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TESIS DOCTORAL

CARACTERIZACIÓN DE ALIMENTOS FUNCIONALES MEDIANTE METODOLOGÍAS SEPARATIVAS AVANZADAS Y APLICACIONES A TECNOLOGÍA DE ALIMENTOS

presentada por

Ana María Gómez Caravaca

para optar al grado de

Doctor Europeo en Química

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CERTIFICA:

Que el trabajo que se presenta en esta tesis doctoral bajo el título: "**CARACTERIZACIÓN DE ALIMENTOS FUNCIONALES MEDIANTE METODOLOGÍAS SEPARATIVAS AVANZADAS Y APLICACIONES A TECNOLOGÍA DE ALIMENTOS",** 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 de la Universidad de Granada; también, parcialmente, en las instalaciones de la Universidad de Bologna (Italia) (Dipartimento di Scienze degli Alimenti, sede de Cesena), en la Universidad de Ioannina (Grecia) (Centro de NMR) y en la Universidad Autónoma de Barcelona (Servicio de Resonancia Magnética Nuclear), reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dª. Ana María Gómez Caravaca pueda optar al grado de Doctor Europeo en Química.

Y para que conste, expido y firmo el presente certificado en Granada a 2 de Marzo de 2009.

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Resumen

En esta memoria se reúnen los resultados obtenidos durante la realización de la tesis doctoral titulada "Caracterización de alimentos funcionales mediante metodologías separativas avanzadas y aplicaciones a la tecnología de alimentos". Se ha dividido en una "INTRODUCCIÓN", que incluye información acerca de los alimentos funcionales, compuestos bioactivos y compuestos fenólicos como compuestos bioactivos de interés, además de las técnicas separativas y de detección empleadas (CE y HPLC con detección UV, MS, y NMR).

A continuación, la sección de "PARTE EXPERIMENTAL. RESULTADOS Y DISCUSIÓN" se desglosa en cuatro bloques, correspondientes a las cuatro matrices estudiadas: aceite de oliva, miel, nuez y *Teucrium polium*. En cada uno de ellos se hace una introducción a la matriz estudiada.

El BLOQUE I (aceite de oliva) está dividido en 5 capítulos (Capítulos 1-5).
El capítulo 1 es un trabajo de revisión bibliográfica donde se recoge una visión amplia y clara acerca de la importancia de los compuestos fenólicos en el aceite de oliva. Se resume su importancia a nivel sensorial, de salud, su poder antioxidante y las metodologías analíticas puestas a punto en los diez años anteriores a su publicación.

En el segundo capítulo se describe la puesta a punto de un método para la determinación de los compuestos fenólicos del aceite de oliva mediante CE-UV-Vis. Esta metodología se centra en los compuestos mayoritarios de esta fracción fenólica, llegándose a identificar ocho analitos, cuatro de los cuales (pinoresinol, oleuropeína aglicona, ligustrósido aglicona y ácido elenólico) nunca antes habían sido detectados mediante electroforesis capilar. El método se aplicó a 7 variedades de aceite de oliva virgen extra obteniendo resultados interesantes. De esta forma se demuestra la potencialidad y versatilidad de la CE como alternativa a HPLC para el análisis de esta fracción y su aplicabilidad a diferentes variedades de aceite.

El tercer capítulo centra su interés en la misma familia que se estudió en el capítulo 2: los compuestos fenólicos del aceite de oliva. Sin embargo, en este caso se utiliza una metodología electroforética más innovadora empleando únicamente disolventes orgánicos dando lugar a una metodología en medio no acuoso acoplada a detección

mediante espectrometría de masas con tiempo de vuelo (NACE-ESI-MS (TOF)). El gran logro de este método es que permite la inyección directa de aceite en el interior del capilar, y que enriqueciendo este aceite en compuestos fenólicos procedentes de una extracción de aceite, permite la identificación de 16 compuestos.

