the aldehydic form of Ol Agl [44] can be established.

Many studies have provided reliable information, and some of them have helped to clarify the structures of some of these compounds in olive oil. Nevertheless, because of the complexity of the wide group of secoiridoids, many of these important phenolic compounds in olive oil remain unidentified [45].

On the other hand, flavonoids are very common and widespread secondary plant metabolites that include into their chemical structure a C6-C3-C6 configuration. They have a wide range of biological and physiological activities [46, 47] and serve as chemotaxonomic marker compounds [48].

In particular, Rovellini *et al.* [49] reported that flavonoids such as luteolin and apigenin were also phenolic components of virgin olive oil. These compounds are mentioned in the literature, but they have only been identified and quantified in olive oil by HPLC [49–52]. To our knowledge, this is the first time that the flavonoids of virgin olive oil have been identified by CE.

Our first objective was the improvement of the CE methods previously mentioned in order to obtain a very simple, rapid CZE method which allowed the separation of the most important compounds of the SPE-diol phenolic fraction of extra-virgin olive oil. Our second aim was to carry out the identification of most of the compounds which are present in this fraction by using commercial or HPLC-collected standards, by using the information provided by HPLC-MS analyses and taking into account all the UV-spectral information (in fact, the identification of flavonoids and several forms of the secoiridoids which have never been seen before with CE has been done).

Finally, with this study we want to demonstrate that CE is able to obtain the aims that have been traditionally achieved by using HPLC providing an alternative methodology to characterize phenolic compounds from olive oil, and prove 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.

## 2 Materials and methods

### 2.1 Apparatus

The CE instrument used was a Beckman 5500 CE connected to a diode array detector (belonging to the Univer-

sity of Granada). The system comprises a 0–30 kV high-voltage built-in power supply.

All capillaries (fused silica) used were obtained from Beckman instrument (Fullerton, CA, USA) and had an inner diameter (id) of 50 μm, a total length of 47 cm, and an effective separation length of 40 cm. We also did analyses with capillaries of 75 μm id. The temperature was controlled using a fluorocarbon-based cooling fluid. Data acquisition and processing were carried out with GOLD software installed in a PC.

HPLC analyses were performed with an HP 1100 series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system, a degasser, autosampler, diode array UV-Vis detector (DAD), and a mass spectrometer detector (MSD).

The semipreparative HPLC column used was a Phenomenex Luna ($C_{18}$) column (10 μm, 25 cm × 10 mm) and the flow rate was 3 mL/min.

The analytical HPLC column used was a $C_{18}$ Luna column (5 μm, 25 cm × 3.0 mm) (Phenomenex, Torrance, CA), with a $C_{18}$ precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min.

## 2.2 Reagents and reference compounds

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma and was used as running buffer at different concentrations and pHs.

Methanol, ACN, and n-hexane were acquired from Panreac (Montcada I Reixac, Barcelona, Spain) and were HPLC grade.

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

SPE cartridges DSC-Diol were obtained from Supelco (Bellefonte, PA, USA).

During the development of this method, we have used reference compounds that can be divided in two groups: commercially available standards and HPLC-isolated standards.

### 2.2.1 Commercially available standards

The phenolic acids (4-hydroxyphenylacetic acid), gallic acid (3,4,5-trihydroxybenzoic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), caffeic acid (3,4-dihydroxycinnamic acid), dopac (3,4-dihydroxyphenylacetic acid), p-coumaric acid (4-hydroxycinnamic acid), sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid), 2,5-dihydroxybenzoic acid, trans-cinnamic acid, o-coumaric acid (2-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methox-

ycinnamic acid), and gentisic acid (2,5-dihydroxybenzoic acid) standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Rutin, vanillin (4-hydroxy-3-methoxybenzaldehyde), and quercetin (3,3′,4′,5,7-pentahydroxyflavone) were also acquired from Sigma-Aldrich. TY, 4-hydroxybenzoic acid, 3-methoxybenzoic acid, and homovanillic acid were acquired from Fluka. HYTY, dihydrocaffeic acid, the flavanonol (+)-taxifolin (3,3′,4′,5,7-pentahydroxyflavanone), and the flavonoids, luteolin and apigenin, were obtained from Extrasynthèse (Genay, France) and all the analytes were used as received.

### 2.2.2 HPLC-collected standards

The following compounds were isolated: EA, decarboxylated oleuropein aglycon (DOA), (+)-pinoresinol (Pin), (+)-1-acetoxypinoresinol (Ac Pin), Ol Agl, and Lig Agl by using semipreparative HPLC [37] (see Section 2.5).

It is important to highlight that the composition of the phenolic fraction of each sample is different, and the isomeric forms present in each variety of olive oil (and their relative concentration) may also be different. The collected standards were isolated from a commercial Bertolli extra-virgin olive oil, so the composition of these standards depends on the original chemical characteristics of this oil.

## 2.3 Samples

Extra-virgin olive oil samples were obtained from two Spanish geographic zones. Each of them was from a unique variety of olive fruit called Picual or Hojiblanca (January 2005).

To carry out the HPLC experimental work and the isolation of the standard by semipreparative HPLC, commercial extra-virgin olive oil samples were used (from Bertolli, Unilever Bestfoods Italia SpA, Inveruno-MI, Italy).

## 2.4 SPE procedure

In order to isolate the phenolic fraction, we used SPE with Diol cartridges; the SPE protocol was carried out with the extraction conditions and amounts of oil which are described in a recent paper of our research group [31].

Extra-virgin oil (60 g) was dissolved in 60 mL of hexane and was passed through the column. The solvent goes through, leaving the sample on the solid phase. The cartridge was washed with three portions (of 5 mL) of hexane, which were then discarded in order to remove the nonpolar fraction of the oil. Finally, the sample was recovered by passing through eight portions (of 5 mL) of methanol and brought to dryness in a rotary evaporator under reduced pressure and a temperature of 35°C. The residue was dissolved with 2 mL of methanol/water

(50:50 v/v) and filtered through a 0.25 µm filter before the CE analysis.

## 2.5 HPLC isolation and analysis of phenolic compounds

To isolate the previously mentioned phenolic compound the mobile phases were water with acetic acid (0.5%) (phase A) and ACN (phase B), and the solvent gradient changed according to the conditions described by Carrasco-Pancorbo *et al.* [37]. The injection volume for the isolation of the reference compounds was 100 µL of extracts of Bertolli olive oil in methanol/water (50:50 v/v). The wavelengths were set at 240, 280, and 340 nm.

The analysis of the isolated compounds was done with the analytical column and an MS detection system to check their purity and confirm their identity, using the same gradients and other instrumental conditions used in the semipreparative HPLC method as have been described elsewhere [37].

## 2.6 Electrophoretic procedure

CE separation was carried out on a fused-silica capillary (50 µm id, 375 µm od, total length 47 cm, a detection window was created at 40 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of $100 \times 200$ µm). Every time a new capillary was used, it was preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5-min rinse with Milli-Q water, and 15 min with buffer. For the following analyses the capillary was rinsed with 0.1 M NaOH for 2 min, followed by a 2-min flush with Milli-Q water to assure good repeatability. The capillary was equilibrated with the running buffer (45 mM sodium tetraborate adjusted to pH 9.3) for 3 min before each sample injection. After each analysis, the capillary tubing was rinsed for 2 min with Milli-Q water. All solutions and samples were filtered through a 0.25 µm syringe filter. The running buffer was changed after four runs.

Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 psi) for 8 s (1 psi = 6895 Pa) and also using electrokinetic (EK) injection (see Section 3.1.5).

Electrophoretic separations were performed at 28 kV for 10 min. The temperature was maintained at 22°C.

UV detection was performed in all cases at 200, 240, 280, and 330 nm, although diode-array detection was used over the range of 190–600 nm to achieve spectral data. Peak identification was done by comparing both migration time and spectral data obtained from olive oil samples and standards (commercial standards or isolated compounds by HPLC), with spiked methanol-water extracts of olive oil with HPLC-collected compounds and commercially available standards at several concentra-

tion levels. We have also used the information previously reported [24, 25, 27, 31].

## 3 Results and discussion

### 3.1 Optimization of separation conditions

The optimization of CZE involves identification of the separation conditions under which the analysis can be performed in the shortest time at sufficient resolution. Typical parameters to be optimized involve voltage, effective length of capillary, capillary temperature, time and type of sample injection, and composition of running buffer. The last parameter involves conductivity/ionic strength and pH or any other parameter used to affect and thus distinguish effective mobilities of the compounds under study.

### 3.1.1 Selection of capillary length and diameter

Separation complexity normally dictates capillary length and efficiency, while resolution and detection limit dictate capillary diameter. The electropherograms obtained using two different sizes of internal diameter of the capillary are shown in Fig. 1. The main advantage resulting from increasing the capillary id is the enhancement in detection sensitivity due to the increased pathlength. However, a decrease in the surface-to-volume ratio accompanies an increase in diameter and this may lead to less efficient dissipation of Joule heat.

In the figure, the same injection time and pressure were used for both capillary diameters, it means that different volumes of samples were injected (corresponding to about 10 nL injected (1.1% of the capillary) in the case of 50 µm id and approximately 50 nL (2.5% of the capillary) in the case of 75 µm id). When both injection volumes were comparable, the relative resolution improved in the electropherograms obtained with the broader capillary, but considering the analysis time the narrower one was better.

We have used 40 cm (effective length) $\times$ 50 µm id in the rest of experimental work to get good efficiency, high resolution, and acceptable UV detection limits.

### 3.1.2 Selection of the capillary temperature

The temperature of the analysis may sometimes be important in CE, as fluctuations in the temperature may affect the viscosity of the running buffer, leading to higher analyte electrophoretic mobilities and shorter analysis time. The temperature changes can also affect the pH of the buffer. The lower the temperature used the more effective the heat dissipation, and therefore the wider the workable region before Joule heating becomes a problem. In this case, we obtained the best results working at 22°C.
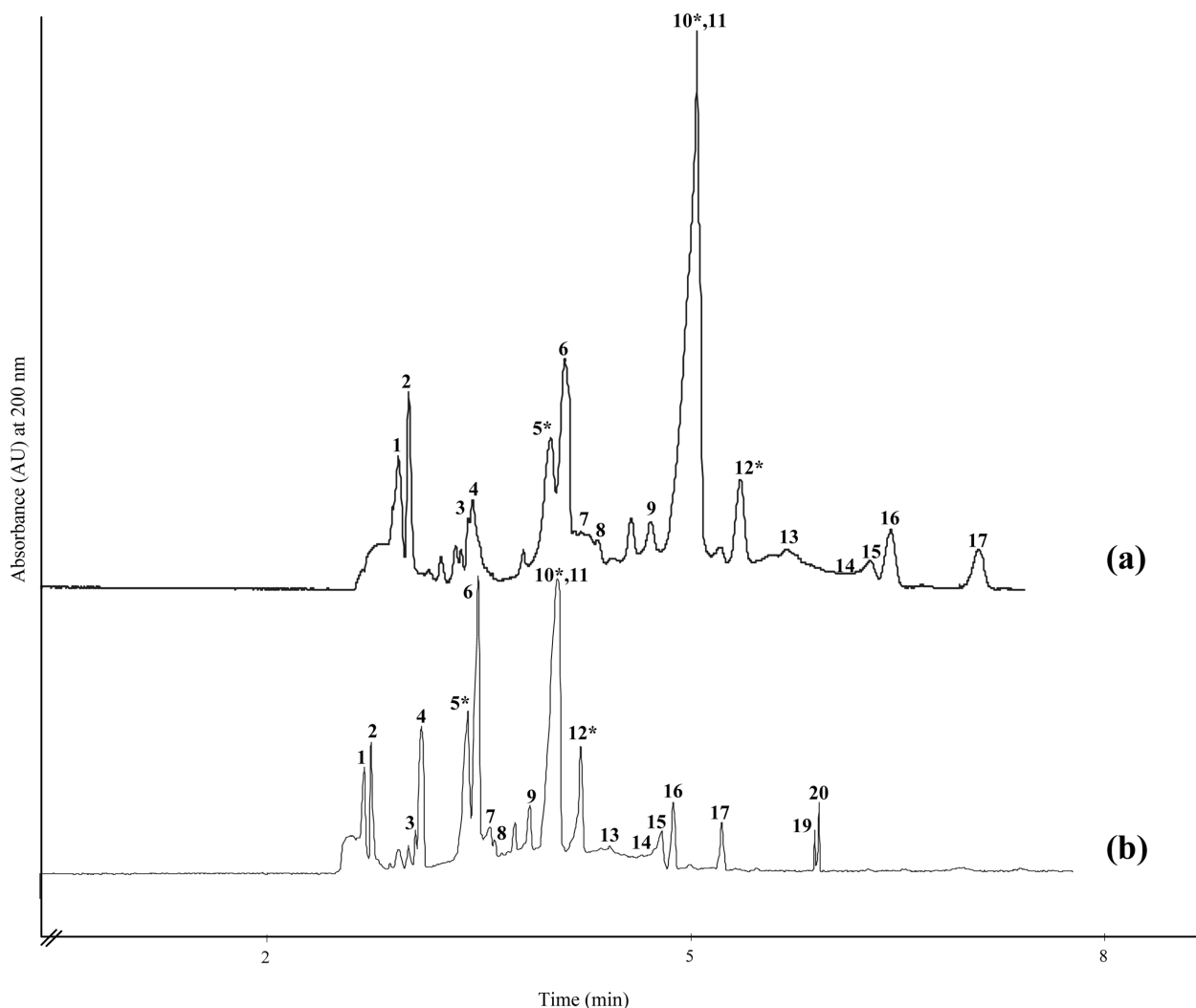
**Figure 1.** Capillary zone electropherograms obtained using (a) id of the capillary of 75 μm and (b) id of 50 μm for an extract of Hojiblanca extra-virgin olive oil. Other separation conditions: capillary, 47 cm (40 cm of effective length); applied voltage, 28 kV; applied temperature, 22°C; buffer, 45 mM sodium tetraborate (pH 9.30); HD injection, 0.5 psi for 8 s. Detection was performed at 200 nm. Peak identification numbers: **1**, Lig Agl (a); **2**, TY; **3**, Pin; **4**, Ac Pin; **5**, Ol Agl (a) + DOA (a); **6**, DOA (b); **7**, Lig Agl (b); **8**, Ol Agl (b); **9**, EA (a); **10**, Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b, c); **11**, HYTY; **12**, DOA (d) + EA (d); **13**, EA (e); **14**, *trans*-cinnamic acid; **15**, 4-hydroxyphenylacetic acid; **16**, sinapinic acid; **17**, gentisic acid; **18**, *o*-coumaric acid; **19**, luteolin; and **20**, apigenin. * Peaks overlapped.

### 3.1.3 Optimization of pH and concentration of the running buffer

The composition of the running buffer should be the first experimental parameter considered when optimizing separation conditions, since the other parameters depend strongly on this.

It has previously been reported that sodium tetraborate is the best buffer to carry out the separation of the phenolic compounds extracted from virgin olive oil [24–28, 31]. The effect of pH was tested by adjusting the buffer (sodium tetraborate) pH between 8.5 and 10.5 by adding the necessary amount of 1.0 M HCl or 1.0 M NaOH. A more detailed study between pH 9.0 and 10.0 was carried out (in steps of 0.1) and the optimum value was 9.3.

The buffer concentration was investigated in a range between 10 and 100 mM (in steps of 5). We found that 45 mM of sodium tetraborate buffer at pH 9.3 represented the best compromise for the resolution of the compounds studied and a satisfactory analysis time, as the increase of the ionic strength of the separation buffer increases the thickness of the ionic double layer, and is the effect of decreasing the EOF, hence, increasing the analysis time. In this case, buffer concentrations higher than 45 mM of sodium tetraborate did not increase the resolution.
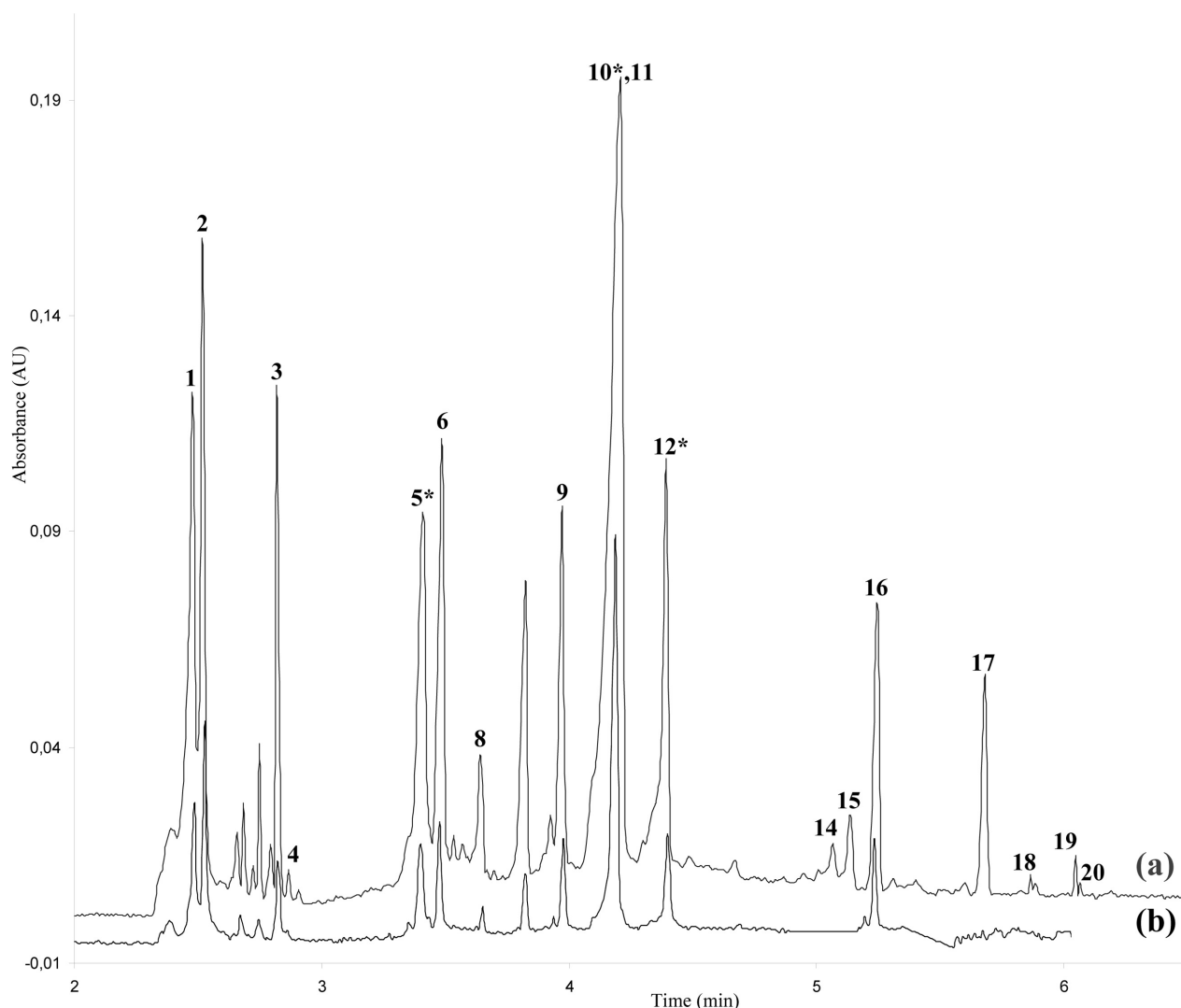
**Figure 2.** Electrophoretic profiles of an extract of Picual extra-virgin olive oil obtained using (a) HD (b) and EK injection. (a) HD injection, 0.5 psi for 4 s. (b) EK injection, −5 kV for 15 s. For separation conditions and peak identification numbers see Fig. 1. Inner diameter: 50 μm. Detection was performed at 200 nm.

### 3.1.4 Optimization of separation voltage

It is advisable to produce an Ohm's Law plot (voltage *versus* current) with the desired buffer from 0 to 30 kV in steps of 2.5 kV. For a given buffer and capillary, the Ohm's Law plot indicates the voltage that will give the fastest separation with optimum efficiency and resolution. Separation efficiency, as measured by the number of theoretical plates of an analyte peak, is proportional to the voltage applied, so the analyst should use the highest voltage possible within the relatively linear range of the plot.

After carrying out the optimization of this parameter, the voltage used to obtain the shorter analysis time and maintain a good resolution was 28 kV.

### 3.1.5 Selection of type of injection

We have used two type of injections: hydrodynamic (HD) injection and EK injection. For the HD injection, we used injections from 2 to 12 s, choosing 8 s as optimum injection time to detect several compounds that are scarcely present in extra-virgin olive oil and to maintain a good resolution. For the EK injections, the polarity was inverted because of the negative charge of the compounds under study in the buffer solution and we used from −5 to −15 kV during 5–30 s. To compare the two types of injection the samples were dissolved in buffer solution (because in methanol/water, 50:50 v/v, these phenolic compounds do not have negative charge).

In CE, EK injection is a highly controversial sampling technique. It is a simple mode of sample introduction

which is suitable for online preconcentration of the analytes, but its precision and accuracy are more strongly affected by experimental conditions compared to HD injection. We have observed that the repeatability of the measurements was better using HD injection; and reproducibility over time was also better for HD, this is because the change of the condition of the capillary affects the EK injection much more readily.

To show the differences between the two types of injection, Fig. 2 is added.

Finally, we decided to work with HD injection (8 s) for all the reasons previously mentioned and because, in general, it is more appropriate for routine quantitative analysis [53].

## 3.2 Identification of phenolic compounds in the polar extract of extra-virgin olive oil

### 3.2.1 Use of commercially available standards to carry out the identification

Several of the peaks present in the electrophoretic profile of Picual extra-virgin olive oil could be identified with the use of commercially available standards. Peak identification was done by comparing both migration time and spectral data obtained from olive oil samples and standards, and with spiked methanol/water extracts of olive oil with commercially available standards at several concentration levels.

In this way we were able to identify the following compounds: TY, HYTY, *trans*-cinnamic acid, 4-hydroxyphenylacetic acid, sinapinic acid, gentisic acid, *o*-coumaric acid, luteolin, apigenin, vanillic acid, 4-hydroxybenzoic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, gallic acid, and protocatechuic acid (see Fig. 3).

TY and HYTY were identified for the first time in the phenolic fraction extracted from an extra-virgin olive oil by Bendini *et al.* [24]; then, in several papers, it is possible to see these compounds identified in the profiles of extracts of virgin olive oils [25, 26, 31]. Furthermore, several families of phenolic acids have been determined by Carrasco-Pancorbo *et al.* [27, 54] and Buiarelli *et al.* [28]. However, it is important to point out that this method offers the possibility to identify, for the first time using CE, both phenylethylalcohols and phenolic acids in the same run in a very short time with a satisfactory resolution.

This method provides another important advantage, since it permits the identification of flavonoids.

Specific chromatographic methods for flavonoid analysis have demonstrated the presence of some of these substances in olive leaves, fruits, and oils. Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. These compounds are mentioned in the literature, but they have only been identi-

fied and quantified in olive oil by HPLC [49–52]. To our knowledge, this is the first time that the flavonoids of virgin olive oil have been determined by CE.

In the UV absorption data for methanol solutions of these compounds, we found that the maxima for luteolin were 200 (approximately), 253, 275, and 347; and for apigenin were 200 (approximately), 268, 300, and 337 nm.

In the electropherograms obtained at 340 nm, it is possible to see that at this wavelength we can only detect the flavonoids (this fact will be commented in the Section 3.2.3).

### 3.2.2 Use of HPLC-isolated compounds to carry out the identification. Identification of secoiridoids: isomeric forms

As we commented before, after the isolation of several phenolic compounds by HPLC, their analysis was done with an analytical column to check the purity of the isolated compounds and confirm their identity, using two different gradients. The detection in HPLC was carried out using UV (240, 280, 340 nm) and MS (APCI and ESI). We proved that the isolated phenolic compounds produced only one peak and gave clean and not noisy MS spectra (data not shown).

After the optimization of the electrophoretic method, we also analyzed the HPLC-isolated compounds with this CZE method. The results for four of the compounds obtained by HPLC at two wavelengths (200 and 240 nm) are shown in Fig. 4. In our opinion, it is not necessary to show the electropherograms for the other two isolated compounds (lignans: Pin and Ac Pin), because they produced only one peak in CZE and their identification was carried out in a previous paper [31]. That means that this section is only about secoiridoids.

Several previous studies [19, 55, 56] support the conclusion that intact secoiridoids, such as oleuropein glucoside and ligstroside glucoside, are almost completely absent in olive oil because of their high solubility in water. They undergo enzymatic hydrolysis during olive storage and olive oil extraction, producing first Ol Agl and Lig Agl upon removal of the attached glucose moiety and then a number of secoiridoid derivatives upon further molecular transformations *via* ring opening and rearranged reclosure [57]. This enzymatic hydrolysis explains the presence of so many isomeric or very related forms in this family of compounds. Other recent studies have described a little more in depth the compounds of this family of secoiridoids [22, 58, 59] using HPLC-NMR, HPLC-MS, GC-MS, or even with a CE-MS method [60]. According to De la Torre-Carbot *et al.* [19] nine basic models to describe and classify the structure of ligstroside and Ol Agls are found in the bibliography; each model shares the same EA derivative ring structure.
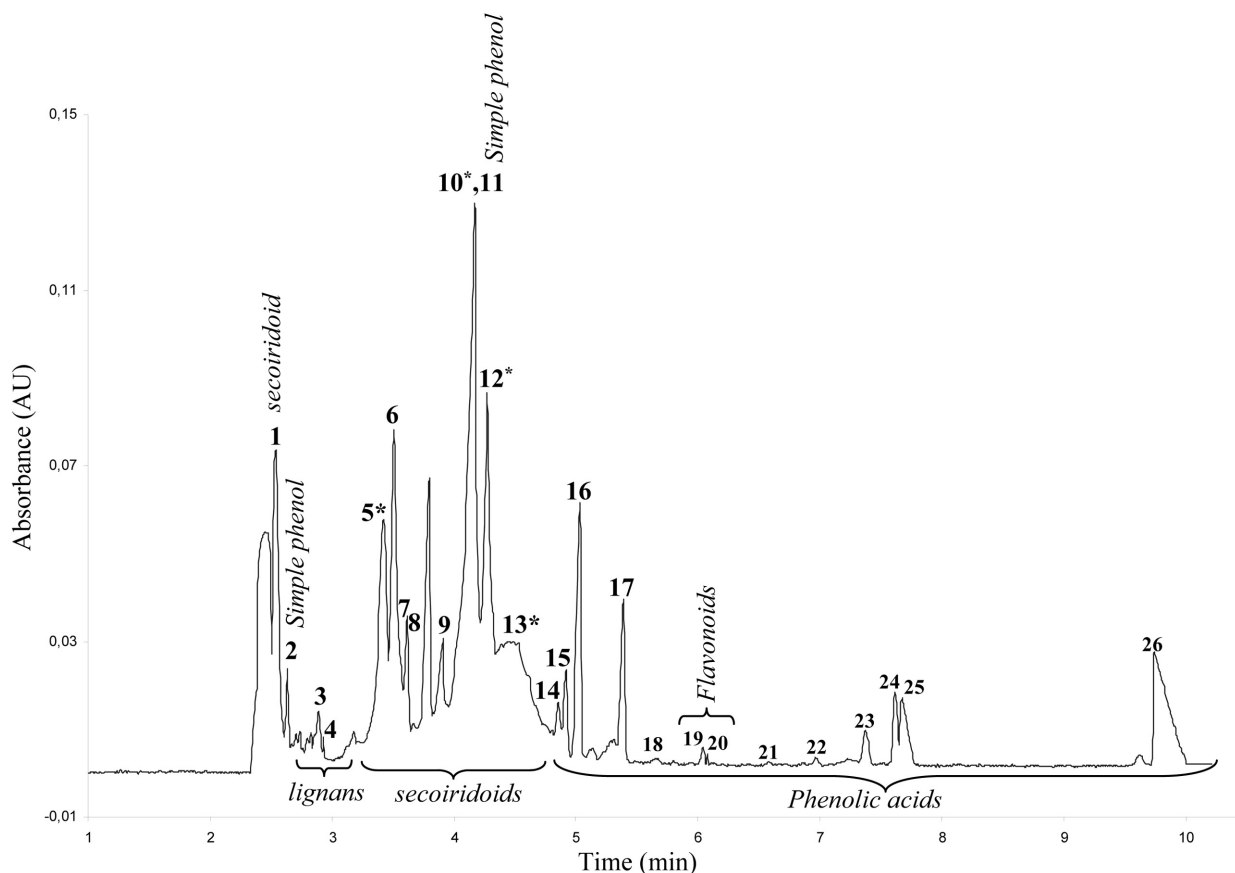
**Figure 3.** CZE of extra-virgin olive oil (Picual variety) sample under optimized conditions. Separation conditions and peak identification numbers as in Fig. 1. Detection was performed at 240 nm. Other peak identification numbers: **21**, vanillic acid; **22**, 4-hydroxybenzoic acid; **23**, caffeic acid; **24**, 3,4-dihydroxyphenylacetic acid; **25**, gallic acid; and **26**, protocatechuic acid.

The identification of the secoiridoids was carried out using the HPLC-isolated standards and we could observe the potential resolution of CE with respect to HPLC, as CE was able to separate compounds which produce only one peak using HPLC and having the same molecular mass, *i.e.*, they are isomers (or at least very related compounds, since they have the same migration time in HPLC analyses and the MS detector was not able to distinguish different mass spectra).

As we can see in Fig. 4, the electrophoretic analysis of the HPLC-standard corresponding to Lig Agl produces four peaks at 240 nm and three at 200 nm. At 240 nm, the peaks a, c, and d have never been detected before by using CE. The latter two are overlapped with forms of Ol Agl (c and d forms) and EA (peaks (b+c) and e). At 200 nm, only three peaks can be detected, as the last peak detected at 240 nm does not absorb the UV radiation at this wavelength. This fact is logical because this compound seems to be very related with one of the peaks of EA (peak e, which has only a maximum of absorbance at 240 nm). In one of the papers previously mentioned [59],

the authors described that the compound with *m/z* 361 (Lig Agl) gave five peaks in the trace chromatogram in full-scan and in ion-scan mode of *m/z* 361 (in negative mode); it means that this compound may have five isomeric forms.

In the case of DOA, it is possible to observe two peaks at 240 nm and four peaks at 200 nm. The first at 240 nm (peak a) has the same migration time of peak a of Ol Agl, and the second was previously identified by our research group [31].

At 200 nm, the same can be observed for the two peaks with shorter migration time; however, peak c is overlapped with the highest peak of the Ol Agl (peak c) at this wavelength, and the last (peak d) is overlapped with one of the peaks of the EA (peak d).

This compound is an oleuropein derivative in decarboxylated form with *m/z* 319 (in negative polarity in an MS detector) and the bibliography gives three different models of oleuropein derivatives with this mass molecule. In

● *Peaks corresponding to secoiridoids derivatives or related compounds*

⟶ *Peaks overlapped*
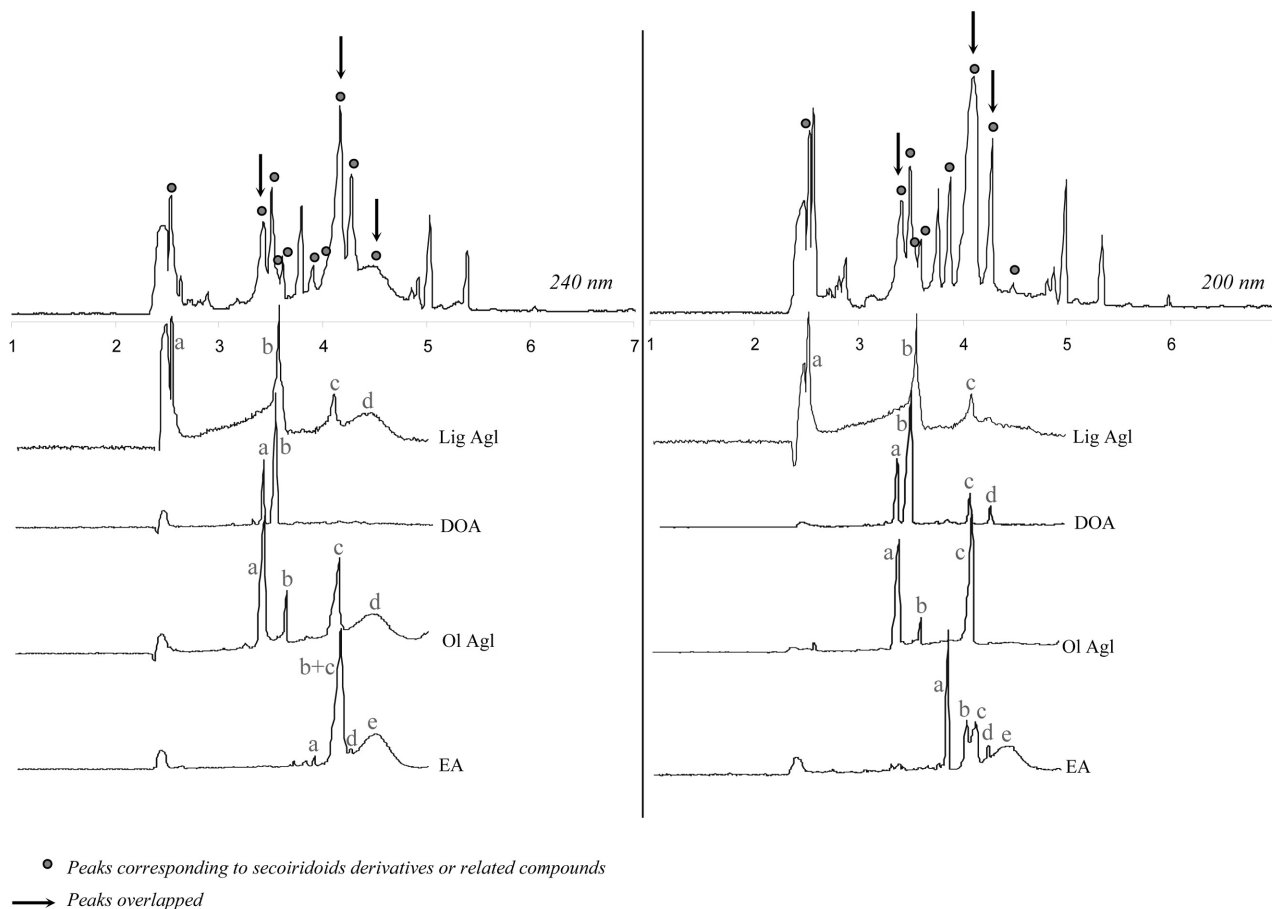
**Figure 4.** Electropherograms of an extract of Picual extra-virgin olive oil at two wavelenghts (240 and 200 nm) and the isolated standards of the secoiridoids obtained by HPLC. For separation conditions see Fig. 1.

our experience, it is possible to detect until four compounds related with DOA.

Ol Agl gives four peaks at 240 nm and three at 200 nm. De la Torre-Carbot *et al.* [59] observed that at *m/z* 377 (in the negative polarity of the mass spectrometer) four peaks could be detected, a fact that agrees with the result expected from the described keto-enolic isomerization of the secoiridoid moiety of oleuropein[20, 56, 61]. At 240 nm, peak a of Ol Agl is related with peak a of DOA (they have the same migration time), the second (peak b) is a compound which has never been detected before by CE, peak c was determined by our research group [31] and is overlapped with forms of the EA (peak (b + c)) and one of the Lig Agl (peak c), and the fourth is also related with the EA and Lig Agl (peaks e and d, respectively). The information obtained at 200 nm is similar, however in this case, peak d does not appear.

At 240 nm, in the electropherogram of EA, we can see four peaks. At 200 nm, the same compound generates five peaks, as peak b at 240 nm appears now as two peaks.

In general, the presence or absence of aldehyde, carboxyl, and/or methyl groups and the open or closed form of the EA ring structure indicate the differences between aglycons.

Figure 5 shows the CZE-UV spectra for the secoiridoids determined in this study.

### 3.2.3 Using four wavelengths to facilitate and confirm the identification of phenolic compounds

Detecting these phenolic compounds from virgin olive oil at four wavelengths, it was very useful to confirm and facilitate the identification of almost all the compounds present in this polar fraction, as well as to check which wavelength would be better to identify or even quantify each family of analytes. In Fig. 6, it is possible to observe how the electrophoretic profile changes as a function of the wavelength. The absorbance scales are different in each case, because if the electropherograms obtained at 280 and 340 nm are represented in the same scale as that
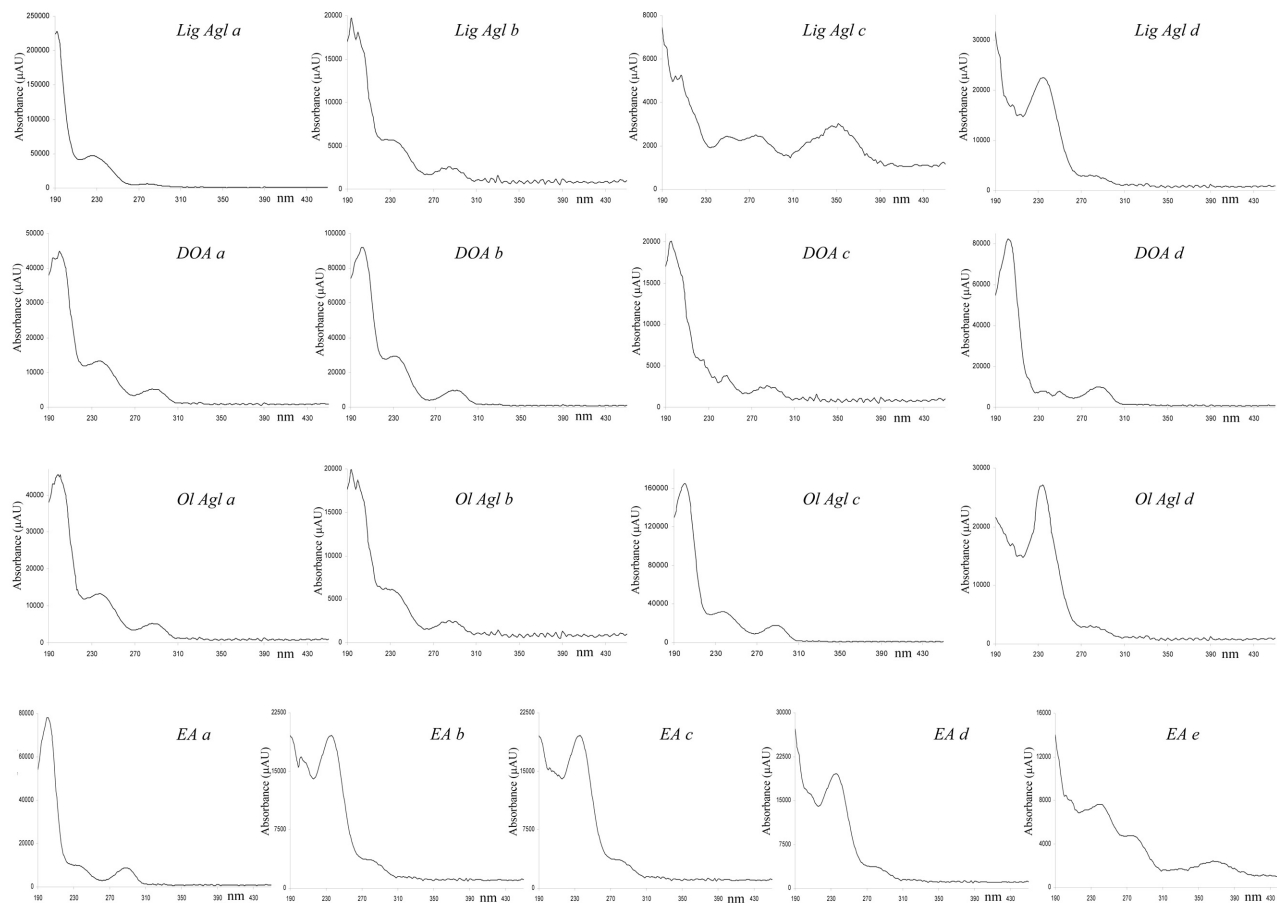
**Figure 5.** CZE online spectra of several isomeric forms of secoiridoid compounds.

obtained at 200 nm the peaks could be difficult to observe.

At 200 nm, it is possible to study simple phenols, lignans, secoiridoids, several phenolic acids, and flavonoids obtaining bigger absorbance signals than by using the other wavelengths. It is interesting to note that 240 nm would be the optimum value of the wavelength to determine the EA derivatives and phenolic acids, and to quantify one of the peaks of the secoiridoid derivatives (which is overlapped with HYTY having a minimum of absorbance at this wavelength; $t_{\mathrm{mig}}$ = 4.07 min). For some of these compounds, 280 nm is the most specific value of wavelength but the absorbance signals are smaller. In the same figure 340 nm is shown as well because it was really useful for carrying out the identification of the flavonoids. In the electropherogram shown in Fig. 6d, it is possible to verify that at this wavelength we can only detect the flavonoids and see a very small signal produced by the other phenolic compounds present in the methanol/water extract from a virgin olive oil. Figure 6d also shows the CZE-UV spectra for the two flavonoids determined in this study. The Diol-SPE protocol, in this

case, was done using the same quantity of olive oil (60 g) and conditions as in the other cases [31], but the residue after the rotary evaporator was dissolved with 0.5 mL of methanol/water (50:50 v/v) to obtain more concentrated extract in terms of phenolic compounds.

## 4 Concluding remarks

This paper reports a complete and rapid CZE method for the qualitative determination of extra-virgin olive oil phenolic compounds. The Diol-SPE extracts from extra-virgin olive oil were analyzed in less than 10 min and we were able to study several families of phenolic compounds present in this foodstuff, such as simple phenols, lignans, complex phenols, phenolic acids, and flavonoids.

The described method is not completely original regarding the electrophoretic conditions, but its novelty is clear in terms of identification of phenolic compounds in virgin olive oil, since, to our knowledge, this is the first paper to show the determination of flavonoids and several isomeric forms of secoiridoid compounds in olive oil

**Figure 6.** Electropherograms obtained for a methanol/water extract of a Picual extra-virgin olive oil using the DAD detection at four wavelenghts (200, 240, 280, and 340 nm). The Diol-SPE protocol, in (d) (340 nm), was done using the same quantity of olive oil (60 g) and conditions as in the other cases, but the residue after the rotary evaporator was dissolved with 0.5 mL of methanol/water (50:50 v/v) to obtain more concentrated extract in terms of phenolic compounds.

samples using a CE method. Furthermore, as the extraction protocol uses a considerable quantity of oil (60 g), it is possible to detect in the same run 26 compounds belonging to both majority and minority families which, to date, have not been detected simultaneously in any CE method.

In this study, we have demonstrated the qualitative potential of the described method; its usefulness to carry out quantitative determinations will be considered as future direction.

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Si consideramos la figura que se adjuntó en el anexo del capítulo 2, recordaremos que en ese entonces sólo pudimos estudiar la zona que ahora señalamos en rojo. Sin embargo, nos vimos obligados a decir, que la primera zona, de compuestos polihidroxilados (ahora resaltada con una línea negra discontinua) era una zona "incógnita".
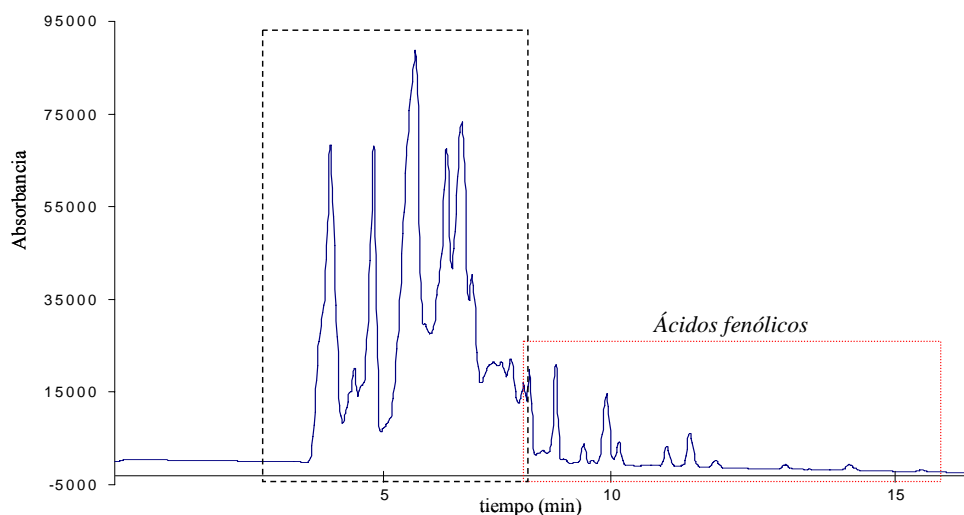


*Figura*. Electroferograma de un extracto de una muestra de aceite de oliva virgen extra de la variedad Arbequina.

------- Zona de los compuestos polihidroxilados (compuestos mayoritarios del aceite de oliva)
·············· Zona de los ácidos fenólicos

Con el desarrollo del método aquí descrito, pudimos estudiar la mayoría de los compuestos presentes en ambas zonas con un excelente tiempo de análisis.

Después de haber puesto a punto este método y para proseguir nuestra investigación, pensamos que sería muy útil utilizar esta herramienta para hacer un trabajo aplicado consistente en estudiar aceites con diferentes orígenes, para comprobar si la información obtenida con esta metodología electroforética permitiría discriminar entre unos aceites y otros. Los resultados obtenidos en estos experimentos se reúnen en el siguiente capítulo.

# Capítulo 7

## Cuantificación de la fracción fenólica de aceites de oliva vírgenes españoles mediante CE con detección UV

Una vez puesto a punto el método descrito en el capítulo 6, que permitía de forma sencilla y rápida caracterizar fenoles en la fracción más polar del aceite, quisimos demostrar su utilidad comparando varias muestras de aceites de oliva virgen-extra españoles llevando a cabo la cuantificación (tanto individual como en grupos teniendo en cuenta las distintas categorías de compuestos fenólicos presentes en el perfil electroforético) de catorce compuestos que pertenecían a las familias de fenoles simples, lignanos, fenoles complejos y flavonoides. Decidimos considerar sólo los seis primeros minutos de cada análisis, puesto que a tiempos más largos aparecían los ácidos fenólicos y estos ya habían sido estudiados en varios de los capítulos anteriores. Además, creímos que estos seis minutos eran suficientes porque todos los compuestos mayoritarios de esta fracción atravesaban la ventana del detector antes de ese intervalo de tiempo.