El capítulo 4 pone de manifiesto cómo se afecta el contenido fenólico del aceite después de diversos procesos de filtración convencionales (algodón y filtro de papel acompañado de sulfato sódico anhidro) y cómo esto afecta a la estabilidad oxidativa del aceite de oliva y su contenido en agua. El trabajo experimental incluido en este capítulo se desarrolló en la Universidad de Bolonia, Departamento de Ciencia de los Alimentos. Los aceites utilizados fueron aceites provenientes de diferentes regiones de Italia, producidos en años diferentes (por lo que se ve afectado el estado oxidativo), obtenidos mediante tecnologías diferentes y almacenados de forma distinta. El perfil fenólico fue analizado mediante HPLC-DAD-MS, la estabilidad oxidativa medida mediante OSI y el contenido en agua mediante valoración Karl-Fischer. Los resultados llevaron a la conclusión que a pesar de la disminución del contenido en agua de los aceites, la concentración de fenoles aumentaba tras la filtración, probablemente debido a su mayor afinidad por el disolvente de extracción al encontrarse dentro de una matriz más apolar que en un principio. A pesar de esto la estabilidad oxidativa disminuye después de la filtración, encontrándose explicación en la paradoja polar: los compuestos fenólicos, por su polaridad, tienen mayor actividad en las emulsiones de agua en aceite.

El capítulo 5 muestra otra parte del trabajo desarrollado en el Departamento de Ciencia de los Alimentos de la Universidad de Bolonia. En este capítulo se estudia cómo el ataque de la aceituna por la mosca del olivo (*Bactrocera oleae*) afecta a diversos parámetros químicos del aceite de oliva y al contenido fenólico. Además se trata de evaluar qué parámetro es el mejor para estimar el estado de salud de la aceituna con que se produjo un aceite. Los parámetros estudiados fueron acidez libre e índice de peróxidos (PV), composición en ácidos grasos, índice de estabilidad oxidativa (OSI), determinación del poder antioxidante mediante una técnica electroquímica, y compuestos fenólicos mediante HPLC-DAD-MS. Las muestras de aceite pertenecían a diversas variedades de aceituna, obtenidas mediante procesos tecnológicos diferentes, y con distinto porcentaje de ataque de la mosca, pero todas ellas cultivadas en la misma

región (Abruzzo, Italia). Como conclusión se obtuvo que la acidez libre era el parámetro más adecuado para juzgar la calidad de un aceite justo después de su producción ya que no depende de tantos factores externos como el contenido fenólico.

El BLOQUE II (miel) está compuesto por 3 capítulos (Capítulos 6-8).
El capítulo 6 resume los métodos analíticos descritos para la determinación de los compuestos fenólicos en productos derivados de las abejas (miel y propóleo). La realización del review que compone este capítulo 6 nos dio una idea clara de qué había ya hecho, qué sería interesante hacer y puso de manifiesto la escasez de datos obtenidos mediante CE hasta ese momento.

El capítulo 7 describe el desarrollo de un método para la determinación de compuestos fenólicos en miel mediante CE-UV-Vis. En la puesta a punto del método y sobre todo a la hora de realizar la identificación mediante UV aparecen una serie de problemas que dificultan este trabajo. La complejidad de la matriz miel (que da lugar a muchas interferencias) y la universalidad y baja selectividad y sensibilidad de la detección UV-Vis dificultaron en gran medidad la identificación de los compuestos fenólicos. Por ello, y a pesar de los esfuerzos realizados, sólo pudieron identificarse 5 compuestos.

Con el afán de mejorar los resultados obtenidos en el capítulo anterior, el capítulo 8 muestra el desarrollo de otra metodología para el análisis de la misma familia de compuestos en miel, pero esta vez mediante CE-ESI-MS (IT) de forma que se pudiera adquirir información estructural de los compuestos objeto de estudio. En el momento de su publicación era la primera vez que se emplaba la espectrometría de masas acoplada a CE para este tipo de análisis. La nueva metodología permite un análisis rápido en el que se llegan a detectar 13 compuestos, de los que se identifican 9.