Además, usando estadística multivariante, intentamos también lograr distinguir con claridad los aceites de oliva estudiados considerando su contenido fenólico. El número de muestras no fue excesivo (8 aceites x 7 repeticiones de cada muestra), ya que nuestro propósito no era distinguir aceites de oliva monovarietales obtenidos de diferentes variedades de aceituna, sino más bien comprobar la capacidad discriminatoria que podrían ofrecen las variables electroforéticas determinadas en este estudio; es decir, queríamos demostrar que podíamos diferenciar unas muestras de otras considerando la fracción fenólica cuantificada mediante CE.

# Rapid Quantification of the Phenolic Fraction of Spanish Virgin Olive Oils by Capillary Electrophoresis with UV Detection

ALEGRIA CARRASCO-PANCORBO,[†] ANA MARIA GÓMEZ-CARAVACA,[†]
LORENZO CERRETANI,[‡] ALESSANDRA BENDINI,[‡]
ANTONIO SEGURA-CARRETERO,*[,†] AND ALBERTO FERNÁNDEZ-GUTIÉRREZ*[,†]

Department of Analytical Chemistry, Faculty of Sciences, University of Granada,
C/Fuentenueva s/n, E-18071 Granada, Spain, and Department of Food Science,
University of Bologna, P.zza Goidanich 60, 47023 Cesena (FC), Italy

A rapid and reliable capillary zone electrophoresis method was used as a tool to obtain both qualitative and quantitative information about simple phenols, lignans, complex phenols (isomeric forms of secoiridoids), phenolic acids, and flavonoids in the solid-phase separation extracts from different Spanish extra-virgin olive oil in a short time (less than 6 min). Peak identification was done by using commercial and HPLC-isolated standards, studying the information of the electropherograms obtained at several wavelengths and also using the information previously reported. For the quantification of lignans and complex phenols (secoiridoid derivatives), we used a reference compound (oleuropein glucoside) at two different wavelengths (200 and 240 nm) and for the quantification of tyrosol and flavonoids, we used their commercially available standards.

## INTRODUCTION

The traditional Mediterranean diet is characterized by an abundance of plant foods (fruits, vegetables, cereals, legumes) in which olive oil is the principal source of fat.

Olives (*Olea europaea* L.) and virgin olive oil provide a rich source of natural antioxidants. These include carotenoids, tocopherols, and phenolic compounds, which may act, by different mechanisms, as an effective defense against reactive species (*1*).

The content of phenolic compounds is an important factor to be considered when evaluating the quality of virgin olive oil (*2*), since members of this family of compounds have potent antioxidant activity and contribute significantly to the extraordinary stability of olive oil against oxidation (*3*). Several epidemiological studies have shown that phenolic compounds afford considerable protection against cancer (skin, breast, and colon), coronary heart disease, and aging by inhibiting oxidative stress (*4*−*6*). Also, phenolic compounds are related to the sensory and nutritional qualities of virgin olive oil (*7*−*9*).

Therefore, it would be very interesting to have a simple, rapid, and reliable method in order to quantify these compounds. Traditionally, separation and determination of individual phenolic compounds in the extracts obtained from olive oil have been carried out by high-performance liquid chromatography (HPLC) analysis coupled to different detection systems (*10*, *11*) and gas chromatography (*10*, *11*). Both of them have obtained reliable results, but they show some limitations:  the long time necessary for the analysis and the partial separation in cases where components have a complex structure (such as secoiridoid compounds) for HPLC and the problem dealing with the sample derivatization for GC.

On the other hand, capillary electrophoresis (CE) has proven to be a fast, valid, and reliable tool for food analysis (*12*, *13*). It is a powerful technique that affords rapid and high-resolution separations ($10^4$−$10^6$ theoretical plates) while requiring only few microliters of sample. Futhermore, a wide range of compounds are amenable to separations by CE.

In particular, this technique has recently been used for the analysis of phenolic compounds of extra-virgin olive oil (*14*−*21*), olive mill wastewater (*22*), and alperujo (*23*).

In almost all the mentioned papers that study the phenolic compounds present in extracts of extra-virgin olive oil the detection system was UV (*14*−*17*, *19*, *21*), only one of them used MS as detection system (*20*). The electrophoretic conditions for the methods which use UV detection are not drastically different regarding the experimental and instrumental variables. However, the differences can be found when the extraction system used and the families under study are observed.

Phenolic acids have been the family of interest in three of these research works (*17*−*19*); in the others (*14*−*16*, *20*, *21*), the aim of the authors was to study the complete polyphenolic fraction of the olive oil, attempting to determine several families of phenols simultaneously.

* Authors to whom correspondence should be addressed: Fax: +34 958249510. E-mail: ansegura@ugr.es (A.S.-C.) or albertof@ugr.es (A.F.-G.).
† University of Granada.
‡ University of Bologna.

Bendini et al. (*14−16*) identified several important compounds of the polyphenolic fraction in virgin olive oil samples (tyrosol, hyodroxytyrosol, lignans, and decarboxylated oleuropein zone) by using a fast electrophoretic method. Then, in a published paper by our group (*21*), the number of identified compounds in an electrophoretic profile of an extract of virgin olive oil increased using standards obtained by semipreparative HPLC, although the analysis time in this case was long.

Recently, the characterization of the polar extracts of olive oil has been improved a bit more by using a recent method that is able to determine 20 compounds belonging to simple phenols, lignans, complex phenols (isomeric forms of secoiridoids), phenolic acids, and flavonoids in the solid-phase separation extracts from extra-virgin olive oil in a short time (about 6 min) (*24*).

So, the aim of this work was to demonstrate the usefulness of the latter method (*24*) to compare several Spanish extra-virgin olive oil samples by carrying out the quantitation (individually and in groups taking into account the different categories of phenolic compounds) of 14 phenolic compounds belonging to simple and complex phenols, lignans, and flavonoids. Using multivariate statistics, we also tried to distinguish the oils under study taking into account their phenolic content.

## MATERIALS AND METHODS

**Apparatus.** The CE instrument used was a Beckman 5500 capillary electrophoresis connected to a diode-array detector (belonging to the University of Granada). The system comprises a 0−30 kV high-voltage built in power supply.

All capillaries (fused silica) used were obtained from Beckman instrument, Inc. (Fullerton, CA) and had an inner diameter (i.d.) of 50 $\mu$m, a total length of 47 cm, and an effective separation length of 40 cm. The temperature was controlled using a fluorocarbon-based cooling fluid. Data acquisition and processing were carried out with GOLD software (Beckman instrument Inc.) installed on a personal computer.

To carry out the reproducibility studies, the CE instrument used was a Beckman 5500 capillary electrophoresis connected to a diode-array detector (belonging to the University of Bologna) and the capillaries (fused silica) used were obtained from Composite Metal Services (Worcester, England) and had the same inner diameter and length as those described above.

**Reagents, Stock Solutions, and Reference Compounds.** 2-(4-Hydroxyphenyl)ethanol (tyrosol (TY)) was acquired from Fluka (Buchs, Swiss) and oleuropein (oleuropein glucoside) was obtained from Extrasynthèse (Genay, France). The stock solution containing these two analytes was prepared in methanol/water (50/50, v/v) at a concentration of 500 $\mu$g/mL in the case of tyrosol and 8000 $\mu$g/mL for oleuropein glucoside. Tyrosol was used for the quantification of this compound present in the extracts of olive oil, and oleuropein glucoside was used to make the calibration curves for the quantification of lignans and complex phenols.

The flavonoids luteolin and apigenin were obtained from Extrasynthèse and all the analytes were used as received. The standards of luteolin and apigenin were used for the quantification of these two compounds in olive oil; the stock solution containing them was prepared in methanol/water (50:50, v/v) at a concentration of 250 $\mu$g/mL in the case of luteolin and 100 $\mu$g/mL for apigenin.

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma-Aldrich (St. Louis, MO) and was used as running buffer at different concentrations and pHs.

Methanol, acetonitrile, and *n*-hexane were acquired from Panreac (Montcada I Reixac, Barcelona, Spain) and were HPLC grade.

Doubly deionized water with a conductivity of 18.2 M$\Omega$ was obtained by using a Milli-Q system (Millipore, Bedford, MA).

DSC-Diol solid-phase separation cartridges were obtained from Supelco (Bellefonte, PA).

**Samples.** Spanish extra-virgin olive-oil samples were obtained from a unique variety of olive fruit called picual, hojiblanca, lechín de Sevilla, arbequina, and cornicabra (January 2005). An organic olive oil was obtained in the supermarket, where we also acquired two types of picual olive oil ["suave" (mild) and "intenso" (strong flavor)].

**Solid-Phase Extraction (SPE) Procedure and Preparation of Oil Spiked with Phenolic Extract.** To isolate the phenolic fraction, we used SPE with DSC-Diol cartridges; the SPE protocol was carried out with the extraction conditions and amounts of oil that are decribed in a recent paper of our research group (*21*).

**Electrophoretic Procedure.** CE separation was carried out on a fused silica capillary (50 $\mu$m i.d., 375 $\mu$m o.d., total length 47 cm; a detection window was created at 40 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of 100 $\times$ 200 $\mu$m). Every time a new capillary was used it was preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5 min rinse with Milli-Q water and 15 min with buffer. For the following analyses the capillary was rinsed with 0.1 M NaOH for 2 min, followed by a 2-min flush with Milli-Q water to ensure good repeatability. The capillary was equilibrated with the running buffer (45 mM sodium tetraborate adjusted to pH 9.3) for 3 min before each sample injection. After each analysis, the capillary tubing was rinsed for 2 min with Milli-Q water. All solutions and samples were filtered through a 0.25-$\mu$m syringe filter. The running buffer was changed after four runs.

Samples were injected hydrodynamically in the anodic end in low-pressure mode (0.5 psi) for 8 s (1 psi = 6895 Pa). Electrophoretic separations were performed at 28 kV for 10 min, and the temperature was maintained at 22 °C.

UV detection was performed in all cases at 200, 240, 280, and 340 nm, although diode-array detection was used over the range of 190−600 nm to collect spectral data. The quantification of the phenolic compounds determined in this study was carried out at 200 and 240 nm, but the detection at four wavelengths and the complete spectral information were used to facilitate the identification of all the analytes (*24*).

Peak areas and migration velocity (cm/min) were used for the quantification of the analytes versus oleuropein glucoside in some cases (complex phenols and lignans), and peak areas were used for the quantification of tyrosol, luteolin, and apigenin versus the corresponding commercial standards.

The electropherogram obtained for an extract of extra-virgin olive oil of picual variety at optimized conditions is presented in **Figure 1**.

**Statistical Analysis.** The results reported in this study are the averages of at least three repetitions ($n = 3$), unless otherwise stated. Tukey's honest significant difference (HSD) 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 analyses 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 AND DISCUSSION

**Analytical Parameters of the Method Proposed.** We carried out a study to check the repeatability and reproducibility of the proposed method, as well as to establish the calibration curves to quantify the compounds under study and calculate the detection, quantification limit, and precision [as relative standard deviation (RSD) of the intermediate concentration value of linear range] of the method.

**Repeatability and Reproducibility Study.** For carrying out this study extracts from the same extra-virgin olive oil (picual variety) were prepared.

Repeatability was studied by performing a series of separations using the optimized method on one of the samples on the same day (intraday precision, $n = 12$) and on three consecutive days (interday precision, $n = 36$). The relative standard deviations (RSDs) of peak areas/migration time and migration times were determined considering five of the compounds present in
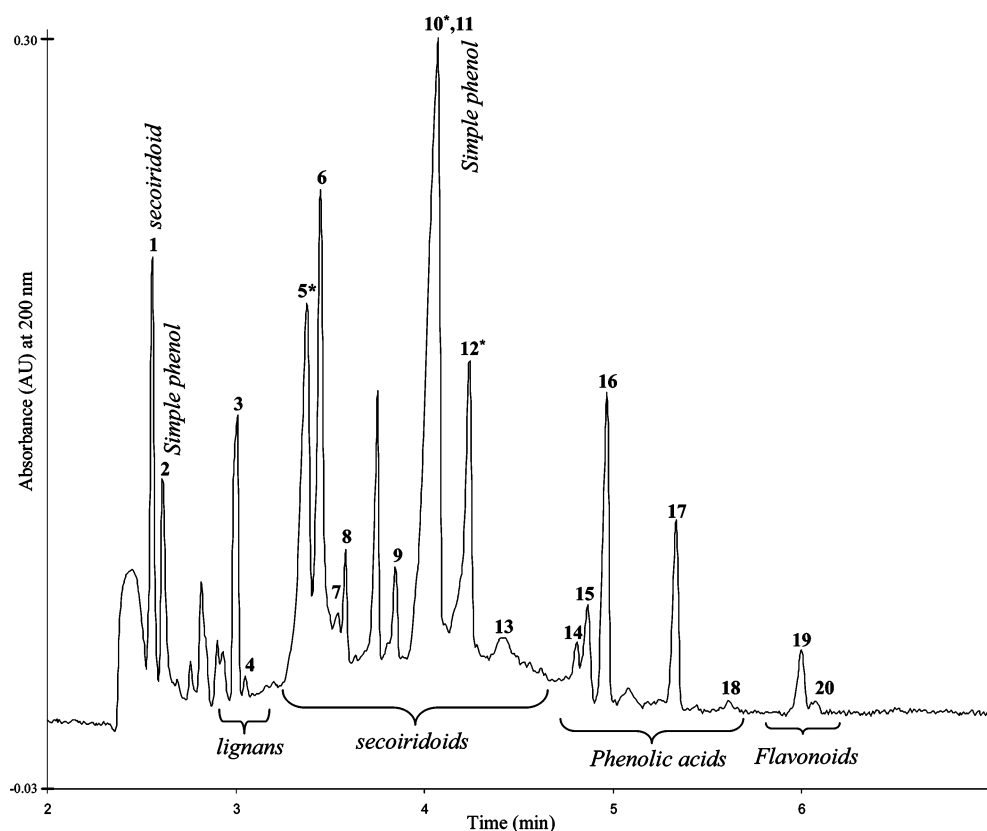
**Figure 1.** CZE of extra-virgin olive oil (picual variety) sample under optimized conditions. Detection was performed at 200 nm. Peak identification numbers: 1, Lig Agl (a); 2, TY; 3, Pin; 4, Ac Pin; 5, Ol Agl (a) + DOA (a); 6, DOA (b); 7, Lig Agl (b); 8, Ol Agl (b); 9, EA (a); 10, Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b,c); 11, HYTY; 12, DOA (d) + EA (d); 13, EA (e); 14, *trans*-cinnamic acid; 15, 4-hydroxyphenylacetic acid; 16, sinapinic acid; 17, gentisic acid; 18, *o*-coumaric acid; 19, luteolin; and 20, apigenin. *Peaks overlapped. The letters (a), (b), (c), (d), and (e) are used to distinguish the different isomeric forms of secoiridoids.

**Table 1.** RSD Values of the Peak Area and Migration Time Obtained in the Study of Repeatability and Reproducibility

| | repeatability | | | | reproducibility ($n = 20$) | |
| | intraday ($n = 12$) | | interday ($n = 36$) | | | |
| compound | peak area/migration time | migration time | peak area/migration time | migration time | peak area/migration time | migration time |
|---|---|---|---|---|---|---|
| tyrosol | 2.32 | 0.48 | 4.99 | 0.89 | 6.29 | 0.91 |
| (+)-pinoresinol | 1.26 | 0.49 | 2.87 | 0.82 | 3.03 | 0.95 |
| DOA | 2.03 | 0.62 | 3.25 | 0.91 | 3.35 | 1.25 |
| 4-HFA | 1.32 | 0.51 | 2.32 | 0.81 | 2.45 | 0.87 |
| luteolin | 1.25 | 0.91 | 2.41 | 1.61 | 2.93 | 2.57 |

the extracts [tyrosol, (+)-pinoresinol, decarboxylated oleuropein aglycon (peak b), 4-hydroxyphenylacetic acid, and luteolin].

All the data obtained in this study are summarized in **Table 1**. Both the intraday and the interday repeatabilities calculated on the migration time for these five analytes (expressed as RSD) and the intraday and interday repeatability values on the total peak area/migration time (expressed as RSD) were acceptable.

The reproducibility was studied by performing 20 separations of another extract, under the same conditions but with a different operator, different capillary, and different CE instrument (belonging to the University of Bologna). The results obtained for the migration times and for the total peak area/migration time (expressed as RSD) considering the five analytes previously mentioned were satisfactory.

**Calibration Curves.** Standard calibration graphs for lignans and complex phenols were prepared using oleuropein (oleuropein glucoside) at two wavelengths (200 and 240 nm). An external standard methodology (with oleuropein glucoside) was used for the quantification of lignans and complex phenols (as there are no commercially available standards of these compounds).

Using this methodology, time-corrected area counts are necessary for quantitative CE. Since separation is dependent on analytes migrating at different rates, the separated analytes will pass the detector at different rates. Time-corrected area counts are the product of the area counts and the velocity for each peak in the electropherogram.

Thus, peak areas and migration velocity (cm/min) were used to calculate the time-corrected area counts to compensate the differences in migration velocities among oleuropein glucoside, lignans, and complex phenols.

Lig Agl (a), Pin, Ac Pin, Ol Agl (a) + DOA (a), DOA (b), Lig Agl (b), Ol Agl (b), EA (a), and DOA (d) + EA (d) were quantified with the data obtained for the calibration curve of oleuropein obtained at 200 nm. However, for the quantification of Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b+c) and Lig Agl (d) + Ol Agl (d) + EA (e) the curve of oleuropein at 240 nm was used. Although oleuropein aglycon [peak c and other secoiridoid forms overlapped, Lig Agl (c) + EA (b + c)] and hydroxytyrosol have the same retention time (overlap), it was possible to calculate (approximately) the concentration of secoiridoids with this migration time ($t_{mig} = 4.07$ min) in the virgin olive oil extracts using the curve of calibration of oleuropein at 240 nm, since hydroxytyrosol has a minimum of absorbance at this wavelength (approximately) (*24*).

Tyrosol was quantified using the calibration curve of its commercial standard at 200 nm, and flavonoids were quantified

**Table 2.** Analytical Parameters of Proposed Method

| analyte | RSD[a] (%) (intermediate value) | detection limit ($\mu$g/mL) | quantification limit ($\mu$g/mL) | calibration range ($\mu$g/mL) | calibration equations | $r^2$ |
|---|---|---|---|---|---|---|
| tyrosol[b] | 2.53 | 0.836 | 2.787 | 2.787–500 | $y = 1.88 \cdot 10^{-5}x + 1.37 \cdot 10^{-5}$ | 0.994 |
| oleuropein glucoside[b] $\lambda = 200$ nm | 2.21 | 2.085 | 6.950 | 6.950–6500 | $y = 1 \cdot 10^{-5}x + 1 \cdot 10^{-4}$ | 0.996 |
| oleuropein glucoside[c] $\lambda = 200$ nm | 2.01 | 0.104 | 0.348 | 0.348–6500 | $y = 2 \cdot 10^{-4}x + 9 \cdot 10^{-4}$ | 0.997 |
| oleuropein glucoside[b] $\lambda = 240$ nm | 4.93 | 4.170 | 13.901 | 13.901–2000 | $y = 5 \cdot 10^{-6}x + 6 \cdot 10^{-6}$ | 0.998 |
| oleuropein glucoside[c] $\lambda = 240$ nm | 1.73 | 0.348 | 1.158 | 1.158–2000 | $y = 6 \cdot 10^{-5}x - 2 \cdot 10^{-4}$ | 0.995 |
| luteolin[b] | 3.06 | 0.355 | 1.183 | 1.183–200 | $y = 5.88 \cdot 10^{-5}x + 5.67 \cdot 10^{-5}$ | 0.992 |
| apigenin[b] | 2.23 | 0.311 | 1.037 | 1.037–200 | $y = 6.7 \cdot 10^{-5}x + 4.8 \cdot 10^{-5}$ | 0.995 |

[a] RSD: relative standard deviation. LD $= 3\sigma_b/b$ ($\sigma_b = 6.95 \times 10^{-6}$, calculated using 100 data); LQ $= 10\sigma_b/b$. Data obtained using the software Microcal Origin, Microcal Software, Inc. [b] $y = bx \pm a$, where $y$ is the peak area (AU), $x$ is the concentration ($\mu$g/mL), $a$ is the $y$ intercept, and $r^2$ is the correlation coefficient. [c] $y = bx \pm a$, where $y$ is the [time corrected area counts $=$ peak area (AU) $\times$ migration velocity (cm/min)].

using the calibration curves obtained at 200 nm for commercial standards of luteolin and apigenin respectively. Peak areas were used for the quantification of these three compounds versus the corresponding commercial standards.

The determination of the phenolic acids in olive oil has been carried out by other research groups and by our own group (17–19) (for this reason we do not show the data corresponding to the calibration curves and quantification results for this family of compounds). However, it is the first time in which flavonoids (luteolin and apigenin) and several secoiridoid compounds from olive oil are identified and quantified using capillary zone electrophoresis (CZE). We have also obtained the quantification in terms of families of phenolic compounds (simple phenols, complex phenols, lignans, and flavonoids) present in olive oil.

The detection, quantification limit, and precision (as relative standard deviation of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie (25). Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. The calibration plots indicate good correlation between peak areas and analyte concentrations in the case of tyrosol and flavonoids, and between time-corrected area counts and analyte concentrations for the secoiridoids and lignans; regression coefficients were higher than 0.994 for tyrosol and oleuropein (at the two wavelengths), and were higher than 0.992 for luteolin and apigenin. All the features of the proposed method are summarized in **Table 2**.

**Recovery of Secoiridoid Compounds and Flavonoids.** As far as the recoveries of these compounds are concerned using solid-phase extraction, it is important to explain that we have used the protocol described by Gómez-Caravaca et al. (21), and we have spiked a refined sunflower oil with an exactly specified dose of a phenolic extract previously obtained. The results are summarized in **Table 3**.

**Analysis of Several Extra-Virgin Olive Oil Samples.** Extracts of eight extra-virgin olive oils were analized. Five of them were of monovarietal extra-virgin olive oils: picual, hojiblanca, lechín de Sevilla, arbequina, and cornicabra (January 2005). An organic olive oil and two types of picual olive oil ("suave" and "intenso") were acquired from a supermarket. All samples were injected in the CE instrument seven times ($n = 7$).

Using the described SPE system and CZE method, all the virgin olive oils were analyzed. The polyphenolic profiles are shown in **Figure 2**. The absorbance scales in the electrophero-

**Table 3.** Recovery (%) of Phenolic Compounds (Flavonoids and Secoiridoids) Isolated by Solid-Phase Extraction from Reference Sunflower Oil Samples ($n = 3$)

| | method 1[a] | SD[b] | method 2[c] | SD |
|---|---|---|---|---|
| | | flavonoids | | |
| luteolin | 89.34 | 2.43 | 89.56 | 2.29 |
| apigenin | 91.01 | 1.98 | 92.12 | 2.45 |
| | | secoiridoids | | |
| Lig Agl (a)[d] | 71.80 | 2.57 | 72.07 | 1.66 |
| Ol Agl (a) + DOA (a)[d] | 72.34 | 1.45 | 72.45 | 1.56 |
| Ol Agl (b)[d] | 87.41 | 2.31 | 88.54 | 2.00 |
| EA (a)[e] | 65.67 | 1.67 | 66.99 | 3.01 |
| EA (d) + DOA (d)[e] | 93.33 | 3.12 | 93.66 | 2.48 |
| EA (d)[f] | 92.43 | 2.43 | 92.12 | 1.12 |

[a] Refined sunflower oil spiked with 1 mL of extract of virgin olive oil phenols. [b] SD $=$ standard deviation  [c] Refined sunflower oil spiked with 0.5 mL of extract of virgin olive oil phenols. [d] Calculated using the information at 200 and 240 nm. [e] Calculated using the information at 200 nm. [f] Calculated using the information at 240 nm.

grams are not exactly the same, in order to show each electrophrogram at its maximum of absorbance and see with clarity and ease all the peaks. The quantitative results are presented in **Table 4**.

TY was found in the range of 6781.0–11457.0 $\mu$g of analyte/kg of olive oil in this study, although for lechín de Sevilla it was possible to see the highest peak of this compound.

(+)-1-Acetoxypinoresinol was very abundant in arbequina and hojiblanca oils, while in the picual variety and commercial oils its quantity was low. In terms of (+)-pinoresinol, picual and cornicabra were the richest varieties.

As far as the amount of complex phenols is concerned, it is important to highlight that Lig Agl (peak a) was abundant in cornicabra and lechín de Sevilla olive oils, while Lig Agl (peak b) was found at high concentrations in the commercial and cornicabra oils. In terms of Ol Agl (c) + DOA (a), cornicabra was the richest with twice the amount of these compounds as in organic, picual suave, arbequina, and hojiblanca oils. This oil was the richest in terms of DOA (b) as well; however one of the commercial olive oils (picual intenso) and the oil obtained from olives of picual variety had the highest quantities of DOA (d) + EA (d). Ol Agl (b) was not found in all the olive oils analized and EA (a) were in the range of 2607.3–20521.5 $\mu$g of analyte/kg of olive oil.

Keeping in mind the two compounds which are quantified at 240 nm [(Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b,c) and
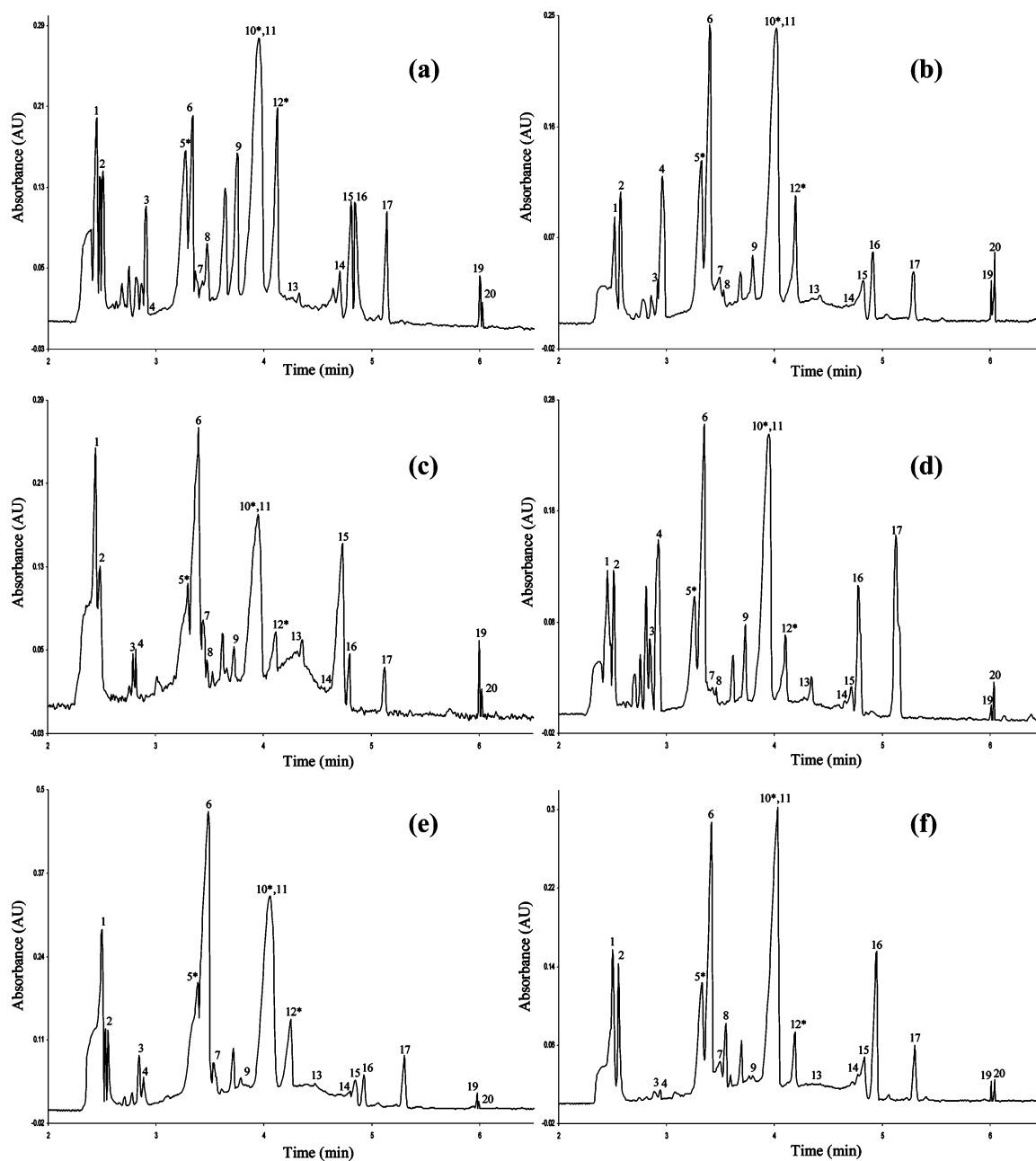
**Figure 2.** CZE electropherogram of phenolic fraction extracted from extra-virgin olive oil samples by solid-phase extraction: (**a**) picual, (**b**) hojiblanca, (**c**) lechín de Sevilla, (**d**) arbequina, (**e**) cornicabra, and (**f**) organic oil. For identification of the compounds, see **Figure 1**. Detection was performed at 200 nm. The absorbance scales (AU) in the electropherograms are different. (Instrumental and experimental parameters as in **Figure 1**).

EA(e) + Lig Agl (d) + Ol Agl (d)], they were significantly more abundant in cornicabra, lechín de Sevilla, and picual intenso, which had very similar quantities.

Flavonoids were found in the range of 657.9−7598.9 μg of analyte/kg of olive oil for the luteolin, and 365.8−2203.8 μg of analyte/kg of olive oil for the apigenin. In hojiblanca extra-virgin olive oil, the quantity of apigenin was greater than that of luteolin.

Considering the families of phenolic compounds that are present in olive oil and doing the quantification in terms of simple phenols, lignans, complex phenols, and flavonoids, the results are those which are shown in **Table 5**.

In our opinion, it is also interesting to observe in detail the differences between picual suave and intenso. As mentioned before, polyphenols are an important functional minor components of virgin olive oils that are responsible for the key sensory

characteristics of bitterness, pungency, and astringency (*7*). The intensity of the bitterness of virgin olive oil has been related to the presence of phenolic compounds derived from the hydrolysis of the oleuropein. It is known that oleuropein is the bitter principle found in olives; however, it is not found at significant concentrations in olive oils, but oleuropein aglycon and isomers of oleuropein are. In several papers it has been concluded that the greater the intensity of bitterness for an olive oil, the greater the content of dialdehydic and aldehydic forms of decarboxy-methyl oleuropein aglycon and the dialdehydic form of decar-boxymethyl ligstroside aglycon (*8, 26, 27*).

We have observed that the quantities found in picual suave and intenso are very similar in terms of simple phenols (TY). Lignans were present in a bigger quantity in picual suave. However, in our opinion the most valuable information can be obtained by taking into account the concentrations of the

**Table 4.** Results ($\mu$g of analyte/kg of olive oil) of the Analysis of Real Samples; Quantification of the Individual Components ($n = 7$) ($\pm$SD)

| analyte[a] | picual | hojiblanca | lechín de Sevilla | arbequina | cornicabra | organic oil | picual suave | picual intenso |
|---|---|---|---|---|---|---|---|---|
| Lig Agl (a)[c] | 26200.8 ± 786.8 | 11946.8 ± 298.6 | 41286.9 ± 1238.6 | 22691.6 ± 907.6 | 44434.4 ± 1692.7 | 24102.5 ± 990.6 | 15311.3 ± 566.5 | 23632.2 ± 562.1 |
| TY[b] | 9492.2 ± 456.6 | 7278.4 ± 348.5 | 11457.0 ± 373.4 | 7726.1 ± 191.6 | 6781.0 ± 128.1 | 9417.5 ± 277.9 | 8148.8 ± 300.7 | 8173.8 ± 404.6 |
| Pin[c] | 8110.5 ± 324.4 | 3027.2 ± 75.7 | 2289.9 ± 45.8 | 4944.9 ± 199.8 | 6223.3 ± 242.9 | 1372.8 ± 38.9 | 2111.3 ± 65.1 | 473.7 ± 22.6 |
| Ac Pin[c] | 299.2 ± 14.0 | 11972.6 ± 359.2 | 2630.7 ± 92.1 | 15694.3 ± 627.8 | 3504.9 ± 136.3 | 485.5 ± 26.1 | 370.1 ± 6.7 | 335.2 ± 16.0 |
| Ol Agl (a) + DOA (a)[c] | 31390.6 ± 1176.2 | 20949.2 ± 733.2 | 25349.9 ± 1069.7 | 17149.8 ± 524.9 | 45604.1 ± 1582.5 | 20074.5 ± 850.3 | 17887.8 ± 734.5 | 29477.3 ± 1146.7 |
| DOA (b)[c] | 19089.9 ± 572.7 | 25638.9 ± 1261.9 | 42233.2 ± 1123.4 | 26547.9 ± 753.4 | 80246.1 ± 2383.3 | 29111.7 ± 753.9 | 15151.1 ± 424.1 | 39762.7 ± 1500.6 |
| Lig Agl (b)[c] | 4496.5 ± 89.9 | 5086.0 ± 203.4 | 5566.3 ± 192.0 | 2706.2 ± 121.3 | 7400.3 ± 273.1 | 6548.8 ± 218.3 | 6767.1 ± 163.2 | 7443.9 ± 322.0 |
| Ol Agl (b)[c] | 5540.8 ± 110.8 | 1675.7 ± 67.0 | 1718.5 ± 79.0 | 783.7 ± 31.3 | nd[f] | 5143.4 ± 231.4 | 1670.6 ± 44.3 | 2486.9 ± 71.0 |
| EA (a) [c] | 20521.5 ± 820.8 | 5464.3 ± 56.6 | 6545.4 ± 327.3 | 5541.6 ± 257.7 | 6236.5 ± 217.0 | 2607.3 ± 49.5 | 5155.5 ± 219.9 | 13726.5 ± 508.6 |
| Ol Agl (c) + Lig Agl (c) + DOA (c) + EA(b,c)[d] | 87397.1 ± 3295.9 | 132407.5 ± 5296.3 | 207546.4 ± 10149.0 | 118984.6 ± 2260.7 | 265177.2 ± 7341.5 | 117671.5 ± 4585.6 | 96880.7 ± 2718.3 | 204555.5 ± 4394.6 |
| DOA (d) + EA (d)[c] | 17073.9 ± 341.5 | 9182.6 ± 367.3 | 7430.8 ± 221.4 | 5612.3 ± 224.5 | 17691.2 ± 577.6 | 4944.9 ± 107.7 | 8532.0 ± 315.64 | 16189.70 ± 634.6 |
| EA (e) + Lig Agl (d) + Ol Agl (d)[d] | 57821.7 ± 2023.7 | 88259.6 ± 3530.4 | 233478.9 ± 9689.3 | 89602.4 ± 4659.3 | 208604.3 ± 6953.5 | 137049.7 ± 5402.9 | 145426.5 ± 2668.4 | 246587.7 ± 9343.2 |
| Lut[e] | 5255.8 ± 223.8 | 657.9 ± 31.1 | 7598.9 ± 187.5 | 2454.8 ± 88.0 | 2213.5 ± 77.6 | 699.9 ± 23.6 | 5123.4 ± 156.7 | 4879.0 ± 123.5 |
| Apig[f] | 879.0 ± 43.8 | 2203.8 ± 109.5 | 897.7 ± 34.7 | 1256.3 ± 46.8 | 365.8 ± 12.0 | 609.9 ± 19.9 | 765.7 ± 33.3 | 658.0 ± 30.8 |

[a] TY, tyrosol; Pin, (+)-pinoresinol; Ac Pin, 1-(+)-acetoxypinoresinol; DOA, decarboxylated oleuropein aglycon; Lig Agl, ligstroside aglycon; Ol Agl, oleuropein aglycon; EA, elenolic acid; Lut, luteolin; Apig, apigenin. [b] Quantified with a calibration curve of tyrosol at $\lambda = 200$ nm. [c] Quantified with a calibration curve of oleuropein glucoside at $\lambda = 200$ nm (time corrected area counts vs concentration). [d] Quantified with a calibration curve of oleuropein glucoside at $\lambda = 240$ nm (time corrected area counts vs concentration). [e] Quantified with a calibration curve of luteolin at $\lambda = 200$ nm. [f] Quantified with a calibration curve of apigenin at $\lambda = 200$ nm. [g] nd: not detected.

**Table 5.** Results (mg of analyte/kg of olive oil) of the Analysis of Extra-Virgin Olive Oil Samples; Quantification in Terms of Families of Phenolic Compounds ($n = 7$) ($\pm$SD)

| family of compounds[a] | picual | hojiblanca | lechín de Sevilla | arbequina | cornicabra | organic oil | picual suave | picual intenso |
|---|---|---|---|---|---|---|---|---|
| simple phenols | 9.49 ± 0.39 | 7.28 ± 0.28 | 11.46 ± 0.44 | 7.73 ± 0.25 | 6.78 ± 0.12 | 9.42 ± 0.31 | 8.15 ± 0.19 | 8.17 ± 0.31 |
| complex phenols (A) | 124.31 ± 3.03 | 799.43 ± 35.64 | 130.13 ± 4.15 | 81.03 ± 2.56 | 201.61 ± 5.01 | 92.53 ± 4.57 | 70.48 ± 3.15 | 132.72 ± 4.21 |
| complex phenols (B) | 145.22 ± 4.21 | 220.77 ± 6.29 | 441.03 ± 11.21 | 208.59 ± 5.55 | 473.78 ± 10.09 | 254.72 ± 6.21 | 242.31 ± 5.12 | 451.14 ± 18.04 |
| lignans | 8.41 ± 0.42 | 15.00 ± 0.28 | 4.92 ± 0.13 | 20.64 ± 0.30 | 9.73 ± 0.23 | 1.86 ± 0.11 | 2.48 ± 0.04 | 0.81 ± 0.03 |
| flavonoids | 6.27 ± 0.29 | 3.16 ± 0.12 | 8.61 ± 0.38 | 4.05 ± 0.11 | 2.63 ± 0.09 | 1.39 ± 0.05 | 5.99 ± 0.28 | 5.62 ± 0.20 |

[a] Simple phenols = tyrosol (quantified with a calibration curve of tyrosol at $\lambda = 200$ nm). Complex Phenols = (A) secoiridoid forms quantified with the calibration curve of oleuropein glucoside at $\lambda = 200$ nm; (B) secoiridoid forms quantified with the calibration curve of oleuropein glucoside at $\lambda = 240$ nm. Lignans = 1-(+)-acetoxypinoresinol + (+)-pinoresinol (quantified with a calibration curve of oleuropein glucoside at $\lambda = 200$ nm). Flavonoids = luteolin + apigenin (quantified with a calibration curve of luteolin at $\lambda = 200$ nm).

secoiridoid compounds. When we express the results using the quantification in terms of families of phenolic compounds, picual intenso is richer than picual suave in complex phenols (both those complex phenols quantified with the calibration curve of oleuropein glucoside at $\lambda = 200$ nm and those quantified with the calibration curve of oleuropein glucoside at $\lambda = 240$ nm). Regarding individual secoiridoid compounds, in all the cases the concentrations of these compounds present in picual intenso were greater than those of the picual suave. These results demonstrate the essential role played by the compounds mentioned before in the bitter taste of virgin olive oil. As far as the flavonoids luteolin and apigenin are concerned, the quantities found

in these two virgin olive oils were very similar. The differences between these two commercial olive oils can be seen clearly in **Tables 4** and **5**.

It is possible to say that, in general, the results obtained by this method are in good agreement with those previously reported obtained by using other techniques (*5, 28−31*), although the comparison can often be hard because the results are not expressed in identical units (in terms of the same reference standard).

**Statistical Analysis.** To evaluate the possibility of differentiating the samples, taking into account the phenolic compounds identified and quantified by this electrophoretic method, we

Phenols from Spanish Virgin Olive Oil by CE Analysis     **G**



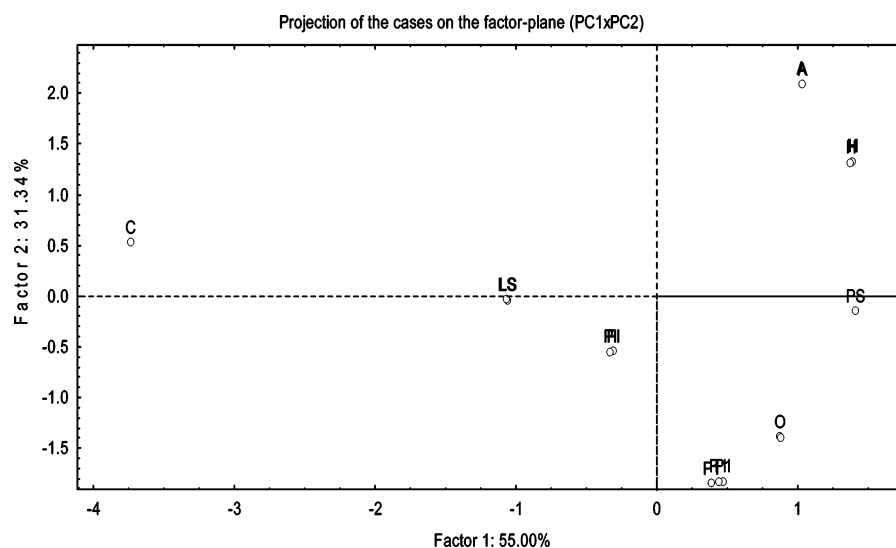**Figure 3.** Score plot for the two principal components: picual, P1; hojiblanca, H; lechín de Sevilla, LS; arbequina, A; cornicabra, C; organic oil, O; picual suave, PS; picual intenso, PI.

chose a multivariate statistical approach. This statistical analysis, applied to a limited number of samples, did not have the purpose of distinguishing monovarietal olive oils obtained from different cultivars but to check the discrimination capacity achieved by using the electrophoretic variables considered; that means our aim was to prove that we could differentiate the samples considering the phenolic fraction quantified by CE. All the phenolic compounds quantified were considered to identify the two principal factors. Five variables [Ac Pin, DOA (b), Lig Agl (a), Ol Agl (a) + DOA (a), Ol Agl (b)] that had factor loading higher than 0.7 (evaluated by factors extraction test) were selected for the PCA and the explained variance was higher than 80%. The relative standard deviations (RSD) obtained in the study of repeatability were lower than 0.91% for the migration times and 2.32% for the peak areas/migration time (intraday study).

A map of samples (score plot) for the two principal components is shown in **Figure 3**. Extra-virgin olive oils made from cornicabra olives are quite different from the others, since these samples lie far from those olive oils obtained from olives of other cultivars or those commercial olive oil samples. This fact can be explained by taking into account the amount of Ol Agl (a) + DOA (a) and DOA (b) present in the cornicabra extra-virgin olive oils under study. Picual intenso and lechín de Sevilla contain high concentrations of Ol Agl (a) + DOA (a) and DOA (b) as well, but not as high as cornicabra olive oil; thus, they can be found in the third section of the plot.

Arbequina and hojiblanca oils, however, are very close in the score plot shown (in the first section or quadrant) because of their high Ac Pin content. Picual, picual suave, and the organic olive oil are located in the fourth section. Picual and organic olive oil have a similar Ol Agl (b) content, and picual suave is situated in this fourth quandrant very near the X axis because of its low content of DOA (b), Lig Agl (a), and Ol Agl (a) + DOA (a).

It is important to highlight the discriminant capacity of the function of the selected variables to distinguish the samples under study.

This is the first time in which an electrophoretic method was demonstrated to have the ability to detect and quantify simultaneously members of five different families of phenolic compounds present in olive oil. Its usefulness has been demonstrated by analyzing five monovarietal extra-virgin olive oils (January 2005), an organic olive oil, and two types of picual olive oils with different commercial names related to their sensorial properties, and very interesting results were found. CE joined to statistical analysis permits the discrimination among different olive oils. In our opinion, to carry out a wider study taking into account the phenolic composition of olive oils obtained from the main varieties of Spanish, Italian, and Greek olives would be really interesting. This topic will be the next step in our research.

## ABBREVIATIONS USED

TY, tyrosol; Pin, (+)-pinoresinol; Ac Pin, 1-(+)-acetoxypinoresinol; DOA, decarboxylated oleuropein aglycon; Lig Agl, ligstroside aglycon; Ol Agl, oleuropein aglycon; EA, elenolic acid; HYTY, hydroxytyrosol; Apig, apigenina; and Lut, luteolin.

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Como novedad en la parte aplicada del método descrito en este capítulo, podemos ver que los aceites analizados no fueron únicamente monovarietales como en anteriores trabajos recogidos en la presente memoria. Se estudiaron en profundidad 8 aceites; 5 de ellos sí que fueron monovarietales procedentes de aceitunas de tipo Picual, Hojiblanca, Lechín de Sevilla, Arbequina y Cornicabra, pero en los otros 3 aceites podríamos resaltar algunas connotaciones particulares. Los tres se compraron en el supermercado; dos de ellos eran de la variedad Picual, pero se distinguían por atributos sensoriales (uno era suave y otro picante), el tercero fue un aceite ecológico.

Incluyendo estos tres aceites, pudimos enriquecer los resultados experimentales y conclusiones obtenidas, ya que:

∗ Comprobamos que dos aceites de la misma variedad clasificados con una etiqueta comercial diferente en lo que a características sensoriales se refiere (Picual "Suave" y Picual "Intenso"), diferían bastante en el contenido de algunos de los compuestos fenólicos identificados y cuantificados en este estudio. Los datos cuantitativos se incluyeron en el artículo, pero en este anexo mostramos una gráfica donde los perfiles electroforéticos de los extractos de ambos aceites se comparan.