El BLOQUE III (nuez) consta de un capítulo (Capítulo 9).
En el capítulo 9 se puso de manifiesto la potencialidad de la CE acoplada a MS (TOF) para la caracterización de la fracción fenólica de la nuez, una de las matrices de origen vegetal que se ha descrito que contiene mayor cantidad de compuestos fenólicos. Esta metodología arroja muy buenos resultados, los análisis son muy rápidos (menos de 15 min), es capaz de identificar y cuantificar 11 compuestos y se ha llegado a la

identificación de un nuevo compuesto nunca antes determinado en nuez (ácido 8-hidroxi-2,7-dimetil-2,4-decadien-1,10-dioico 6-O-β-D-glucopiranosilester). Para la identificación, además de los datos de masa exacta proporcionados por ESI-MS (TOF), se realizaron experimentos de MS/MS mediante ESI-MS (IT). Esta metodología se aplicó al analisis de tres variedades diferentes de nuez demostrándose su capacidad para la caracterización de la fracción fenólica en diversas variedades de nuez.

El BLOQUE IV (*Teucrium polium*) lo constituye un capítulo (Capítulo 10).
En el capítulo 10 se describe el desarrollo de una metodología para la caracterización de la fracción fenólica de una planta medicinal denominada *Teucrium polium* conocida comunmente como zamarrilla. Esta metodologia se llevó a cabo mediante HPLC acoplada en línea a un módulo de extracción en fase sólida, donde se recogían las fracciones separadas por HPLC y que posteriormente se transferían a un detector NMR también acoplado en línea. Esta instrumentación posee un alto potencial ya que permite obtener información estructural precisa de los compuestos bajo estudio llegándose a la correcta identificación de cada uno de ellos.

Por último se recoge un anexo acerca del la aplicación de LC-SPE-NMR a la determinación de la fracción fenólica del aceite de oliva. Este anexo no se muestra como capítulo ya que el trabajo no ha sido aún concluído, pero al haberse llevado a cabo durante el preiodo de tesis, se ha incluído un resumen del trabajo realizado y de las perspectivas futuras.

Riassunto

In questo paragrafo sono riportati i risultati ottenuti durante lo svolgimento della tesi di dottorato intitolata "Caracterización de alimentos funcionales mediante metodologías separativas avanzadas y aplicaciones a la tecnología de alimentos". La tesi è stata redatta in più parti. La prima è una "INTRODUZIONE", all'interno della quale sono comprese informazioni riguardanti gli alimenti funzionali, i composti bioattivi e i composti fenolici come composti bioattivi di largo interesse; inoltre è presente una carrellata delle tecniche analitiche di separazione e i detector utilizzati durante lo svolgimento della tesi (CE e HPLC con detector UV-DAD, MS ed NMR).

Successivamente, la parte di "PARTE SPERIMENTALE. RISULTATI E DISCUSSIONE" si suddivide in 4 sezioni corrispondenti alle quattro matrici alimentari studiate: olio di oliva, miele, noci e *Teucrium polium.* In ogni sezione è presente un'introduzione della matrice studiata.

La SEZIONE I (olio di oliva) è suddivisa in 5 capitoli (capitoli 1-5).
Il capitolo 1 rappresenta una revisione bibliografica all'interno della quale è possibile trovare una vasta e chiara descrizione dell'importanza dei composti fenolici nell'olio di oliva. Tale capitolo tiene conto degli aspetti sensoriali, salutistici, del potere antiossidante e delle metodologie analitiche messe a punto nella decade precedente alla pubblicazione della review.

Il secondo capitolo descrive la messa a punto di un nuovo metodo di determinazione dei composti fenolici dell'olio di oliva mediante CE-UV-Vis. Questa metodologia si è focalizzata sui composti maggioritari della frazione fenolica, permettendo la identificazione di otto analiti, quattro dei quali (pinoresinolo, oleuropeina aglicone, ligstroside aglicone e acido elenolico) non erano mai stati identificati mediante elettroforesi capillare. Il metodo messo a punto è stato impiegato per la determinazione dei composti fenolici in sette differenti varietà di olio extravergine di oliva, mostrando interessanti risultati. In questa maniera è stata dimostrata la potenzialità e versatilità della tecnica elettroforetica (CE) come alternativa alla cromatografia liquida (HPLC) per la determinazione dei composti fenolici nell'olio di oliva proveniente da diverse varietà.

Il terzo capitolo focalizza il suo interesse sulla stessa classe di composti studiati nel capitolo 2, ovvero i composti fenolici dell'olio di oliva. Però, in questo caso, la metodologia analitica utilizzata risulta essere più innovativa rispetto a quella vista nel capitolo 2. Tale metodologia elettroforetica prevede l'uso di soli solventi organici e la rivelazione degli analiti mediante spettrometria di massa con analizzatore a tempo di volo (NACE-ESI-MS (TOF)). Il vantaggio di questo metodo sta nel fatto che permette l'iniezione diretta dell'olio nel capillare; di fatti l'olio viene utilizzato come solvente per ricostituire un estratto fenolico di olio e viene iniettato direttamente in CE. Questo ha permesso l'identificazione di 16 composti.

Il capitolo 4 evidenzia come diversi procedimenti convenzionali di filtrazione dell'olio (cotone, carta da filtro + sodio solfato anidro) influenzano il contenuto di composti fenolici e il contenuto in acqua dell'olio stesso e conseguentemente la stabilità ossidativa dell'olio. Il lavoro sperimentale incluso in questo capitolo è stato effettuato presso l'Università di Bologna, Dipartimento di Scienze degli Alimenti. I campioni d'olio utilizzati, provenivano da diverse regioni italiane, sono stati prodotti in differenti annate (questo ha consentito la valutazione dello stato ossidativo), ottenuti con diversi sistemi di estrazione. Il profilo fenolico è stato valutato mediante l'utilizzo di HPLC-DAD-MS, la stabilità ossidativa è stata valutata mediante OSI e il contenuto in acqua è stato determinato attraverso un titolatore Karl-Fisher. I risultati hanno evidenziato come una diminuzione del contenuto in acqua dell'olio era correlata ad una concentrazione dello stesso in composti fenolici, probabilmente dovuto ad una maggiore affinità con il solvente d'estrazione al cospetto di un ambiente più apolare rispetto a quello di partenza. Tuttavia, la stabilità ossidativa diminuiva con il processo di filtrazione giustificando il paradosso polare: i composti fenolici, per la loro polarità, hanno una maggior attività in una emulsione di acqua e olio.

Il capitolo 5 riporta un'altra parte del lavoro maturato presso il Dipartimento di Scienze degli Alimenti dell'Università di Bologna. In questo capitolo è stato studiato come l'attacco della mosca dell'olivo (*Bactrocera oleae*) condiziona alcuni parametri chimici e il contenuto in composti fenolici dell'olio di oliva. Inoltre, è stato valutato quale è il parametro in grado di determinare lo stato di salute dell'oliva con la quale verrà prodotto l'olio. I parametri considerati sono stati l'acidità libera e il numero di perossido

(PV), la composizione in acidi grassi, la stabilità ossidativa valutata mediante OSI test, la determinazione del potere antiossidante determinato mediante tecnica elettrochimica e la valutazione dei composti fenolici mediante HPLC-DAD-MS. I campioni in esame provenivano dalla regione Abruzzo (Italia) da diverse varietà di olivo, ottenuti con diverso processo estrattivo, e presentavano una diversa percentuale di attacco da mosca. In conclusione è stato notato che il parametro dell'acidità libera si è rivelato il più adeguato a stabilire la qualità dell'olio al momento della produzione, in quanto tale indice non dipende da variabili esterne come accade per il contenuto in composti fenolici.

La SEZIONE II (miele) è composta da 3 capitoli (capitoli 6-8).
Il capitolo 6 riporta i metodi analitici impiegati per la determinazione dei composti fenolici in alcuni prodotti dell'alveare (miele e propoli). La stesura della review che compone il capitolo 6 ha dato la possibilità di evidenziare lo stato dell'arte riguardo questo argomento e stilare una strategia di lavoro futura. Per di più, ha evidenziato la limitata presenza di risultati ottenuti mediante CE fino a quel momento.

Il capitolo 7 descrive lo sviluppo di un metodo di determinazione dei composti fenolici del miele mediante CE-UV-Vis. Durante la messa a punto, e soprattutto al momento dell'identificazione mediante detector UV sono insorti una serie di problemi che hanno reso difficile il totale completamento del lavoro. La complessità della matrice (che presenta una serie di sostanze interferenti) e la bassa sensibilità e selettività, nonché la universalità del detector UV-Vis hanno reso ostica la identificazione dei composti fenolici. Per questo motivo è stato possibile identificare solo 5 composti fenolici.