*Figura*. Electroferogramas obtenidos al analizar los extractos fenólicos de dos aceites comerciales de la variedad Picual usando las condiciones óptimas del método de CZE descrito en este capítulo. (a) Aceite Picual "Intenso"; (b) Aceite Picual "Suave". La detección se realizó a 200 nm. Los números que identifican cada número siguen la misma codificación que la que se muestra en el artículo.
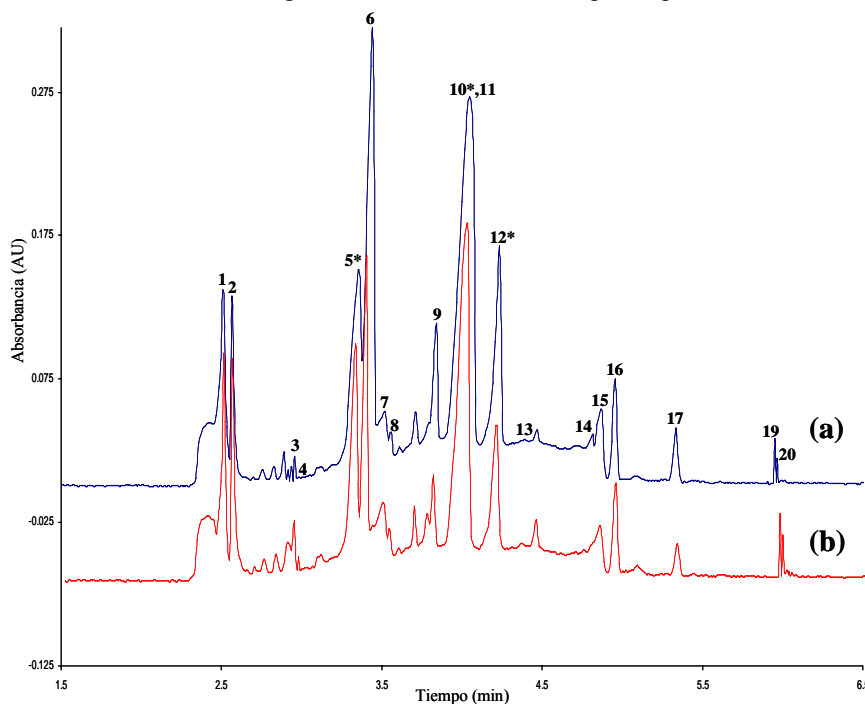
Este hecho nos confirmó la importancia que los compuestos fenólicos pueden tener en los atributos sensoriales del aceite, cosa que ya había sido puesta de manifiesto por otros investigadores utilizando técnicas cromatográficas. Por ello, se siguió ahondando en este argumento, cosa que dio lugar a parte del trabajo que constituye el capítulo siguiente (capítulo 8).

∗ Llevamos a cabo por primera vez el análisis de un aceite ecológico [352] mediante CE.

La producción del aceite de oliva ecológico, supone el máximo respeto al medio ambiente, al utilizarse en el cultivo del olivar, únicamente productos naturales y por tanto, estos cultivos están libres del uso de productos químicos que tantos perjuicios pueden provocar al ecosistema y a la salud de las personas. Comprar aceite de oliva

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[352] Reglamento (CEE) nº 2092/91 del Consejo de la Unión Europea, de 24 de junio de 1991, sobre la producción agraria ecológica y su indicación en los productos agrarios y alimenticios, modificado por última vez por el reglamento (ce) n o 2254/2004 de la comisión de 27 de diciembre de 2004

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ecológico certificado, conlleva unos rigurosos controles de calidad previos que garantizan mediante la etiqueta ecológica que el aceite de oliva es realmente ecológico. Igualmente, los procesos de extracción en frío del aceite de oliva ecológico, así como las avanzadas técnicas para no producir alpechín en las almazaras, hacen que el resultado de todo este proceso sea un producto más respetuoso con la naturaleza.

La concienciación cada día mayor de la población hacia los productos ecológicos está haciendo que la demanda crezca, lo que hace, obviamente, aumentar la producción. Por todo ello, nos pareció bastante interesante llevar a cabo la caracterización de este aceite en términos de compuestos fenólicos, ya que permitiría comprobar si la producción ecológica afectaba notablemente a esta fracción del aceite de oliva tanto desde un punto de vista cualitativo, como cuantitativo.

# Capítulo 8

**Estudio comparativo del contenido fenólico de aceites de oliva virgen-extra pertenecientes a diferentes DOPs y su relación con las propiedades sensoriales**

En el vasto mercado europeo existe una gran riqueza y variedad de productos pero cuando un producto adquiere cierta reputación fuera de sus fronteras se puede encontrar con usurpaciones e imitaciones. Esta competencia desleal no sólo desalienta a los productores sino que también engaña a los consumidores. Por ello, en 1992, la UE creó unos sistemas conocidos como DOP e IGP (Denominación de Origen Protegida e Indicación Geográfica Protegida) para promover y proteger productos agroalimenticios [353]. Para tener estas etiquetas de calidad el producto debe cumplir una serie de especificaciones: nombre y descripción del mismo, definición del área geográfica, métodos de preparación, detalles del etiquetado y requerimientos legislativos…

Pertenecer a una DOP o a una IGP es, además, muy importante en la industria alimentaria porque uno de los problemas más importantes que se da hoy en día en dicha industria es la puesta a punto de herramientas que puedan determinar y asegurar la trazabilidad, de modo que sea relativamente sencillo conocer qué ocurre con un alimento en toda la cadena industrial hasta que llega al consumidor. Los productos incluidos en estas categorías tienen garantizada la calidad, así como otras características esenciales y exclusivas debido al particular ambiente geográfico de donde proceden.

La **DOP** designa el nombre de un producto cuya producción, transformación y elaboración deben realizarse en una zona geográfica determinada, con unos conocimientos específicos reconocidos y comprobados. Mientras que, en la **IGP** el vínculo con el medio geográfico sigue presente en al menos una de las etapas de la producción, de la transformación o de la elaboración.

España es uno de los productores de aceite más importantes y actualmente posee nueve DOPs (5 en Andalucía, 2 en Cataluña, una en Castilla la Mancha y otra en Aragón), mientras que en otro de los países que más aceite produce, Italia, encontramos hasta 36 DOPs.

Viendo la importancia que para estos dos países tiene la producción del aceite de oliva y su comercialización, y habiendo establecido una colaboración (mencionada anteriormente) con un grupo de investigación italiano mediante una estancia en la Universidad de Bolonia, creímos muy interesante realizar un estudio comparativo entre una DOP española y una italiana.

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[353] Reglamento (CEE) 2081/92 del Consejo, de 14 de julio de 1992, relativo a la protección de las indicaciones geográficas y las denominaciones de origen de los productos agrícolas y alimenticios.

Así, el objetivo planteado fue analizar los componentes fenólicos contenidos en la fracción insaponificable de aceites de oliva virgen extra, intentando diferenciar los aceites provenientes de una DOP española (Sierra de Segura) y una italiana (Chianti Classico) considerando este contenido polifenólico y utilizando estadística multivariante. En particular se analizaron 16 aceites pertenecientes a la DOP española y 9 pertenecientes a la italiana.

A su vez, se persiguió el establecer un paralelismo entre el análisis del aceite mediante CE y el proceso organoléptico de cata del mismo. Se intentó determinar qué compuestos eran aquellos que con su presencia, o que al estar presentes en una determinada concentración en el aceite, pudieran otorgarle un atributo característico al aceite (picante, amargo, afrutado…).

# Comparative study of the phenolic profiles of extra-virgin olive oil belonging to different Spanish and Italian PDOs and their relation with the sensorial properties

A. Carrasco-Pancorbo[1], S. Cortacero-Ramírez[1], A. M. Gómez-Caravaca[1], L. Cerretani[2*], A. Bendini[2], F. Moreno-Ballesteros[3], A. Segura-Carretero[1*] and A. Fernández-Gutiérrez[1].

[1] *Department of Analytical Chemistry, Faculty of Sciences, University of Granada.*
*C/Fuentenueva s/n, E-18071 Granada, Spain*

[2] *Department of Food Science, University of Bologna.*
*P.zza Goidanich 60, 47023 Cesena (FC), Italy.*

[3] *CRDO Sierra de Segura*
*P. I. Llanos de Armijo, 23360 La Puerta de Segura, Jaén, Spain*

**Abstract**

To belong to a Protected Designations of Origin (PDO) has become very important for food, as one today´s major problems in the agricultural-food industry is to set down objective tools in order to determine the traceability of raw materials as well as finished products so that we can follow the products from the producer to the consumer. There is a great interest in foods that belong to a PDO because the quality and other essential and exclusive characteristics due to a particular geographical environment are guaranteed.
We have used a capillary electrophoretic method for the simple, rapid and simultaneous characterization and quantification of the polyphenolic fraction of extra-virgin olive oil from different PDOs. To demonstrate the usefulness of this method, we have analyzed sixteen samples of a Spanish PDO and other nine samples belonging to an Italian PDO (n=5). In this way, it was possible to compare the phenolic profiles of the oils of different zones of the same PDO, as well as the phenolic profiles of Spanish and Italian extra-virgin olive oils. Multivariate statistics was used for differenciating the oils produced in each PDO.

The correlations among several of the phenolic compounds present in the extracts of olive oil and its sensorial properties were checked as well.

*Keywords:* Polyphenols; Quantification; Capillary zone electrophoresis; Olive oil; PDO.

*Author to whom correspondence should be addressed: Dr. L. Cerretani, Dipartimento di Scienze degli Alimenti, Università di Bologna, P.zza Goidanich 60, 47023 Cesena, (FC) Italy.

E-mail: lorenzo.cerretani2@unibo.it or ansegura@ugr.es

Tel. +390547338121; Fax +390547382348

## 1. Introduction

Climatic and pedologic factors, cultivation and agronomic techniques, harvesting, carriage and storage systems of olives, ripening degree of drupes, genetic factors (cultivar), and processing techniques, affect the analytical characteristics of oil [1,2,3,4]. They are also influenced by the kind of container used to store the product [5,6].

The chemical composition of oil is in large measure attributed to the agronomic or geographical origin [7]. In fact, certain zones are renowned for the chemical and sensory characteristic of their oils. In order to certify the origin zone of typical oils with excellent characteristics, the EU identifies two types of food quality names based on their geographical origin: the protected geographical indication (PGI) and the protected designation of origin (POD) [8]. Once these names are registered, they are protected against the sale of any other competing imitation product seeking to use the reputation of the name of origin. To qualify for a PGI or PDO designation, a product must comply with a specification containing the following: the name and description of the product; the definition of the geographical area; the methods of preparation; factors relating to the geographic environment; the inspection bodies; details of labelling and any legislative requirements that must be met. The type of link between the product and the geographical location is more stringent in the case of the PDO designation, the quality or other characteristics being due essentially or exclusively to its geographical environment [8].

Spain is one of the most important olive oil producers and currently has nine PDOs (five in Andalusia, two in Catalonia, one in Castilla la Mancha and one in Aragon). On the other hand, in Italy there are 36 PDOs and one PGI of olive oil among 155

alimentary products which are certificated in this country. As the PDO label defines the origin of the oils and the varieties used and guarantees the production and transformation of the product in its geographic areas, the products included in the same PDO have some exceptional characteristics in common, including both analytical specifications and organoleptic properties [9].

Extensive sensory and analytical analyses (free acidity, peroxide value, $K_{232}$ and $K_{270}$ among others) have to be followed [10,11] to comply with the specific quality criteria of the European Union (EU) directives and International Olive Oil Council (IOOC) [10,11, 12]. Sensory assessment requires highly trained tasters, whilst the analytical characterization requires the application of several techniques (separative or spectroscopic). Other techniques such as headspace-mass spectrometry [13] and nuclear magnetic resonance [14] have also been applied for this purpose.

In particular among all its components, the phenolic compounds in olive oil have also been a subject of considerable interest, both because of its chemoprotective effect in human beings [15,16,17] and because it is a major factor in the high stability (shelf-life) of olive oils [18,19,20,21,22]. Furthermore, phenols also contribute to the organoleptic properties of VOOs and are commonly described as bitter and astringent [20,23,24,25] and responsible of organoleptic characteristics in general [26]. Less commonly, phenolic molecules are associated with pungency, that is, peppery, burning, or hot sensations [20, 27 ]. Unfortunately, the relationships between individual hydrophilic phenols of VOO and its sensory characteristics is not totally defined.

In spite of the interest in these compounds, to the date, official methods to analyze phenolic compounds from the olive oil do not exist. The polar phenolic fraction of virgin olive oil consists of a mixture of compounds, which differ in chemical properties and impact on quality of virgin olive oil [7,18,28]. Analytical procedures for the characterization and quantification of the complete profile of phenolic components of olive oils usually entail extraction (liquid-liquid or solid-phase) of this compounds from the oil, followed by a separative technique (RP-HPLC, GC or CE) [24,29,30,31,32,33,34,35,36,37,38,39] or colorimetric assay [40].

Among the different separative techniques that can be used to analyze foods and food compounds, the use of CE has emerged as a good alternative since this technique provides fast and efficient separations in this type of analysis [41]. CE is based on the different electrophoretic mobilities of substances in solution under the action of an electric field. The main properties of CE are high speed of analysis, high separation

efficiencies, great variety of applications, and additionally reduced sample and solvents comsumption.

In this work we focus on olive oils from the two PDOs; one of Italy (Chianti Classico) and the other one of Spain (Sierra de Segura). The oils belonging to the Italian PDO were poly-varietal (*Moraiolo, Leccino and Frantoio cv.*), however, the spanish oils were obtained from a unique variety of olive fruit called Picual. The main purpose of this work was to investigate if CZE could effectively give reliable information about the amount of phenolic compounds and the composition of this fraction in olive oils belonging to two different PDOs. Another aim, using multivariate statistics, was to differentiate the oils produced in each PDO of those under study. Then, comparing the CE data with those obtained after the sensory analysis carried out by a expert panel of tasters, we tried to find correlations between several sensory properties (fruity, bitter or pungent) and the concentrations of one or several phenolic compounds present in the polar extract of the oils.

## 2. Materials and methods

### 2.1. Apparatus

Experiments were performed with a Beckman P/ACE[TM] System MDQ capillary electrophoresis instrument connected to a diode array detector. A GOLD software installed in a personal computer was used for system control and data handling. The instrument comprises a 0-30 kV high-voltage built in power supply. All capillary tubing (uncoated fused silica) was provided from Beckman Instruments, Inc. (Fullerton, CA, USA). The internal diameter of the capillary cartridge was 50 μm with a total length of 47 cm and an effective separation length of 40 cm. The temperature (22ºC) was controlled by the use of a fluorocarbon based cooling fluid.

### 2.2. Reagents, stock solutions and reference compounds

2-(4-hydroxyphenyl)ethanol (tyrosol (TY)) was acquired from Fluka  (Buchs, Swiss) and oleuropein (oleuropein glucoside) was obtained from Extrasynthèse (Genay, France). The stock solution containing these two analytes was prepared in methanol/ water (50/50, v/v) at a concentration of 500 μg/ml in the case of tyrosol and 2000 μg/ml for oleuropein glucoside. Tyrosol was used for the quantification of this compound present in the

extracts of olive oil and oleuropein glucoside was used to make the calibration curves for the quantification of lignans and complex phenols.

The phenolic acids 4-hydroxyphenylacetic acid, sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid), trans-cinnamic acid and gentisic acid (2,5-dihydroxybenzoic acid) standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Each phenolic acid was used for the quantification of this compound present in the extracts of olive oil; the stock solutions containing these analytes were prepared at a concentration of 100 µg/ml.

Hydroxytyrosol (HYTY) and luteolin were obtained from Extrasynthèse (Genay, France). The first one was used for the quantification of this compound and luteolin was used for the quantification of the flavonoids (as a sum of luteolin and apigenin). Their stock solutions were prepared in methanol/water (50:50, v/v) at a concentration of 300 and 250 µg/ml, respectively. All the analytes were used as received.

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma and was used as running buffer at different concentrations and pHs.

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

Solid phase separation (SPE) cartridges DSC-Diol were obtained from Supelco (Bellefonte, PA, USA).

*2.3 Samples*

Extra-virgin olive-oil samples were obtained from two PODs, one of Italy (Chianti Classico) and the other one of Spain (Sierra de Segura). We studied the phenolic fraction of 9 Italian and 16 Spanish olive oils.

Different mills for making olive oil belonging to the Italian PDO produced the olive oils used in this study: Castello di Ama, Poggio Torselli, Azienda Giacomo Grassi, Il Casalone, Rocca di Castagnoli, Castello di Cacchiano, Villa Branca, Monteraponi, Fattoria le Fonti, and Castello di Gabbiano. Belonging to the Spanish PDO the mills for making olive oil which produced the oils used in this study were: S.C.A. Agrosegura, Potosí 10 S.A., S.C.A. NTRA. SRA. Nazaret, S.C.A. San Juan de la Cruz, S.C.A. San Francisco, Aceites El Carrascal S.A., S.C.A. Virgen del Campo de Génave. One of the

samples was produced in S.C.A. Agrosegura; other one in S.C.A. Virgen del Campo de Génave; two extra virgin olive oils were obtained from Potosí 10 S.A.; the mill S.C.A. NTRA. SRA. Nazaret produced two of the samples under study; in S.C.A. San Juan de la Cruz three samples were made; one oil had its origin in the mill Aceites El Carrascal S.A., and all the other samples were obtained from S.C.A. San Francisco (see Table 1).

All these samples were subjected to the electrophoretic analyses and the evaluation of sensory scores.

The Fig. 1 shows where these PDOs are situated in their origin countries and a specific map of the zone of these PDOs. The different producer mills in each PDO were selected in order to have representative olive oil samples belonging to the area which complies with the specifications in each case.

## 2.4. Solid phase extraction (SPE) procedure

In order to isolate the phenolic fraction, we used SPE with Diol-cartridges; the SPE protocol was carried out with the extraction conditions and amounts of oil which are described in another paper of our research group [42].

Briefly, extra-virgin oil (60 g) was dissolved in 60 ml of hexane and was passed through the column. The solvent goes through, leaving the sample on the solid phase. The cartridge was washed with three portions (of 5 ml) of *n*-hexane, which were then discarded in order to remove the non-polar fraction of the oil. Finally, the sample was recovered by passing through eight portions (of 5 ml) of methanol and brought to dryness in a rotary evaporator under reduced pressure and a temperature of 35ºC. The residue was dissolved with 2 ml of methanol/water (50/50 v/v), diluted 1:8 with methanol/water (50/50 v/v) and filtered through a 0.25 μm filter before the CE analysis.

## 2.5. Sensory characterization

The fruity, bitter and pungent intensities were evaluated according to the European method (EC, Regulation, 2002) [43]. The descriptors of the official panel were set up for describing the most remarkable sensory perceptions for the extra-virgin olive oil samples. A set of positive and negative sensory attributes were evaluated, and the oils were graded on a numerical scale related to the perception of their flavour stimuli, according to the judgement of a group of trained assessors working as a panel.

## 2.6. Electrophoretic procedure

CE separation was made according to the experimental and instrumental conditions described by Carrasco-Pancorbo et al. [39]. Briefly, the separations were carried out on a fused silica capillary (50 μm i.d., 375 μm o.d., total length 47 cm, a detection window was created at 40 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of 100x200 μm). Everytime a new capillary was used it was preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5 min rinse with Milli-Q water and 15 min with buffer. For the following analyses the capillary was rinsed with 0.1 M NaOH for 2 min, followed by a 2 min flush with Milli-Q water to assure good repeatability. The capillary was equilibrated with the running buffer (45 mM sodium tetraborate adjusted to pH 9.3) for 3 min before each sample injection. After each analysis, the capillary tubing was rinsed for 2 min with Milli-Q water. All solutions and samples were filtered through a 0.25 μm syringe filter. The running buffer was changed after 4 runs.

Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 p.s.i.) for 8 s (1 p.s.i.= 6895 Pa).

Electrophoretic separations were performed at 28 kV for 7 min, resulting in a current of ~80 μA. The temperature was maintained at 22ºC.

UV detection was performed in all cases at 200 nm, 240 nm, 280 nm and 340 nm, although diode-array detection was used over the range of 190 to 600 nm to achieve spectral data.

Peak areas and migration velocity (cm/min) were used for the quantification of the analytes versus oleuropein glucoside in some cases (complex phenols and lignans), and peak areas were used for the quantification of simple phenols (TY and HYTY), flavonoids (as sum of luteolin and apigenin (versus the standard of luteolin)) and phenolic acids (trans-cinnamic, 4-hydroxyphenylacetic, sinapic and gentisic acids) versus the corresponding commercial standards.

## 2.7. Statistical analysis

The results reported in this study are the averages of at least three repetitions (n=3), unless otherwise stated. Tukey's honest significant difference (HSD) 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 analyses 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).

## 3. Results and discussion

### 3.1. Determination of phenolic compounds in the extra virgin olive oils belonging to the Italian and Spanish PDOs

#### 3.1.1. Analytical parameters of the method proposed

We carried out a study to establish the calibration curves to quantify the compounds under study and calculate the detection (DL), quantification limit (QL) and precision (as relative standard deviation (RSD) of the intermediate concentration value of linear range) of the method.

Standard calibration graphs for lignans and complex phenols were prepared using oleuropein (oleuropein glucoside) at two wavelengths (200 nm and 240 nm). An external standard methodology (with oleuropein glucoside) was used for the quantification of lignans and complex phenols.

Using this methodology, time-corrected area counts are necessary for quantitative CE. Since separation is dependent on analytes migrating at different rates, the separated analytes will pass the detector at different rates. Time-corrected area counts are the product of the area counts and the velocity for each peak in the electropherogram.

Thus, peak areas and migration velocity (cm/min) were used to calculate the time-corrected area counts to compensate the differences in migration velocities among oleuropein glucoside, lignans and complex phenols.

Lig Agl (a), Pin, Ac Pin, Ol Agl (a) + DOA (a), DOA (b), Lig Agl (b), Ol Agl (b), EA (a), Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b+c) and DOA (d) + EA (d) were

quantified with the data obtained for the calibration curve of oleuropein obtained at 200 nm. However, for the quantification of Lig Agl (d) + Ol Agl (d) + EA (e) the curve of oleuropein at 240 nm was used.

Tyrosol was quantified using the calibration curve of its commercial standard at 200 nm; hydroxytyrosol was quantified with the calibration curve of its commercially available standard at the same wavelength as the other simple phenol (200 nm); phenolic acids were quantified using the calibration curves obtained at 200 nm for commercial standards of 4-hydroxyphenylacetic acid, sinapinic acid, trans-cinnamic acid and gentisic acid respectively, and flavonoids were quantified using the calibration curve of the luteolin (as a sum of luteolin and apigenin). Peak areas were used for the quantification of all these compounds versus the concentration of the corresponding commercial standards.

The detection (DL), quantification limit (QL) and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie [44]. Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. The calibration plots indicate good correlation between peak areas and analyte concentrations in the case of simple phenols, phenolic acids and flavonoids, and between time-corrected area counts and analyte concentrations for the secoiridoids and lignans; regression coefficients were higher than 0.993 in all the cases. All the features of the proposed method are summarized in Table 2.

*3.1.2. Analysis of Italian and Spanish extra-virgin olive oil samples*

Extracts of 25 extra-virgin olive oils were used in the current study. Nine of them were from the Italian PDO and the others from the Spanish PDO (January 2005). All samples were injected in the CE instrument three times (n=3).

Using the described Diol-SPE system and the CZE method, all the virgin olive oils were analyzed. The electrophoretic profiles of an Italian extra virgin olive oil and another Spanish are shown in Fig. 2. The absorbance scales in the electropherograms are not the same, in order to show each electrophrogram at its maximum of absorbance and see

with clarity and ease all the peaks. The quantitative results are presented in Tables 3 (Italian PDO) and 4 (Spanish PDO).

If we observe all the data summarized in these tables, it is impossible to say that all extra-virgin olive oils belonging to the same POD have the same composition concerning phenolic compounds. However, the homogeneity can be found among oils produced in the same mill for making olive oil of the POD (in the case of Spanish oils). For example the results for the samples number 2 and 3 (S.C.A. NTRA. SRA. Nazaret), samples 6, 7 and 8 (belonging to  S.C.A. San Juan de la Cruz ), and the five olive oils from S.C.A. San Francisco (samples 9, 10, 11, 12, 13 and 14) were very similar taking into account the concentration of phenolic compounds.

In spite of the differences found for the phenolic composition of the olive oils belonging to the same POD, we can point out several characteristics which could help to distinguish the olive oils from Chianti Classico and those produced by Sierra de Segura. To compare the Italian oils with those obtained from the Spanish PDO, we also show Table 5 and 6 which contain the quantification in terms of families of phenolic compounds.

Both TY and HYTY were found in higher concentrations in Italian olive oils. TY was in a range between 0.47 and 49.61 mg analyte /kg olive oil in the oils belonging to Chianti Classico PDO, and between 3.42 and 13.45 mg/kg in the case of the Spanish oils. On the other hand, the concentration of HYTY in the Italian oils was found from 4.52 to 84.34 mg/kg, whilst for the Sierra de Segura olive oils its concentration varied between 1.28 and 16.57 mg/kg.

In general, we can say that the concentration of the lignans for the italian oils was higher. The Ac Pin was the responsible of the differences in the lignans concentration, since it was found in the range from 41.65 to 128.18 mg analyte/kg olive oil for the Italian PDO and from 2.27 to 8.53 for the Sierra Segura PDO. Other authors [45] have already evidenced the low Ac Pin content on Spanish virgin olive oil produced from Picual cultivar, in the mentioned work they propose this evidence as a varietal marker. They found Ac Pin in Picual olive oils in a concentration around 2 mg/kg and Pin concentration was of 40 mg/kg (approx).

Several secoiridoid compounds (complex phenols) were found in a higher concentration in the oils from the Italian POD (peaks 6,7,10 and 13). However, peaks 8 and 9 were more abundant in Spanish olive oils; in fact, the peak number 9 (EA (a)) was

not found in any Italian sample, whilst it was found in the all samples belonging to the Spanish PDO in a range that varied from non quantified to 5.84 mg analyte/kg olive oil.

Considering all the secoiridoids that are quantified at 200 nm, the Italian oils were richer than the Spanish, since the concentrations varied from 212.99 to 529.59 mg analyte/kg olive oil in the Chianti Classico oils and from 72.00 to 395.20 mg analyte/kg olive oil in the Sierra de Segura olive oils.

Furthermore, we found in the electrophoretic profiles of all the Italian oils an unknown peak (with migration time of 4.025 min); however, it was not found in the major part of the Spanish oils, and when it was present, the concentration was low.

Concerning individual phenolic acids, their concentrations were (for the four phenolic acids under study) bigger in those oils obtained from the Chianti Classico PDO. When their quantification was carried out in terms of families using the calibration curve of gentisic acid at 200 nm, the ranges found were 4.72-11.35 mg/kg for the Spanish PDO and 7.32-15.69 mg/kg for the Italian one.

The flavonoids were quantified as mg luteolin/kg olive oil considering the sum of the areas of luteolin and apigenin. The quantity of flavonoids present in three samples of each PDO was lower than the minimum that this method can quantify. The tendency for the other samples was that, in general, the Italian oils had higher concentrations than the Spanish oils. The concentration range in the Chianti Classico PDO was from 1.32 to 6.29 mg/kg, whilst in Sierra de Segura PDO was from 0.53 to 2.51 mg/kg.

As commented before, it would be really interesting to find possible "phenolic markers" or a "quantity of a specific phenolic compound" which could distinguish the oils from different PDOs. For this purpose, 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 electrophoretic analyses of the quali- and quantitative-phenolic profile. All the phenolic compounds quantified (18 compounds) have been considered in order to identify the two principal factors. In Table 7 it is possible to see all the 18 variables or loadings; we point out these variables which had factor loadings higher than 0.7 with regard to the single factor (PC1 or PC2). The explained variance of these two factors considering the 18 variables was 50.4%.

After that, a statistical analysis of principal components was carried out keeping in mind only the 8 variables highlighted in the latter table.

A map of samples (score plot) for the two principal components is shown in Figure 3. Samples lying close to each other are similar and samples far from each other are

different regarding the 8 variables considered. It is important to stand out the discriminant capacity of the function of the selected variables to distinguish the samples of the different PDOs; in fact, olive oils belonging to Chianti Classico PDO were found in the first and fourth region of the plot, whilst olive oils from Sierra de Segura PDO were found in the second and third sections.

The explained variance was 75.1%; the horizontal axis (PC1) describes more than 58% of the variation in the data.

*3.2. Correlation among the sensory characteristics of the oils and the phenolic compounds present in their extracts*

As commented before, the fruity, bitter and pungent intensities were evaluated according to the European method. The descriptors of the official panel were set up for describing the most remarkable sensory perceptions for the extra-virgin olive oil samples.

The importance of the phenolic compounds in connection with the sensory attributes of an olive oil has been previously studied. Various secoiridoid derivatives of hydroxytyrosol and tyrosol have been shown to contribute to the bitterness of olive oil [24,27,46,47,48]. In a study carried out by Mateos et al. [49] correlations between bitterness and concentrations of secoiridoid derivatives were determined, with a linear relationship found between bitterness and the aldehydic form of oleuropein aglycone (3,4-DHPEA-EA). García et al. [47] studied the reduction of oil bitternes by heating of olive fruits, and a good correlation between oil bitterness and content of hydroxytyrosol secoiridoid derivatives was found. Furthermore, it was demonstrated by Andrewes et al [27] that the pungency of virgin olive oil is caused by decarboxymethyl ligstroside aglycone (p-HPEA-EDA), a finding which has recently been confirmed in an independent study of Beauchamp and co-workers [50,51]. They attributed ibuprofen-like activity to p-HPEA-EDA in this study and speculated on the potential specific health effects of this aglycone [50].

Moreover, for instance, several authors associated the off-flavour note of "fusty (atrojado)" with the presence of phenolic acids in VOO [52], but other studies did not show relation between bitter sensory note and phenolic acid content in a VOO [53].

In this research work we carried out a statistical study considering the fruity, bitter and pungent intensities of each oil and their relation with the concentration of the 18

phenolic compounds quantified. If the Table 8 is observed, it is possible to see that the peak 6 (DOA (b)) is the compound more related with the bitterness of the oils (r=0.61 for p<0.05). This fact is in good agreement with the findings obtained by several authors [24]. In that case, they concluded that the peaks corresponding to the dialdehydic and aldehydic forms of decarboxymethyl oleuropein aglycone and the dialdehydic form of decarboxymethyl ligstroside aglycone were those mainly responsible for the bitter taste of virgin olive oil. The correlation was higher than in our study, fact that can be caused, in our opinion, because in that case all the olive oils under study were Spanish.

Regarding the correlation with the attribute "pungency", the values for Ac Pin (peak number 4) and the unidentified peak with $t_{mig}$ 4.025 min (peak *) were the highest. Other authors, however, have found the compound decarboxymethyl ligstroside aglycon (also named oleocanthal) as a key contributor to pungency [27,50]. This compound has not still been identified in the electrophoretic profiles described in the current paper; this topic will be taken into account as future direction.

Considering the correlation with the fruitiness of the oils, we can stand out the compound Ac Pin which had the highest value. Other compounds with considerable correlation were the peak with migration time 4.025 min and the peak number 13. The correlation was also significant (with negative sign) in the case of peak 9.


## 4. Conclusions


Apparently, this is the first time in which an electrophoretic method demonstrates to have the ability to detect and quantify simultaneously so many phenolic compounds present in olive oil belonging to five different families. The quantitative data obtained in this study are in good agreement with those previously reported by using HPLC. This technique could improve the characterization of this polar fraction and determine the geographical origin of olive oils or detect possible "POD markers". Furthermore, CE joined to statistical analysis permits the discrimination among olive oils belonging to two different PDOs.

A statistical study considering three sensory atributes of each oil and their relation with the concentration of the 18 phenolic compounds quantified was carried out as well, and DOA (b) (peak 6) was the compound more related with the bitterness of the oils, Ac Pin (peak number 4) and the unidentified peak with $t_{mig}$ 4.025 min played an important

role in the pungent taste of the oils, and these two latter compounds and the peak 13 seemed to be related with the fruitiness of the oils.

The results shown in this paper could clarify the relationship among polyphenols and olive oil pungency, bitterness and fruitiness.

## 5. Abbreviations used

**TY**: tyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **HYTY**: hydroxytyrosol; **Apig,** apigenina**; and Lut,** luteolin**.**

## 6. Acknowledgements

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Figure 1

a)



a<sub>1</sub>)



b)



b<sub>1</sub>)

Figure 2

Figure 3



Projection of the cases on the factor-plane (PC1 x PC2)
Labelling variable: Country (S=Spain, I=Italy)
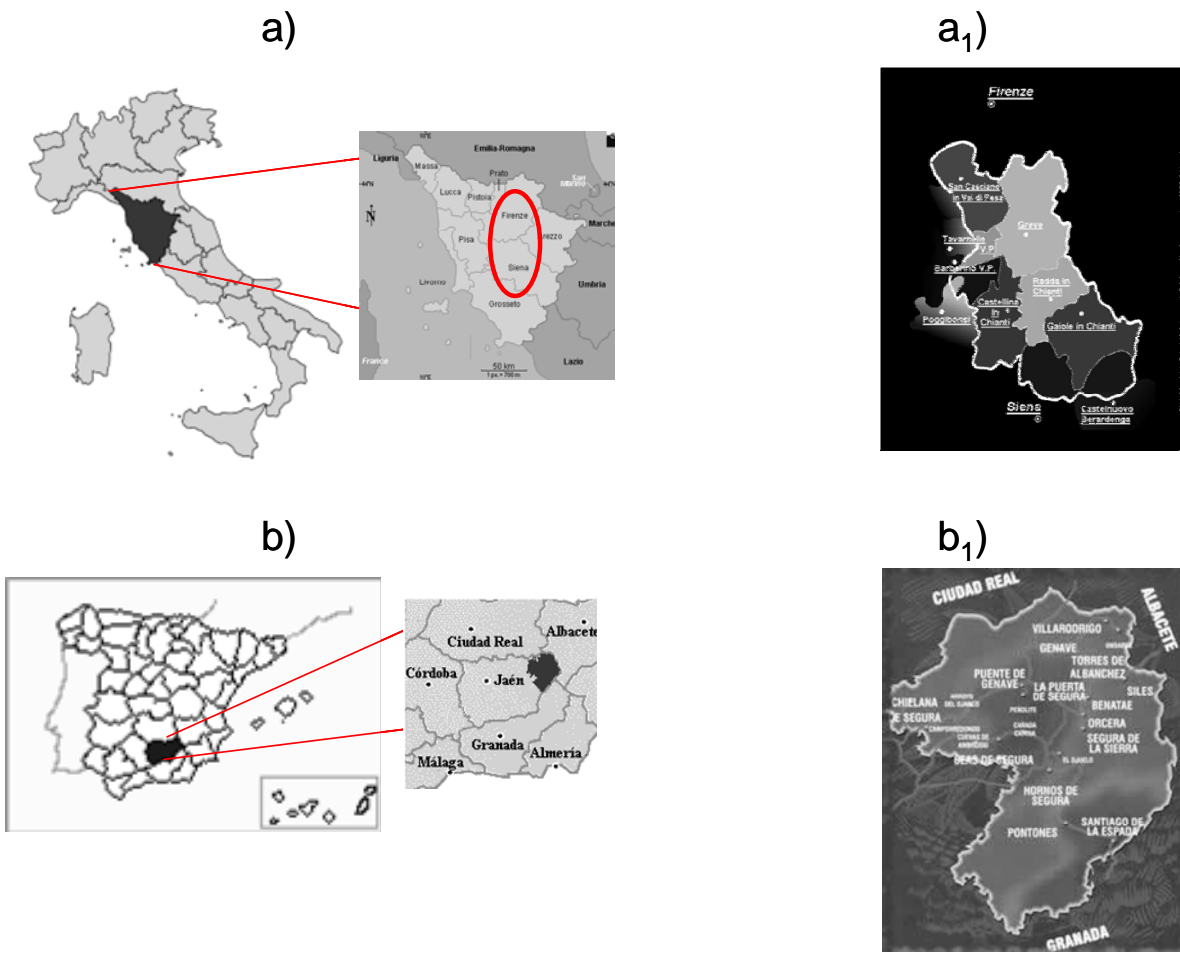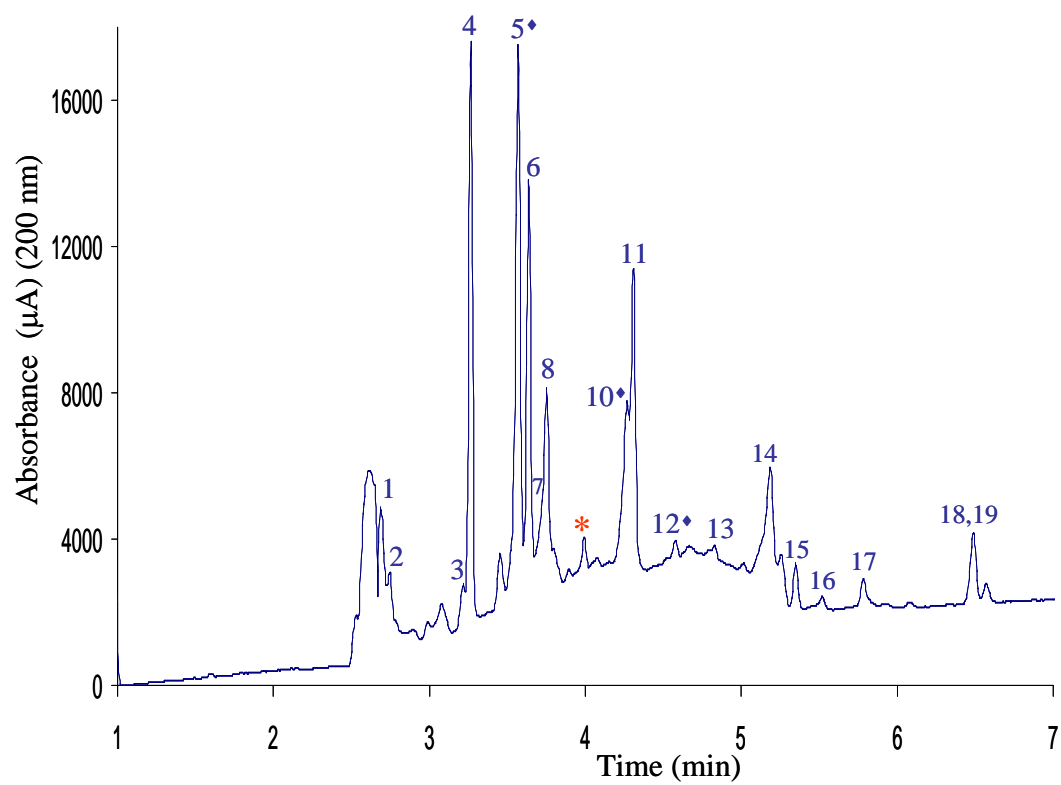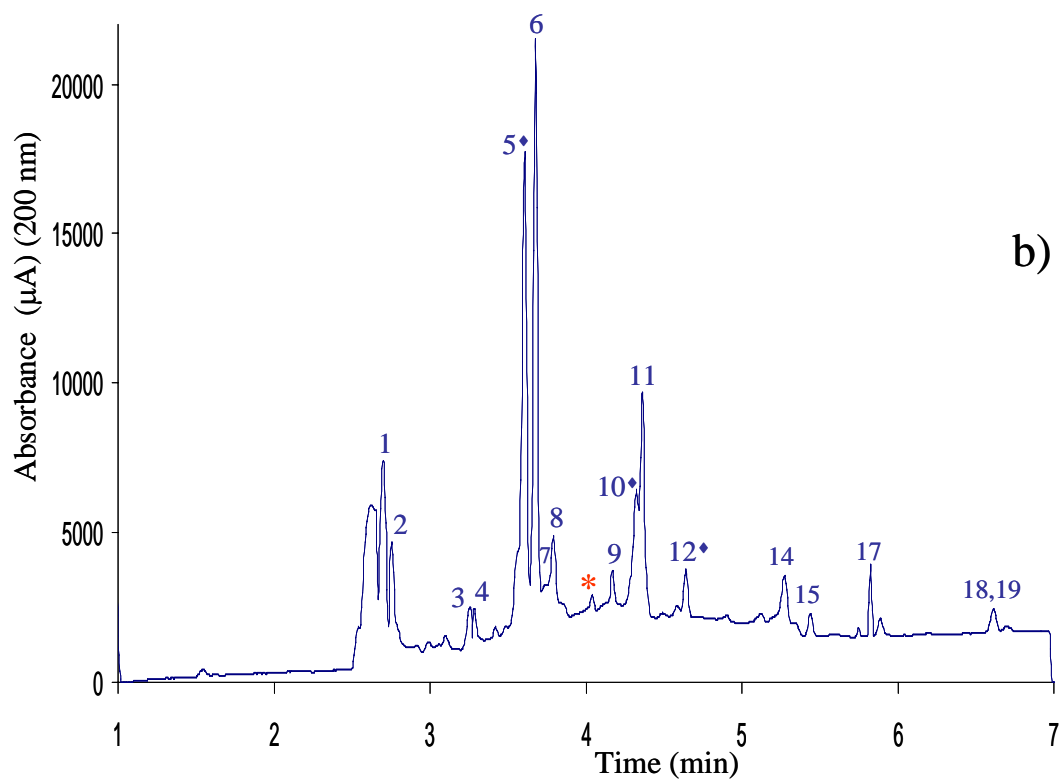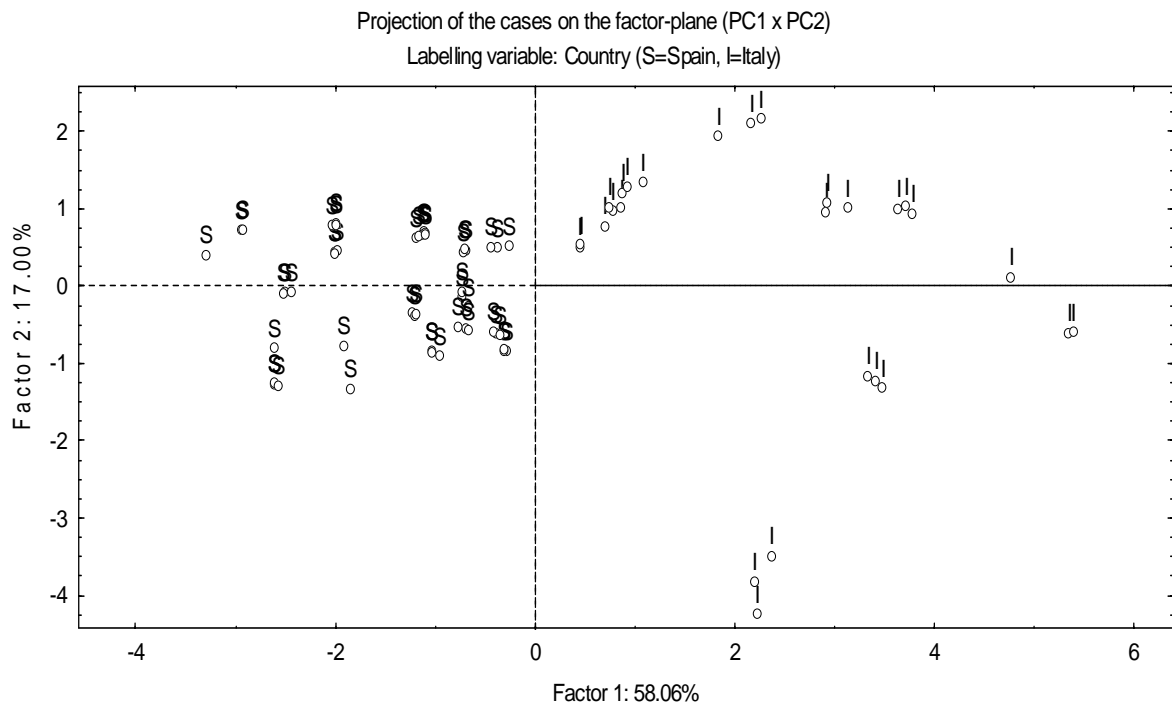
**Caption to figures**

Fig. 1. Geographical location of the two PDOs under study in their origin countries and specific maps of the area where each PDO is located.

Fig. 2. Electrophoretic profiles obtained at optima conditions for olive oils of the two PDOs under study. a) Extract of an extra-virgin olive oil produced in Azienda Giacomo Grassi (Chianti Classico PDO), *sample CC2*; b) extract of an extra-virgin olive oil produced in S. C. A. San Francisco (Sierra de Segura PDO), *sample SS13*.

Separation conditions: capillary, 47 cm (40 cm of effective length) x 50 μm of i.d.; applied voltage, 28 kV; applied temperature, 22 ºC; buffer, 45 mM Sodium tetraborate (pH 9.30); hydrodynamic injection, 0.5 p.s.i. for 8 s. Detection was performed at 200 nm.

Peak identification numbers: **1**, Lig Agl (a); **2**, TY, **3**, Pin; **4**, Ac Pin; **5**, Ol Agl (a) + DOA (a); **6**, DOA (b); **7**, Lig Agl (b); **8**, Ol Agl (b); **\***, $t_{mig}$ 4.025 min; **9**, EA (a); **10**, Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b,c); **11**, HYTY; **12**, DOA (d) + EA (d); **13**, EA (e); **14**, trans-cinnamic acid; **15**, 4-hydroxyphenylacetic acid; **16**, sinapinic acid; **17**, gentisic acid; **18**, luteolin; and **19**, apigenin.

♦ Peaks overlapped

Fig. 3. Score plot for the two principal components.