Con il fine di migliorare e completare i risultati ottenuti nel precedente capitolo, il capitolo 8 descrive una nuova metodologia analitica (CE-MS (IT)) in grado di fornire informazioni strutturali riguardo la composizione in composti fenolici del miele. Al momento della pubblicazione, questo si presentava come il primo metodo di determinazione dei composti fenolici del miele mediante separazione elettroforetica e rivelazione con spettrometro di massa. Questa nuova metodologia ha permesso di determinare 13 composti fenolici, dei quali 9 sono stati identificati.

La SEZIONE III (noci) è formata da un capitolo (capitolo 9).
Il capitolo 9 ha confermato le potenzialità della CE accoppiata ad un sistema MS (TOF) per la caratterizzazione della frazione fenolica in campioni di noci, che rappresentano una delle matrici vegetali che è considerata tra quelle con più alto contenuto di composti fenolici. Questa metodologia ha riportato dei buoni risultati, le analisi sono risultate rapide (meno di 15 minuti a corsa), sono stati identificati e quantificati 11 composti, tra i quali è stato identificato per la prima volta un composto non riportato in bibliografia quale l'acido 8-idrossi-2,7-dimetil-2,4-decadien-1,10-dioico 6-O-β-D-glucopiranosilestere. Per l'identificazione, i dati di massa esatta ottenuti mediante ESI-TOF-MS, sono stati comparati con una serie di determinazioni effettuate mediante ESI-IT-MS mediante la quale è stato possibile effettuare delle determinazioni di MS^n. La metodologia è stata applicata a tre differenti varietà di noci in maniera tale da verificare la capacità del metodo a discriminare la frazione fenolica in campioni diversi.

La SEZIONE IV (*Teucrium polium*) è costituita da un capitolo (capitolo 10).
Nel capitolo 10 viene descritto lo sviluppo di un metodo analitico per la caratterizzazione di una pianta medicinale chiamata *Teucrium polium* comunemente conosciuta in Italia come *Camedrio Polio*. Questo metodo analitico ha previsto l'uso di un HPLC accoppiato in linea con un sistema di estrazione in fase solida, all'interno del quale avveniva la concentrazione delle frazioni separate mediante HPLC, le quali venivano successivamente eluite in un detector NMR accoppiato in linea. Questa strumentazione possiede un alto potenziale in quanto permette di ottenere informazioni circa la struttura della molecola oggetto di studio e permettendo la esatta identificazione di ogni analita.

Per ultimo, si riporta un'appendice relativa all'applicazione di un metodo LC-SPE-NMR per la determinazione della frazione fenolica dell'olio di oliva. Tale appendice non è riportata come capitolo in quanto presenta una parte preliminare del lavoro che non è ancora stato terminato. Tuttavia, essendo stato svolto all'interno del periodo di dottorato, viene riportato un riassunto del lavoro svolto e una prospettiva futura di ricerca.

Summary

This work is a summary of all the results obtained during the PhD thesis: "Caracterización de alimentos funcionales mediante metodologías separativas avanzadas y aplicaciones a la tecnología de alimentos". The current work can be divided in several sections; the first one is the "INTRODUCTION", which includes outstanding information about functional food, bioactive compounds, phenolic compounds and two separative techniques (CE and HPLC) and several detection systems (UV, MS and NMR).

Then, we can see the "EXPERIMENTAL SECTION. RESULTS AND DISCUSSION" section, divided in four sections related to every matrix that has been studied: olive oil, honey, walnut and *Teucrium polium*. Every section contains an introduction about the matrix studied.

SECTION I (olive oil) comprises 5 chapters (Chapters 1-5).
Chapter 1 includes an overview where the importance of the phenolic fraction of olive oil is widely described. Aspects as sensorial properties, health aspects, oxidative stability, new analytical approaches to characterization of the phenolic profile and applied studies carried out during the last decade are summarized.

The second chapter describes the development of a method for the determination of phenolic compounds from virgin olive oil by CE-UV-Vis. This methodology is focused in the determination of the principal compounds of the phenolic fraction of olive oil. Eight of these compounds could be identified, and it is important to highlight that four of them (pinoresinol, oleuropein aglycon, ligstroside aglycon and elenolic acid) had never been identified before by CE. The method was applied to seven different varieties of extra virgin olive oil obtaining very interesting results. Therefore it is demonstrated that CE is a powerful technique able to be used as an alternative to HPLC for the analysis of this fraction of olive oil and it can be applied to different varieties of olive oils.

Chapter 3 deals with the same family studied in the latter chapter: phenolic compounds from olive oil. However, in this study we develop an innovative electrophoretic methodology using exclusively organic solvents obtaining a non aqueous capillary

electrophoresis method coupled to a time-of-flight mass spectrometer (NACE-ESI-MS (TOF)). This method allows the direct injection of olive oil in capillary. The injection of "enriched" olive oil makes possible the determination of 16 compounds, which allows the comparation of this method to the aqueous CE method.

In chapter 4 it is shown how the phenolic content of olive oil is affected by different filtration systems (cotton or filter paper plus sodium sulphate anhydrous) and the correlation of this fact with the water content and oxidative stability under forced conditions. This work was carried out in the Department of Food Science of the University of Bologna. Olive oils used differed in the production year (oxidative state), production plant (traditional and continuous), and storage conditions. The determination of the phenolic fraction was performed using HPLC-DAD-MS, oxidative stability by OSI and water content by Karl Fischer titration. Filtration with either cotton or paper plus anhydrous sodium sulphate led to an apparent increase in the phenolic content. These apparently contradictory data can be explained by considering that the reduction of the water content permits a higher availability of phenolic compounds that remain in oil, and are extracted with the methanol-water mixture. Despite this fact antioxidant activity decreases after filtration, the explanation can be found in the "polar paradox": phenolic compounds, being polar molecules, have a higher activity in a water-in-oil emulsion.

Another part of the work carried out in the Department of Food Science of the University of Bologna is shown in chapter 5. The attack of the fly attack (*Bactrocera oleae*) has been studied in order to evaluate its influence on the quality of virgin olive oil (free acidity, peroxide value, fatty acid composition, water content, oxidative stability, phenolic compounds by HPLC-DAD-MS, and antioxidant power of phenolic fraction by electrochemical analysis). The aim of this work was to find the most appropriate method to know if an olive oil comes from olives attacked by the olive fly. Samples differed in the percentage of fly attack, variety of olive cultivars, and technological system used (pressure or centrifugation, with or without a destoning phase) but all of them were cultivated in the same region Abruzzo, Italy). There has been evidence that a simple parameter such as free acidity is stronger and more useful

for judging the quality of an olive oil right after production because it is independent of all of the other technological parameters.

SECTION II (honey) is divided in 3 chapters (Chapters 6-8).
Chapter 6 represents an overview of analytical methods for the measurement of polyphenols in honey and propolis. This work was useful to know what research had been carried out previously and what would be interesting to study in the future. This study also showed that only a few works had been done by CE.

Chapter 7 explains the development of a method for the determination of phenolic compounds in honey by CE- UV-Vis. During the optimization of the method and especially during the identification by UV-Vis appeared some problems that made difficult those steps. The complicated nature of the matrix (that causes interferences) and the universality, low selectivity and sensibility of this kind of detector made difficult the identification of the compounds that belong to the phenolic fraction of honey. This fact caused that despite the effort carried out, only 5 compounds could be identified.

To improve the results obtained in the latter chapter, chapter 8 describes another methodology for the analysis of the same family of compounds in honey. The diference appears in the detection system used, this time analysis were achive by CE-MS (IT) in order to obtain some structural information of the compounds. This was the first time that MS was used coupled to CE for this kind of analysis. The new methodology allows a fast analysis where 13 compounds are detected, from which 9 are identified.

SECTION III (walnut) is constituted for 1 chapter (Chapter 9).
CE-MS (TOF) was used in chapter 9 to characterize the phenolic fraction of walnut which has been decribed to be one of the dietary plants with a higher content of phenolic compounds. The results obtained are very interesting; fast analysis (less than 15 min), ability to detect and quantify simultaneously 11 compounds, identification of a new compound that had never been identified before in walnut ((2E,4E)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6-O-d-glucopiranosyl ester). The exact mass data provide by ESI-MS (TOF) and MS/MS data supply by ESI-MS (IT) were used to

perform the identification. Three different varieties of walnut were studied using this method that has demonstrated to be a good tool to characterize the phenolic fraction in different varieties of walnut.