Table 1. Codes for the Italian and Spanish samples analyzed in this study.

| PDO | Mills for making olive oil | Code |
|---|---|---|
| *Chianti Classico (CC)* | Castello di Ama | CC1 |
| | Azienda Giacomo Grassi | CC2 |
| | Fattoria le Fonti | CC3 |
| | Castello di Gabbiano | CC4 |
| | Villa Branca | CC5 |
| | Il Casalone | CC6 |
| | Castello di Cacchiano | CC7 |
| | Roca di Castagnoli | CC8 |
| | Poggio Torselli | CC9 |
| | | |
| *Sierra de Segura (SS)* | S.C.A. Agrosegura | SS1 |
| | Potosí 10 S.A. | SS2 |
| | | SS3 |
| | S.C.A. NTRA. SRA. Nazaret | SS4 |
| | | SS5 |
| | S.C.A. San Juan de la Cruz | SS6 |
| | | SS7 |
| | | SS8 |
| | S.C.A. San Francisco | SS9 |
| | | SS10 |
| | | SS11 |
| | | SS12 |
| | | SS13 |
| | | SS14 |
| | Aceites El Carrascal S.A. | SS15 |
| | S.C.A. Virgen del Campo de Génave | SS16 |

Table 2

Analytical parameters of proposed method. y=bx±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. (*y=bx±a, where y is the (Time corrected area counts=peak area (AU) x migration velocity (cm/min)).

| Analyte | RSD (%) (intermediate value) | Detection limit (DL) (μg/ml) | Quantification limit (QL) (μg/ml) | Calibration range(μg/ml) | Calibration equations | $r^2$ |
|---|---|---|---|---|---|---|
| **Tyrosol** | 2.04 | 0.660 | 2.201 | QL-500 | y = 226.12x + 9685.4 | 0.993 |
| **Hydroxytyrosol** | 2.34 | 0.307 | 1.023 | QL-300 | y = 486.65x + 2438.7 | 0.996 |
| **\*Oleuropein glucoside λ=200 nm** | 2.23 | 0.110 | 0.367 | QL-2000 | y = 1358.4x + 21989 | 0.995 |
| **\*Oleuropein glucoside λ=240 nm** | 1.53 | 0.288 | 0.960 | QL-2000 | y = 518.89x – 10651 | 0.995 |
| **Trans-cinnamic acid** | 1.89 | 0.446 | 1.487 | QL-100 | y = 335.12x – 559.32 | 0.997 |
| **4-HFA acid** | 1.67 | 0.241 | 0.803 | QL-100 | y = 619.75x – 1178.2 | 0.998 |
| **Sinapic acid** | 3.24 | 0.484 | 1.613 | QL-100 | y = 308.45x – 754.11 | 0.994 |
| **Gentisc acid** | 3.13 | 0.291 | 0.971 | QL-100 | y = 513.95x – 1675.4 | 0.994 |
| **Luteolin** | 1.91 | 0.300 | 0.998 | QL-100 | y = 498.79x + 1184.3 | 0.998 |

RSD: Relative Standard Deviation        LD=3$\sigma_b$/b ($\sigma_b$= 49.78, calculated using 50 data); LQ=10$\sigma_b$/b

Table 3. Results of the analysis of Italian samples. Quantification of the individual components (n=3)

($Value = \overline{X}$ ) (in all the cases SD was lower than 5% of the mean value)

**mg/kg = mg analyte/kg olive oil**

| Analyte | CC1 | CC2 | CC3 | CC4 | CC5 | CC6 | CC7 | CC8 | CC9 |
|---|---|---|---|---|---|---|---|---|---|
| **Lig Agl (a) [2]** | 94.77 | 52.75 | 124.21 | 106.50 | 107.00 | 65.15 | 82.04 | 114.24 | 129.91 |
| **TY [1]** | 3.76 | 0.47 | 4.84 | 11.78 | 9.35 | 1.29 | 6.52 | 7.07 | 49.61 |
| **Pin [2]** | 2.77 | 3.19 | 3.17 | 4.16 | 11.78 | 3.81 | 6.87 | 0.01 | 0.02 |
| **Ac Pin [2]** | 59.72 | 75.42 | 128.18 | 84.27 | 41.65 | 69.36 | 44.74 | 46.78 | 86.08 |
| **Ol Agl (a) + DOA (a) [2]** | 85.52 | 87.42 | 157.08 | 56.21 | 81.14 | 57.49 | 64.39 | 44.38 | 75.41 |
| **DOA (b) [2]** | 146.76 | 50.77 | 147.39 | 149.51 | 294.93 | 82.67 | 50.95 | 114.09 | 104.56 |
| **Lig Agl (b) [2]** | 29.38 | 33.23 | 53.46 | 11.25 | 14.04 | 16.87 | 3.28 | 10.68 | 13.04 |
| **Ol Agl (b) [2]** | 0.16 | 0.33 | 4.88 | 0.26 | 0.35 | 0.46 | 2.05 | 0.19 | 0.28 |
| **Min 4.025 (*)[2]** | 9.66 | 1.46 | 11.00 | 7.75 | 5.59 | 2.98 | 4.97 | 2.60 | 3.35 |
| **EA (a) [2]** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Ol Agl (c)+Lig Agl (c)+DOA (c)+EA(b,c) [2]** | 24.66 | 22.00 | 34.39 | 21.99 | 14.30 | 7.03 | 9.78 | 8.98 | 27.38 |
| **HYTY[4]** | 13.22 | 10.03 | 15.76 | 39.85 | 11.27 | 4.52 | 9.12 | 5.64 | 84.34 |
| **DOA (d) + EA (d) [2]** | 0.29 | n.q. | 8.18 | 0.25 | 0.15 | 0.16 | 0.50 | 1.55 | 0.30 |
| **EA (e) + Lig Agl (d) + Ol Agl (d) [3]** | 122.43 | 158.31 | 167.16 | 82.77 | 62.89 | 76.61 | 82.77 | 71.30 | 122.45 |
| **Trans-cinnamic[5]** | 7.52 | 12.49 | 3.82 | 2.00 | 0.95 | 1.55 | 0.56 | 1.45 | 1.55 |
| **4-HFA[6]** | 1.54 | 2.25 | 2.30 | 1.87 | 1.05 | 1.17 | 0.85 | 1.19 | 1.12 |
| **Sinapic[7]** | 1.46 | 1.36 | 1.30 | 1.03 | 3.07 | 1.90 | 3.32 | 0.81 | 0.85 |
| **Gentisic[8]** | 3.75 | 2.39 | 3.65 | 4.62 | 6.42 | 2.44 | 2.29 | 2.97 | 6.03 |

**TY**: tyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **HYTY**: hydroxytyrosol.

[1]: quantified with a calibration curve of tyrosol at λ=200 nm.

[2]: quantified with a calibration curve of Oleuropein glucoside at λ=200 nm. (Time corrected area counts vs concentration)

[3]: quantified with a calibration curve of Oleuropein glucoside at λ=240 nm. (Time corrected area counts vs concentration)

[4]: quantified with a calibration curve of hydroxytyrosol at λ=200 nm.

[5]: quantified with a calibration curve of trans-cinnamic acid at λ=200 nm.

[6]: quantified with a calibration curve of 4-HFA at λ=200 nm.

[7]: quantified with a calibration curve of Sinapic acid at λ=200 nm.

[8]: quantified with a calibration curve of Gentisic at λ=200 nm.

n.d.: non detected

n.q.: non quantified

Table 4. Results of the analysis of Spanish samples. Quantification of the individual components (n=3)

($Value = \overline{X}$) (in all the cases SD was lower than 5% of the mean value)

## mg/kg = mg analyte/kg olive oil

| Analyte | SS1 | SS2 | SS3 | SS4 | SS5 | SS6 | SS7 | SS8 | SS9 | SS10 | SS11 | SS12 | SS13 | SS14 | SS15 | SS16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lig Agl (a) [2] | 85.40 | 34.18 | 50.58 | 33.67 | 76.52 | 100.65 | 94.41 | 64.22 | 100.97 | 65.20 | 79.31 | 75.56 | 79.13 | 79.59 | 78.45 | 33.19 |
| TY [1] | 13.45 | 3.42 | 5.16 | 4.12 | 7.05 | 6.82 | 6.47 | 7.48 | 5.98 | 5.50 | 7.95 | 7.30 | 6.17 | 6.33 | 3.98 | 7.61 |
| Pin [2] | n.q. | 5.05 | 4.44 | 1.03 | 2.16 | 1.32 | 4.29 | 2.61 | 14.09 | 4.59 | 3.06 | 1.68 | 6.04 | 1.64 | n.q. | n.q. |
| Ac Pin [2] | n.q. | 2.78 | 11.01 | 5.21 | 1.81 | 5.09 | 2.27 | 3.98 | 4.09 | 5.61 | 3.50 | 5.29 | 5.41 | 8.53 | n.q. | n.q. |
| Ol Agl (a) + DOA (a) [2] | 124.03 | 54.62 | 70.79 | n.q. | 152.14 | 83.37 | 91.00 | 85.20 | 116.89 | 66.75 | 83.49 | 85.61 | 80.37 | 90.47 | 56.04 | n.q. |
| DOA (b) [2] | 16.83 | 40.38 | 40.65 | 29.52 | 115.69 | 68.28 | 77.41 | 59.67 | 134.16 | 64.62 | 80.84 | 87.54 | 91.69 | 102.81 | 25.07 | 38.56 |
| Lig Agl (b) [2] | 1.48 | 15.15 | 29.01 | 6.14 | 14.11 | 29.96 | 28.00 | 9.35 | 20.35 | 6.25 | 10.90 | 8.15 | 1.30 | 12.64 | 11.56 | n.q. |
| Ol Agl (b) [2] | 10.20 | n.q. | n.q. | n.q. | 0.01 | n.q. | 0.12 | 0.08 | n.q. | n.q. | n.q. | n.q. | 12.70 | n.q. | n.q. | n.d. |
| Min 4.025 (*) [2] | 5.32 | n.d. | n.q. | n.q. | n.q. | n.d. | 0.98 | 0.87 | n.q. | n.q. | 1.52 | 1.32 | 1.23 | 1.16 | n.q. | n.q. |
| EA (a) [2] | n.d. | 1.30 | 0.78 | n.q. | 0.86 | n.q. | 0.91 | 3.28 | 0.97 | 0.19 | 4.67 | 5.84 | 3.56 | 5.53 | n.q. | n.q. |
| Ol Agl (c)+Lig Agl (c)+DOA (c)+EA(b,c) [2] | 21.41 | 8.87 | 9.77 | 8.39 | 24.83 | 18.83 | 18.84 | 17.91 | 19.61 | 9.19 | 8.75 | 10.70 | 20.04 | 21.96 | 6.44 | 0.25 |
| HYTY [4] | 16.57 | 2.22 | 3.23 | 5.96 | 9.46 | 2.86 | 7.55 | 9.63 | 6.18 | 4.37 | 9.52 | 9.63 | 8.59 | 9.38 | 1.50 | 1.28 |
| DOA (d) + EA (d) [2] | 5.33 | 0.15 | 0.11 | 4.30 | 2.65 | n.q. | 0.22 | 1.20 | 2.25 | 1.15 | 4.96 | 3.03 | 0.45 | 1.51 | 0.30 | n.q. |
| EA (e) + Lig Agl (d) + Ol Agl (d) [3] | 61.51 | 34.18 | 33.31 | 32.82 | 88.85 | 72.45 | 66.98 | 62.09 | 71.12 | 34.18 | 79.43 | 69.99 | 75.01 | 73.78 | 47.85 | 31.44 |
| Trans-cinnamic [5] | 0.79 | 5.25 | 4.83 | 2.07 | 0.61 | 4.05 | 4.08 | 2.55 | 0.81 | 1.60 | 0.45 | 0.56 | 5.09 | 3.78 | 0.49 | 0.38 |
| 4-HFA [6] | 5.73 | 0.99 | 0.74 | 0.82 | 0.58 | 0.63 | 1.00 | 1.05 | 0.65 | 1.11 | 0.51 | 0.69 | 0.88 | 0.82 | 0.81 | 0.45 |
| Sinapic [7] | 0.78 | 1.08 | 1.19 | 1.43 | 2.52 | 0.81 | 0.85 | 0.67 | 2.32 | 0.76 | 2.74 | 2.59 | 2.50 | 2.79 | 0.75 | 0.78 |
| Gentisc [8] | 1.79 | n.q. | n.q. | 1.24 | 1.60 | n.q. | 1.45 | 1.51 | 1.92 | 1.58 | 1.94 | 1.97 | 2.80 | 2.27 | 1.17 | 1.44 |

**TY**: tyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **HYTY**: hydroxytyrosol.

[1]: quantified with a calibration curve of tyrosol at λ=200 nm.

[2]: quantified with a calibration curve of Oleuropein glucoside at λ=200 nm. (Time corrected area counts vs concentration)

[3]: quantified with a calibration curve of Oleuropein glucoside at λ=240 nm. (Time corrected area counts vs concentration)

[4]: quantified with a calibration curve of hydroxytyrosol at λ=200 nm.

[5]: quantified with a calibration curve of trans-cinnamic acid at λ=200 nm.

[6]: quantified with a calibration curve of 4-HFA at λ=200 nm.

[7]: quantified with a calibration curve of Sinapic acid at λ=200 nm.

[8]: quantified with a calibration curve of Gentisic at λ=200 nm.

n.d.: non detected

n.q.: non quantified

Table 5. Results for the analysis of Italian samples. Quantification in terms of families of phenolic compounds (n=3) (*Value* = $\overline{X}$ ) (in all the cases SD was lower than 5% of the mean value)

**mg/kg = mg analyte/kg olive oil**

| *Family of compounds* | CC1 | CC2 | CC3 | CC4 | CC5 | CC6 | CC7 | CC8 | CC9 |
|---|---|---|---|---|---|---|---|---|---|
| Simple phenols | 28.89 | 19.20 | 35.10 | 90.24 | 30.57 | 8.98 | 23.42 | 17.02 | 217.19 |
| Complex phenols(A) | 381.25 | 246.50 | 529.59 | 345.97 | 511.91 | 229.83 | 212.99 | 294.11 | 350.88 |
| Complex phenols(B) | 122.43 | 158.31 | 167.16 | 82.77 | 62.89 | 76.61 | 82.77 | 71.30 | 122.45 |
| Lignans | 62.49 | 78.61 | 131.35 | 88.43 | 53.43 | 73.17 | 51.61 | 46.79 | 86.10 |
| Phenolic acids | 13.04 | 15.69 | 11.33 | 10.45 | 11.79 | 7.66 | 7.32 | 7.47 | 10.53 |
| Flavonoids | 1.32 | 6.29 | 5.36 | 3.21 | n.q. | n.q. | n.q. | 2.26 | 3.26 |

**TY**: tyrosol; **HYTY**: hydroxytyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **Lut**: luteolin; **Apig**: apigenin.

**Simple phenols** = TY + HYTY (quantified with a calibration curve of tyrosol at λ=200 nm)
**Complex Phenols** = (A) Secoiridoid forms quantified with the calibration curve of Oleuropein glucoside at λ=200 nm
                 (B) Secoiridoid forms quantified with the calibration curve of Oleuropein glucoside at λ=240 nm
**Lignans** = Ac Pin + Pin (quantified with a calibration curve of Oleuropein glucoside at λ=200 nm)
**Phenolic acids** = Trans-cinnamic acid + 4-HFA + Sinapic acid + Gentisic acid (quantified with a calibration curve of Gentisic acid at λ=200 nm)
**Flavonoids** = Lut + Apig (quantified with a calibration curve of Luteolin at λ=200 nm)

Table 6. Results of the analysis of Spanish samples. Quantification in terms of families of phenolic compounds (n=3) ($Value = \overline{X}$ ) (in all the cases SD was lower than 5% of the mean value)

**mg/kg = mg analyte/kg olive oil**

| Family of compounds | SS1 | SS2 | SS3 | SS4 | SS5 | SS6 | SS7 | SS8 | SS9 | SS10 | SS11 | SS12 | SS13 | SS14 | SS15 | SS16 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Simple phenols | 45.29 | 6.52 | 10.27 | 14.71 | 24.65 | 11.19 | 20.25 | 25.42 | 17.00 | 12.90 | 25.66 | 25.25 | 22.01 | 23.77 | 5.64 | 8.82 |
| Complex phenols(A) | 264.68 | 118.65 | 201.69 | 82.02 | 386.81 | 301.09 | 310.91 | 240.91 | 395.20 | 213.35 | 272.92 | 276.43 | 289.24 | 314.51 | 177.86 | 72.00 |
| Complex phenols(B) | 61.51 | 34.18 | 33.31 | 32.82 | 88.85 | 72.45 | 66.98 | 62.09 | 71.12 | 34.18 | 79.43 | 69.99 | 75.01 | 73.78 | 47.85 | 31.44 |
| Lignans | 7.86* | 7.83 | 15.45 | 6.24 | 3.97 | 6.41 | 6.56 | 6.59 | 18.18 | 10.02 | 6.56 | 6.97 | 11.45 | 10.17 | 8.45* | 5.45* |
| Phenolic acids | 11.35 | 6.92 | 6.41 | 6.07 | 5.88 | 5.54 | 7.46 | 6.57 | 6.23 | 6.10 | 6.35 | 6.29 | 10.33 | 9.11 | 4.72 | 4.70 |
| Flavonoids | n.q. | n.q. | 1.15 | 0.79 | 1.39 | 0.88 | 1.13 | 1.10 | 2.51 | 0.91 | 1.47 | 1.43 | 1.79 | 1.38 | 0.53 | n.q. |

**TY**: tyrosol; **HYTY**: hydroxytyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **Lut**: luteolin; **Apig**: apigenin.

**Simple phenols** = TY + HYTY (quantified with a calibration curve of tyrosol at $\lambda$=200 nm)
**Complex Phenols** = (A) Secoiridoid forms quantified with the calibration curve of Oleuropein glucoside at $\lambda$=200 nm
        (B) Secoiridoid forms quantified with the calibration curve of Oleuropein glucoside at $\lambda$=240 nm
**Lignans** = Ac Pin + Pin (quantified with a calibration curve of Oleuropein glucoside at $\lambda$=200 nm)
       *Only quantified in term of family of lignans (not individually because of the overlapping)
**Phenolic acids** = Trans-cinnamic acid + 4-HFA + Sinapic acid + Gentisic acid (quantified with a calibration curve of Gentisic acid at $\lambda$=200 nm)
**Flavonoids** = Lut + Apig (quantified with a calibration curve of Luteolin at $\lambda$=200 nm)

Table 7. Table that shows the 18 variables which have been considered to define the 2 principal factors.

**Method for factor extraction:**
Missing data were substituted by means
  75 cases were processed (selected)
  75 valid cases were accepted
  Correlation matrix was computed for 18 variables
  Maximum no. of factors 2
  Minimum eigenvalue 2

*Factor Loadings (Unrotated)*
*Extraction: Principal components*
*(Marked loadings are >0.7)*

| Variable | Factor 1 | Factor 2 |
|---|---|---|
| LigAgl | **-0.747** | 0.353 |
| TY | -0.306 | 0.319 |
| Pin | -0.204 | 0.423 |
| AcPin | **-0.852** | -0.105 |
| Ol Agl (a)+DOA(a) | -0.552 | -0.084 |
| DOA | -0.630 | 0.547 |
| LigAgl (b) | -0.542 | -0.480 |
| OlAgl (b) | -0.203 | -0.011 |
| $t_{mig}$ 4.025 | **-0.809** | 0.087 |
| EA | **-0.782** | 0.069 |
| Ol Agl (c)+LigA(c)+DOA (c)+EA(b.c) | **-0.736** | -0.154 |
| HYTY | -0,634 | 0,075 |
| DOA(d)+EA(d) | -0.268 | -0.698 |
| EA(e)+LigAgl(d)+OlAgl(d) | **-0.854** | -0.339 |
| Trans-cinnamic | -0.301 | **-0.747** |
| 4-HFA | -0.349 | -0.332 |
| Sinapic | -0.124 | 0.480 |
| Gentisic | **-0.761** | 0.463 |
| Expl.Var | 5.870 | 2.706 |
| Prp.Totl | 0.345 | 0.159 |

Table 8. Correlation among the attributes "fruity, bitter and pungent" and the concentration of the 18 phenolic compounds determined in this study.

| | fruity | | bitter | | pungent | |
|---|---|---|---|---|---|---|
| Lig Agl (a) | ,3011 | p=,144 | ,3863 | p=,056 | ,1769 | p=,397 |
| TY | ,0637 | p=,762 | -,0091 | p=,966 | -,2731 | p=,187 |
| Pin | ,2645 | p=,201 | **,4018** | **p=,046** | ,2702 | p=,191 |
| Ac Pin | **,7561** | **p=,000** | ,3802 | p=,061 | **,6661** | **p=,000** |
| Ol Agl (a) + DOA (a) | ,1928 | p=,356 | ,3373 | p=,099 | ,1960 | p=,348 |
| DOA (b) | **,4578** | **p=,021** | **,6187** | **p=,001** | **,4007** | **p=,047** |
| Lig Agl (b) | **,5009** | **p=,011** | ,3894 | p=,054 | **,5044** | **p=,010** |
| Ol Agl (b) | -,1610 | p=,442 | -,1538 | p=,463 | ,1178 | p=,575 |
| Min 4.025 (*) | **,6077** | **p=,001** | **,5442** | **p=,005** | **,6595** | **p=,000** |
| EA (a) | **-,5656** | **p=,003** | -,3950 | p=,051 | -,3628 | p=,075 |
| Ol Agl (c)+Lig Agl (c)+DOA (c)+EA(b,c) | ,2881 | p=,163 | **,4340** | **p=,030** | ,3457 | p=,090 |
| HYTY | ,3329 | p=,104 | ,1331 | p=,526 | ,1199 | p=,568 |
| DOA (d) + EA (d) | -,1795 | p=,391 | ,0448 | p=,831 | -,0613 | p=,771 |
| EA (e) + Lig Agl (d) + Ol Agl (d) | **,5342** | **p=,006** | **,4216** | **p=,036** | **,5624** | **p=,003** |
| Trans-cinnamic | ,3282 | p=,109 | ,2443 | p=,239 | ,3958 | p=,050 |
| 4-HFA | ,0632 | p=,764 | -,0387 | p=,854 | ,0568 | p=,787 |
| Sinapic | ,0709 | p=,736 | **,4160** | **p=,039** | ,2580 | p=,213 |
| Gentisc | **,4708** | **p=,018** | **,4330** | **p=,031** | ,3541 | p=,082 |

The represented values are "r". The positive correlations are marked in boldface letter.
Correlations (Spreadsheet7) Marked correlations are significant at p < ,05000 N=25 (Casewise deletion of missing data)

# Capítulo 9

## Evaluación de la influencia de la oxidación térmica en la composición fenólica y en la actividad antioxidante de aceites de oliva virgen-extra

El consumo de alimentos fritos es una práctica que se realiza desde la antigüedad, especialmente en los países mediterráneos. La fritura de alimentos en baño de aceite, favorecida en parte por el aumento de consumo de comidas preparadas o precocinadas, se ha convertido en los últimos años en una de las técnicas culinarias más extendidas del mundo. De todos los posibles, el aceite de oliva es el producto que ha demostrado mayor idoneidad para la frituras.

La fritura es un proceso culinario que consiste en introducir un alimento en un baño de aceite o grasa caliente a temperaturas elevadas (150-200º C), donde el aceite actúa de transmisor del calor produciendo un calentamiento rápido y uniforme del producto. Con el aumento de la temperatura se aceleran todos los procesos químicos y enzimáticos. Por lo tanto, una grasa o aceite calentados se degradan con bastante rapidez, sobre todo si hay residuos que potencian las reacciones de alteración actuando como catalizadores. Los principales cambios y alteraciones químicas que los aceites calentados pueden sufrir son: la hidrólisis que provoca la ruptura del enlace éster de los triglicéridos; la oxidación y auto-oxidación que consiste en la acción del oxígeno sobre los ácidos grasos; la termo-oxidación que se produce por el efecto de las elevadas temperatura, de modo que se favorece aún más la alteración oxidativa; y la polimerización, dada por la presencia de radicales libres que se combinan entre sí o con los ácidos grasos forman polímeros lineales o cíclicos.

Muchos investigadores han estudiado los compuestos fenólicos en aceite de oliva virgen crudo o incluso después de un almacenamiento largo; también han intentado determinar la influencia de los polifenoles en la estabilidad y propiedades organolépticas de los aceites, así como sus beneficios nutricionales; sin embargo, no hay muchos datos disponibles acerca de cómo varía el perfil polifenólico de un aceite después de someterlo a un tratamiento consistente en alta temperatura durante un tiempo variable, es decir, un tratamiento muy similar a lo que se definiría como una fritura en tecnología de los alimentos. Además, en los casos en los que se dispone de esta información, se obtuvo la misma mediante el empleo de métodos colorimétricos y con HPLC.

Por ello, nos pareció muy interesante comparar los resultados obtenidos mediante HPLC-UV, HPLC-MS y CE-UV para caracterizar el deterioro del aceite de oliva virgen extra durante un calentamiento que simulara la temperatura de fritura (180º C) durante

diferentes intervalos de tiempo. Se determinó la concentración de compuestos fenólicos pertenecientes a cuatro familias (fenoles simples, lignanos, fenoles complejos y ácidos fenólicos) mediante ambas técnicas. Del mismo modo, se observó la formación de "nuevos" compuestos ("unkonwn") que se podían detectar en los perfiles de los aceites sometidos al tratamiento; estos compuestos se estudiaron mediante HPLC-MS y se cuantificaron. El índice de peróxidos y la capacidad antioxidante de la fracción fenólica (mediante el test OSI) se determinaron tras cada intervalo del tratamiento térmico.

# Evaluation of the Influence of Thermal Oxidation in the Phenolic Composition and in Antioxidant Activity of Extra-Virgin Olive Oils

ALEGRÍA Carrasco-Pancorbo[1], LORENZO Cerretani[2*], ALESSANDRA Bendini[2], ANTONIO Segura-Carretero[1], GIOVANNI Lercker[2], and ALBERTO Fernández-Gutiérrez[1].


[1] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.


[2] Department of Food Science, University of Bologna, P.zza Goidanich 60, 47023 Cesena (FC), Italy.



**Running title header**: Effect of Thermal Treatment in Phenols from Olive Oils


[*] Author to whom correspondence should be addressed: Tel. +390547338121 Fax +390547382348

e-mail: lorenzo.cerretani2@unibo.it

## ABSTRACT

A comparison between the results obtained by using HPLC-UV, HPLC-MS and CE-UV for characterizing the deterioration of extra-virgin olive oil during heating, simulating frying temperature, was investigated. The polar extracts of a commercial extra-virgin olive oils were analyzed by both techniques to determine changes in the phenolic compounds present in the oil as a result of heating at 180 ºC during six time intervals (30-180 min). The concentration of several compounds belonging to four families of phenols (simple phenols, lignans, complex phenols and phenolic acids) was determined in the samples after the thermal treatment by both techniques. Several "unknown" compounds were determined in the phenolic profiles of the oils after the thermal treatment and their presence was confirmed in refined olive oils.

The antioxidant activity performed by the phenolic fraction of virgin olive oil was indirectly measured by the OSI test and, for each oil sample, we also determined the peroxide value.

## INTRODUCTION

Besides its fatty acid composition, virgin olive oil contains many minor compounds with a great importance among which polyphenols stand out (1,2,3]). These phenolic compounds have been reported as influencing sensory quality (4,5,6,7), as having beneficial biological activity (8,9,10,11) and oxidative stability of olive oil (12,13,14). The major phenolic compounds identified and quantified in olive oil belong to five different classes: simple phenols (hydroxytyrosol, tyrosol), secoiridoids (oleuropein, ligstroside, and their hydrolysis derivatives), lignans ((+)-pinoresinol and (+)-acetoxypinoresinol), flavonoids (luteolin and apigenin) and phenolic acids (*p*-coumaric acid, vanillic acid, etc.) (15).

Virgin olive oil may be consumed raw in toast, salads, and other foodstuffs, but it is also consumed after domestic heating, such as frying, boiling, and microwave heating (16,17). Virgin olive oil is considered to be excellent for applications involving high temperatures, as it fulfills all the fatty acid criteria of the stable healthful frying oils, i.e., being rich in monounsaturated fatty acids, low in saturated and polyunsaturated fatty acids, very low in linolenic acid and containing practically no *trans* fatty acids (18). Moreover, olive oil is considered to be a premium frying oil with added advantages linked to its relatively low melting point. That means that it drains from the dried food easily leading to a low content of oil in the fried food (19).

During the thermal treatments, due to the high temperature and the absorption of oxygen and water, triacylglycerols in the oil suffer a series of reactions, namely hydrolysis, oxidation, isomerization, and polymerization (20). The thermal treatments are influenced by a large number of variables among which the type of process, i.e., continuous or discontinuous, the surface-to-oil volume ratio, the food (in the case that there is some food in contact with the oil), the addition of fresh oil or not, the

Capillary electrophoresis (CE) is another separative technique which offers to the analyst a number of key advantages for the analysis of the compounds of foods. CE offers better resolution than HPLC and is more adapted at the simultaneous separation of a number of components of different chemistries within a single matrix (41). CE has been used for the analysis of phenolic compounds in olive oil and related samples and its results represent a good compromise between analysis time and characterization of some classes of phenols in virgin olive oil (42,43,44,45,46,47,48).

The aim of this work was to study the changes in the phenolic profile of extra-virgin olive oil during thermal oxidation at frying temperatures and during different periods of time by using two separative techniques (HPLC and CE). The concentration of several compounds belonging to four families of phenols (phenolic alcohols or simple phenols, phenolic acids, secoiridoids and lignans) was determined in the samples after the thermal treatment by both techniques, as well as the concentration of several "unknown" compounds which appeared after the treatment. The peroxide value trend during heating was evaluated and changes in the antioxidant capacity of the phenolic fraction of each virgin olive oil after different time intervals of treatment was indirectly measured by the OSI test.

**MATERIALS AND METHODS**

**Instruments.** HPLC analyses were performed with a HP 1100 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump delivery system, a degasser, an autosampler, a diode array UV-VIS detector (DAD), and a mass spectrometer detector (MSD).

The HPLC column used was a $C_{18}$ Luna column, 5 μm, 25 cm x 3.0 mm (Phenomenex, Torrance, CA), with a $C_{18}$ pre-column (Phenomenex) filter. The mobile phase flow rate was 0.5 mL $min^{-1}$.

The CE instrument used was a Beckman 5500 capillary electrophoresis connected to a diode array detector. The system comprises a 0-30 kV high-voltage built in power supply.

All capillaries (fused silica) used were obtained from Beckman instrument, Inc. (Fullerton, CA) and had an inner diameter (i.d.) of 50 μm, a total length of 47 cm and an effective separation length of 40 cm. Data acquisition and processing were carried out with GOLD software installed in a personal computer.

The oxidative stability of the samples was evaluated by the oxidative stability index (OSI), using an eight-channel oxidative stability instrument (Omnion, Decatur, IL).

**Reagents, stock solutions and reference compounds.** 2-(4-Hydroxyphenyl)ethanol (tyrosol) was acquired from Fluka, dopac (3,4-dihydroxyphenylacetic acid) was acquired from Sigma Aldrich (St. Louis, MO), and oleuropein (oleuropein glucoside) and hydroxytyrosol were obtained from Extrasynthèse (Genay, France). The stock solutions containing these analytes were prepared in methanol/ water (50/50, v/v) at a concentration of 2000 μg $mL^{-1}$ for tyrosol, 500 μg $mL^{-1}$ for dopac, 10000 μg $mL^{-1}$ for oleuropein and 1500 μg $mL^{-1}$ for hydroxytyrosol. These compounds were used to do the calibration curves.

Methanol, acetonitrile and *n*-hexane HPLC-grade were from Merck (Darmstadt, Germany).

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

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma and was used as running buffer at different concentrations and pHs.

**Samples.** Bertolli commercial extra-virgin olive oil samples were used for this study (from Bertolli, Unilever Bestfoods Italia S.p.A., Inveruno-MI, Italy). We also used a commercial refined olive oil (Sagra, SALOV, Lucca, Italy) and a crude refined olive oil after and before decolorization phase.

Refined olive oil is the olive oil obtained from virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 grams per 100 grams of oil (0.3%) and its other characteristics correspond to those fixed for this category in this standard. The refined olive oil, on its turn, is the so-called virgin olive oil 'lampante' which, because of faults and serious alterations (as acidity higher than 2%) cannot be eaten, needs of an industrial process of deacidification, deodorizing, and decolouring, becoming in this way a neutral product both in taste and in colour.

**Liquid-liquid Extraction (LLE) of Phenolic Compounds from Olive Oils** LLE system was used to extract the phenolic compounds present in VOO. The LLE was carried out with the same extraction conditions and amounts of oil that are related by Carrasco Pancorbo et al. (45). Briefly, 60 g of oil ($\pm$0.001 g) were dissolved in 60 mL of hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v) solution. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure and a temperature of 40 °C. Finally, the residue was redissolved in 0.5 mL of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter.

**Thermal treatment.** We compared changes in the antioxidant capacity of olive oil and its phenolic composition, due to degradation of antioxidant compounds, after thermal treatment, simulating frying temperatures (180 ºC) during different time intervals (30, 60, 90, 120, 150 and 180 min). The samples (100 mL) were poured into an appropriate glass beakers (15 cm diameter), thus providing an excellent control of the surface-to-oil volume ratio. The samples were then removed form the oven, cooled to room temperature and analyzed.

**Peroxide value**. Evaluation of primary auto-oxidation products was carried out by the determination of the peroxide value (PV) according to the official method described in Regulation EEC 2568/91 (49). We measured this parameter after intervals of thermal treatment of 30 min.

**Oxidative Stability of Oil Samples.** The instrumental conditions were set following the analytical protocol described by Jebe et al. (50). To obtain the oxidative stability index, a stream of purified air (120 mL min$^{-1}$ air flow rate) was passed through the sample (5 g) and the effluent air for the oil sample is then bubbled through a vessel containing deionised water. The effluent air contains volatile organic acids swept form the oxidising oil, which increase the conductivity of the water. The temperature to carry out this test was 110 ºC. The OSI index (or OSI time) was expressed in hours (n=4). The OSI time was controlled after intervals of thermal treatment of 30 min.

**HPLC analysis.** The mobile phases were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the conditions described by Carrasco-Pancorbo et al. (14).

The injection volume was 10 μL. The wavelengths were set at 240, 280 and 330 nm. The detection was made using MS as well, and the analyses were carried out using an electrospray (ESI) interface operating in positive mode using the following conditions:

drying gas flow, 9.0 L min$^{-1}$; nebulizer pressure, 50 psi; gas drying temperature, 350 ℃; capillary voltage, 3000 V; fragmentor voltage, 60 V; and also using atmospheric pressure chemical ionization (APCI) interface operating in negative mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 30 psi; gas drying temperature, 350 ℃; vaporizer temperature, 450 ℃; capillary voltage, 3000 V; fragmentor voltage, 60 V. The polarity of ESI and APCI and all the parameters of MS detector were optimized using the height of the MS signal for the phenolic compounds isolated previously from the methanol-water extracts of extra-VOO as an analytical parameter.

**Calibration curves and quantification in HPLC.** To carry out the quantification in HPLC, five standard calibration curves were done using four commercial reference compounds, tyrosol, hydroxytyrosol, dopac (3,4-dihydroxyphenylacetic acid) and oleuropein (oleuropein glucoside). Tyrosol and hydroxytyrosol were quantified using the calibration curves of their corresponding commercial standards at 280 nm; other simple phenol (hydroxytyrosol-acetate) was quantified using the calibration curve at dopac at 280 nm; (+)-pinoresinol, (+)-1-acetoxypinoresinol, and complex phenols or secoiridoid derivatives (decarboxymethyl oleuropein aglycon, oleuropein aglycon and ligstroside aglycon) were quantified with the calibration curve of oleuropein obtained at 280 nm. However, the curve of oleuropein at 240 nm was used for the quantification of elenolic acid.

The "unknown" compounds which appeared after the thermal treatment in the phenolic profiles of the oil were quantified using the calibration curve of dopac at 280 nm. The quantification was carried out in all the cases using UV detection, but the MS detection was used as well in all the analyses to confirm the identity of the analytes.

The detection (DL), quantification limit (QL) and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie (51). Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. All the features of the proposed method are summarized in **Table 1**.

**Electrophoretic procedure.** CE method was that previously described by Carrasco-Pancorbo et al. (48). CE separation was carried out on a fused silica capillary (50 μm i.d., 375 μm o.d., total length 47 cm, a detection window was created at 40 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of 100x200 μm). Everytime a new capillary was used it was preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5 min rinse with Milli-Q water and 15 min with buffer. For the following analyses the capillary was rinsed with 0.1 M NaOH for 2 min, followed by a 2 min flush with Milli-Q water to assure good repeatability. The capillary was equilibrated with the running buffer (45 mM sodium tetraborate adjusted to pH 9.3) for 3 min before each sample injection. After each analysis, the capillary tubing was rinsed for 2 min with Milli-Q water. All solutions and samples were filtered through a 0.25 μm syringe filter. The running buffer was changed after 4 runs.

Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 psi) for 3 s (1 psi= 6895 Pa). Electrophoretic separations were performed at 28 kV for 7 min and the temperature was maintained at 22 ºC. UV detection was performed in all cases at 200 nm, 240 nm, 280 nm and 330 nm.

**Calibration curves and quantification in CE.** Standard calibration graphs for lignans and complex phenols were prepared using oleuropein (oleuropein glucoside) at

two wavelengths (200 nm and 240 nm). An external standard methodology (with oleuropein glucoside) was used for the quantification of lignans and complex phenols.

Using this methodology, time-corrected area counts are necessary for quantitative CE. Since separation is dependent on analytes migrating at different rates, the separated analytes will pass the detector at different rates. Time-corrected area counts are the product of the area counts and the velocity for each peak in the electropherogram.

Thus, peak areas and migration velocity (cm/min) were used to calculate the time-corrected area counts to compensate the differences in migration velocities among oleuropein glucoside, lignans and complex phenols.

Lig Agl (a), Pin, Ac Pin, Ol Agl (a) + DOA (a), DOA (b), Lig Agl (b), Ol Agl (b), EA (a), Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b+c) and DOA (d) + EA (d) were quantified with the data obtained for the calibration curve of oleuropein obtained at 200 nm. However, for the quantification of Lig Agl (d) + Ol Agl (d) + EA (e) the curve of oleuropein at 240 nm was used.

Tyrosol was quantified using the calibration curve of its commercial standard at 200 nm; hydroxytyrosol was quantified with the calibration curve of its commercially available standard at the same wavelength as the other simple phenol (200 nm). Peak areas were used for the quantification of these compounds versus the corresponding commercial standards.

The "unknown" compounds which appeared after the thermal treatment in the phenolic profiles of the oil were quantified using the calibration curve of dopac at 200 nm taking into account the time-corrected area counts versus time.

The detection (DL), quantification limit (QL) and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie

(51). Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. The calibration plots indicate good correlation between peak areas and analyte concentrations in the case of tyrosol and hydroxytyrosol, and between time-corrected area counts and analyte concentrations for dopac and oleuropein; regression coefficients were higher than 0.992. All the features of the proposed method are summarized in **Table 2**.

## RESULTS AND DISCUSSION

**Peroxide value.** In the case of the Bertolli commercial extra-virgin olive oil the peroxide value without any thermal treatment was 10.54 meqO$_2$ kg$^{-1}$ (n=5), then we measured this parameter as commented before after intervals of thermal treatment of 30 min as can be seen in **Figure 1a**. It can be observed that the peroxide values during this study changed in the range of 6.26-10.79 meqO$_2$ kg$^{-1}$, at the beginning a decrease was observed in this parameter (from 0 to 1 h), remaining almost stable during the next 90 minutes, and finally, it was possible to observe an increase of this value after 3 hours of heating at 180 ºC.

**Oxidative Stability of Oil Samples.** To determine the oxidative stability of extra-VOOs (OSI) the conductivity was measured as a function of time. The trend of the oxidation process can be evaluated by the determination of secondary oxidation products by this test, since volatile oxidized products such as formic acid are absorbed in the deionized water, thus enhancing the conductivity of the solution. This test involves the measurement of an induction period, wich is defined as the time during

which the olive oil´s natural resistance to oxidation, due to the presence of naturally occuring antioxidants, inhibits the oxidation.

In **Fig. 1b** the difference of the value obtained in each case minus the media of five determinations for the induction time of an olive oil without any treatment (24.28 hours) has been represented versus time. The trend is clear, the induction time decreased when the heating was longer.

**HPLC analyses.** All components decreased in concentration with an increase in the duration of the thermal treatment, although the rate of loss depended on chemical structure and antioxidant activity (see **Figure 2**).

When Bertolli commercial extra-virgin olive oil was studied we observed that HYTY, in the first step of the thermal treatment (180 ºC during 30 minutes), was reduced from 9.83 to 3.79 mg kg$^{-1}$ (in a 61.49%), then it continued to decrease during the heating (see **Table 3**). After 3 h it was impossible to detect this compound with the HPLC method in the polar extract of this olive oil. The beaviour of TY was totally different, as it only decreased in a percentage of 19.92 after 30 min. The amount of TY which remained in the olive oil after the thermal treatment of 90, 120 and 150 minutes was practically the same, this evidence is in according to Cheikhousman et al. (17). Moreover, other authors (14) determined the lower antioxidant activity or even pro-oxidant effect of this compound in olive oil during an accelerated oxidation test. After 3 h at 180 ºC, the concentration of TY determined in the extract was almost the 30% of the initial quantity.

HYTY-Ac is a compound very related with HYTY, however it was more resistent to these treatments than HYTY. The determined concentration of this compound in Bertolli olive oil without treatment was 1.66 mg kg$^{-1}$, and after heating the olive oil during three hours we found 0.36 mg kg$^{-1}$.

The decrease of EA was very drastic, since the 56% (approx.) of the initial concentration disappeared after the first step. After two hours it was possible to find only the 12.53% of the initial concentration and after 3 h, the 6.22%.

Regarding individual secoiridoid compounds studied by using this HPLC method, we can distinguish two families; the secoiridoids which are HYTY derivatives and those that derive from TY. The tendecies for DOA and Ol Agl were very similar, decreasing more or less in the same proportion until the final step. After three hours at 180 ºC, the percentages of the initial amount of these compounds present in Bertolli olive oil were 5.02 and 6.25%, respectively. However, Lig Agl seemed to be one of the compounds more resistant to this kind of thermal treatment. These results are in good agreement with the data found in bibliography where in general, when olive oil and other vegetable oils are subjected to thermal treatments, hydroxytyrosol derivatives are the first antioxidants that are lost during thermal oxidation and tyrosol derivatives seem to be the most stable compounds (17). This means that hydroxytyrosol derivatives are the first compounds to be oxidized, providing therefore oxidative stability to the oil (14,37,39,40). This observed trend is also consistent with the high antioxidant activity of hydroxytyrosol and its secoiridoid derivatives in virgin olive oil, as compared to these compounds belonging to the TY family (12,52), since antioxidants act by reacting rapidly with lipid radicals and are thereby consumed.

Furthermore, the level of hydroxytyrosol and its derivatives in virgin olive oil have been reported to correlate well with the oxidative stability of the oil, as determined by the Rancimat method (53,54). In this case, after one hour of treatment the 73.81, 42.81 and 46.62% of the initial amount of HYTY, DOA and Ol Agl respectively had been consumed; and after two hours, the 91.47, 77.25 and 85.30%. This fact can explain the shape of the graph which represents the OSI results.

It is important to highlight the behaviour found when other family of phenolic compounds (lignans) present in olive oil was studied; considering the concentration of Pin it was really stable during the first two hours, since the amount of this compound only changed from 90.57 to 78.16 mg kg$^{-1}$. The decrease was a little bit sharpened when the thermal treatment took more than 2 hours, and after 3 hours we could still determined in the polar extracts of Bertolli olive oil concentrations which represented the 45.25% of the initial quantity. Ac Pin was stable during the first hour of heating (approx), then the decrease was faster and finally, after three hours the 37.68% of the initial quantity remained in the oil. Lignans demonstrated to be very resistant to thermal treatment in the studies carried out by Brenes et al. (16), whilst in another study of Gómez-Alonso et al. (38) using another kind of frying, both the content of lignans and the elenolic acid fell drastically in the first frying operation. The results obtained in the research carried out by Brenes et al. seem to be agree with those published by Carrasco-Pancorbo et al. (14) who demonstrated the lignans show a low antioxidant activity in comparison with the antioxidant activity showed by other phenolic compound, such as HYTY, DOA or Ol Alg.

**"Unknown" compounds detected in the chromatographic profiles and their presence in other oils.** When the chromatographic profiles shown in **Figure 2** are observed, it is also possible to see that there are several peaks (six peaks in particular) which are higher when the thermal treatment is longer, that means there are several unknown peaks which appear in the chromatogram after the treatment. Their retention time, absorption maxima, and the fragmentation patterns using the two interfaces are shown in **Table 4**. We also made their quantification the results are summarized in **Table 3**.

Then, we analyzed a commercial refined olive oil and a crude refined olive oil after and before decolorization phase. To get a refined oil thermal treatments are employed, so we thought it could be possible to find the "unknown" compounds in the polar fraction of these oils. In **Figure 3** the HPLC analyses of these three oils are shown. One of the unknown compounds was found in the commercial refined olive oil and three were detected in the crude refined olive oil after and before decolorization phase. In the profile of the commercial refined olive oil six of the eight phenolic compounds present in a normal extra-VOO were found, that means that the treatment in this case was probably softer than in the other two oils under study, as the alterations in the phenolic profile were not really marked.

**CE analyses.** The analyses of the extracts of extra-virgin olive oil with and without thermal treatment were carried out by CE as well. As commented before, all the components decreased in concentration with an increase in the duration of the thermal treatment, although the rate of loss depended on chemical structure and antioxidant activity (see **Figure 4**).

Taking into account the compounds which are quantified in terms of their commercial standard (tyrosol and hydroxytyrosol), we can say that the results obtained by both techniques are in good agreement (see **Table 5**). Considering the lignans (Pin and Ac Pin), it was also possible to observe very similar concentrations determined by HPLC and CE. In the lignans zone, considerable changes took place, since after the treatment this area appeared as one of the richest containing a lot of separated compounds.

Differences can be found when the results for the secoiridoids are studied, but the cause of these apparent discrepancy could be that CE is able to separate several isoforms of the secoiridoids (48). So, the secoiridoids that appear in the HPLC profiles as one peak, can be separated in several peaks with the CE method.

Besides these compounds the CE method was able to detect and determine several phenolic acids that appear within 7 minutes of analysis (4-HFA, sinapinic and gentisic acid). The chromatographic method used was not able to detect any phenolic acid after three hours of treatment at high temperature.

**"Unknown" compounds detected in the electrophoretic profiles and their presence in other oils.** Apparently, for controlling new peaks which appear in the phenolic profile of the extra-virgin olive oil under study, HPLC seemed to be more appropriate, since in the electrophoretic data only one unknown peak could be detected with clarity without taking into account the lignans area.

As commented in the HPLC section we proceeded to analyze a commercial refined olive oil and a crude refined olive oil after and before decolorization phase. The results can be observed in the **Figure 5** and the differences in the profiles of the three applications were noticeable. Different areas are indicated with a red line in the figure, in these zones there were not compounds in the profile of an extra-VOO without treatment. In the olive oils (a) and (b) several peaks were detected before and after of the zone of the lignans; although in (c) only the peak which appears after the Ac Pin was detected. Moreover, in the crude refined olive oil obtained after the decolorization phase, several peaks were detected just before of the unknown peak found in the extra-VOO after thermal treatment which was also detected. This unknown peak detected also in the Bertolli extra-VOO after the thermal treatment was found in the commercial refined olive oil. The crude refined olive oil obtained after the decolorization phase (b) had in its composition other two compounds which appeared in the electropherogram after 5 minutes; they were the highest peaks in the profile and were not found in the other two refined oils. Other compound with a migration time of 4.53 min was found

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when the analyses of the crude refined olive oil obtained before the decolorization phase were studied; it was not detected in the other two oils under study.