SECTION IV (*Teucrium polium*) comprises 1 chapter (Chapter 10).
Chapter 10 includes the characterization of the phenolic fraction of a medicinal plant known as *Teucrium polium.* HPLC coupled on–line to an SPE system was used. The SPE system collected the separated fractions from HPLC and afterwards the fractions were transferred to the NMR detector that was also coupled *on-line*. This structure of hyphenated techniques is a powerful tool that allows obtaining structural information about the compounds under study, information that is very useful for the identification step.

Finally an appendix section summarizes the aplication of LC-SPE-NMR to the determination of the phenolic fraction of olive oil. This appendix has not the same structure as the rest of the chapters because the work explained on it is not concluded. Anyway it has been performed during the PhD. period we summarize the work carried out and future perspectives.

Objeto de la memoria

De un tiempo a esta parte lo relacionado en general con la alimentación ha cobrado gran importancia, generando un interés cada vez mayor, y no sólo como modo de cubrir los requerimientos nutricionales y energéticos indispensables para la supervivencia, sino como forma de mejorar el estado de salud y disminuir el riesgo de sufrir enfermedades tales como diabetes, obesidad, enfermedades cardiovasculares, cáncer, hipertensión arterial, etc.

Los alimentos funcionales son aquellos que aportan algún tipo de beneficio para la salud, por ello su estudio y caracterización resulta de gran interés. Entre los compuestos que otorgan estas características funcionales a los alimentos se encuentran los compuestos fenólicos que han despertado gran interés debido a su poder antioxidante, que les dota de un efecto quimioprotector en seres humanos y les hace tener gran influencia en la estabilidad a la oxidación que presentan los alimentos. Además influyen en las propiedades organolépticas de estos. Por ello, su identificación y cuantificación son una buena vía para la caracterización de los alimentos que los contienen.

Por todo ello, el objetivo que se planteó en esta Tesis Doctoral, fue caracterizar el perfil fenólico de diferentes alimentos de origen vegetal presentes ampliamente en la dieta Mediterránea: aceite de oliva, miel, nuez y *Teucrium polium*. El interés científico por caracterizar estas matrices requiere el empleo de técnicas analíticas avanzadas que puedan desarrollar métodos rápidos, robustos y fiables para la determinación de este tipo de compuestos, decidiéndonos para ello por la electroforesis capilar y la cromatografía líquida de alta resolución acopladas a diferentes sistemas de detección: UV-Vis, espectrometría de masas y espectrometría NMR. Las dos últimas usadas, de forma complementaria a la detección óptica, son capaces de dar información estructural y complementaria muy útil a la hora de la identificación de los analitos de interés.

De forma desglosada los objetivos planteados fueron los siguientes:

- Demostrar la potencialidad de la electroforesis capilar y la cromatografía líquida de alta resolución para la separación, identificación y cuantificación de la fracción fenólica de matrices vegetales, poniendo a punto metodologías apropiadas para este fin y, en el caso del aceite de oliva, estudiando algunos parámetros tecnológicos que afectan al perfil fenólico.
- Llevar a cabo la identificación de los compuestos fenólicos de la forma más fiable posible empleando para ellos detectores potentes capaces de determinar la estructura de los compuestos de la forma más exacta posible (espectrometría de masas (IT y TOF) y NMR).