Changes in the phenolic extracts obtained from extra-virgin olive oil caused by thermal treatments were controlled by HPLC-UV and HPLC–MS and by CE-UV-Vis. To our knowledge it is the first time in which these phenolic compounds are quantified individually after the treatment by both techniques. The results obtained for the two separative techniques were in good agreement and confirmed other data previously published. Furthermore, the CE method was able to determine three phenolic acids and to separate several isoforms of the secoiridoids which appeared as one peak in the chromatograms.

HYTY, EA, DOA and Ol Agl reduced their concentration with the thermal treatment quicker than other phenolic compounds present in olive oil. HYTY-Ac and Lig Agl demonstrated to be quite resistant to this kind of treatment. The behaviour of lignans could be outstanding as they belong to the family most resistant to thermal treatments.

The peroxide values found during the study varied in the range of 6.26-10.79 meq $O_2$ kg$^{-1}$, observing a little increase after 3 hours of treatment; the trend for the results of the OSI test was clear, since the induction time decreased when the heating was longer.

Moreover, several new unknown compounds were detected and quantified in the phenolic fraction of the oil and their most relevant characteristics were determined. We studied several refined olive oils looking for the unknown compounds detected in the extra-VOO subjected to thermal oxidation and several of them were found.

**ABBREVIATIONS USED**

**VOO:** Virgin olive oil**;** **TY**: tyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxylated oleuropein aglycon; **Lig Agl**: ligstroside

aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **HYTY**: hydroxytyrosol; **4-HFA**: 4-hydroxyphenylacetic acid; **Sinapinic**: sinapinic acid; **Gentisic**: gentisic acid.

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temperature and the oil selected, are of particular relevance (21). Due to the difficulties found in defining and/or controlling such variables and the additional strong interactions between them (22), it is not easy to replicate results from different laboratories.

The analytical methods used to evaluate fat degradation, i.e., carbonyl value (23,24) iodine value (25), viscosity (25,26), volatiles (27), room odour and flavour scores (25,28), triacylglycerol oxidation (29), polar compounds (22,30), and others, may also have contributed significantly to the variability found in the results. These results would suggest interactions between the variables of the thermal process and dependence on the analytical method applied.

Even though many researches have studied polyphenols in raw and stored virgin olive oils (31,32,33), their influence on the stability and organoleptic properties of the oil (34,35), and nutritional benefits (8), there are not many data that report the availability of these compounds after domestic heating of the oil (16,36,37,38, 39). In these cases, the phenolic compounds have been determined colorimetrically (36,37) and also by using HPLC (16,38,39,40). In these previously mentioned research works different quantities of olive oil have been subjected to very different thermal treatments in various recipients inside distinct fryers or ovens (frying operation with potatoes (39); frying temperatures (160-190 ℃) and heating times typical of domestic frying conditions (0.5-2 h) (37); thermal oxidation at 60 and 100 ℃ in an oven during a long time (40); twelve frying operations at 180 ℃ with potato slices into pieces (38); thermal oxidation of oils at 180 ℃ in a Rancimat apparatus, boiling a mixture of virgin olive oil and water in a pressure cooker for 30 min, and microwave heating of oils for 10 min (39), etc...) and for this reason it is very difficult to compare all the data, although it is true that several general considerations can be taked into account.

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**Caption to figures**

**Figure 1**. **(a)** Changes in the Peroxide value after thermal treatment (180 ℃) from 0 (without thermal treatment) to 3 hours. (n=5)

**(b)** Trend of the oxidation process evaluated by the determination of volatile secondary oxidation products by OSI test. The difference of the value obtained in each case minus the media of five determination for the induction time of an olive oil without any treatment has been represented versus time. (n=5)

**Figure 2**. Chromatograms which show the evolution in the phenolic profile of Bertolli extra-VOO after thermal treatment at 180 ℃. a) Extra-virgin olive oil without treatment; b) the same oil after 1 hour at the mentioned temperature; c) the same oil after 2 hours at 180 ℃; d) the same oil after 3 hours at the mentioned temperature.

(The "unknown compounds" appeared after the heating are marked with an asterisk in red an a letter)

**Figure 3**. HPLC analyses of three refined olive oils looking for the unknown compounds detected in the extra-VOO subjected to thermal treatment. (a) A commercial refined olive oil, (b) a crude refined olive oil after decolorization phase and (c) a crude refined olive oil before decolorization phase.

(the injection time was in the three examples 50 μl to provide peaks with a good intensity for being observed with clarity)

**Figure 4**. Electropherograms obtained for a) Bertolli extra-virgin olive oil without treatment; b) the same oil after 1 hour at the mentioned temperature; c) the same oil after 2 hours at 180 ℃; d) the same oil after 3 hours at the mentioned temperature.

(The "unknown compound" appeared after the heating which can be detected using the CE method is marked with an asterisk in red)

**Figure 5**. CE profiles of three refined olive oils used as applications of the methods looking for the unknown compounds detected in the extra-VOO subjected to thermal treatment. (a) A commercial refined olive oil, (b) a crude refined olive oil after decolorization phase and (c) a crude refined olive oil before decolorization phase.

(the injection time was in the three examples 9 s to provide peaks with a good intensity for being observed with clarity)

**TABLES**

**Table 1.** Analytical parameters of HPLC method

| Analyte | RSD (%) (intermediate value) | Detection limit (DL) ($\mu g\ mL^{-1}$) | Quantification limit (QL) ($\mu g\ mL^{-1}$) | Calibration range($\mu g\ mL^{-1}$)* | Calibration equations | $r^2$ |
|---|---|---|---|---|---|---|
| **Hydroxytyrosol** | 3.24 | 0.19 | 0.63 | 1500 | y=25.76x-200.67 | 0.997 |
| **Tyrosol** | 3.02 | 0.40 | 1.33 | 2000 | y=12.11x-43.92 | 0.996 |
| **3,4 Dihydroxyphenylacetic acid** | 3.78 | 0.23 | 0.77 | 500 | y=21.29x-16.55 | 0.997 |
| **Oleuropein glucoside λ=280 nm** | 3.22 | 1.08 | 3.60 | 10000 | y=4.45x+15.93 | 0.997 |
| **Oleuropein glucoside λ=240 nm** | 4.21 | 0.29 | 0.97 | 10000 | y=16.44x+1587.60 | 0.998 |

RSD: Relative Standard Deviation
* From QL to the value in the table

**Table 2**

Analytical parameters of proposed method. y=bx±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. (*y=bx±a, where y is the (Time corrected area counts=peak area (AU) x migration velocity (cm/min)). (Data obtained using the software Microcal$^{TM}$ Origin$^{TM}$, Microcal Software, Inc, USA)

| Analyte | RSD (%) (intermediate value) | Detection limit (DL) ($\mu g\ mL^{-1}$) | Quantification limit (QL) ($\mu g\ mL^{-1}$) | Calibration range ($\mu g\ mL^{-1}$) | Calibration equations | $r^2$ |
|---|---|---|---|---|---|---|
| **Tyrosol** | 2.03 | 1.14 | 3.80 | QL-1500 | $y=7 \cdot 10^{-6}x + 5 \cdot 10^{-6}$ | 0.994 |
| **\*Oleuropein glucoside λ=200 nm** | 2.51 | 0.10 | 0.33 | QL-10000 | $y=8 \cdot 10^{-5}x + 3 \cdot 10^{-4}$ | 0.997 |
| **\*Oleuropein glucoside λ=240 nm** | 2.04 | 0.40 | 1.33 | QL-10000 | $y=2 \cdot 10^{-5}x - 8 \cdot 10^{-5}$ | 0.995 |
| **Hydroxytyrosol** | 2.34 | 0.40 | 1.33 | QL-500 | $y=2 \cdot 10^{-5}x + 8 \cdot 10^{-6}$ | 0.992 |
| **\*Dopac** | 1.67 | 0.04 | 0.13 | QL-2000 | $y=2 \cdot 10^{-4}x + 4.8 \cdot 10^{-3}$ | 0.997 |

RSD: Relative Standard Deviation   LD=$3\sigma_b/b$ ($\sigma_b$= 2.66x10$^{-6}$, calculated using 100 data); LQ=$10\sigma_b/b$

**Table 3**
Results of the analysis of Bertolli extra-virgin olive oil samples before and after thermal treatment (HPLC). Quantification of the individual components. (n=7) ($Value = \overline{X}$ ) (in all the cases SD was lower than 5% of the mean value)

## mg/kg = mg analyte/kg olive oil

| *Analyte* | **Bertolli** | Bertolli 30 min | Bertolli 1h | Bertolli 90 min | Bertolli 2 h | Bertolli 150 min | Bertolli 3 h |
|---|---|---|---|---|---|---|---|
| **HYTY** [1] | 9.83 | 3.79 | 2.57 | 2.26 | 0.84 | 0.73 | n.d |
| **TY** [2] | 17.32 | 13.87 | 10.04 | 8.86 | 8.85 | 8.62 | 4.66 |
| **HYTY Ac** [3] | 1.66 | 1.58 | 1.38 | 0.86 | 0.64 | 0.37 | 0.36 |
| **EA** [4] | 197.17 | 86.14 | 61.63 | 26.24 | 24.70 | 19.03 | 12.27 |
| **DOA** [5] | 109.84 | 78.72 | 62.82 | 49.59 | 24.99 | 14.19 | 5.51 |
| **Pin** [5] | 22.61 | 22.58 | 22.30 | 20.47 | 19.52 | 11.58 | 10.23 |
| **Ac Pin** [5] | 122.51 | 118.88 | 104.62 | 88.72 | 53.41 | 48.21 | 46.16 |
| **Ol Agl** [5] | 58.91 | 43.99 | 31.45 | 16.51 | 8.66 | 4.15 | 3.68 |
| **Lig Agl** [5] | 7.31 | 7.08 | 6.91 | 6.62 | 6.27 | 5.96 | 5.53 |
| | | | | | | | |
| **"Unknown" a** [3]([5]) | 0.22 (0.98) | 0.28 (1.41) | 0.32 (1.44) | 0.36 (1.66) | 0.67 (3.15) | 0.87 (4.11) | 0.95 (4.49) |
| **"Unknown" b** [3]([5]) | 1.66 (7.87) | 2.05 (10.87) | 2.55 (12.15) | 3.05 (15.82) | 3.11 (14.80) | 3.83 (18.27) | 4.34 (20.51) |
| **"Unknown" c** [3]([5]) | 1.16 (5.46) | 1.18 (5.55) | 1.22 (5.81) | 1.23 (6.38) | 1.80 (8.57) | 1.98 (9.45) | 2.08 (9.83) |
| **"Unknown" d** [3]([5]) | 2.33 (10.96) | 4.50 (21.17) | 5.70 (27.14) | 6.35 (32.94) | 7.83 (37.28) | 8.43 (40.23) | 9.18 (43.38) |
| **"Unknown" e** [3]([5]) | 0.81 (3.81) | 1.65 (7.76) | 1.95 (9.28) | 2.31 (10.89) | 3.37 (16.04) | 3.75 (17.90) | 4.28 (20.23) |
| **"Unknown" f** [3]([5]) | 0.72 (3.39) | 1.67 (7.85) | 1.82 (8.66) | 2.10 (9.90) | 3.34 (15.90) | 3.24 (15.47) | 3.88 (18.34) |

**HYTY:** hydroxytyrosol; **TY**: tyrosol; **HYTY-Ac**: hydroxytyrosol-acetate; **EA**: elenolic acid; **DOA**: decarboxilted oleuropein aglycon; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **Ol Agl**: oleuropein aglycon; **Lig Agl**: ligstroside aglycon.

[1]: quantified with a calibration curve of hydroxytyrosol at λ=280 nm.

[2]: quantified with a calibration curve of tyrosol at λ=280 nm.

[3]: quantified with a calibration curve of dopac at λ=280 nm.

[4]: quantified with a calibration curve of Oleuropein glucoside at λ=240 nm.

[5]: quantified with a calibration curve of Oleuropein glucoside at λ=280 nm.

n.d.: non detected

**Table 4**. Retention times, absorption maxima, and the fragmentation patterns using the two interfaces for the unknown compounds found in the chromatographic profiles after the thermal treatment.

| Analyte | $t_r$ (min) | $\lambda_{max}$ (nm) | Major fragments APCI negative | Major fragments ESI positive |
|---|---|---|---|---|
| unknown **a** | 27.41 | 282 | Non detectable in APCI neg | 249.0 / 475.2 |
| unknown **b** | 32.15 | 235/280 | 261.1 | 221.1 / 181.1 |
| unknown **c** | 42.51 | 226/282 | 227.1 / 309.1 / 319.2/ 85.1 | 321.1 / 361.2 / 241.3 / 121.1 |
| unknown **d** | 44.40 | 244/276 | 319.1 / 181.1 | 343.0 / 500.1 / 121.1 |
| unknown **e** | 49.09 | 280 | 125.2 / 169.0 / 85.1 / 173.1 / 329.2 | 221.2 / 261.1 / 349.2 |
| unknown **f** | 49.92 | 280 | 329.2 / 349.1 | 315.1 / 349.2 / 373.2 /239.1 /221.1 / 121.1 |

**Table 5**
Results of the analysis of Bertolli extra-virgin olive oil samples before and after thermal treatment (CE). Quantification of the individual components. (n=7) ($Value = \overline{X}$ ) (in all the cases SD was lower than 5% of the mean value)

## mg/kg = mg analyte/kg olive oil

| Analyte | Bertolli | Bertolli 30 min | Bertolli 1h | Bertolli 90 min | Bertolli 2 h | Bertolli 150 min | Bertolli 3 h |
|---|---|---|---|---|---|---|---|
| Lig Agl (a) [2] | 43.50 | 42.86 | 42.20 | 26.42 | 23.89 | 23.64 | 6.89 |
| TY [1] | 18.26 | 13.60 | 11.47 | 9.68 | 9.61 | 8.18 | 5.91 |
| Pin [2] | 19.98 | 19.65 | 19.22 | 17.98 | 17.21 | 10.19 | 8.91 |
| Ac Pin [2] | 118.12 | 114.34 | 100.34 | 85.78 | 51.65 | 46.63 | 44.60 |
| Ol Agl (a) + DOA (a) [2] | 11.44 | 9.81 | 7.50 | 3.45 | 2.55 | 1.87 | n.d. |
| DOA (b) [2] | 66.78 | 48.38 | 31.39 | 11.01 | 6.27 | 5.46 | n.d. |
| Lig Agl (b) [2] | 6.43 | 5.49 | 4.92 | 1.62 | n.d. | n.d. | n.d. |
| Ol Agl (b) [2] | 5.04 | 4.55 | 3.95 | 3.73 | 2.98 | 2.86 | 1.20 |
| EA (a) [2] | 4.33 | 3.56 | 2.84 | 1.28 | n.q. | n.q. | n.d. |
| Ol Agl (c)+Lig Agl (c)+DOA (c)+EA(b,c) [2] | 37.03 | 31.23 | 23.47 | 8.66 | 5.20 | 3.87 | 3.18 |
| HYTY [4] | 8.27 | 4.11 | 2.89 | 2.50 | 0.89 | 0.73 | 0.58 |
| DOA (d) + EA (d) [2] | 14.56 | 11.10 | 9.50 | 8.65 | 8.15 | 8.05 | n.d. |
| EA (e) + Lig Agl (d) + Ol Agl (d) [3] | 122.04 | 63.54 | 57.32 | 15.86 | 15.29 | 11.78 | 7.60 |
| 4-HFA [5] | 2.62 | 2.34 | 2.18 | n.d. | n.d. | n.d. | n.d. |
| Sinapinic [5] | 4.45 | 4.01 | 3.15 | 2.26 | 1.32 | n.d. | n.d. |
| Gentisic [5] | 3.14 | 3.12 | 3.10 | 2.68 | 2.32 | 2.09 | n.d. |
| "Unknown" [2] | - | 54.91 | 71.91 | 83.82 | 92.95 | 108.96 | 116.89 |
| "Unknown" [5] | - | 21.60 | 28.29 | 33.06 | 36.71 | 43.11 | 46.24 |

**Lig Agl**: ligstroside aglycon; **TY**: tyrosol; **Pin**: (+)-pinoresinol; **Ac Pin**: 1-(+)-acetoxypinoresinol; **DOA**: decarboxilated oleuropein aglycon; **Ol Agl**: oleuropein aglycon; **EA**: elenolic acid; **4-HFA**: 4-hydroxyphenylacetic acid; **Sinapinic**: sinapinic acid; **Gentisic**: gentisic acid.

[1] quantified with a calibration curve of tyrosol at $\lambda$=200 nm.

[2] quantified with a calibration curve of Oleuropein glucoside at $\lambda$=200 nm. (Time corrected area counts vs concentration)

[3] quantified with a calibration curve of Oleuropein glucoside at $\lambda$=240 nm. (Time corrected area counts vs concentration)

[4] quantified with a calibration curve of hydroxytyrosol at $\lambda$=200 nm.

[5] quantified with a calibration curve of dopac at $\lambda$=200 nm. (Time corrected area counts vs concentration)

**FIGURES**

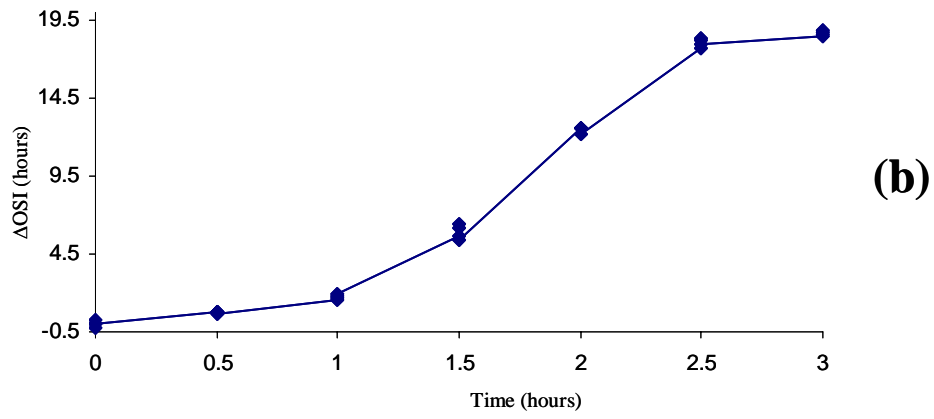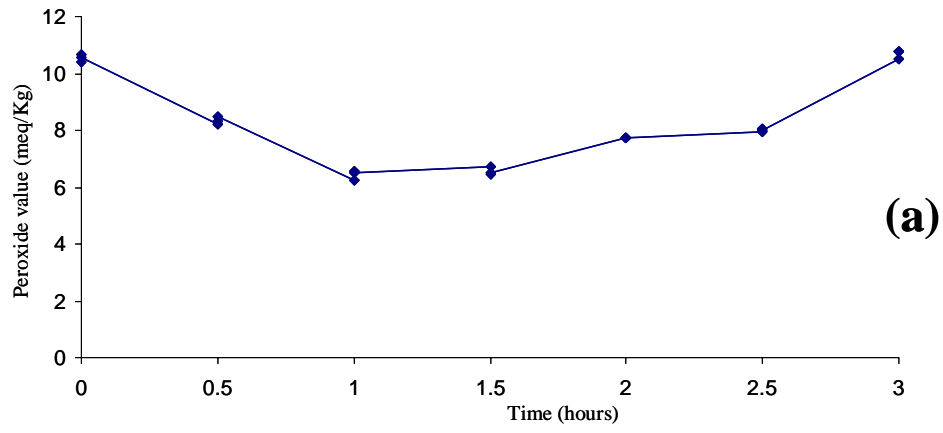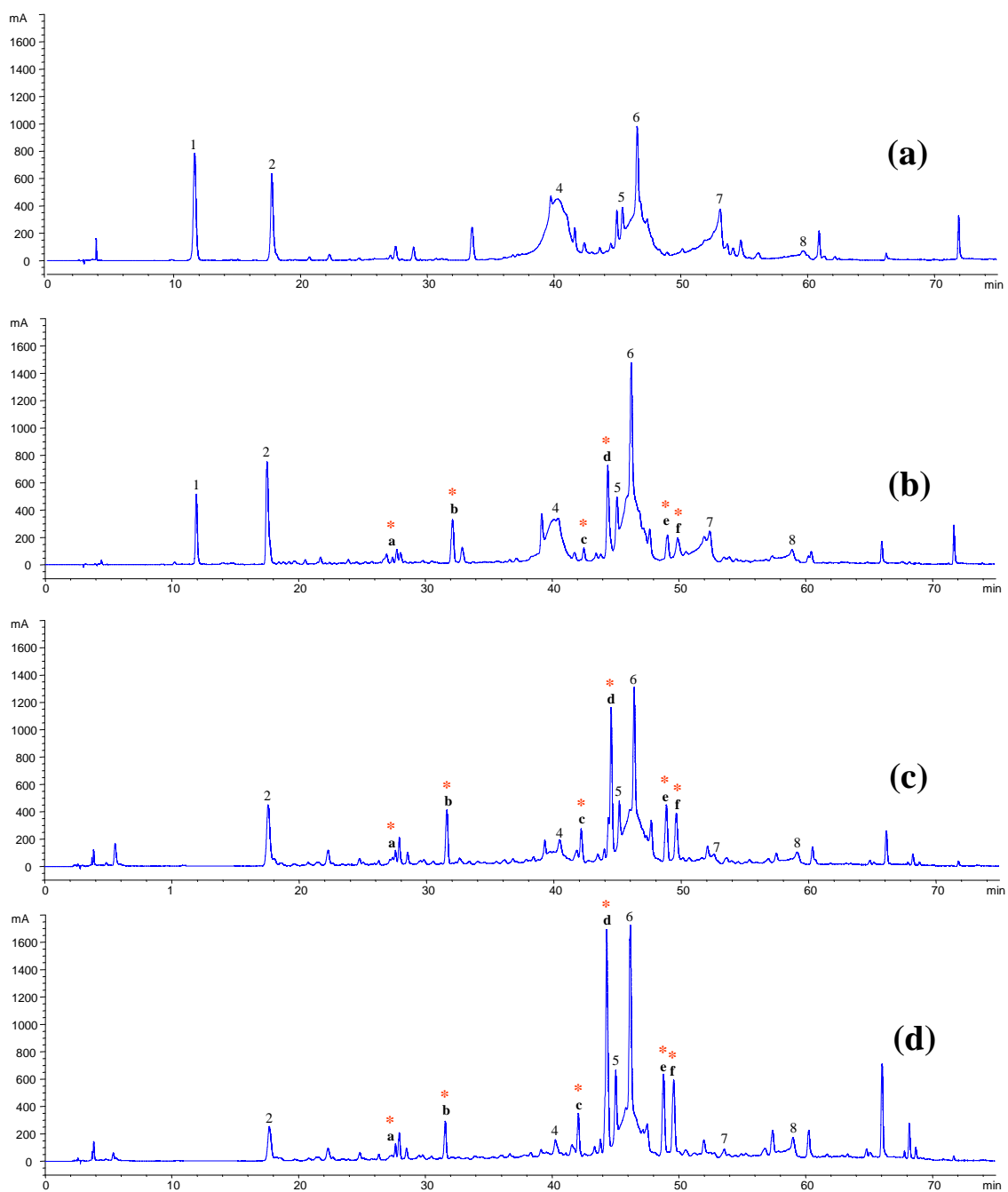**Figure 1**
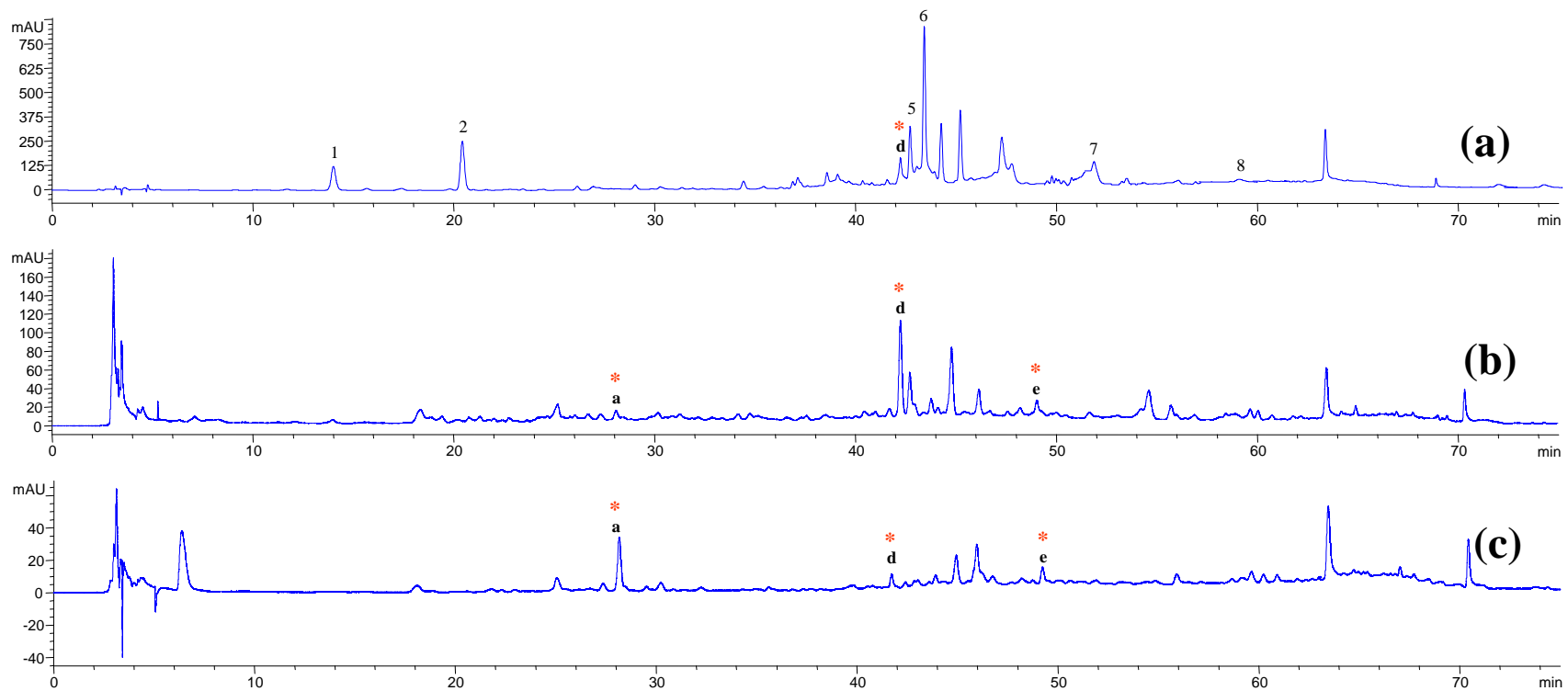


(a)

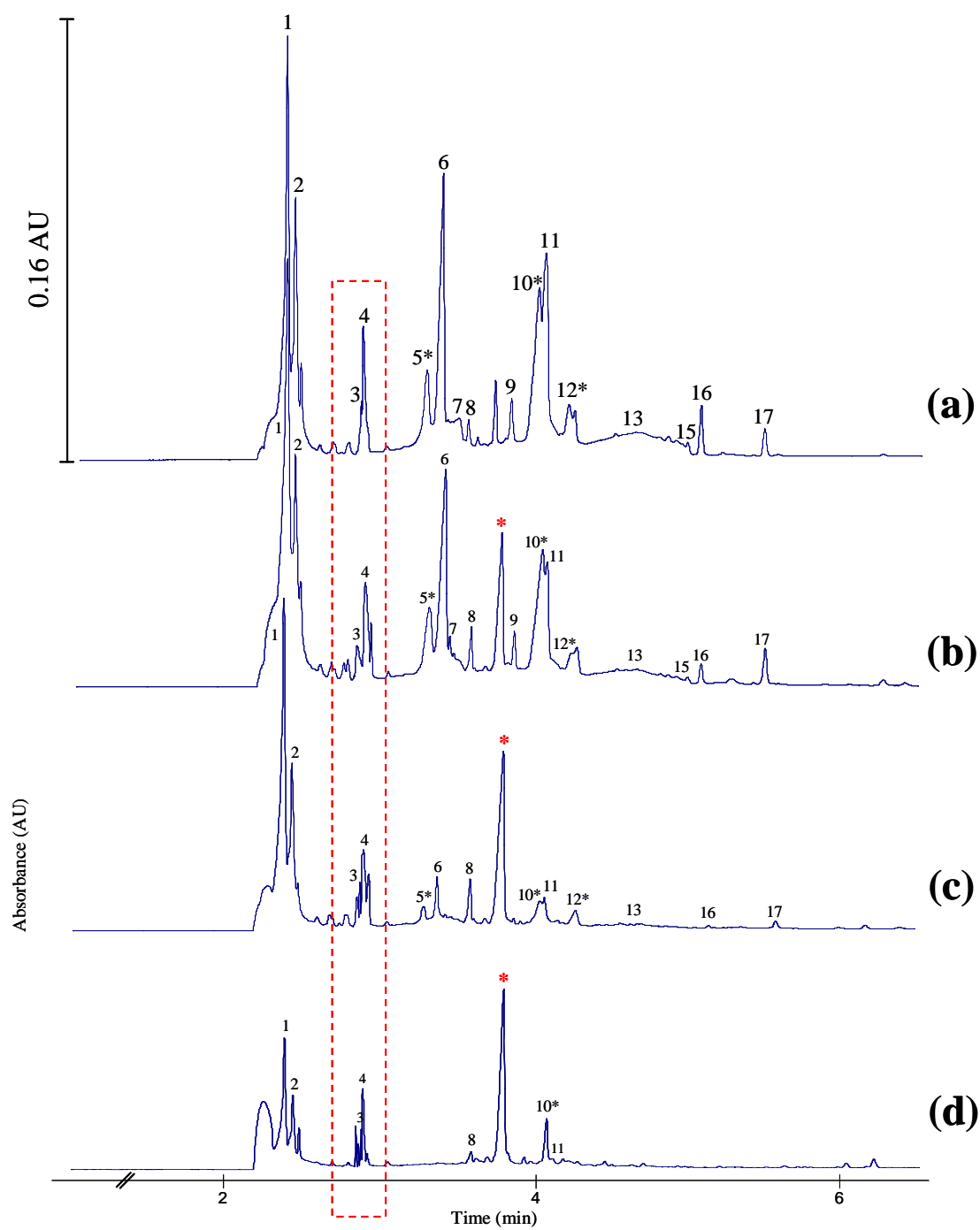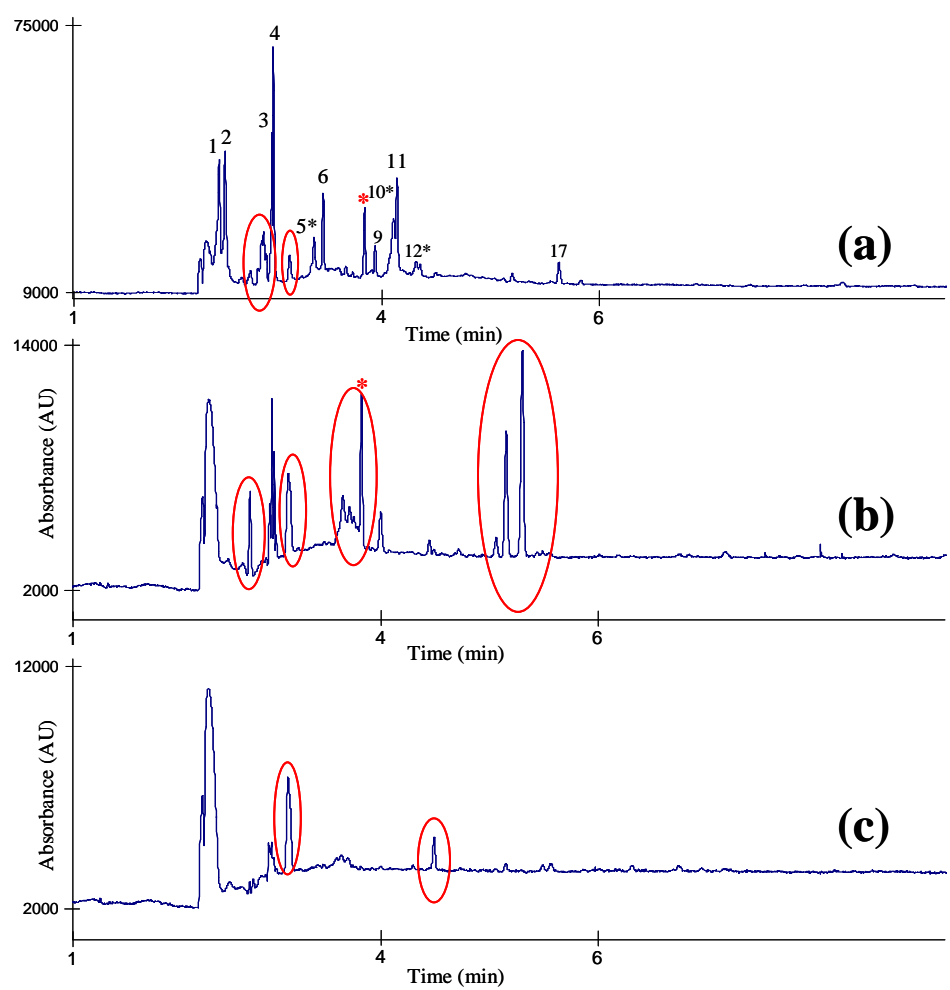

(b)

**Figure 2**

**Figure 3**

**Figure 4**

**Figure 5**

# Capítulo 10

# Método CE-ESI-MS para determinar la fracción fenólica del aceite de oliva virgen-extra

Como se mencionaba en la introducción, en los últimos años el acoplamiento entre la CE como técnica analítica de separación y la espectrometría de masas como sistema de detección ha despertado un gran interés en la comunidad científica. Ese interés es debido sobretodo a que en el acoplamiento CE-MS se combinan la rapidez del análisis, el alto poder de resolución, y el consumo muy pequeño de muestra y disolventes proporcionados por la CE, con la selectividad del análisis y la información estructural que proporciona la espectrometría de masas.

En el caso particular de la fracción fenólica del aceite de oliva, es muy importante disponer de un detector que sea capaz de proporcionar información estructural debido a que la ausencia de patrones comerciales puede dificultar en gran medida la identificación de alguno de sus componentes. Además, con el uso de la MS se podrían identificar nuevos compuestos que aún no hayan sido caracterizados en la fracción más polar de esta compleja matriz.

La adquisición de un espectrómetro de masas (IT) por parte del grupo de investigación al que pertenezco, posibilitó la puesta a punto de un método CE-ESI-IT-MS para el análisis de la fracción polifenólica del aceite de oliva. El objetivo era caracterizar el mayor número de compuestos fenólicos presentes en un extracto (SPE-Diol) de aceite de oliva virgen extra. Para demostrar el poder cuantitativo de la MS, se compararon también los perfiles polifenólicos de tres aceites obtenidos de variedades de aceituna diferentes.

**Alegría Carrasco-Pancorbo**
**David Arráez-Román**
**Antonio Segura-Carretero**
**Alberto Fernández-Gutiérrez***

Department of Analytical Chemistry,
Faculty of Sciences,
University of Granada,
Granada, Spain

## Research Article

# Capillary electrophoresis-electrospray ionization-mass spectrometry method to determine the phenolic fraction of extra-virgin olive oil

We describe the first analytical method involving SPE and CZE coupled to ESI-IT MS (CZE-ESI-MS) used to identify and characterize phenolic compounds in olive oil samples. The SPE, CZE and ESI-MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity of their determination. To this end we have devised a detailed method to find the best conditions for CE separation and the detection by MS of the phenolic compounds present in olive oil using a methanol–water extract of Picual extra-virgin olive oil (VOO). Electrophoretic separation was carried out using an aqueous CE buffer system consisting of 60 mM $NH_4OAc$ at pH 9.5 with 5% of 2-propanol, a sheath liquid containing 2-propanol/water 60:40 v/v and 0.1% v/v triethylamine. This method offers to the analyst the chance to study important phenolic compounds such as phenolic alcohols (tyrosol (TY), hydroxytyrosol (HYTY) and 2-(4-hydroxyphenyl)ethyl acetate), lignans ((+)-pinoresinol and (+)-1-acetoxypinoresinol), complex phenols (ligstroside aglycon (Lig Agl), oleuropein aglycon, their respective decarboxylated derivatives and several isomeric forms of these (dialdehydic form of oleuropein aglycon, dialdehydic form of ligstroside aglycon, dialdehydic form of decarboxymethyl elenolic acid linked to HYTY, dialdehydic form of decarboxymethyl elenolic acid linked to TY) and 10-hydroxy-oleuropein aglycon) and one other phenolic compound (elenolic acid) in extra-VOO by using a simple SPE before CE-ESI-MS analysis.

**Correspondence:** Dr. Antonio Segura-Carretero, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain
**E-mail:** ansegura@ugr.es
**Fax:** +34-958-249510

**Abbreviations: Ac Pin**, (+)-1-acetoxypinoresinol; **decarboxylated-Lig Agl** and **decarboxylated-Ol Agl**, decarboxylated derivatives of Lig Agl and Ol Agl; **decarboxylated-dialdehydic-Ol Agl**, dialdehydic form of decarboxymethyl elenolic acid linked to HYTY; **decarboxylated-dialdehydic-Lig Agl**, dialdehydic form of decarboxymethyl elenolic acid linked to TY; **dialdehydic-Ol Agl**, dialdehydic form of oleuropein aglycon; **dialdehydic-Lig Agl**, dialdehydic form of ligstroside aglycon; **EA**, elenolic acid; **10-H-Ol Agl**, 10-hydroxy-oleuropein aglycon; **HYTY**, hydroxytyrosol; **HYTY-Ac**, 2-(4-hydroxyphenyl)ethyl acetate; **Lig Agl**, ligstroside aglycon; **Ol Agl**, oleuropein aglycon; **Pin**, (+)-pinoresinol; **TEA**, triethylamine; **TY**, tyrosol; **VOO**, virgin olive oil

## 1 Introduction

Virgin olive oil (VOO), the juice of the olive obtained by pressing, is one of the few oils that are consumed without any further refining process. For this reason, VOO contains several substances, such as phenolic compounds, which are generally acknowledged to be of considerable importance [1, 2]. The antioxidant potential of phenolic compounds in olive oil has been a subject of great interest, both because of its chemoprotective effect in human beings [3, 4] and because it is a major factor in the high stability (shelf-life) of olive oils [3, 5, 6]. The antioxidant activity of VOO components has been related to the pro-

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* Additional corresponding author: Alberto Fernández Gutiérrez, E-mail: albertof@ugr.es.

tection for important chronic and degenerative diseases as coronary hearth diseases (CHDs), ageing neuro-degenerative diseases and tumors of different localizations [7, 8]. Phenolic compounds also contribute to the organoleptic properties of VOOs and are commonly described as bitter and astringent [9, 10] and responsible of organoleptic characteristics in general [11, 12].

Qualitative and quantitative determinations of both simple and complex phenolic substances in oils are therefore essential. One method used for the quantitative determination of total phenolic compounds in VOO is the colorimetric assay, based on the reaction of Folin-Ciocalteu reagent [13], but unfortunately this method is nonspecific, and both simple and complex phenols are detected indistinctly. To devise more specific methods several researchers have developed different extraction systems from oils. Traditionally the protocol consists of the extraction of the oil dissolved in a lipophilic solvent (usually hexane) with several portions of methanol or methanol/water (with different proportions of water ranging between 0 and 40%) [14–17], although $C_{18}$-cartridges [18–20], and anionic exchange cartridges [21] have also been assayed. After these extraction systems, GC [22] and HPLC have been used for the analysis of simple phenols [15, 23], and complex phenolic substances [14, 24, 25].

Even though the characterization of phenolic compounds from olive oils has been successfully carried out using GC and HPLC, CE has become an alternative or complement to chromatographic separations because it needs no derivatization step, requires only small amounts of sample and buffer and has proved to be a high-resolution technique.

The use of CE as analytical separation technique coupled to MS as detection method can provide important advantages in food analysis because of the combination of the high separation capabilities of CE and the power of MS as identification and confirmation method [26]. In general, if a separation technique is coupled with MS the interpretation of the analytical results can be more straightforward [27–29]. Consequently, ESI has emerged as a highly useful technique which allows direct coupling with electrophoretic separation techniques [30]. Furthermore, collisionally induced dissociation can be used to obtain fragment ions of structural relevance for identifying target compounds in a highly complex matrix.

Although CE offers the analyst a number of key advantages for the analysis of food components, only a few data are available to date on the application of this technique to the phenolic contents of olive oil [24, 31–36] and waste products from the olive oil industry (olive-mill wastewater [37] and alperujo (a semisolid waste from the olive oil industry) [38]). Only in one previously published paper is the use of CE described with MS as detector [37].

The aim of our work has been to develop the first simple SPE-CE-ESI-MS method for the identification and characterization of phenolic compounds in olive oil samples. We used several standards obtained by semipreparative HPLC in order to confirm the identity of the phenolic compounds and in this way were able to identify by CZE-ESI-MS three simple phenols, two lignans, several complex phenols and other phenolic compounds as the main components of extra-VOO.

## 2 Materials and methods

### 2.1 Chemicals and samples

All chemicals were of analytical reagent grade and used as received. Ammonium acetate from Panreac (Barcelona, Spain) and ammonia from Merck (Darmstadt, Germany) were used for preparing the CE running buffers at different concentrations and pH values. Buffers were prepared by weighting the appropriate amount of ammonium acetate at the concentrations indicated and adding ammonium hydroxide (0.5 M) to adjust the pH. The buffers were prepared with doubly deionized water, stored at 4°C and brought to room temperature before use.

Doubly deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA). Triethylamine (TEA) from Aldrich (Steinheim, Germany) and 2-propanol HPLC-grade from Panreac were used in the sheath flow. Sodium hydroxide was from Panreac. SPE DSC-Diol cartridges were from Supelco (Bellefonte, PA). Extra-VOO samples were obtained from three Spanish varieties of olive: Picual, Hojiblanca and Lechín de Sevilla, all of which were harvested at the same time of year (January 2005). (Extra-VOO is a VOO which has a free acidity, expressed as oleic acid, of not more than 0.8 g *per* 100 g, and the organoleptic characteristics of which correspond to those fixed for this category.)

### 2.2 SPE of polyphenols from extra-VOO

A Diol-SPE extraction system was used to recover the phenolic compounds present in VOO. The SPE protocol was carried out with the extraction conditions and quantities of oil described by Gómez-Caravaca *et al.* [36]. Extra-virgin oil (60 g) was dissolved in 60 mL of hexane and was passed through the column. The solvent goes through, leaving the sample on the solid phase. The cartridge was washed with three portions (of 5 mL) of hexane, which

were then discarded in order to remove the nonpolar fraction of the oil. Finally, the sample was recovered by passing through eight portions (of 5 mL) of methanol and brought to dryness in a rotary evaporator under reduced pressure and a temperature of 35°C. The residue was dissolved with 2 mL of methanol/water (50/50 v/v) and filtered through a 0.25 μm filter before the CE analysis. These extracts were used in the optimization of the experimental conditions.

## 2.3 HPLC isolation and analysis of polyphenolic compounds

### 2.3.1 Apparatus

HPLC analyses were made with an HP 1100 series (Agilent Technologies, Palo Alto, CA) equipped with a binary pump delivery system, a degasser, autosampler, diode array UV-VIS detector (DAD) and a mass spectrometer detector (MSD).

The semipreparative HPLC column used was a Phenomenex Luna ($C_{18}$) column, 10 μm, 25 cm × 10 mm and the flow rate was 3 mL/min.

The analytical HPLC column used was a $C_{18}$ Luna column, 5 μm, 25 cm × 3.0 mm (Phenomenex, Torrance, CA), with a $C_{18}$ precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min.

### 2.3.2 Isolation of polyphenols using semipreparative HPLC

In the semipreparative 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 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 [36].

The injection volume for the isolation of the reference compounds was 100 μL of extracts in methanol:water 50/50 v/v. All of the analyses were carried out at room temperature. The wavelengths were set at 240, 280 and 330 nm. The following compounds were isolated: hydroxytyrosol (HYTY), tyrosol (TY), elenolic acid (EA), deacetoxy oleuropein aglycon, (+)-pinoresinol (Pin), (+)-1-acetoxypinoresinol (Ac Pin), oleuropein aglycon (Ol Agl) and ligstroside aglycon (Lig Agl).

### 2.3.3 Analysis of the isolated compounds using analytical HPLC

After isolation these compounds were analysed with the analytical column to check their purity and confirm their identity, using the same gradient as in the semipreparative HPLC method. The injection volume was 10 μL. The wavelengths were set at 240, 280 and 330 nm. Detection was also made using MS, the analyses being carried out using an electrospray (ESI) interface operating in positive mode under the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psi; drying gas temperature, 350°C; capillary voltage, 3000 V; fragmentor voltage, 60 V; and also using an atmospheric pressure chemical ionization (APCI) interface operating in negative mode under the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 30 psi·g; drying gas temperature, 350°C; vaporizer temperature, 300°C; capillary voltage, 3000 V; fragmentor voltage, 60 V. The polarity of ESI and APCI and all the parameters of the MS detector were optimized using the intensity of the MS signal for the main compounds detected in the methanol–water extracts of extra-VOO as an analytical parameter.

Using the same mobile phases, another gradient was used to check the purity of the isolated compounds, bearing in mind the fact that they only produced one peak in the chromatograms. The solvent gradient changed according to the following conditions: from 0 to 10 min, 90% (A): 10% (B) to 70% (A): 30% (B); from 10 to 20 min, 70% (A): 30% (B) to 65% (A): 35% (B); from 20 to 30 min, 65% (A): 35% (B) to 55% (A): 45% (B); from 30 to 40 min, 55% (A): 45% (B) to 50% (A): 50% (B); from 40 to 45 min, 50% (A): 50% (B) to 5% (A): 95% (B); from 45 to 48 min, 5% (A): 95% (B) to 95% (A): 5% (B). This last value was maintained for 5 min [36].

## 2.4 CE

The analyses were made with a P/ACE™ System MDQ (Beckman Instruments, Fullerton, CA, USA) CE apparatus equipped with an UV-Vis detector working at 214 nm and coupled to the MS detector by an orthogonal electrospray interface (ESI).

A commercial coaxial sheath-flow interface was used (see bellow). Bare fused-silica capillaries of 50 μm id came from Beckman Coulter (Fullerton, CA, USA). A detection window was created at 7 cm for the UV detector and 100 cm was the total length (corresponding to the MS detection length). UV detection was performed at 200 nm.

Capillary conditioning of the columns was done by flushing for 2 min with water, and then for 5 min with the separation buffer (60 mM ammonium acetate adjusted to pH 9.3 and 5% of 2-propanol) using during all the capillary conditioning 20 psi.
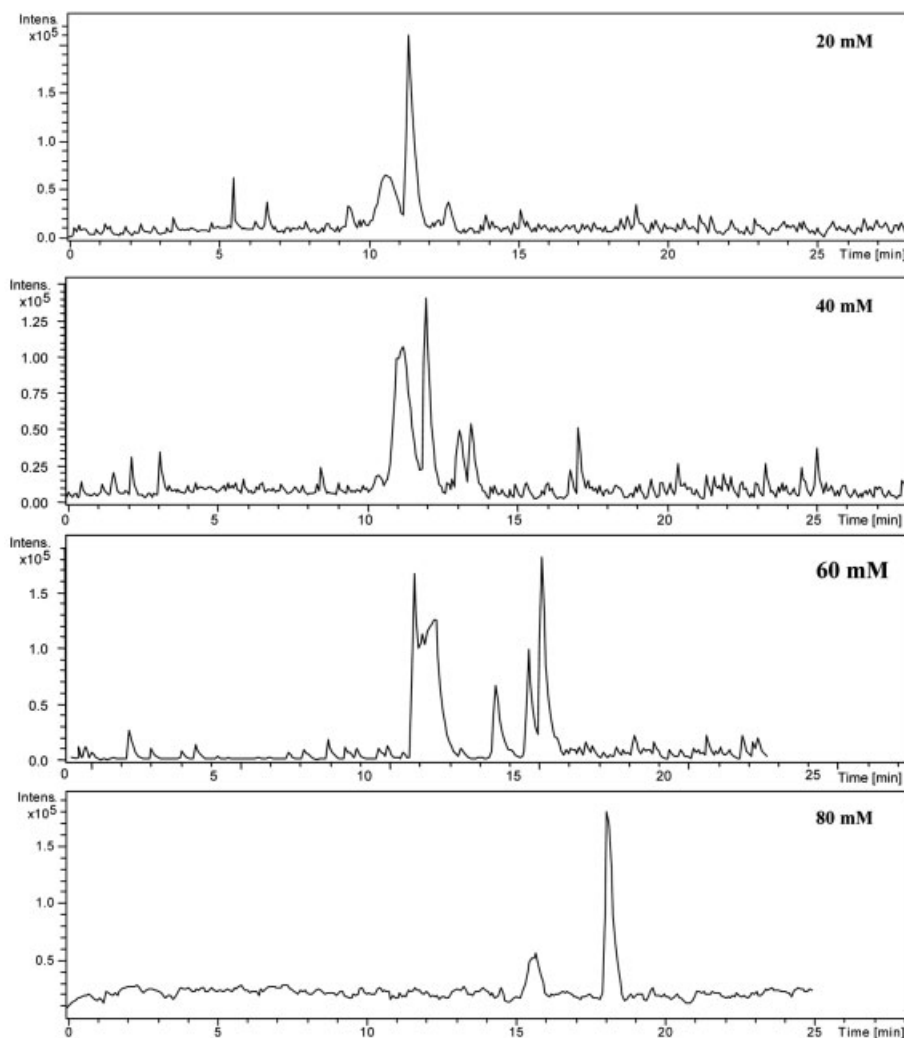
**Figure 1.** CE-ESI-MS electropherograms of a methanol/water extract obtained from an extra-VOO from the Picual variety of olive at different concentrations of ammonium acetate. Separation conditions: capillary, 100 cm × 50 μm; applied voltage, 25 kV; pH value, 9.5; hydrodynamic injection, 0.5 psi for 10 s. For ESI parameters, see text.

Before first use, the bare capillaries were conditioned with 0.1 M NaOH for 20 min followed by a water rinse for another 10 min. At the end of the day the capillary was flushed with water for 10 min and air for 5 min. The instrument was controlled by a PC running the 32Karat System software.

## 2.5 MS

MS experiments were performed on a Bruker Daltonics Esquire 2000™ IT mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies). Electrical contact at the electrospray needle tip was established *via* a sheath liquid by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA). The composition of this sheath liquid was 60:40 v/v 2-propanol/water with 0.1% v/v of TEA. For the connec-

tion between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation capillary was fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid established the electrical contact between capillary effluent and water for electrospray needle. The mass spectrometer was run in the negative ion mode and the capillary voltage was set at 4000 V. The IT scanned at 50–650 *m/z* range at 13 000 U/s during the separation and detection. The maximum accumulation time for the IT was set at 5.00 ms, the target count at 20 000 and the trap drive level at 100%. Electrospray operating conditions were optimized as described in Section 3, and the optimum values of the ESI-MS parameters were: drying gas temperature, 300°C; drying gas flow, 5 L/min; nebulizing gas pressure, 5 psi; compound stability, 25%; and sheath liquid flow, 0.30 mL/h. The instrument was controlled by a PC running the Esquire NT software.
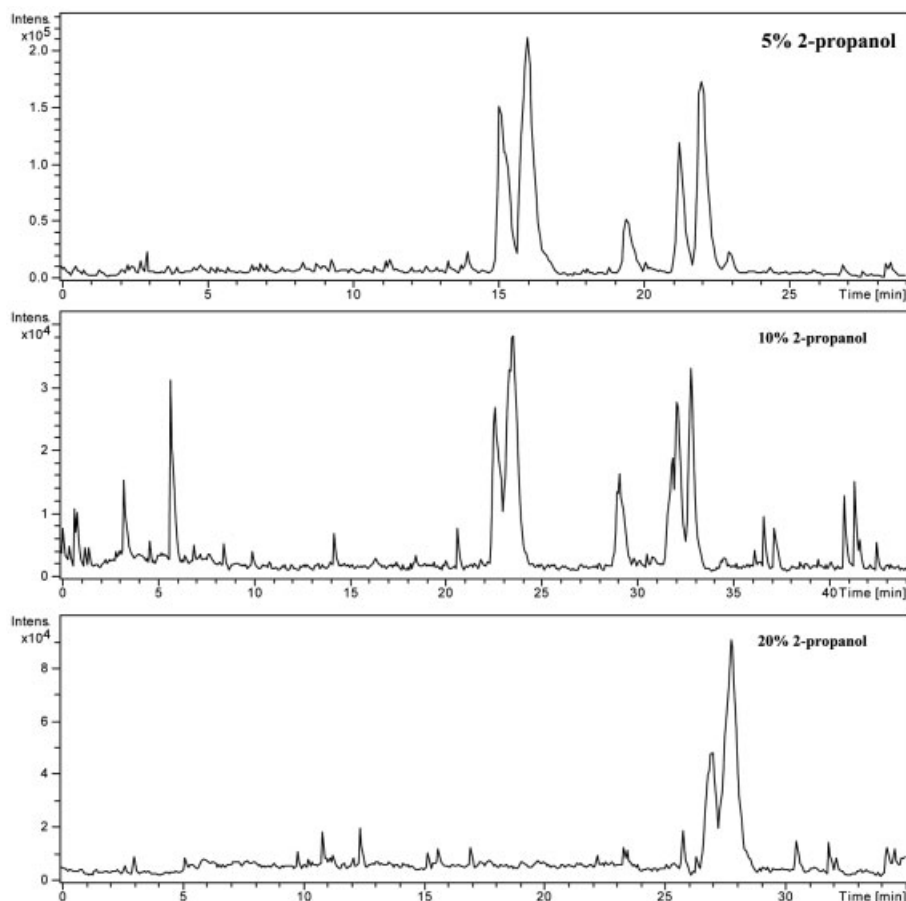
**Figure 2.** Optimization of the separation of the phenolic compounds present in an extract of Picual extra-VOO adding different percentages of 2-propanol. Separation conditions: capillary, 100 cm × 50 μm; applied voltage, 25 kV; ammonium acetate/ammonium hydroxide 60 mM pH 9.5; hydrodynamic injection, 0.5 psi for 10 s. For ESI parameters, see text (in the electropherograms, the intensity scales and the time scales are different).

## 3 Results and discussion

### 3.1 Development of the CE-ESI-MS method

The methanol–water extracts of a Picual extra-VOO obtained as described in Section 2.2 were used to optimize the electrophoretic and MS conditions.

Initially, the electrophoretic conditions were optimized according to the following criteria: migration behavior, sensitivity, analysis time and peak shape. First, buffers containing different concentrations of ammonium acetate at high pH values were tested. Due to the simple composition of volatile buffer solution it is essential to decrease the background noise and not to suppress the ionization efficiency of analytes in ESI. Amonium acetate concentration was varied from 10 to 80 mM (in steps of 10) in an attempt to improve the resolution and minimize the analysis time (Fig. 1); pH values from 8 to 11 (in steps of 0.5), and concentrations of 2-propanol from 0 to 15% (in steps of 2.5%) were assayed (Fig. 2) to obtain the optimum peak shape and best resolution and efficiency among the phenolic compounds. The optimum parameters turned out to be 60 mM ammonium acetate at pH 9.5 and 5% 2-propanol. The volt-

age applied was varied between 10 and 30 kV; a voltage of 25 kV was finally chosen in order to afford the best resolution together with satisfactory current and analysis time. The injections were made at the anodic end using an $N_2$ pressure of 0.5 psi for 10 s (1 psi = 6894.76 Pa). These conditions were chosen for the subsequent optimization of the ESI parameters. During buffer optimization we used the optimum values for the ESI parameters obtained in the preliminary studies: a sheath liquid containing 2-propanol/water 60:40 v/v and 0.1% v/v of TEA at a flow rate of 0.28 mL/h, a drying gas flow rate of 5.5 L/min at 300°C, nebulizer gas pressure of 5 psi and a compound stability of 25%.

It has often been demonstrated that optimization of the ESI parameters plays a key role in the achievement of adequate MS signals for any analyte. To optimize the detection of the compounds extracted from extra-VOO we applied a univariant method.

It is also well known that the choice of sheath liquid has a significant effect on the sensitivity and electrical contact between CE and ESI [39, 40]. Generally, small amounts of volatile TEA or ammonium hydroxide can be used for ESI-negative detection [41].
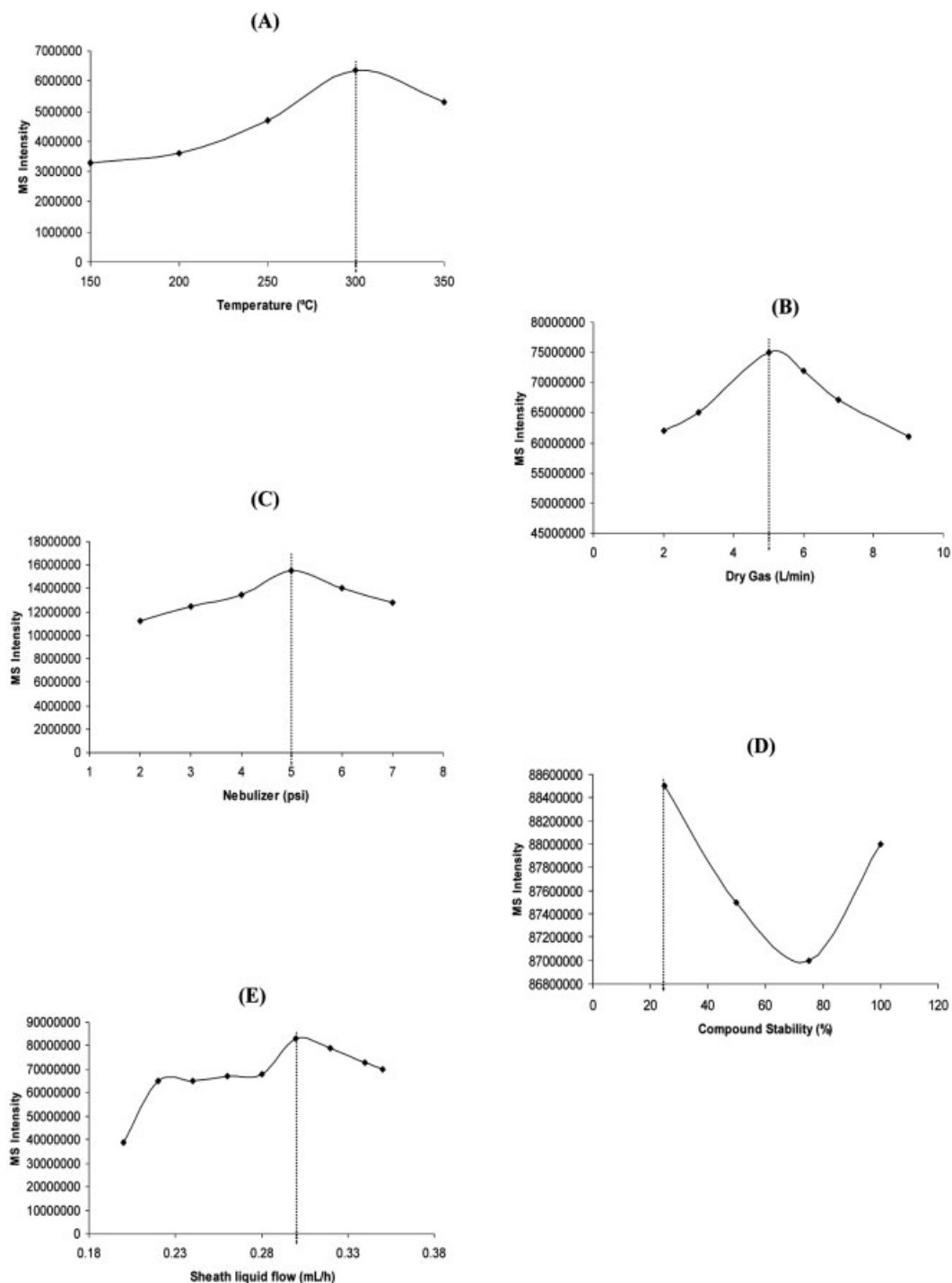
**Figure 3.** Optimization of ESI-MS parameters. For details see text. Dry gas temperature (A) and flow (B), nebulizing gas pressure (C), compound stability (D) and sheath liquid flow (E).

The ESI-MS operating conditions were optimized by adjusting the needle-counter electrode distance, sheath liquid composition, nebulizer gas flow rate and applied electrospray potentials while a sample solution was injected and separated in the CE-ESI-MS system.

Initially we tested six different types of sheath-flow liquids (after checking in the preliminary studies that the best results were obtained with 2-propanol as organic modifier): 2-propanol/water (50:50 v/v); 2-propanol/water (60:40 v/v); and 2-propanol/water (80:20 v/v), with and

**Figure 4.** Base peak electropherogram of the diol-SPE extract obtained from a Picual extra-VOO under optimized conditions.

without 0.1% v/v TEA. Using 80:20 sheath liquid with and without TEA, the current broke down after about 5 min, possibly due to poor electrical contact between the CE and ESI, which may have been due to the high organic content of the solution. The use of a sheath liquid of 60:40 v/v 2-propanol/water plus 0.1% v/v TEA however provided higher current stability and MS signal. Therefore, 60:40 v/v 2-propanol/water with 0.1% v/v TEA was selected as the sheath liquid.

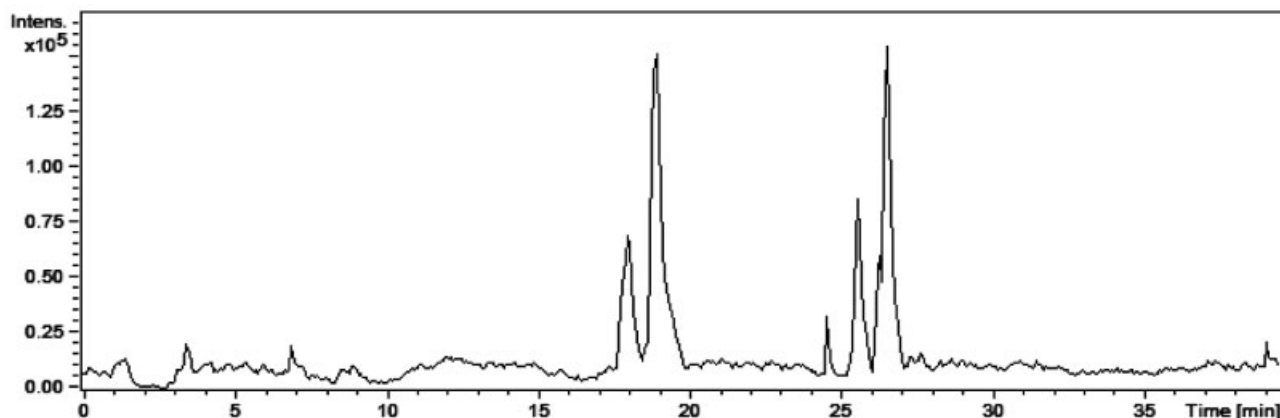We then optimized the other ESI-MS parameters, drying gas temperature and flow, nebulizing gas pressure, compound stability and sheath liquid flow (Figs. 3A–E) using the height of the MS signal for the main compounds detected in the methanol–water extracts of VOO as our analytical parameter. Initially the value for each parameter was the optimum found in the preliminary studies; after reoptimizing each parameter we then used the new optimum value to complete the optimization of the other parameters.

As can be seen, a temperature of 300°C provided the best signal (Fig. 3A), optimum drying gas flow was achieved at 5 L/min (Fig. 3B) and optimum nebulizer gas pressure was obtained at 5 psi (Fig. 3C).

It can also be seen in Fig. 3D that compound stability plays an important role in detecting olive-oil compounds. Thus, at higher percentages of compound stability the MS signal decreases due to the low number of molecules transferred into MS, whilst at lower percentages most of the compounds become 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 is the voltage applied by the MS instrument and, therefore, the higher is the solute fragmentation that can take place at that point. We chose 25% as optimum value.

The optimum sheath-liquid flow was one of 0.30 mL/h (Fig. 3E). This effect has also been mentioned in the literature [42]; at low sheath-liquid flows the ionization yield is reduced because of the instability of the spray whilst at higher flows the increased dilution of the electrophoretic bands emerging from the capillary may be excessive and the intensity of the MS signal for these compounds is therefore reduced.

Under these conditions CE-ESI-MS separations such as the one shown in Fig. 4 were obtained for methanol–water extracts of VOO.

Repeatability was studied by performing series of separations using the optimized method on one of the methanol–water extracts on the same day (intraday precision, *n* = 12) and on three consecutive days (interday precision, *n* = 36). The RSDs of peak areas/migration time and migration times were determined for two compounds (Pin and 10-hydroxy-oleuropein aglycon (10-H-Ol Agl)).

The intraday repeatabilities of the migration time for Pin and 10-H-Ol Agl (expressed as RSD) were 0.45 and 0.82%, respectively, whilst the interday repeabilities of the migration time were 0.89 and 1.26%. The intraday repeatabilities of the total peak area/migration time (expressed as RSD) were 0.93 and 1.53%, whilst the interday repeatabilities of the total peak area/migration time were 1.32 and 2.04% for Pin and 10-H-Ol Agl, respectively.

## 3.2 Characterization of extra-VOO phenolic compounds by CE-ESI-MS

The potential of the CE-ESI-MS method was checked by characterizing the Diol-SPE extracts obtained from an extra-VOO from Picual olives.

The peaks of the main polyphenols of extra-VOO were identified by comparing both migration time and MS data obtained from olive-oil samples and compounds isolated by HPLC, with spiked methanol–water extracts of olive oil with HPLC-collected compounds at several concentration levels, taking the elution order into account and bearing in mind all the data reported in the literature. We also checked that the fragmentation patterns for all the phenolic compounds present in the olive oil and their corresponding standards (isolated by HPLC) were the same. With the use of these standards we were able to confirm that there was no contamination from common fragments from comigrating compounds (data not shown).

Figure 5 shows the extracted ion electropherograms of several compounds identified in the methanol–water extract of an extra-VOO of Picual olives. The following compounds were identified: three simple phenols (TY, HYTY and 2-(4-hydroxyphenyl)ethyl acetate (HYTY-Ac)); two lignans (Pin and Ac Pin); several complex phenols (Lig Agl, Ol Agl and their respective decarboxylated derivatives (decarboxylated-Lig Agl and decarboxylated-Ol Agl); and several isomeric forms of these (dialdehydic form of oleuropein aglycon (dialdehydic-Ol Agl), dialdehydic form of ligstroside aglycon (dialdehydic-Lig Agl), dialdehydic form of decarboxymethyl elenolic acid linked to HYTY (decarboxylated-dialdehydic-Ol Agl), dialdehydic form of decarboxymethyl elenolic acid linked to TY (decarboxylated-dialdehydic-Lig Agl)) and 10-H-Ol Agl); and one other phenolic compound (EA). The structures of the phenolic compounds in question can be seen in Fig. 6.

Fragmentation patterns for all the compounds described are set out in Table 1; Fig. 7 demonstrates that it is possible to obtain satisfactory MS spectra for all the analytes. It is important to bear in mind that the detection in negative polarity is more selective than in positive mode, but we can also highlight the difficulty of ESI-MS analysis in the negative mode in terms of sensitivity. Some of the MS spectra given in the previous figure are quite noisy – fact that can be explained first – for the concentration of several of the compounds under study in the sample of extra-VOO and second, because of carrying out the detection in negative mode.

The major simple phenols found in the extracts of Picual VOO were HYTY and TY. HYTY-Ac has been described elsewhere in Spanish olive varieties [25] and has also been identified in the Picual variety using this CE-ESI-MS method.

As has been reported in previous papers, Ac Pin is present in small quantities in this variety, and on occasions has been used as a tool to authenticate Picual olive oil [43]. We also found Pin in the Picual oils that we analysed. According to Owen *et al.* [44] these compounds (lignans) are the main components of the phenolic fraction of the olive seed and are practically absent from the pulp, leaves and limbs, and therefore their presence in the oil must be due to the pits' being broken when the olives are crushed.

It is interesting to study the isomeric forms of the secoiridoids in some depth. In order to understand this phenomenon, the possible biochemical mechanism for the formation of secoiridoid derivatives starting from oleuropein or ligstroside *via* the action of β-glucosidase and then methylesterase is shown in Fig. 8. This mechanism has been explained by several authors [45]. Oleuropein is present in olive oils in the form of aglycon due to the action of hydrolytic enzymes released during their preparation. This hydrolysis also causes partial modification of the aglycon due to a keto-enolic tautomeric equilibrium that involves the ring opening of secoiridoids.

Two peaks can be seen in the extract ion electropherogram of Ol Agl, one corresponding to the mono-aldehydic form of Ol Agl and the other to its dialdehydic form. Whenever we found two peaks with the same mass spectra, we presumed that the first peak corresponds to the dialdehydic form and the other to the aldehydic form (closed-ring structure) of the compound. In the case of oleuropein derivatives (HYTY moiety derivatives) the migration times of the two peaks are farther apart, which might be put down to the double negative charge of the HYTY part in this molecule. The same fact can be observed in the extract ion electropherogram of Lig Agl. Two peaks always appear with the decarboxylated forms of these compounds, corresponding once more to the monoaldehydic and dialdehydic forms.

As mentioned above, several authors have observed this phenomenon using chromatographic techniques. Angerosa *et al.* [22], for example, found that although in their chromatograms (made using GC-MS) there was a peak corresponding to the silylated form of decarbomethoxyligstroside aglycon, three other peaks also appeared, all with the same mass spectrum. Their conclusion was that two of them probably corresponded to a closed-ring structure and the other to silylated enolic forms of mono or dialdehydic forms of the elenolic moiety. In the same way, Caruso *et al.* [46] observed that four peaks were present in the ion chromatogram of ions at *m/z* 377 (Ol Agl). Other interesting results have also been published on the same subject [47–49].

It is important to note that we also detected 10-H-Ol Agl showing *m/z* 393 in Picual olive oil. Another fragment that can be observed for this compounds is *m/z* 363.0, which corresponds to the loss of the $CH_3OH$ group.
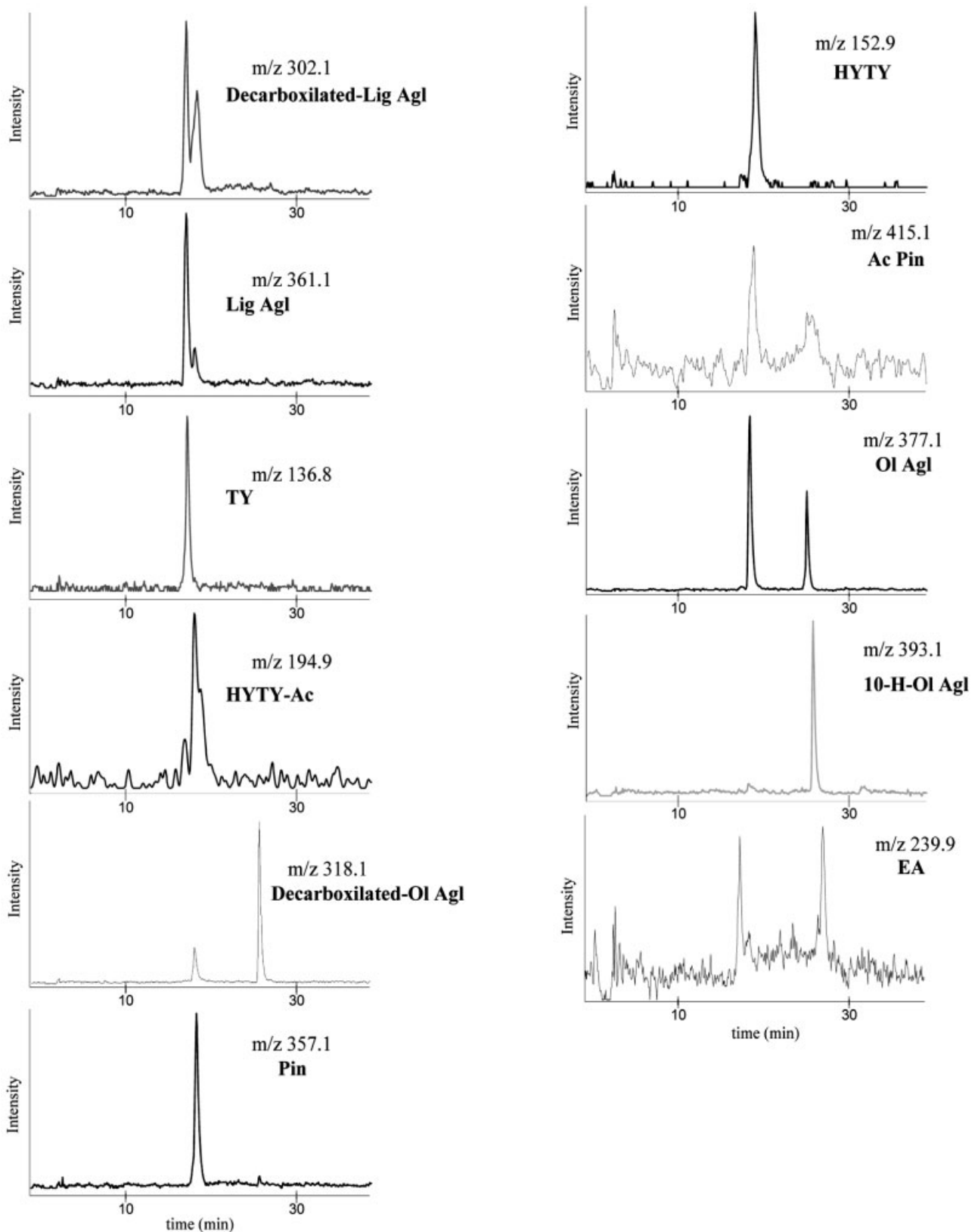
**Figure 5.** Extracted ion electropherograms of the compounds identified in the methanol–water extract of an extra-VOO of Picual variety.
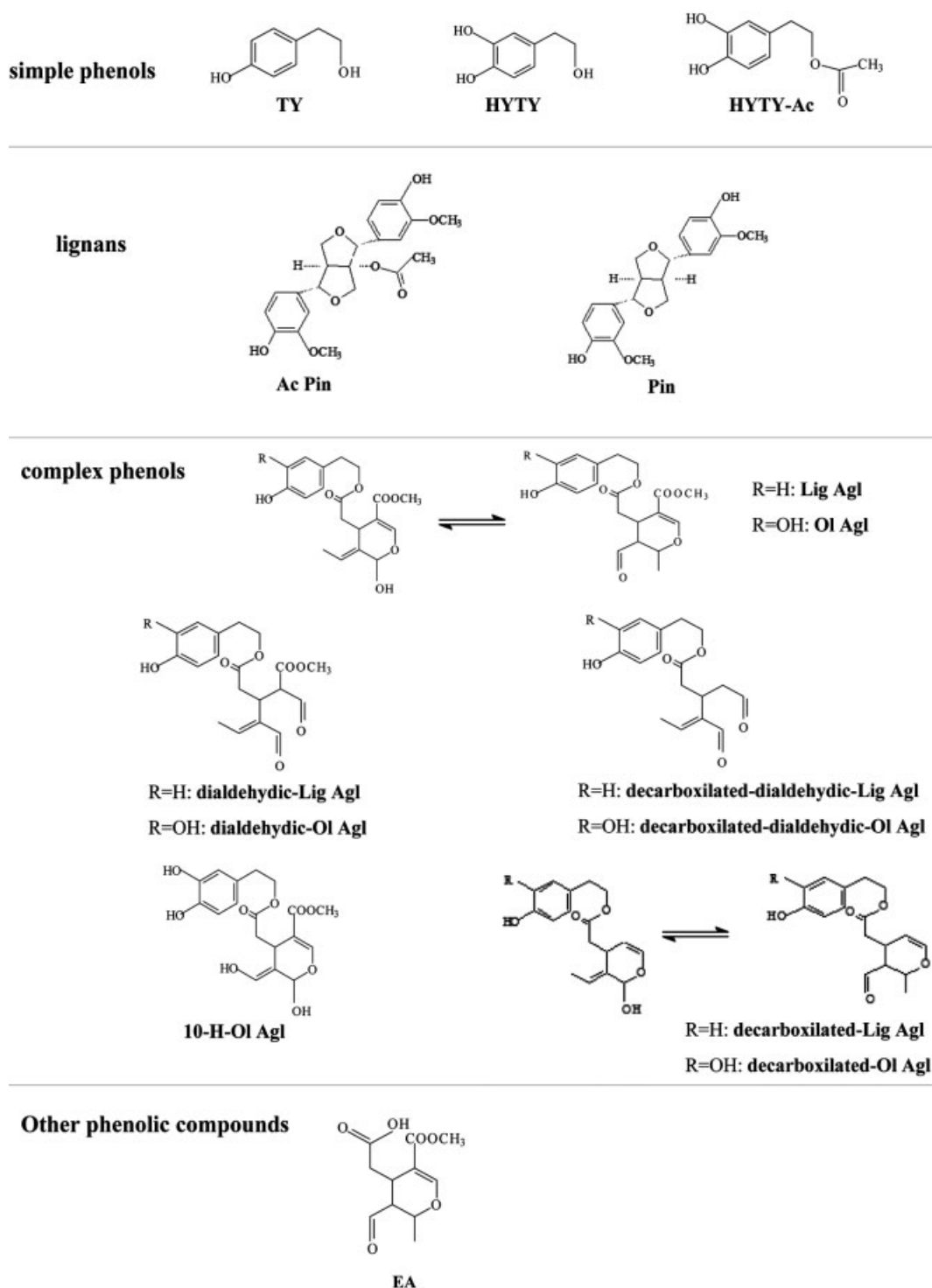
**Figure 6.** Chemical structures of phenolic compounds under study. TY, tyrosol; HYTY, hydroxytyrosol; HYTY-Ac, 2-(4-hydroxyphenyl)ethyl acetate; Ac Pin, (+)-1-acetoxypinoresinol; Pin, (+)-pinoresinol; Lig Agl, ligstroside aglycon; Ol Agl, oleuropein aglycon; dialdehydic-Lig Agl, dialdehydic form of ligstroside aglycon; dialdehydic-Ol Agl, dialdehydic form of oleuropein aglycon; decarboxylated-dialdehydic-Lig Agl, dialdehydic form of decarboxymethyl elenolic acid linked to TY; decarboxylated-dialdehydic-Ol Agl, dialdehydic form of decarboxymethyl elenolic acid linked to HYTY; 10-H-Ol Agl, 10-hydroxy-oleuropein aglycon; decarboxylated-Lig Agl, decarboxylated form of ligstroside aglycon; decarboxylated-Ol Agl, decarboxylated form of oleuropein aglycon; EA, elenolic acid.
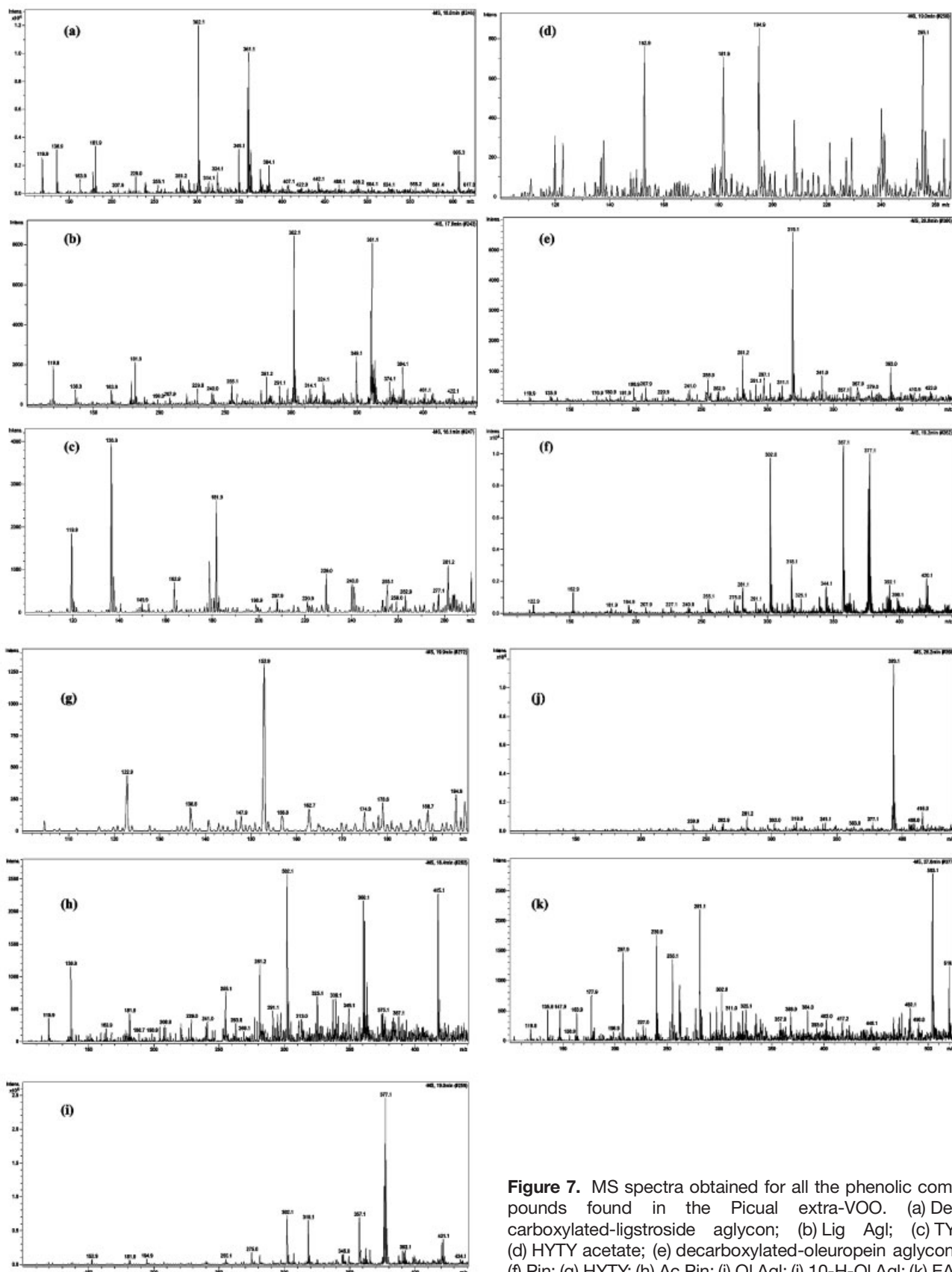
**Figure 7.** MS spectra obtained for all the phenolic compounds found in the Picual extra-VOO. (a) Decarboxylated-ligstroside aglycon; (b) Lig Agl; (c) TY; (d) HYTY acetate; (e) decarboxylated-oleuropein aglycon; (f) Pin; (g) HYTY; (h) Ac Pin; (i) Ol Agl; (j) 10-H-Ol Agl; (k) EA.

**Table 1.** Fragmentation patterns for all the phenolic compounds under study

| Analyte | Molecular mass | Major ESI fragments | | | |
|---|---|---|---|---|---|
| | | $[M-H]^-$ | $[2M-H]^-$ | $[M-OH-H]^-$ | Other |
| Decarboxylated-Lig Agl | 304.3 | 302.1 | 605.3 | | 119.9 |
| | | | | | 163.9 |
| Lig Agl | 362.4 | 361.1 | | | 119.9 |
| Tyrosol | 138.2 | 136.8 | | 119.9 | |
| HYTY acetate | 196.2 | 194.9 | | | 122.9 [M–COCH$_3$–CH$_2$OH] |
| | | | | | 152.9 [M–COCH$_3$] |
| Decarboxylated-Ol Agl | 320.3 | 318.1 | 637.3 | 302.1 | 122.9 |
| | | | | | 152.9 |
| | | | | | 227.1 |
| Pin | 358.4 | 357.1 | | | |
| HYTY | 154.2 | 152.9 | | 136.8 | 122.9 [M–CH$_2$OH] |
| Ac Pin | 416.4 | 415.1 | | | |
| Ol Agl | 378.4 | 377.1 | | | 122.9 |
| | | | | | 152.9 |
| | | | | | 240.0 |
| | | | | | 345.1 [M–CH$_3$OH] |
| 10-H-Ol Agl | 394.4 | 393.1 | | | 363.0 [M–CH$_3$OH] |
| EA | 242.2 | 239.9 | 482.1 | | |

### 3.3 Differences among three varieties of extra-VOO

The potential of this CE-ESI-MS method to characterize the extracts obtained from extra-VOO was demonstrated during the optimization and all the analyses of the extracts of a Picual olive oil. We were subsequently able to compare the results obtained for three monovarietal extra-VOOs: Picual, Lechín de Sevilla and Hojiblanca. All samples were injected into the CE instrument seven times (*n* = 7). The peaks were identified by comparing both migration times and MS data obtained from olive oil samples and compounds isolated by HPLC, and with spiked extracts of olive oil with compounds collected by HPLC at several concentrations.

The differences in the polyphenolic profiles can be seen in Table 2, where the quantitative results are presented as values of area. If we observe the areas for the simple phenols, the differences among these three varieties are not very significant; only in the case of HYTY-Ac is it possible to highlight that Lechín de Sevilla olive oil is the richest in terms of this compound. As we expected, smaller quantities of Ac Pin were present in the Picual oil compared to Lechín de Sevilla and Hojiblanca.

This is the first time that the isomeric forms of several secoiridoids have been determined and semiquantified using CE with MS as detector. With another CE method developed by our research group [36] we observed that the most representative and abundant peak in Lechín de

Sevilla extracts was that of decarboxylated-Ol Agl. In this case, it is important to consider that the first of the isomeric forms of this compound that can be determined with this method is more abundant in the same variety; nevertheless, the highest quantity of the second peak was found in Hojiblanca oil. Lechín de Sevilla is the richest variety if the second peak of EA is taken into account.

There were no significant differences among these extra-VOOs in terms of the first peak of Ol Agl, but the area for the second form of this compound was between seven and ten times bigger in Hojiblanca than in the other two.

Finally, similar concentrations of Lig Agl were found in all three oils studied, but in Picual it was significantly more abundant when taking the second peak into account.

## 4 Concluding remarks

We describe a qualitative and semiquantitative CE-ESI-MS method to study phenolic alcohols, lignans and several complex phenols in olive-oil samples after their extraction by Diol-SPE. The compounds were identified using the electrophoretic results, the molecular weight, the structural information of MS obtained using HPLC and EC, and the isolated phenolic compounds by HPLC.

Repeatability of the method was studied and the RSDs of peak areas/migration time (determined for two compounds, Pin and 10-H-Ol Agl) were 0.93 and 1.53% in the
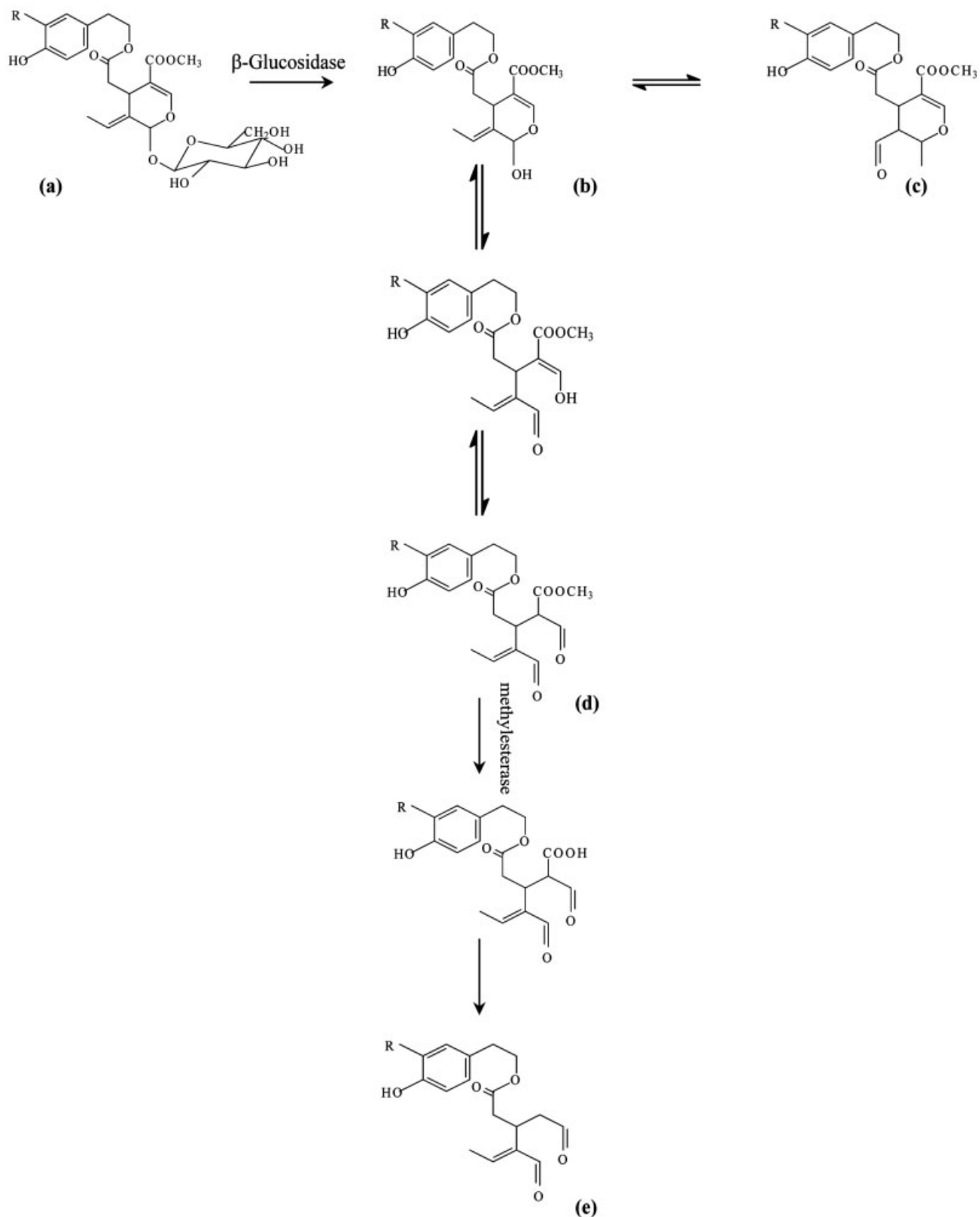
**Figure 8.** Proposed biochemical mechanism for the formation of secoiridoid derivatives: (a) R=H: ligstroside; R=OH: oleuropein; (b) R=H: Lig Agl; (c) R=OH: Ol Agl; (d) R=H: dialdehydic-Lig Agl; R=OH: dialdehydic-Ol Agl; (e) R=H: de-carboxylated-dialdehydic-Lig Agl; R=OH: decarboxylated-dialdehydic-Ol Agl.

**Table 2.** Results of the analysis of three monovarietal extra-VOO samples (*n* = 7) (Value = area $\times 10^{-3}$)

| Analyte | Picual | Lechín de Sevilla | Hojiblanca |
|---|---|---|---|
| | | | 640.2[a] |
| Decarboxylated-Lig Agl | 2 215.1[a] | 994.2[a] | |
| | 2 410.7[b] | 594.8[b] | 2 962.0[b] |
| Lig Agl | 3 047.4[a] | 4 254.6[a] | 4 940.4[a] |
| | 590.6[b] | 1 448.1[b] | 128.3[b] |
| TY | 640.7 | 711.3 | 839.3 |
| HYTY-Ac | 208.2 | 1 315.2 | 757.7 |
| Decarboxylated-Ol Agl | 1 005.8[a] | 8 601.2[a] | 507.4[a] |
| | 3 368.9[b] | 6 131.8[b] | 8 723.4[b] |
| Pin | 1 567.1 | 912.5 | 803.8 |
| HYTY | 690.8 | 980.8 | 998.1 |
| Ac Pin | 237.9 | 3 695.9 | 971.2 |
| Ol Agl | 3 794.3[a] | 5 224.9[a] | 2 006.3[a] |
| | 1 656.0[b] | 1 063.5[b] | 11 895.0[b] |
| 10-H-Ol Agl | 1 334.1 | 6 596.8 | 8 723.4 |
| EA | 206.6[a] | 766.6[a] | 564.1[a] |
| | 266.2[b] | 1 182.3[b] | 727.6[b] |

RSD in all the cases ≤5%.
For the compounds that have two isomeric forms:
a) Area corresponding to the first of the peaks of the isomeric forms.
b) Area corresponding to the second of the peaks of the isomeric forms.

intraday study, and 1.32 and 2.04% in the interday study for Pin and 10-H-Ol Agl, respectively.

After demonstrating the potential of this CE-ESI-MS method to characterize the extracts obtained from an extra-VOO, it was used to compare the phenolic content in three monovarietal extra-VOOs: Picual, Lechín de Sevilla and Hojiblanca.

This method also permits to observe the isomeric forms of secoiridoid derivatives of olive oil for first time using CE-ESI-MS.

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# Capítulo 11

# CE- y HPLC-ESI-TOF para la caracterización de compuestos fenólicos en aceite de oliva

Al igual que el capítulo anterior (capítulo 10), se empleó la espectrometría de masas como sistema de detección en el trabajo experimental que aquí se recoge. La diferencia radica en que, en este caso, el analizador no fue la trampa de iones (IT), sino el (tiempo de vuelo) TOF; además no se utilizó una sola técnica separativa, sino que se compararon CE y HPLC.

La IT nos permite realizar espectros MS/MS en tiempo real, proporciona buena sensibilidad, una limpia información espectral y es aplicable a un amplio abanico de muestras. Sin embargo, para poder continuar caracterizando este grupo de compuestos, es necesario emplear sistemas de detección aún más potentes, que sean capaces de dar al analista la masa de los analitos con un error mínimo (casi total certeza) que nos permita estar seguros de la fórmula molecular y ayudar a comprender las posibles vías de fragmentación de los mismos y con ello, las posibles estructuras.

Micro-TOF es un detector relativamente nuevo todavía, que está ofreciendo resultados muy satisfactorios y que presenta ciertas ventajas respecto a la trampa de iones. 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.

Desarrollando una estancia de investigación en una de las sedes de Bruker Daltonik (Bremen, Alemania) tuvimos la oportunidad de profundizar en el estudio de la fracción fenólica del aceite de oliva usando CE y HPLC con ESI-MicroTOF. Así pudimos comparar los resultados obtenidos por las dos técnicas separativas, viendo qué compuestos eran capaces de determinar y cuál era más apropiada para cada uno de ellos. Se lograron determinar más de 45 compuestos mediante cada uno de los dos métodos optimizados. De esos 45, un gran número de compuestos pertenecían a familias que han sido descritas por muchos autores (alcoholes fenólicos, ácidos fenólicos, lignanos, flavonoides y secoiridoides), pero también hubo un gran número de compuestos determinados que eran "nuevos".

# Capillary electrophoresis- and High performance liquid chromatography-time of flight-mass spectrometry used to characterize of phenolic compounds in olive oil

**A. Carrasco-Pancorbo[1], C. Neusüβ[2], M. Pelzing[2], A. Segura-Carretero[1], A. Fernández-Gutiérrez[1].**

[1] Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.

[2] Bruker Daltonik GmbH, Fahrenheitstr. 4, 28359 Bremen, Germany.

## Abstract

We present an easy and rapid method for the analysis of phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis (CZE) coupled to electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS). Optimum electrophoretic separation was obtained using a basic carbonate electrolyte. We thus achieved the determination of several important families (phenyl alcohols, phenyl acids, lignans, flavonoids and secoiridoids) of the polar fraction of the olive oil. Furthermore, other "unknown" compounds were also identified.

In addition to the CZE method, HPLC analyses were made, separating compounds belonging to the main families present in this polyphenolic fraction, as well as other new compounds. We compared the results obtained with both techniques and found it was possible to determine more than 45 compounds with both methods.

The sensitivity, together with mass accuracy and true isotopic pattern of the TOF-MS, allowed identification of a broad series of known and so far not described phenolic compounds present in extra-virgin olive oil.

**Keywords**: Capillary electrophoresis / HPLC / Electrospray-time of flight-mass spectrometry / Olive oil / Phenolic compounds

[*]Author to whom correspondence should be addressed:

Dr. A. Segura Carretero or A. Fernández Gutiérrez, Research Group FQM-297, Department of Analytical Chemistry, Faculty of Sciences, University of Granada, C/Fuentenueva s/n, E-18071 Granada, Spain.

**E-mail:** ansegura@ugr.es or albertof@ugr.es

**Fax:** +34 958 249510

# 1 Introduction

The chemical composition of olive oil is principally glycerols representing more than 98% of the total weight. Minor components amount to about 2% of the total oil weight and include, among others, aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and several antioxidants [1]. Phenolic compounds represent one of these families of antioxidants which are present in olive oil.

Phenolic compounds are of fundamental importance for their nutritional properties, sensory characteristics, and the shelf life of virgin olive oil [2,3], since they have high anti-oxidative properties and can confer a marked bitter taste or a sweet taste typical of some virgin olive oils [4]. They also play an important role in human nutrition as preventative agents against several diseases [5,6]. The composition of phenolic compounds in virgin olive oil is related to agronomic and technological aspects [7].

The main components of the phenolic fraction of virgin olive oil are hydroxytyrosol, tyrosol, and their derivatives linked to the aldehydic and dialdehydic forms of elenolic acid, which are described as secoiridoids [8,9]. Moreover, significant amounts of the lignans, such as pinoresinol and 1-acetoxypinoresinol are also present [10,11], as well as flavonoids (luteolin and apigenin) [12,13], phenolic acids (such as caffeic, vanillic, syringic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic and *p*-hydroxybenzoic acid) [14,15] and hydroxy-isochromans [16].

Therefore, the qualitative and quantitative determination of these phenolic compounds in oils is very important and several methods have been already used in recent years. Various methods have been developed for the extraction of these substances from oils as well as separation methods for their analysis.

There is an extensive literature concerning the detection and quantification of phenolic compounds in olive oil. Starting from the early days, non-specific analytical methods, such as paper, thin-layer, and column chromatography, as well as UV spectroscopy were applied to polyphenols analysis with limited success [17].

The need to profile and identify individual phenolic compounds meant that traditional methods were replaced and significant progress was achieved when specific analytical methods were used, such as high-resolution gas chromatography (GC) and, in particular, high-performance liquid chromatography (HPLC). Furthermore, CE has been recently applied for the analysis of phenolic compounds in olive oil and has opened up great expectations, especially due to the higher resolution, reduced sample volume and analysis duration.

GC has been used with three different detectors, FID [18], NMR [19] or MS [20,21,22]; in HPLC it is possible to find research works where UV (photodiode array) [23,24], fluorescence [25,26], electrochemical [27,28], biosensors [29], NMR [17] and MS [30,31,32,33] detectors are used; whilst CE has been used with UV as detection system [10,34,35] and, more recently, MS detectors [36].

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.


Of all the HPLC and CE detection methods reported to date, mass spectrometry clearly has the greatest potential. The advantages of MS detection include the capability for both determining molecular weight and providing structural information.

HPLC and CE can be coupled with different MS analyzers (i.e., with quadrupole, ion trap, Time-of-flight, etc.) and use several ionization methods (APCI, ESI, MALDI…). In this paper we have used CE- and HPLC-ESI-TOF for the analysis of the phenolic fraction of extra-virgin olive oil. ESI is one of the most versatile ionization methods and is the natural method of choice for the detection of ions separated by capillary zone electrophoresis. TOF MS provides excellent mass accuracy [37] over a wide dynamic range if a modern detector technology is chosen. The latter, moreover, allows measurements of the correct isotopic pattern [38], providing important additional information for the determination of the elemental composition [39].


The aim of this work was to develop improved methods using CE and HPLC-ESI-TOF for the characterization of phenolic compounds from extra-virgin olive oil. The potential of these two separation techniques coupled with a TOF detector has been evaluated. The methods are compared in terms of selectivity, analysis time, separation efficiency, operating cost, and sensitivity.

# 2 Materials and methods

## 2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogen carbonate and ammonium hydroxide were from Fluka (Buchs, Switzerland), and ammonium acetate from Merck (Darmstadt, Germany).

The organic solvents, methanol, ACN, and 2-propanol, all from Sigma Aldrich (St. Louis, MO), were used for the CE running buffers, mobile phase in HPLC and sheath liquids. 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).

CE Buffers were prepared by weighing ammonium hydrogen carbonate or ammonium acetate at the concentrations indicated and adjusting the pH when necessary by adding ammonium hydroxide. The buffers were stored at 4ºC and warmed to room temperature before use. Sodium hydroxide solution (1.0 N) from Agilent technologies was used for capillary cleaning procedures before each analysis.

All solutions and buffers were degassed by ultrasonication before use.

## 2.2 Samples

Extra-virgin olive oil samples used in the current study were from 5 varieties of olive fruit called Arbequina, Picual, Hojiblanca, Lechín de Sevilla and Cornicabra (January 2005). For simplicity we present in this paper the chromatographic and electrophoretic profiles of extracts of Arbequina extra-virgin olive oil, although we used the other varieties during the optimization of the separation conditions in each method. The reason for using all these varieties during the optimization was to ensure the potential of the presented methodologies (HPLC and CE) for the analysis of these compounds in any kind of olive oil.

To isolate the phenolic fraction, we used SPE with Diol-cartridges; the SPE protocol was carried out with the extraction conditions and amounts of oil which are described

elsewhere [35]. The extract of extra-virgin olive oil was diluted 1:10 with methanol/water (50/50 v/v) before injection into the CE or HPLC, respectively.

## 2.3 Capillary electrophoresis analyses

CE was performed using a Hewlett Packard [3D]CE (Agilent Technologies, Waldbronn, Germany). If not otherwise specified in the text, fused-silica capillaries of 85 cm in length and 50 µm inner diameter (360 µm outer diameter) were used.

Separation was evaluated based on acetate- or carbonate-containing background electrolytes. Different pH (adjusted with $NH_3$), buffer concentrations, as well as different amounts of organic modifiers were tested (see Results and Discussion Section).

After thorough optimization (see below), we chose as a running buffer 25 mM ammonium hydrogen carbonate at pH 9.0.

The separation voltage was set to 30 kV at the inlet of the capillary. Injection was performed hydrodynamically, typically 50 mbar were applied for 10 s, corresponding to about 10 nL injected (0.6 % of the capillary). For CE-MS coupling, a coaxial sheath-liquid sprayer was used (Agilent Technologies). Isopropanol/water (1:1) was applied as sheath-liquid at a flow rate of 4 µL/min delivered by a 5 mL gas-tight syringe (Hamilton, Reno, NV, USA) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). An electrospray potential of + 4.5 kV was applied at the inlet of the MS (negative mode). A nebulizer gas pressure of 0.15 bar was applied to assist the spraying. Drying gas temperature was set at 180ºC; and drying gas flow at 5 L/min.

All new capillaries were conditioned before their first use by flushing with 1 M NaOH for 10 min followed by a rinse with water for 20 min. Initially the capillary washing routine between runs consisted of 3 min with water followed by 3 min with running buffer (all rinses done using $N_2$ at a pressure of 20 psi). However, we observed that resolution and reproducibility of separation were lost after five injections. Therefore, conditioning was completed by flushing the capillary with sodium hydroxide solution, water and running buffer for 3, 2 and 4 min, respectively.

Using this protocol, the %RSD values for migration times of analytes were lower than 0.93% for five consecutive runs, indicating an adequate capillary reconditioning between runs. Therefore, this latter protocol was used (see Repeatability study section).

## 2.4 HPLC analyses

The separation of the phenolic compounds from extra-virgin olive oil was performed using an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Gernamy) consisting of vacuum degasser, autosampler, and a binary pump equipped with a reversed-phase $C_{18}$ analytical column (2.1 x 100 mm, 3.5 μm particle size; XTerra MS). A volume of 5 μL of the 1:10 diluted (methanol/water 1:1) methanolic extracts of olive oil was injected. Mobile phases A and B consist of water with 0.1% acetic acid, and acetonitrile, respectively. The chromatographic method consisted of a linear gradient from 0 to 100% B during 30 min, followed by a cleaning cycle of 8 using min 100% B and 7 min with 0% B (initial conditions). The flow rate used was 0.20 mL/min. The temperature of the column was maintained at 20ºC.

The HPLC system was coupled to a TOF mass spectrometer equipped with an electrospray ionization (ESI) interface operating in negative ion mode using a capillary voltage of + 4 kV. The other optimum values of the ESI-MS parameters were: drying gas temperature, 200ºC; drying gas flow, 8 L/min; and nebulizing gas pressure, 1.5 Bar.

## 2.5 Mass Spectrometry

MS was performed using the microTOF$^{TM}$ (Bruker Daltonik, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOF-MS). Transfer parameters were optimized by direct infusion experiments with extra-virgin olive oil extracts, as well as with several of the most important compounds belonging to this polar fraction of the olive oil which are commercially available (tyrosol and hydroxytyrosol (phenyl alcohols); luteolin and apigenin (flavonoids); dopac, vanillic, caffeic and *o*-coumaric acid (phenolic acids); and oleuropein glucoside (secoiridoid)).

Thus, good sensitivity at a reasonable resolution was obtained (5,000–10,000 at 250 *m/z*). The trigger time was set to 50μs, corresponding to a mass range of 50–800 *m/z*. Spectra were acquired by summarizing 30,000 single spectra, defining the time resolution to 1.5 s.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 3.3 (Bruker Daltonik GmbH), which provided a list of possible elemental formula by using the GenerateMolecularFormula[TM] editor. The GenerateFormula[TM] editor uses 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 (SigmaValue[TM]) for increased confidence in the suggested molecular formula [40]. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm [41].

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

## 3. Results and Discussion

### 3.1 CE-TOF-MS analyses

#### 3.1.1 Optimization of the separation conditions

The methanol-water extracts of an Arbequina extra-VOO were used to optimize the electrophoretic and MS conditions. Initially, the electrophoretic conditions were optimized according to the following criteria: separation selectivity, sensitivity, analysis time and peak shape.

Separation was evaluated using acetate- ($NH_4OAc$) and carbonate ($NH_4HCO_3$)-containing background electrolytes. Based on reports for successful CZE separation of the compounds present in the polar fraction of olive oils [10,34,35,36], the optimum

value of pH to get the best separation had to be between 8.5-10. However, we studied a wider range adjusting the pH value by $NH_3$ to observe the behaviour of these phenolic compounds using a TOF-MS as detector. Using an acetate ($NH_4OAc$) buffer at 25 mM the separation was tested in 12 pH steps between pH 6.8 and 10.4; whilst using a carbonate electrolyte (25mM $NH_4HCO_3$) the studied pH range was 9.0 to 10.4 (6 steps). At a pH value higher than 10.4 and a concentration of 25mM $NH_4HCO_3$, the current was no longer stable. For this reason, electrolytes with 5 mM of salt concentration and increasing concentration of $NH_3$ (up to 4 M of $NH_3$) were used to study the higher pH region. Using these latter buffers, the method did not result in acceptable MS signals. This was probably due to inefficient ionization in the presence of high concentration of ammonium.

In this pH study, each BGE composition was tried out for all the five extra-virgin olive oil varieties, to ensure the potential of the present CE method for the analysis of these compounds in any kind of olive oil (data not shown).

In general, the profiles obtained using carbonate ($NH_4HCO_3$)-containing background electrolytes were better than those obtained with buffers based on acetate- ($NH_4OAc$). The BPE (base peak electropherogram) (50-800 m/z) at different values of pH (using carbonate ($NH_4HCO_3$) buffer) is presented in Fig. 1. The first and the last electropherograms (i.e. pH 9.0 and 10.4) are the best taking into account the number of peaks, analysis time and resolution among the peaks. In Fig. 1a the peaks that are higher in the electropherogram are TY, Pin, Ac Pin, Ol Agl, DOA and Apig. TY, Ol Agl, DOA and Apig continue to be the highest at pH 9.5 (Fig. 1b), but the shape of the lignans is worse, since they appeared as three peaks with a broad central peak. At pH 9.9 (Fig. 1c), DOA is the most representative peak in the BPE. Ac Pin, Pin, Ol Agl and Apig still produce peaks of considerable intensity. The shape of TY, however, changes a lot, as it appears as a peak with a huge width. When the analysis corresponding to a pH value of 10.4 is observed (Fig. 1d), we realize that the time of analysis is a little longer, leading to a better resolution among some peaks.

The differences between the analyses at pH 9.0 and 10.4 can be seen more in detail in Fig. 2. In this figure (section a), we observe the separation among the main peaks, and in section b, among the minor peaks. Lignans, TY, DOA and Ol Agl were the highest peaks at pH 9.0; they were detected with a satisfactory intensity at pH 10.4 as well, although the analysis was longer in the latter case. The pH value 10.4 seemed to be more appropriate for the flavonoids, since their peak shape improved. When the

separation of the minor peaks is observed, Decarbox-Lig Agl appears as one peak at pH 9.0, whilst it appears as two peaks at pH 10.4. However, in the case of Lig Agl it was possible to observe the opposite (two peaks were detected at pH 9.0). The intensity of HYTY and HYTY-Ac was higher at the lowest pH, so we recommend this pH value of pH for the study of simple phenols.

We also studied the behaviour of the phenolic compounds present in the extracts of olive oil using NaOH at low concentrations and NaOH with $NH_3$ as buffers, but the results were not so good. Neither the shape of the peak nor the separation among the compounds of the extract were satisfactory.

Different organic modifier content (i.e. 5%, 10%, 15% of methanol and 2-propanol) did not lead to better results.

The optimum parameters for separating the compounds of the polar extracts of an Arbequina extra-virgin olive oil turned out to be 25 mM ammonium hydrogen carbonate at pH 9.0. The voltage applied was then varied between 20 and 30 kV and we found that a voltage of 30 kV shortened the analysis time and also gave good separation and acceptable current.. The injections were made at the anodic end using a $N_2$ pressure of 50 mBar for 10 s, corresponding to about 10 nL injected (0.6 % of the capillary).

These conditions were chosen for the subsequent optimization of the ESI-TOF-MS parameters. During buffer optimization we used the values for the ESI-TOF parameters obtained in the preliminary studies.

The ESI-MS operating conditions were optimized by adjusting the needle-counter electrode distance, sheath liquid composition, nebulizer gas flow rate and applied electrospray potentials while a sample solution was injected into the CE–ESI-MS system.

It is well known that the choice of sheath liquid has a significant effect on the sensitivity and electrical contact between CE and ESI [42,43]. Generally, small amounts of volatile triethylamine (TEA) or ammonium hydroxide can be used for ESI-negative detection [44], but in our research  these two additives did not lead to a better sensitivity.

Therefore, 50:50 (v/v) 2-propanol/water without any additive was selected as sheath liquid.

**3.1.2 Analysis of extracts of extra-virgin olive oil by CE-ESI-TOF**

Fig. 3 shows the BPE (50-800 m/z) of an extract of Arbequina extra-virgin olive oil, as well as the extracted ion electropherograms (EIE) for a large number of well-known phenolic compounds. These compounds are summarized in Table 1, with their formula, selected ion, m/z experimental and calculated, error, sigma value, migration time, tolerance and the first compound in the list of possibilities (sorted with respect to Sigma value).

Major phenolic compounds previously observed in several studies [6,8,13,23,24,30,31,35,36] were also detected in the present electropherograms. These compounds can be classified as phenyl alcohols, phenyl acids, lignans, flavonoids, and secoiridoids. Regarding the phenyl alcohols family, it is possible to study the following compounds (in elution order) with the electrophoretic method: HYTY, TY and HYTY-Ac, respectively.

Phenolic compounds of the *Oleaceae* family can be characterized by the presence of a number of coumarin-like compounds known as secoiridoids. These compounds are related to the iridoids, which are produced via secondary metabolism of monoterpenes as precursors of various indole alkaloids [9]. 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 [45]. This enzymatic hydrolysis explains the presence of so many isomeric or related forms in this family of compounds. Other recent studies have described in a little more detail the compounds of this family of secoiridoids [17,22,31,36] using HPLC-NMR, HPLC-MS, GC-MS or CE-MS.
Regarding this family, we can detect and identify Lig Agl, Decarbox-Lig Agl, 10-H-Ol Agl, Ol Agl, DOA, and EA. During the optimization of the electrophoretic conditions, we noticed that at different values of pH, several of the compounds that belong to this family appeared as various peaks (isoforms) in the electropherogram.

In addition to the already mentioned phenolic compounds, a further class of antioxidant phenolic compounds was detected in Arbequina extra-virgin olive oil using the CE-

TOF-MS method, namely the <u>lignans</u> H-Pin, Pin, and Ac Pin. They had practically the same migration time, however, they could clearly be identified with low mass errors. H-Pin was found in the Arbequina extra-virgin olive oil extracts in low concentrations, but Pin and Ac Pin were two compounds found in high concentration in these samples.

Specific chromatographic methods for <u>flavonoid</u> analysis have demonstrated the presence of some of these substances in olive leaves, fruits and oils [46,47]. Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. Both of them have been detected in the Arbequina extra-virgin olive oil under study at the end of the electropherogram, with migration times of 9.7 (Apig) and 11.0 min (Lut). Using 25 mM ammonium hydrogen carbonate at pH 9.0 as a separation buffer, the peak shape of Lut was not satisfactory. We studied its changes as a function of the composition and buffer pH and using ammonium hydrogen carbonate between pH values of 9.3 and 9.9, both migration time and shape of the peak were the same as at pH 9.0. However, at pH 10.4 the shape of the peak was much better, but the analysis time was longer (13.0 min). Furthermore, when we used ammonium acetate at the same concentration (25 mM) in a range of pH from 6.8 to 10.4, we observed that at pH values of 6.8 and 7.7 the migration time was shorter and the shape of the peak was better, although the separation among all the other compounds present in the olive oil extracts was worse. The signal intensity for Lut with ammonium acetate buffer at pH 9.9 was higher, as well as the shape of the peak, but these buffer characteristics did not seem to be very appropriate considering the separation among the group of analytes present in the extract of olive oil.

Therefore, 25 mM ammonium hydrogen carbonate at pH 9.0 is a compromise among the resolution of all the phenolic compounds, their signal intensity and the analysis time.

The method is able to detect four compounds belonging to the class of phenolic acids or closely related compounds (vanillin, vanillic acid, *o*-coumaric acid and ferulic acid). In this case, the final confirmation of the proposed species with standards could be carried out, as the commercial standards of these analytes were available. In general, phenolic acids are present in low amounts in fresh extra-virgin olive oils and their contents increase during storage of the oil, probably due to the hydrolysis of other compounds. Since phenolic acids are a minority group in the polyphenolic fraction of olive oil, we do not include their EIEs in Fig. 3 (to limit the length of the paper); however, other data

regarding their formula, selected ion, m/z experimental and calculated, error, sigma value, tolerance and other possible compounds are included in Table 1.

### 3.1.3 "Unknown" phenolic compounds in olive oil determined by CE-TOF-MS

Besides the previously mentioned phenolic compounds detected with this method, it was also possible to study other compounds present in this fraction of the olive oil which have so far not been described in the literature. In Fig. 4 the BPE (50-800 m/z) shows several peaks in grey (phenolic compounds mentioned in the previous section and shown in Fig. 3). In this case we have represented with a point and a number over the peak those compounds which have a molecular mass not previously found in olive oil. Moreover, we have included in this figure the EIEs of each one (in elution order). Table 2 summarizes all results for the unknown compounds including m/z experimental, selected ion, search tolerance (ppm), a list of possibilities, the error and the sigma value for the first compound of the list, etc. Four lines are in boldface print, they are the compounds which were not found using the HPLC-ESI-TOF-MS method (as desribed below). The EIEs corresponding to these four analytes are shown in section b of Fig. 4.

A reduced number of possible elemental compositions are obtained from the accurate mass of the suspected peak. These elemental compositions can then be matched against available databases (The Merck Index, ChemIndex, commercial e-catalogues) using the deduced molecular formula as a search criterion [48]. It is significant that some of the possible elemental compositions calculated within a certain mass accuracy seem to be not chemically coherent. This fact helps in the unequivocal identification of the "unknown" species and the assignment of its correct elemental composition since it reduces the number of possibilities.

### 3.1.4 Repeatability study

Repeatability was studied by performing a series of separations using the optimized method on one of the samples on the same day (intraday precision, n=12) and on three consecutive days (interday precision, n=36). The relative standard deviations (RSDs) of peak areas/migration time and migration times were determined by assaying ten of the

compounds present in the extracts (TY, Pin, Ol Agl, DOA, Apig and compounds with m/z 297.241, 199.062, 187.097, 167.036, 201.040).

The intraday repeatabilities on the migration time for these ten analytes (expressed as RSD) were found to be inside the range from 0.4 to 0.9%, whereas the interday repeabilities on the migration time were between 0.8 and 1.7%.

The intraday repeatabilities on the total peak area/migration time (expressed as RSD) were between 1.4 and 2.1%, whereas the interday repeatabilities on total peak area/migration time were found in the range from 2.0 to 4.0%.

## 3.2 HPLC-TOF MS analyses

All the well-known compounds which were detected and identified by CE-ESI-TOF were also identified using HPLC with the same detector (see Fig. 5). The figure shows the HPLC-TOF-MS chromatogram (Base Peak 50-800 m/z) for the analysis of the Diol-SPE extract of an extra-virgin olive oil (Arbequina variety), as well as the extracted ion chromatograms of 14 phenolic compounds in olive oil which were also detected by the CE method.

These compounds are summarized in Table 3, with their formula, selected ion, m/z experimental and calculated, error, sigma value, tolerance and the first compound in the list of possibilities (when it was not the phenolic compound under study) classified according to Sigma.

In the first category we can observe three phenyl alcohols, HYTY, TY and HYTY-Ac. These three compounds were the first hits in the list of possibilities. If we consider the secoiridoid family, we can detect and identify the following compounds (m/z experimental): EA, DOA, Ol Agl, 10-H-Ol Agl, Decarbox-Lig Agl, and Lig Agl using this method. In all the EIC (m/z ± 0.003) of these analytes several peaks can be observed, which are isomeric forms of the same compound. We studied the three lignans (Pin, Ac Pin and H-Pin) detected by the previously described CE method by HPLC as well. The flavonoids, luteolin and apigenin with retention times of 15.9 and 17.2 min, respectively, were detected.

Regarding phenolic acids (or related compounds) the HPLC method allows the determination of one more phenolic acid (caffeic acid) than those determined by CE (vanillin, vanillic, o-coumaric and ferulic acids).

For simplicity we do not include their EICs in Fig. 5; however, data regarding their formula, selected ion, experimental and calculated mass, mass accuracy, sigma value, search tolerance and other possible compounds are included in Table 3.

### 3.2.1 "Unknown" phenolic compounds in olive oil determined by HPLC-TOF-MS

As commented in the corresponding section of CE (section 3.1.3), besides the 14 phenolic compounds, other "unknown" compounds were determined with this HPLC-MS method. We proceeded in the same way as in the CE method. Fig. 6 includes the BPC (m/z = 50-800) with all the unidentified peaks being marked with a point and a number above them. This figure can be divided in two sections: one of then includes the compounds detected by both methods, and the second one (section b) shows 2 "unknown" compounds which were not detected with the CE method.

We also include Table 4, where it is possible to observe the m/z experimental, selected ion, tolerance (ppm), a list of possibilities, the mass deviation and the sigma value for the first compound.
The two compounds which are not detected by using the CE method (with m/z experimental of 291.1952 and 205.1599) are in boldface letter.

### 3.3 Comparison between the results obtained by CE-TOF-MS and HPLC-TOF-MS methods

The observed mass values are identical for these orthogonal separation techniques within the 5 ppm mass accuracy, giving confidence in the identification.
If we consider simple phenols, we can observe that the three peaks (HYTY, TY, HYTY-Ac) can be detected by both methods; although the difference between signal intensity is really clear in the case of HYTY. It was the highest peak in the BPC, whilst its intensity in the electropherograms was very low. HYTY-Ac appeared in the chromatograms as several isoforms and as only one peak in the electropherograms.

In general, the HPLC method was more appropriate for studying the secoiridoid family, since all of the secoiridoids represented peaks of significant intensity in the central zone

of the chromatogram. Moreover, using the optimum electrophoretic conditions, we could not observe so many isomeric forms as in the chromatograms obtained in HPLC. Lig Agl, Decarbox-Lig Agl, 10-H-Ol Agl were detected with a very low intensity with the CE method.

Taking into account the signal intensities of the EIC of the lignans obtained using both HPLC and CE methods, the described CE optimum conditions are more favourable for detecting Pin and Ac Pin, since their signal in CE are equal or even, higher (in the case of Ac Pin) than those obtained by HPLC.

Concerning flavonoids it is possible to say that the HPLC method provides better results. The CE method was able to detect Apig and Lut, though the shape and intensity of the latter changed considerably with the pH value of the buffer. Even at the optimum electrophoretic conditions, the peak shape of Lut was modest. However, both peaks had a good shape and intensity in HPLC.

Several compounds belonging to the minority group of phenolic acids were also studied by using the two methods. Five phenolic acids were detected with the HPLC method, and four with the electrophoretic method; caffeic acid was not observed in the electrophoretic profiles. The highest phenolic acid in the HPLC analyses was o-coumaric, whilst in the electropherograms this was vanillic acid.

As far as the "unknown" compounds are concerned, 26 compounds were characterized by HPLC, whilst 28 were detected with CE. The HPLC method provides information about two compounds that are not detected by using the CE method (m/z experimental 291.1952 and 205.1599), and CE-MS gives a list of possible molecular formula for four compounds which are not determined by LC-MS (m/z experimental 471.3457, 150.0562, 183.0304 and 201.0400).

Both methods can be successfully applied to the analysis of phenolic compounds in olive oil and both techniques are reliable enough for determining this class of compounds. However, if we understand them as complementary techniques to improve the characterization of this polar fraction, the results will be more complete. Furthermore, the use of TOF MS provides excellent mass accuracy over a wide

dynamic range if modern detector technology is chosen. Moreover, it allows the measurement of the correct isotopic pattern, providing important additional information for the determination of the elemental composition.

# 4 Concluding remarks

The separation by HPLC and CZE with on-line detection by ESI-TOF-MS is successfully applied to the analysis of the phenolic compounds present in extra-virgin olive oil samples for the first time. The two methodologies are able to determine many well-known phenolic compounds present in olive oil and provide information about the presence and relative concentration of minor phenolic compounds. Both CE-MS and LC-MC can determine more than 45 analytes in each run. The full structures corresponding to the elemental composition of the novel phenolic compounds remain to be investigated. This topic will be the next step in our research.

Excellent repeatability of the methods is obtained, with relative standard deviations (RSDs) of peak areas/migration time (determined for ten compounds) between 1.4% and 2.1% in the intraday study, and between 2.0% and 4.0% in the interday study.

Significant differences were found when the analyses of the five varieties used in the optimization of the methods were compared. This fact could be used in future to find potential markers for the geographical origin of the oil or the olive fruit variety.

# 5 Abbreviations used

**SPE**, solid phase extraction; **CZE**, capillary zone electrophoresis; **CE-ESI-TOF-MS**, capillary electrophoresis-electrospray ionization-time of flight-mass spectrometry; **TY**, tyrosol; **HYTY**, hydroxytyrosol; **HYTY-Ac**, 2-(4-hydroxyphenyl)ethyl acetate or hydroxytyrosol acetatet; **10-H-Ol Agl**, 10-hydroxy-oleuropein aglycon; **EA**, elenolic acid; **Pin**, (+)-pinoresinol; **Ac Pin**, (+)-1-acetoxypinoresinol; **H-Pin**, hydroxyl-pinoresinol; **Lig Agl**, ligstroside aglycon; **Ol Agl**, oleuropein aglycon; **decarbox-Lig Agl,** decarboxilated derivatives of Lig Agl; **DOA,** decarboxilated derivatives of Ol Agl; **Apig,** apigenina**;** and **Lut,** luteolin**.**

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**Table 1**. Well-known phenolic compounds determined by CE-ESI-TOF-MS in an extract of Arbequina extra-virgin olive oil.

| Compound | Formula | Selected ion | m/z experimental | m/z calculated | Error (ppm) | Error (mDa) | Sigma Value | Migration time CE (min) | Classification order considering other possibilities | Tolerance (ppm) in Generate Molecular Formula | First compound in the list of possibilities |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *HYTY* | $C_8H_{10}O_3$ | [M-H]⁻ | 153.0579 | 153.0557 | -1.437 | -0.22 | 0.0255 | 5.6 | 1st (1) | 15 | |
| *TY* | $C_8H_{10}O_2$ | [M-H]⁻ | 137.0612 | 137.0608 | -2.918 | -0.40 | 0.0172 | 5.7 | 1st (1) | 15 | |
| *Lig Agl* | $C_{19}H_{22}O_7$ | [M-H]⁻ | 361.1286 | 361.1293 | 1.938 | 0.67 | 0.0361 | 5.7 | 1st (6) | 10 | |
| *Decarbox-Lig Agl* | $C_{17}H_{20}O_5$ | [M-H]⁻ | 303.1238 | 303.1238 | 0.039 | 0.01 | 0.0435 | 5.8 | 1st (4) | 10 | |
| *H-Pin* | $C_{20}H_{22}O_7$ | [M-H]⁻ | 373.1280 | 373.1293 | 3.484 | 1.30 | 0.0132 | 5.96 | 1st (6) | 10 | |
| *Pin* | $C_{20}H_{22}O_6$ | [M-H]⁻ | 357.1338 | 357.1344 | 1.680 | 0.60 | 0.0214 | 5.97 | 2nd (6) | 10 | $C_6H_{13}N_{16}O_3$ |
| *Ac Pin* | $C_{22}H_{24}O_8$ | [M-H]⁻ | 415.1391 | 415.1398 | 1.686 | 0.70 | 0.0283 | 6.01 | 2nd (13) | 10 | $C_{17}H_{23}N_2O_{10}$ |
| *10-H-Ol Agl* | $C_{19}H_{22}O_9$ | [M-H]⁻ | 393.1187 | 393.1191 | 1.018 | 0.40 | 0.0154 | 7.7* | 1st (4) | 10 | |
| *Ol Agl* | $C_{19}H_{22}O_8$ | [M-H]⁻ | 377.1232 | 377.1242 | 2.652 | 1.00 | 0.0314 | 7.4 | 3rd (8) | 10 | $C_{11}H_{13}N_{12}O_4$ |
| *DOA* | $C_{17}H_{20}O_6$ | [M-H]⁻ | 319.1182 | 319.1187 | 1.567 | 0.50 | 0.0338 | 7.6 | 2nd (4) | 10 | $C_{13}H_{15}N_6O_4$ |
| *HYTY-Ac* | $C_{10}H_{12}O_4$ | [M-H]⁻ | 195.0664 | 195.0663 | -0.513 | -0.10 | 0.0360 | 7.9 | 1st (3) | 15 | |
| *EA* | $C_{11}H_{14}O_6$ | [M-H]⁻ | 241.0717 | 241.0718 | 0.415 | 0.10 | 0.0219 | 7.9 | 1st (3) | 10 | |
| *Apig* | $C_{15}H_{10}O_5$ | [M-H]⁻ | 269.0451 | 269.0455 | 1.487 | 0.40 | 0.0312 | 9.7 | 1st (3) | 10 | |
| *Lut* | $C_{15}H_{10}O_6$ | [M-H]⁻ | 285.0410 | 285.0405 | -1.754 | -0.50 | 0.0341 | 11.00 | 1st (5) | 10 | |
| | | | | | | | | | | | |
| *Vanillin* | $C_8H_8O_3$ | [M-H]⁻ | 151.0406 | 151.0401 | -3.744 | -0.56 | 0.0514 | 6.00 | 1st (1) | 20 | |
| *Ferulic acid* | $C_{10}H_{10}O_4$ | [M-H]⁻ | 193.0512 | 193.0506 | -2.940 | -0.57 | 0.0434 | 6.00* | 2nd (1) | 20 | $C_{11}H_5N_4$ |
| *Vanillic acid* | $C_8H_8O_4$ | [M-H]⁻ | 167.0355 | 167.0350 | -3.265 | -0.55 | 0.0075 | 8.8 | 1st (2) | 10 | |
| *o-coumaric acid* | $C_9H_8O_3$ | [M-H]⁻ | 163.0406 | 163.0401 | -3.067 | -0.50 | 0.0576 | 9.6 | 1st (1) | 20 | |

* There are two peaks in the EIC with the same intensity (approx)

**Table 2**. "Unknown" phenolic compounds determined by CE-ESI-TOF-MS in an extract of Arbequina extra-virgin olive oil.

| m/z experimental | Selected ion | Tolerance (ppm) in Generate Molecular Formula | List of possibilities in Generate Molecular Formula (in increasing order of sigma) | Error (ppm) (for the first compound) | Sigma Value (for the first compound) |
|---|---|---|---|---|---|
| 155.0715 | [M-H]$^-$ | 20 | $C_8H_{11}O_3$ | -0.853 | 0.0085 |
| 223.1331 | [M-H]$^-$ | 15 | $C_8H_{19}N_2O_5$ / $C_{13}H_{19}O_3$ / $C_9H_{15}N_6O_1$ / $C_{15}H_{15}N_2$ | 2.444 2nd(3.456) | 0.0134 2nd(0.0265) |
| 171.1389 | [M-H]$^-$ | 10 | $C_{10}H_{19}O_2$ | 1.187 | 0.0644 |
| 199.1704 | [M-H]$^-$ | 10 | $C_{12}H_{23}O_2$ | -0.368 | 0.0772 |
| 315.1228 | [M-H]$^-$ | 5 | $C_{18}H_{19}O_5$ | 3.184 | 0.0360 |
| 315.2535 | [M-H]$^-$ | 10 | $C_{18}H_{35}O_4$/ $C_{14}H_{31}N_6O_2$ / $C_{19}H_{31}N_4$ | 1.759 | 0.0228 |
| **471.3457** | **[M-H]$^-$** | **5** | **$C_{25}H_{47}N_2O_6$ / $C_{30}H_{47}O_4$ / $C_{26}H_{43}N_6O_2$** | **-3.760 2nd(4.774)** | **0.0257 2nd(0.0459)** |
| 299.2584 | [M-H]$^-$ | 10 | $C_{18}H_{35}O_3$ / $C_{13}H_{35}N_2O_5$/ $C_{14}H_{31}N_6O_1$ | 2.673 | 0.0179 |
| 297.2431 | [M-H]$^-$ | 15 | $C_{18}H_{33}O_3$ / $C_{13}H_{33}N_2O_5$ / $C_{14}H_{29}N_6O_1$ | 1.346 | 0.0262 |
| 295.2272 | [M-H]$^-$ | 10 | $C_{18}H_{31}O_3$ / $C_{13}H_{31}N_2O_5$ / $C_{14}H_{27}N_6O_1$ | 2.371 | 0.0563 |
| 311.2219 | [M-H]$^-$ | 10 | $C_{18}H_{31}O_4$/ $C_{13}H_{31}N_2O_6$ / $C_{14}H_{27}N_6O_2$ / $C_{19}H_{27}N_4$ | 2.892 | 0.0611 |
| 407.1356 | [M-H]$^-$ | 5 | $C_3H_7N_{26}$ / $C_{20}H_{23}O_9$ / $C_{21}H_{19}N_4O_5$ / $C_{17}H_{15}N_{10}O_3$ | -0.865 2nd(-2.073) | 0.0103 2nd(0.0123) |
| 391.1404 | [M-H]$^-$ | 5 | $C_{20}H_{23}O_8$ / $C_{16}H_{19}N_6O_6$ / $C_{17}H_{15}N_{10}O_2$ / $C_{21}H_{19}N_4O_4$ | -1.511 | 0.0350 |
| 243.1966 | [M-H]$^-$ | 10 | $C_{14}H_{27}O_3$ | -0.130 | 0.0157 |
| 199.0617 | [M-H]$^-$ | 15 | $C_9H_{11}O_5$ / $C_{10}H_7N_4O_1$ | -2.488 | 0.0587 |
| 225.1488 | [M-H]$^-$ | 15 | $C_{13}H_{21}O_3$ | 3.553 | 0.0476 |
| 335.1134 | [M-H]$^-$ | 5 | $C_{17}H_{19}O_7$ / $C_{14}H_{11}N_{10}O_1$ / $C_{18}H_{15}N_4O_3$ | 0.587 | 0.0264 |
| 143.1082 | [M-H]$^-$ | 20 | $C_8H_{15}O_2$ | -2.795 | 0.0629 |
| 257.0673 | [M-H]$^-$ | 10 | $C_{11}H_{13}O_7$ / $C_8H_5N_{10}O_1$ / $C_{12}H_9N_4O_3$ | -2.389 | 0.0720 |
| 215.0919 | [M-H]$^-$ | 15 | $C_{10}H_{15}O_5$ / $C_{11}H_{11}N_4O_1$ | 2.600 | 0.0652 |
| **150.0562** | **[M-H]$^-$** | **20** | **$C_8H_8N_1O_2$ / $C_4H_4N_7$** | **-0.838** | **0.0069** |
| 187.0970 | [M-H]$^-$ | 15 | $C_5H_{11}N_6O_2$ / $C_9H_{15}O_4$ / $C_{10}H_{11}N_4$ | -10.314 2nd(4.039) | 0.0060 2nd(0.0108) |
| 271.0608 | [M-H]$^-$ | 5 | $C_{15}H_{11}O_5$ / $C_1H_3N_{16}O_2$ / $C_{16}H_7N_4O_1$ | 1.544 | 0.0349 |

20

| | | | | | |
|---|---|---|---|---|---|
| **157.1240** | **[M-H]$^-$** | **20** | **C$_9$H$_{17}$O$_2$** | **-3.496** | **0.0051** |
| **183.0304** | **[M-H]$^-$** | **20** | **C$_8$H$_7$O$_5$ / C$_9$H$_3$N$_4$O$_1$** | **-2.732** | **0.0521** |
| 183.0667 | [M-H]$^-$ | 20 | C$_9$H$_{11}$O$_4$ / C$_{10}$H$_7$N$_4$ | 2.197 | 0.0174 |
| **201.0400** | **[M-H]$^-$** | **10** | **C$_8$H$_9$O$_6$ / C$_5$H$_1$N$_{10}$ / C$_9$H$_5$N$_4$O$_2$** | **2.457** | **0.0527** |
| 299.0561 | [M-H]$^-$ | 5 | C$_2$H$_3$N$_{16}$O$_3$ / C$_5$H$_{11}$N$_6$O$_9$ / C$_{16}$H$_{11}$O$_6$ / C$_{13}$H$_3$N$_{10}$ / C$_{17}$H$_7$N$_4$O$_2$ | 2.803 | 0.0478 |

**Table 3**. Well-known phenolic compounds determined by HPLC-ESI-TOF-MS in an extract of Arbequina extra-virgin olive oil.

| Compound | Formula | Selected ion | m/z experimental | m/z calculated | Error (ppm) | Error (mDa) | Sigma Value | Classification order considering other possibilities | Tolerance (ppm) in Generate Molecular Formula | First compound in the list of possibilities |
|---|---|---|---|---|---|---|---|---|---|---|
| *HYTY* | $C_8H_{10}O_3$ | [M-H]⁻ | 153.0562 | 153.0557 | -3.267 | -0.50 | 0.0012 | 1st (1) | 20 | |
| *TY* | $C_8H_{10}O_2$ | [M-H]⁻ | 137.0609 | 137.0608 | -0.362 | -0.05 | 0.0048 | 1st (1) | 15 | |
| *EA* | $C_{11}H_{14}O_6$ | [M-H]⁻ | 241.0718 | 241.0718 | -0.306 | -0.07 | 0.0181 | 1st (3) | 10 | |
| *HYTY-Ac* | $C_{10}H_{12}O_4$ | [M-H]⁻ | 195.0660 | 195.0663 | 1.415 | 0.28 | 0.0129 | 1st (3) | 15 | |
| *DOA* | $C_{17}H_{20}O_6$ | [M-H]⁻ | 319.1184 | 319.1187 | 0.824 | 0.26 | 0.0178 | 2nd (4) | 10 | $C_{13}H_{15}N_6O_4$ |
| *Ol Agl* | $C_{19}H_{22}O_8$ | [M-H]⁻ | 377.1224 | 377.1242 | 4.703 | 1.77 | 0.0272 | 2nd (8) | 10 | $C_{11}H_{13}N_{12}O_4$ |
| *10-H-Ol Agl* | $C_{19}H_{22}O_9$ | [M-H]⁻ | 393.1183 | 393.1191 | 1.985 | 0.78 | 0.0087 | 1st (4) | 10 | |
| *Decarbox-Lig Agl* | $C_{17}H_{20}O_5$ | [M-H]⁻ | 303.1239 | 303.1238 | -0.212 | -0.06 | 0.0225 | 1st (3) | 10 | |
| *Lig Agl* | $C_{19}H_{22}O_7$ | [M-H]⁻ | 361.1284 | 361.1293 | 2.418 | 0.87 | 0.0105 | 1st (6) | 10 | |
| *Pin* | $C_{20}H_{22}O_6$ | [M-H]⁻ | 357.1338 | 357.1344 | 1.518 | 0.54 | 0.0360 | 2nd (6) | 10 | $C_{15}H_{21}N_2O_8$ |
| *Lut* | $C_{15}H_{10}O_6$ | [M-H]⁻ | 285.0405 | 285.0405 | -0.041 | -0.01 | 0.0118 | 2nd (5) | 10 | $C_{12}H_1N_{10}$ |
| *Ac Pin* | $C_{22}H_{24}O_8$ | [M-H]⁻ | 415.1389 | 415.1398 | 0.942 | 0.39 | 0.0131 | 2nd (10) | 10 | $C_{13}H_{19}N_8O_8$ |
| *Apig* | $C_{15}H_{10}O_5$ | [M-H]⁻ | 269.0449 | 269.0455 | 2.509 | 0.67 | 0.0478 | 2nd (4) | 10 | $C_1H_1N_{16}O_2$ |
| *H-Pin* | $C_{20}H_{22}O_7$ | [M-H]⁻ | 373.1283 | 373.1293 | 2.772 | 1.03 | 0.0198 | 1st (5) | 10 | |
| | | | | | | | | | | Other compounds in the list of possibilities |
| *Vanillin* | $C_8H_8O_3$ | [M-H]⁻ | 151.0406 | 151.0401 | -3.828 | -0.58 | 0.0089 | 1st (1) | 10 | |
| *Vanillic acid* | $C_8H_8O_4$ | [M-H]⁻ | 167.0353 | 167.0350 | -1.996 | -0.33 | 0.0517 | 1st (2) | 10 | $C_9H_3N_4$ |
| *Caffeic acid* | $C_9H_8O_4$ | [M-H]⁻ | 179.0354 | 179.0350 | -2.409 | -0.43 | 0.0580 | 1st (2) | 10 | $C_{10}H_3N_4$ |
| *o-coumaric acid* | $C_9H_8O_3$ | [M-H]⁻ | 163.0405 | 163.0401 | -2.673 | -0.44 | 0.0307 | 1st (1) | 10 | |
| *Ferulic acid* | $C_{10}H_{10}O_4$ | [M-H]⁻ | 193.0512 | 193.0506 | -2.997 | -0.58 | 0.5746 | 1st (2) | 10 | $C_{11}H_5N_4$ |

**Table 4**. "Unknown" phenolic compounds determined by HPLC-ESI-TOF-MS in an extract of Arbequina extra-virgin olive oil.

| m/z experimental | Selected ion | Tolerance (ppm) in Generate Molecular Formula | List of possibilities in Generate Molecular Formula (in increasing order of sigma) | Error (ppm) (for the first compound) | Sigma Value (for the first compound) |
|---|---|---|---|---|---|
| 199.0610 | [M-H]$^-$ | 20 | $C_9H_{11}O_5$ / $C_{10}H_7N_4O_1$ | 3.463 | 0.0084 |
| 155.0714 | [M-H]$^-$ | 25 | $C_8H_{11}O_3$ | -3.058 | 0.0069 |
| 215.0930 | [M-H]$^-$ | 15 | $C_{10}H_{15}O_5$ / $C_{11}H_{11}N_4O_1$ | -2.299 | 0.0148 |
| 183.0667 | [M-H]$^-$ | 20 | $C_9H_{11}O_4$ / $C_{10}H_7N_4$ | 2.197 | 0.0074 |
| 257.0668 | [M-H]$^-$ | 10 | $C_{11}H_{13}O_7$ / $C_8H_5N_{10}O_1$ / $C_{12}H_9N_4O_3$ | -0.355 | 0.0126 |
| 187.097 | [M-H]$^-$ | 15 | $C_9H_{15}O_4$ / $C_5H_{11}N_6O_2$ / $C_{10}H_{11}N_4$ | 1.273 | 0.0066 |
| 335.1134 | [M-H]$^-$ | 5 | $C_{17}H_{19}O_7$ / $C_{14}H_{11}N_{10}O_1$ / $C_{18}H_{15}N_4O_3$ | 0.587 | 0.0224 |
| 271.0608 | [M-H]$^-$ | 5 | $C_{15}H_{11}O_5$ / $C_1H_3N_{16}O_2$ / $C_{16}H_7N_4O_1$ | 1.544 | 0.0249 |
| 299.0561 | [M-H]$^-$ | 5 | $C_{16}H_{11}O_6$ / $C_{13}H_3N_{10}$ / $C_{17}H_7N_4O_2$ | 0.197 | 0.0267 |
| 407.1340 | [M-H]$^-$ | 5 | $C_{20}H_{23}O_9$ / $C_{16}H_{19}N_6O_7$ / $C_{17}H_{15}N_{10}O_3$ / $C_3H_7N_{26}$ | 1.828 | 0.0083 |
| 225.1485 | [M-H]$^-$ | 15 | $C_{13}H_{21}O_3$ | 4.951 | 0.0441 |
| 315.1240 | [M-H]$^-$ | 5 | $C_{18}H_{19}O_5$ / $C_{19}H_{15}N_4O_1$ | 0.082 | 0.0354 |
| 391.1383 | [M-H]$^-$ | 5 | $C_{20}H_{23}O_8$ / $C_{16}H_{19}N_6O_6$ / $C_{17}H_{15}N_{10}O_2$ | 3.866 | 0.0400 |
| 223.1344 | [M-H]$^-$ | 15 | $C_{13}H_{19}O_3$ | -1.750 | 0.0224 |
| 143.1073 | [M-H]$^-$ | 15 | $C_8H_{15}O_2$ | 3.493 | 0.0094 |
| 311.2224 | [M-H]$^-$ | 10 | $C_{18}H_{31}O_4$ / $C_{14}H_{27}N_6O_2$ / $C_{19}H_{27}N_4$ | 1.103 | 0.0485 |
| 157.1240 | [M-H]$^-$ | 20 | $C_9H_{17}O_2$ | -3.990 | 0.0138 |
| 315.2532 | [M-H]$^-$ | 10 | $C_{18}H_{35}O_4$ / $C_{14}H_{31}N_6O_2$ / $C_{19}H_{31}N_4$ | 2.718 | 0.0343 |
| 243.1966 | [M-H]$^-$ | 10 | $C_{14}H_{27}O_3$ | 0.025 | 0.0257 |
| 171.1391 | [M-H]$^-$ | 15 | $C_{10}H_{19}O_2$ | -0.168 | 0.0146 |
| **291.1952** | **[M-H]$^-$** | **10** | **$C_{14}H_{23}N_6O_1$ / $C_{18}H_{27}O_3$ / $C_{13}H_{27}N_2O_5$** | **-6.599 2$^{nd}$(2.623)** | **0.0097 2$^{nd}$(0.0106)** |
| 295.2271 | [M-H]$^-$ | 10 | $C_{14}H_{27}N_6O_1$ / $C_{18}H_{31}O_3$ | -8.209 2$^{nd}$(0.887) | 0.0070 2$^{nd}$(0.0100) |
| 297.2427 | [M-H]$^-$ | 15 | $C_{14}H_{29}N_6O_1$ / $C_{18}H_{33}O_3$ | -10.409 2$^{nd}$(-1.375) | 0.0124 2$^{nd}$(0.0178) |
| **205.1599** | **[M-H]$^-$** | **20** | **$C_{14}H_{21}O_1$** | **-0.463** | **0.0192** |

| 199.1700 | [M-H]$^-$ | 10 | $C_{12}H_{23}O_2$ | 1.837 | 0.0182 |
|---|---|---|---|---|---|
| 299.2594 | [M-H]$^-$ | 10 | $C_{18}H_{35}O_3$ / $C_{14}H_{31}N_6O_1$ | 0.607 | 0.0095 |

**Caption to figures**

**Figure 1.** BPE (50-800 m/z) at different values of pH using carbonate $NH_4HCO_3$ buffers. a) pH 9, b) pH 9.5, c) pH 9.9 and d) pH 10.4.

**Figure 2**. Extracts of Arbequina extra-virgin olive oil analyzed by the CE-ESI-TOF-MS method using carbonate-containing background electrolyte at pH 9.0 and 10.4. a) EIEs of the majority peaks present in the extract, and b) EIEs of the minority peaks.

**Figure 3.** BPE as obtained by CE-ESI-TOF-MS of an Arbequina extra-virgin olive oil extract at the optima electrophoretic and MS conditions, and EIEs of the well-known phenolic compounds detected (containing information about the m/z experimental, molecular formula and the name of the compound).

**Figure 4.** Base peak electropherogram of the Diol-SPE extract obtained from an Arbequina extra-virgin olive oil, and EIEs of the "*unknown*" phenolic compounds detected (with information about the m/z experimental and molecular formula). Section b) "*Unknown*" phenolic compounds which were not found in the extracts by using the HPLC-ESI-TOF-MS method.

*-Peaks in grey represent the phenolic compounds previously shown in Fig. 3.*

• *With a point and a number over the peak, those compounds which have a molecular mass that has not previously been found in olive oil.*
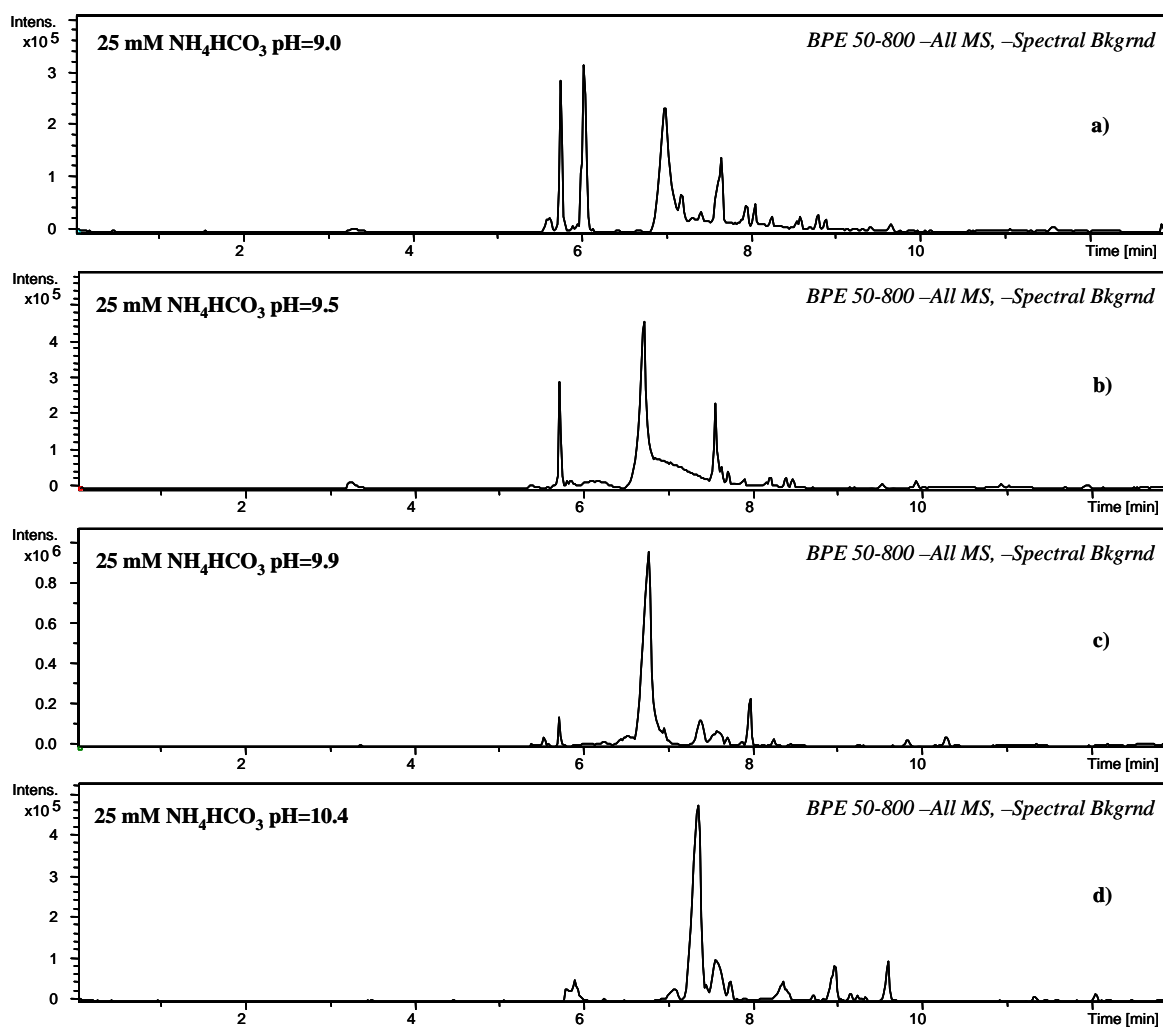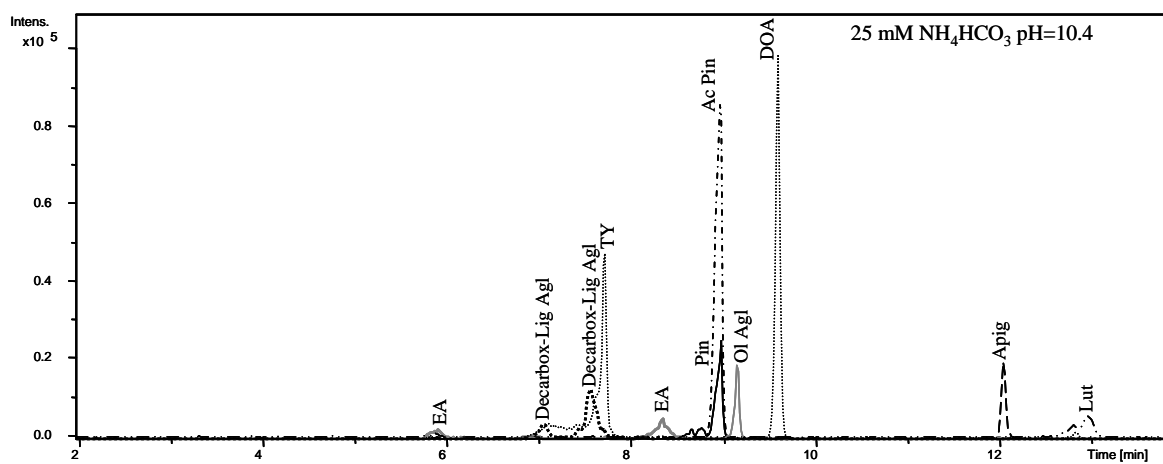
**Figure 5.** BPC (50-800 m/z) as obtained by HPLC-ESI-TOF-MS method of an extract of Arbequina extra-virgin olive oil, and EICs of the well-known phenolic compounds detected (each one contains information about the m/z experimental, molecular formula and the name of the compound).

**Figure 6.** Base peak chromatogram of the Diol-SPE extract obtained from an Arbequina extra-virgin olive oil, and EICs of the "*unknown*" phenolic compounds detected (each one contains information about the m/z experimental and molecular formula). Section b) "*Unknown*" phenolic compounds which were not detected with the CE method.

*-Peaks in grey represent the phenolic compounds previously shown in Fig. 3.*

• *With a point and a number over the peak, those compounds which have a molecular mass that has not previously been found in olive oil.*
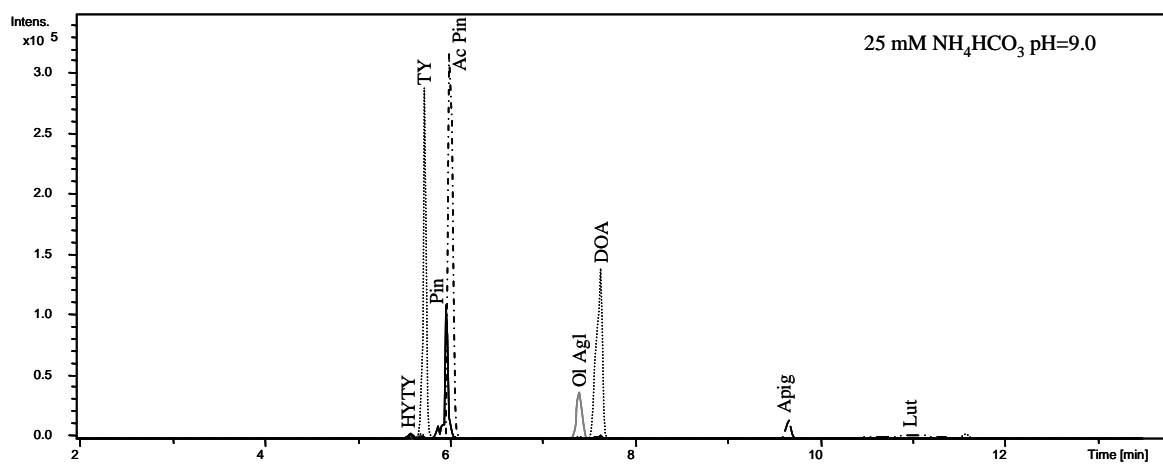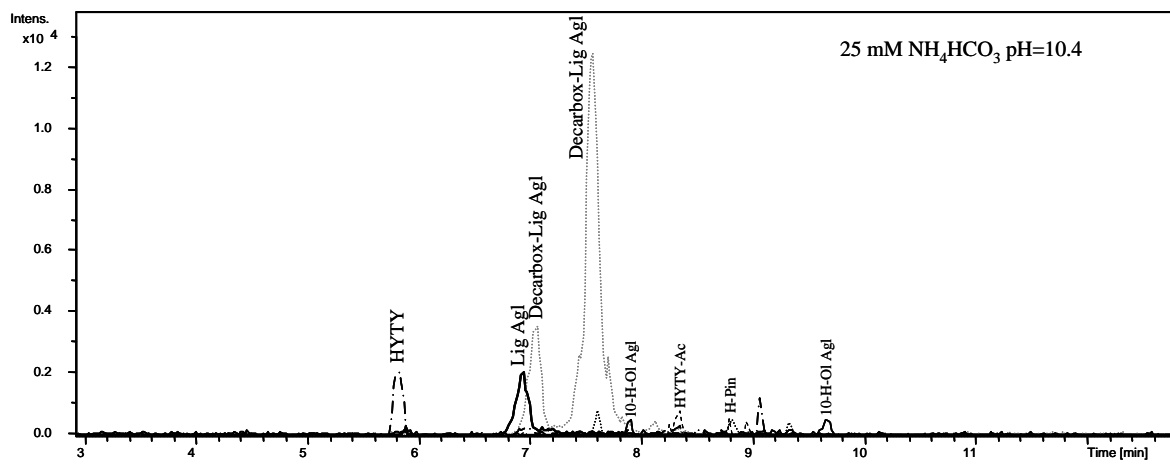
**Figure 1**

**Figure 2**

**a)**

**b)**

# Figure 3



25 mM NH$_4$HCO$_3$ pH=9.0

BPE 50-800 –All Ms

EIE 153.056 ± 0.003 / C8H9O3 / HYTY

EIE 393.119 ± 0.003 / C19H21O9 / 10-H-Ol Agl

EIE 137.061 ± 0.003 / C8H9O2 / Tyrosol

EIE 377.124 ± 0.003 / C19H21O8 / Ol Agl

EIE 361.129 ± 0.003 / C19H21O7 / Lig Agl

EIC 319.119 ± 0.003 / C17H19O6 / DOA

EIE 303.124 ± 0.003 / C17H19O5 / Decarbox-Lig Agl

EIE 195.066 ±0.003 / C10H11O4 / HYTY-Ac

EIE 373.129 ±0.003 / C20H21O7 / H-Pin

EIE 241.072 ±0.003 / C11H13O6 / EA

EIE 357.134 ± 0.003 / C20H21O6 / Pinoresinol

EIE 269.046 ±0.003 / C15H9O5 / Apigenin

EIE 415.140 ± 0.003 / C22H23O8 / Ac Pin
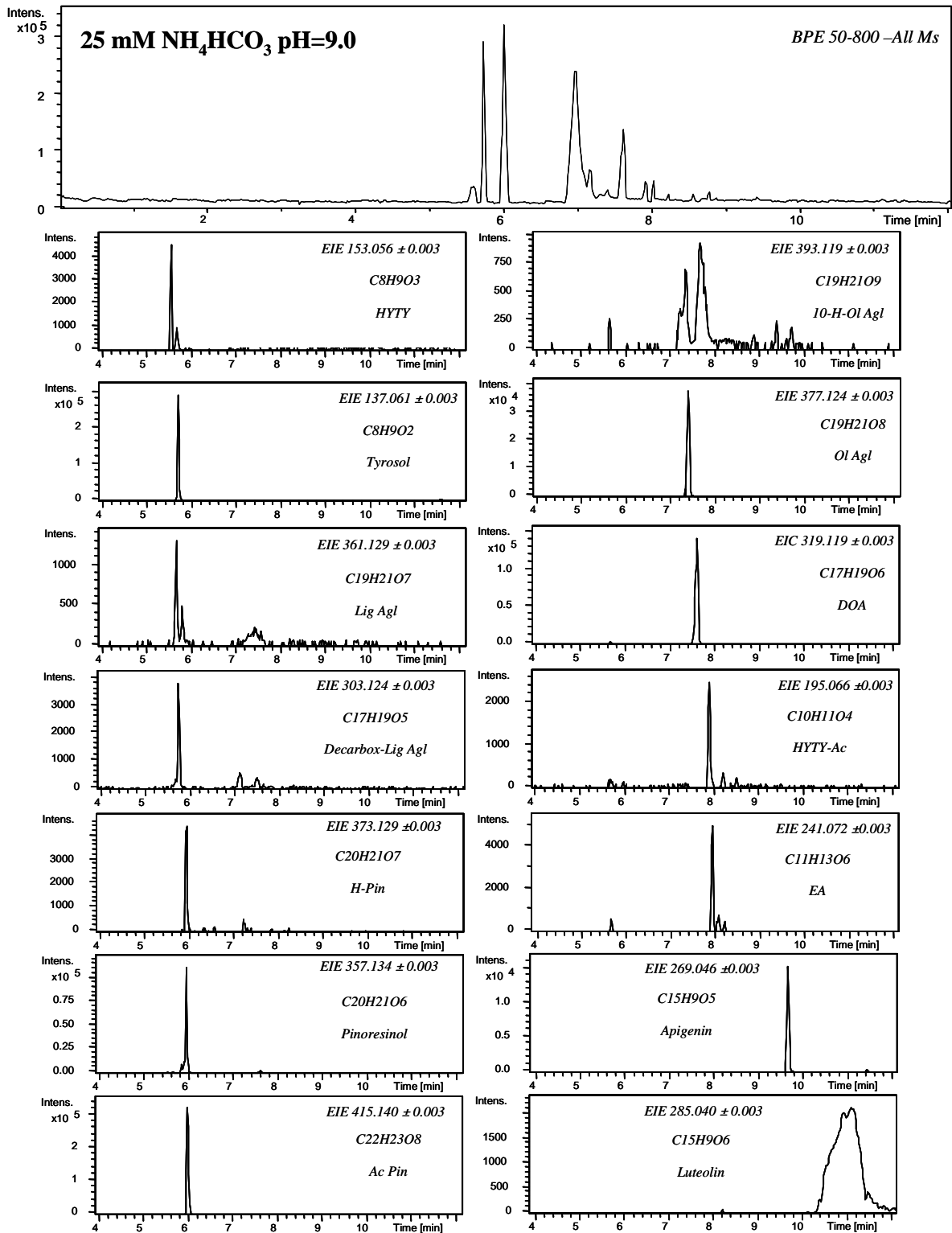
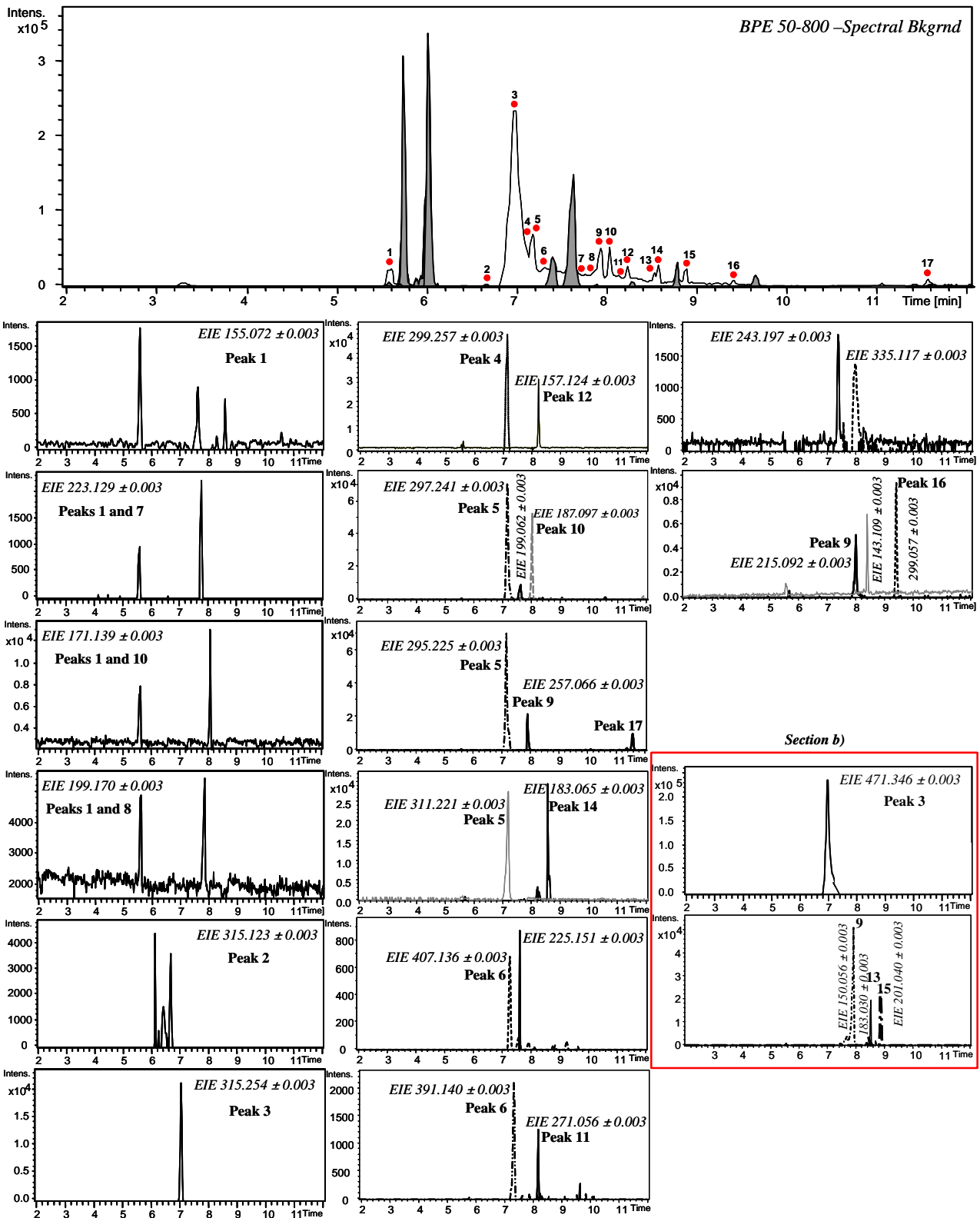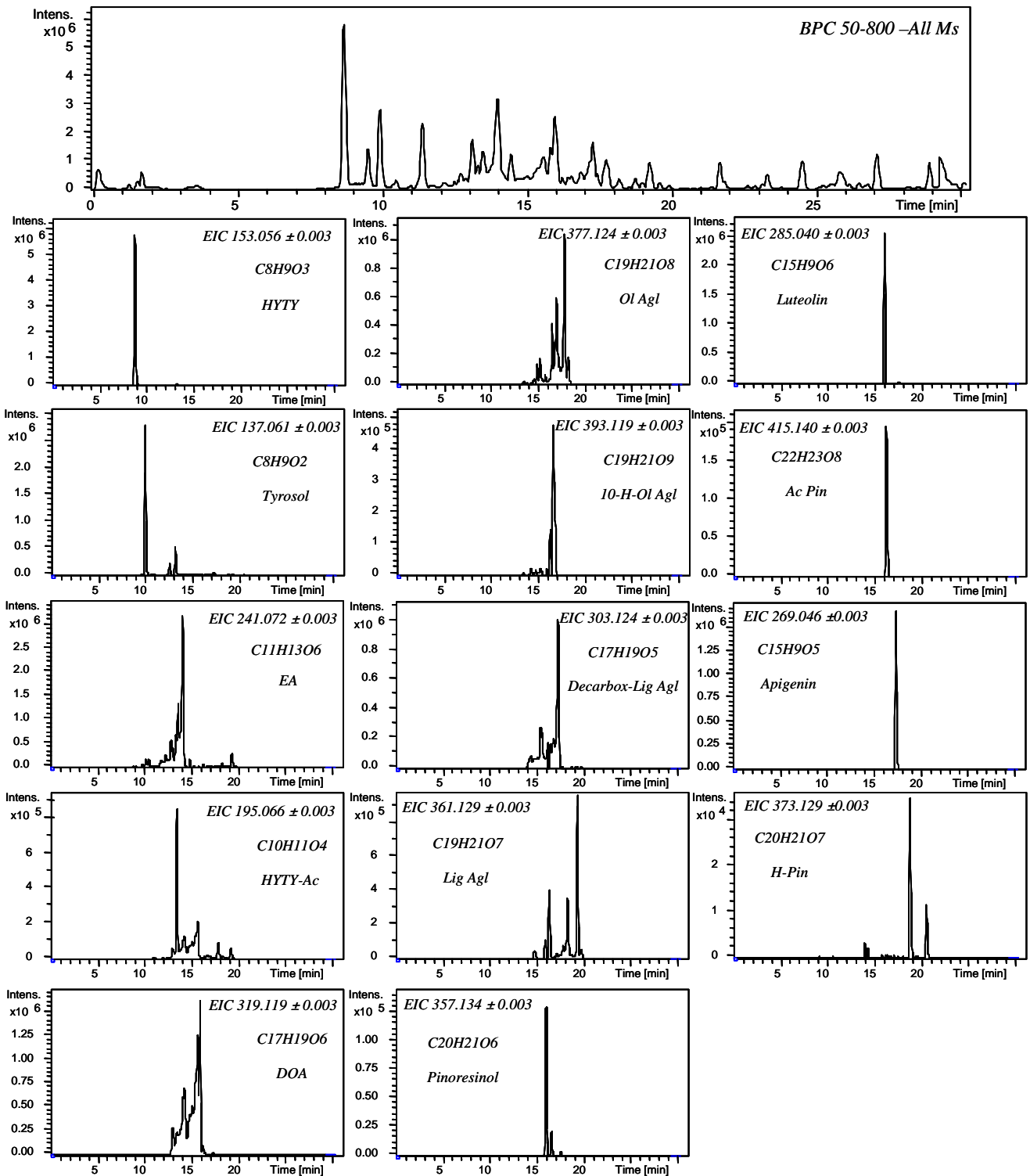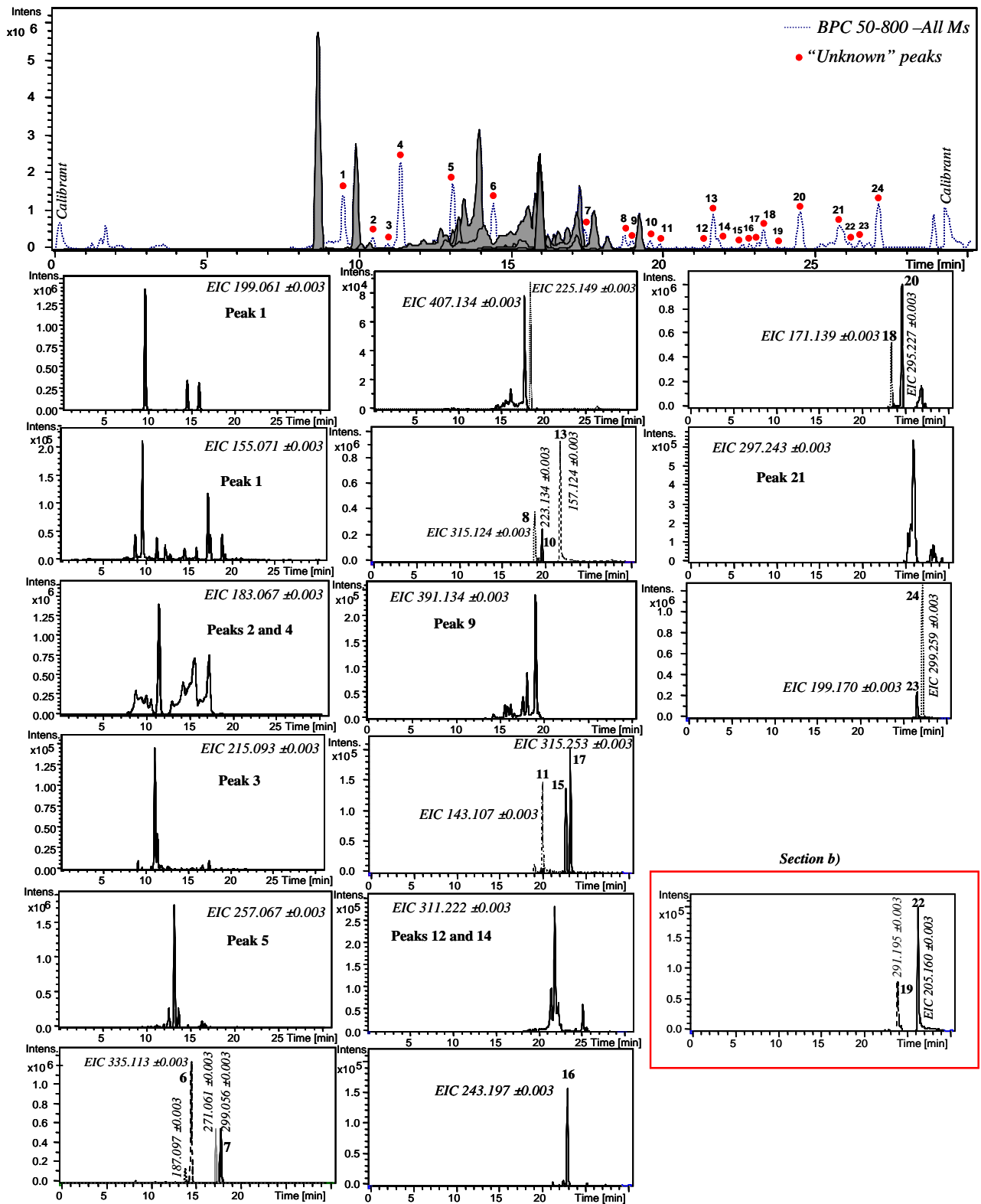EIE 285.040 ± 0.003 / C15H9O6 / Luteolin

**Figure 4**

**Figure 5**

**Figure 6**

# CONCLUSIONES

1) Se ha puesto a punto un método electroforético zonal que permite la identificación y cuantificación de los ácidos fenólicos presentes en la matriz aceite de oliva a una concentración de partes por billón. Se han determinado 14 compuestos en aceites de oliva virgen-extra obtenidos de distintas variedades de aceituna (Arbequina, Picual, Lechín de Sevilla, Hojiblanca, Lechín de Granada y Cornicabra) buscando posibles compuestos que pudieran ser potenciales marcadores varietales; además se han analizado aceites de oliva pertenecientes a diferentes categorías (virgen extra, refinado y mezcla de refinado y aceite de oliva) demostrando las grandes diferencias existentes en lo que a concentración y presencia de ácidos fenólicos se refiere.

2) Se ha desarrollado un método que posibilita la determinación de ácidos fenólicos en aceite de oliva usando modificadores de flujo electroosmótico (HDB) y polaridad invertida que podría evitar las interferencias producidas por los compuestos fenólicos más abundantes del aceite de oliva en la cuantificación de los mencionados ácidos. El método se ha aplicado al análisis de 6 aceites monovarietales mostrando ser muy repetible y robusto, y demostrando que existe una estrecha relación entre la variedad de aceituna de la que procede un aceite y el contenido en ácidos fenólicos del mismo.

3) Se ha llevado a cabo el análisis de un grupo de trece ácidos fenólicos que podrían estar presentes en un gran número de muestras mediante un método electroforético que emplea un novedoso recubrimiento polimérico de la pared interna del capilar. Se demostró que dicho recubrimiento se consigue de un modo rápido y reproducible sin necesidad de que sea añadido a la disolución de separación; además, su uso fue compatible con la presencia de modificadores orgánicos en la disolución reguladora.

4) Se aislaron varios de los compuestos fenólicos mayoritarios del aceite de oliva (HYTY, TY, EA, DAOA, Pin, Ac Pin, Ol Agl y Lig Agl) mediante un método cromatográfico empleando una columna semi-preparativa, cosa que posibilitó tanto el estudio de algunas características de cada compuesto individual coleccionado, como su identificación en los perfiles electroforéticos; la

importancia de estos "estándares aislados" es grande, ya que no se pueden adquirir como patrones comerciales.

5) Se realizaron medidas de la capacidad antioxidante de los ocho compuestos aislados (mencionados en el punto anterior) mediante tres tests basados en técnicas totalmente diferentes (un estudio de "radical scavenging", una oxidación acelerada en sistema lipídico modelo y un nuevo método electroquímico), cosa que permitió comparar los resultados y ver si todas las determinaciones estaban de acuerdo en cuanto a la actividad antioxidante que mostraba cada compuesto. Es la primera vez que se ha llevado a cabo la comparación entre dos tests bastante conocidos y empleados (DPPH y OSI) y uno electroquímico, cosa que da un mayor interés a este estudio.

6) Ha sido posible detectar y cuantificar 26 compuestos fenólicos (perteneciente a 5 familias diferentes: fenoles simples, fenoles complejos, lignanos, ácidos fenólicos y flavonoides) en menos de 10 min empleando un sencillo, pero potente método electroforético. Dicho método ha permitido, por primera vez mediante CE, determinar varias formas isoméricas de los secoiridoides y dos flavonoides en aceite de oliva. Los estudios de repetibilidad y reproducibilidad hacen a este método especialmente idóneo para llevar a cabo análisis de rutina en cualquier laboratorio.

7) El método mencionado en la anterior conclusión, ha servido de útil herramienta para llevar a cabo estudios aplicados, en los que se evaluó el contenido fenólico en varios aceites españoles monovarietales y su relación con las propiedades sensoriales del aceite; y en los que se comparó el perfil polifenólico de los aceites provenientes de dos Denominaciones de Origen Protegidas (DOPs) (una italiana y otra española) logrando diferenciarlos con gran claridad por procedimientos estadísticos.

8) Un aceite comercial se sometió a temperaturas de 180º C (simulación de fritura) durante espacios de tiempo controlados comprendidos entre 30 y 180 minutos; posteriormente se procedió a la extracción de la fracción polar de los mismos utilizando un sistema líquido-líquido, para finalmente realizar la separación y

determinación de los compuestos fenólicos mediante el empleo de HPLC-UV y HPLC-MS y CE-UV. De este modo se pudo evaluar la influencia de la oxidación térmica en la composición fenólica y en la actividad antioxidante de aceites de oliva virgen extra, así como determinar "nuevos" compuestos que aparecían en los perfiles cromatográfico y electroforético de un extracto de aceite tras el tratamiento térmico.

9) Se ha puesto a punto el primer método analítico que incluye una extracción en fase sólida (SPE) y una metodología de electroforesis capilar en zona acoplada a una ionización por electrospray-espectrometría de masas con trampa de iones (CZE-ESI-IT MS) para la identificación y caracterización de compuestos fenólicos en muestras de aceite de oliva. Tras optimizar todos los parámetros del método electroforético y del electrospray persiguiendo la máxima sensibilidad se pudieron determinar tres alcoholes fenólicos, dos lignanos y varios fenoles complejos, y también se pudo llevar a cabo el estudio comparativo de tres aceites monovarietales: Picual, Lechín de Sevilla y Hojiblanca. El poder aportar metodologías que integren la detección por espectrometría de masas es un gran avance, ya que esta técnica facilita en gran medida la identificación de gran número de especies.

10) Se ha profundizado en el estudio de la fracción polifenólica del aceite de oliva usando ESI como interfase y MicroTOF como analizador. Éste posee prestaciones sorprendentes, como son alta sensibilidad y velocidad de barrido, que es aplicable a un rango de masas amplio y que permite la determinación exacta de pesos moleculares. Se pusieron a punto dos métodos, uno empleando como técnica separativa HPLC y otro CE, capaces de determinar unos 50 compuestos en un solo análisis. De hecho, se pudieron determinar por primera vez varios compuestos que nunca antes habían sido descritos en esta fracción del aceite de oliva mediante el empleo de estas técnicas.

# FINAL CONCLUSIONS

1) A CZE method for the quantification of phenolic acids in extra-virgin olive oil extracts at sub-ppm levels has been developed and optimized. Separation and identification of 14 compounds in olive oils of different varieties of olive fruit and in olive oils belonging to different categories was performed with good repeatability and short analysis times. With this method and using a liquid-liquid extraction system (LLE), with recovery values around 95%, it has been possible to detect sub-ppm quantities of phenolic acids in real olive oil samples. To our knowledge, this is the first report showing the quantification of this specific family of phenolic compounds in real virgin olive oil samples by CE.

2) Phenolic acids have been determined in olive-oil samples after the LLE extraction system and using co-electrosmotic capillary electrophoresis mode with UV detection adding a polycationic surfactant (hexadimetrine bromide, HDB) to the electrolyte. The amount of these substances was determined in several extracts obtained from different olive oil varieties. In fact, the distribution of the phenolic compounds shows quantitative differences, which are related to the variety of the olive fruit. The results are very similar to the obtained results with the CZE method (mentioned in the last point) and although more studies are necessary in this way, it is possible to indicate some compounds as potential varietal markers, such as trans-cinnamic acid, dopac, 4-hydroxybenzoic acid and sinapinic acid, that are present only in one or two varieties between all the varieties under study.

3) Co-electroosmotic capillary electrophoresis using DMA-EpyM as stationary polymer can be used for the analysis of phenolic acids which could be present in the extracts of a plenty of products. The developed method provides good repeatability and satisfactory detection limits and demonstrates that DMA-EpyM provides a stable coating even when the separation buffer contains organic modifiers. All these facts make broader the usefulness of this coating.

4) We have isolated several phenolic compounds of extra-virgin olive oil (hydroxytyrosol, tyrosol, elenolic acid, deacetoxy oleuropein aglycon, (+)-pinoresinol, (+)-1-acetoxypinoresinol, oleuropein aglycon and ligstroside aglycon) by semi-preparative high-performance liquid chromatography and identified them

using ultraviolet, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) detection. The purity of these extracts was confirmed by analytical HPLC using two different gradients. These "isolated standards" were really helpful for determining several of their individual characteristics, as well as identifying new compounds in the electrophoretic profiles.

5) The antioxidant activity (unknown until this moment) of different single phenolic compounds (the eight isolated compounds named in the previous point) of virgin olive oil has been evaluated by chemical methods (DPPH), by accelerated oxidation in a lipid model system (OSI=Oxidative Stability Instrument) and by an electrochemical method (FIA-amperometry and cyclic voltammetry). We compared the information to obtain an insight of the chemical groups involved in the antioxidant mechanisms.

6) The Diol-SPE extracts from extra-virgin olive oil have been analyzed with a complete and rapid CZE method in less than 10 min and we were able to study several families of phenolic compounds present in this foodstuff, such as simple phenols, lignans, complex phenols, phenolic acids and flavonoids. It was possible the determination of flavonoids and several isomeric forms of secoiridoid compounds in olive-oil samples using a CE method by first time. Furthermore, as the extraction protocol used a considerable quantity of oil (60 g), the method detected in the same run 26 compounds belonging to both majority and minority families which, to the date, have not been detected simultaneously in any CE method.

7) The method mentioned in the previous conclusion, has been used as a tool to carry out applicative works. The relative standard deviations (RSD) obtained in a study of repeatability of this method were lower than 0.91% for the migration times and 2.32% for the peak areas/migration time (intraday study). Its usefulness has been demonstrated analyzing five monovarietal extra-virgin olive oils (January 2005), an Organic olive oil and two types of Picual olive oils with different commercial names related to their sensorial properties. CE joined to statistical analysis was able to discriminate among different olive oils. Moreover, a comparison among extra-virgin olive oil belonging to two different PDOs (one Spanish and the other one, Italian)

was carried out, and the relation between the phenolic content of an oil and its sensorial attributes was checked. In this sense, the results obtained could clarify the relationship among polyphenols and olive oil pungency, bitterness and fruitiness.

8) Changes in the phenolic extracts obtained from extra-virgin olive oil caused by thermal treatments have been controlled by HPLC-UV and HPLC–MS and by CE-UV-Vis. To our knowledge it is the first time in which these phenolic compounds are quantified individually after the treatment by both techniques. The results obtained for the two separative techniques were in good agreement and confirmed other data previously published. Moreover, several new "unknown" compounds were detected and quantified in the phenolic fraction of the oil and their most relevant characteristics were determined. We also studied several refined olive oils looking for the unknown compounds detected in the extra-VOO subjected to thermal oxidation and several of them were found.

9) We have described a qualitative and semi-quantitative CE-ESI-Ion Trap MS method to study phenolic alcohols, lignans and several complex phenols in olive-oil samples after their extraction by Diol-SPE. Repeatability of the method was studied and the relative standard deviations (RSDs) of peak areas/migration time (determined for two compounds, (+)-pinoresinol (Pin) and 10-hydroxy-oleuropein aglycon (10-H-Ol Agl)) were 0.93% and 1.53% in the intraday study, and 1.32% and 2.04% in the interday study for Pin and 10-H-Ol Agl respectively. After demonstrating the potential of this CE-ESI-MS method to characterize the extracts obtained from an extra-VOO, it was used to compare the phenolic content in three monovarietal extra-VOOs: Picual, Lechín de Sevilla and Hojiblanca. This method also permits to observe the isomeric forms of secoiridoid derivatives of olive oil using CE-ESI-MS.

10) The separation by HPLC and CZE with on-line detection by ESI-TOF-MS has been successfully applied to the analysis of the phenolic compounds present in extra-virgin olive oil samples. The two methodologies are able to determine many well-known phenolic compounds present in olive oil and provide information about the presence and relative concentration of minor phenolic compounds. Both CE-MS and LC-MC can determine more than 45 analytes in each run. Excellent repeatability of the methods is obtained, with relative standard deviations (RSDs) of peak

areas/migration time (determined for ten compounds) between 1.4% and 2.1% in the intraday study, and between 2.0% and 4.0% in the interday study.

# PERSPECTIVAS FUTURAS

Durante la realización de todo el trabajo descrito en esta memoria, se han abierto ante nosotros numerosas posibilidades para futuros trabajos, de entre las cuales, se mencionan algunas a continuación. También se incluyen otras perspectivas que no están relacionadas con la matriz obejto de esta tesis.

- Las últimas tendencias en CE utilizan métodos desarrollados en medios no acuosos (metanol, formamida, N, N-dimetilformamida, mezclas de metanol y acetonitrilo...), que logran reducir el efecto Joule y la adsorción en las paredes del capilar. Estos métodos en non-aqueous CE (NACE) podrían resultar muy apropiados para el análisis de una matriz tan apolar como el aceite de oliva.

- Equipamientos potentes como IT, MicroTOF o incluso MicroTOF-Q, acoplados a técnicas separativas (CE o HPLC), nos brindarán la posibilidad de seguir profundizando en la caracterización de los compuestos fenólicos del aceite de oliva. Aunque ya se ha avanzado bastante en este propósito, hay aún muchos compuestos presentes en esta fracción del aceite de los que sólo se conoce la fórmula molecular, y que se podrían determinar haciendo MS/MS con una trampa de iones o un MicroTOF-Q.

- Dado el tremendo interés que suscitan las llamadas sustancias antioxidantes, y siendo los polifenoles un grupo de compuestos destacado en esta categoría, sería muy interesante, poder colaborar con grupos de investigación de Medicina y áreas relacionadas, y llevar a cabo estudios que pudieran determinar el efecto de cada uno de los compuestos fenólicos del aceite en el organismo humano. De este modo, se podría averiguar qué compuestos influyen más o son más responsables del efecto preventivo que ejercen los polifenoles para diversas enfermedades como el cáncer, la arteriosclerosis o disfunciones cardíacas.

- Siguiendo la línea mencionada en el punto anterior, sería, en nuestra opinión, muy recomendable, intentar abordar el estudio de "biomarcadores" mediante métodos potentes y fiables que aunaran el poder separativo de HPLC o CE

con la capacidad de los detectores de mayores prestaciones (MS, RMN…), para ayudar en el diagnóstico y prevención de muchas enfermedades.