Introducción

A) Alimentos funcionales

1. Alimentación y cultura

La **alimentación** responde a la satisfacción de una necesidad indispensable para la vida, constituye una de las múltiples actividades de la vida cotidiana de cualquier grupo social y, por su especificidad y polivalencia, adquiere un lugar central en la caracterización biológica, psicológica y cultural de la especie humana. Por un lado, las prácticas alimentarias resultan ser imprescindibles para la supervivencia física y el bienestar físico de las personas, pero al mismo tiempo son cruciales para la reproducción social de las sociedades humanas. El hecho de comer está indisolublemente ligado tanto a la biología de la especie humana como a los procesos adaptativos empleados por los humanos en función de sus particulares condiciones de existencia, variables, por otra parte, en el espacio y en el tiempo. La comida puede llegar a considerarse como un elemento básico en el inicio de la reciprocidad y del intercambio interpersonal y, en general, en el establecimiento y mantenimiento de la sociabilidad. Objeto de pactos y conflictos, el comportamiento alimentario marca desde las semejanzas hasta las diferencias étnicas y sociales, clasifica y jerarquiza a las personas y a los grupos sociales, expresa formas de concebir el mundo e incorpora un gran poder de evocación simbólica hasta el punto de evidenciar que en efecto somos lo que comemos. Y no sólo somos lo que comemos, porque los alimentos que ingerimos proporcionan a nuestro cuerpo los nutrientes y la energía necesaria para subsistir, sino porque la incorporación de los alimentos supone también la incorporación de sus propiedades morales y comportamentales, contribuyendo así a conformar nuestra identidad individual y cultural [1,2].

El estudio de la alimentación nos introduce en la investigación de la cultura en su sentido más amplio. Conocer los modos de obtención, distribución de los alimentos, quién y cómo los prepara, aporta un conocimiento extraordinario sobre el funcionamiento de una sociedad. Los comportamientos socioculturales que determinan la alimentación humana son poderosos y complejos: las gramáticas culinarias, las

[1] Contreras, J., Alimentación y cultura: necesidades gustos y costumbres. *Universitat de Barcelona*, Barcelona **1995**.

[2] Gracia-Arnaiz, M. "La alimentación en el siglo XXI: una agenda para la investigación sociocultural en España" en "Somos lo que comemos". Gracia-Arnaiz, M. (Coord.). *Editorial Ariel*, Barcelona **2002** pp. 15-38.

categorizaciones de los diferentes alimentos, los principios de exclusión y de asociación entre tal y cual alimento, las prescripciones y las prohibiciones dietéticas y/o religiosas, los ritos de la mesa y de la cocina, etc. Todo ello estructura las comidas cotidianas. El consumo de alimentos en general, ya sea de comida o bebida, trasciende la pura necesidad de alimentarse en el sentido de nutrirse, pues está tan cargado de significados, de emociones y ligado a circunstancias y acontecimientos sociales que nada tiene que ver con la estricta necesidad de comer. En definitiva, las prácticas alimentarias son una parte integrada de la totalidad cultural.

En este sentido, más allá de la estricta necesidad de comer, el acto de la alimentación lleva intrínseca una serie de funciones socioculturales que podemos resumir en: [3]:

- Satisfacer el hambre y nutrir el cuerpo.
- Iniciar y mantener relaciones personales y de negocios.
- Demostrar la naturaleza y extensión de las relaciones sociales.
- Proporcionar un foco para las actividades comunitarias.
- Expresar amor y afecto.
- Expresar individualidad.
- Proclamar la diferencia de un grupo.
- Proclamar la pertenencia a un grupo.
- Hacer frente al estrés psicológico o emocional.
- Significar status social.
- Recompensas o castigos.
- Reforzar la autoestima y ganar reconocimiento.
- Ejercer poder político y económico.
- **Prevenir, diagnosticar y tratar enfermedades físicas.**
- Prevenir, diagnosticar y tratar enfermedades mentales.
- Simbolizar experiencias emocionales.
- Manifestar piedad o devoción.
- Representar seguridad.

[3] Baas, M. A.; Wakefield, L. M.; Kolasa, K. M., Community nutrition and individual food behaviour. *Burgess Publs*, Minnessota **1999**.

- Expresar sentimientos morales.
- Significar riqueza.

Como puede observarse, de los veinte usos considerados en esta lista, todos menos uno son usos no nutricionales; y es que, como ya se ha dicho, los condicionamientos socioculturales relativos a la alimentación son poderosos y complejos.
Por tanto, habiendo quedado demostrada la importancia de la alimentación a nivel social, no debemos olvidar que se trata de un proceso mediante el cual cada individuo cubre sus requerimientos nutricionales y energéticos indispensables para la supervivencia de la especie humana. Bajo este punto de vista la alimentación, a) aporta la energía necesaria para que se mantenga la integridad y el perfecto funcionamiento de las estructuras corporales; b) proporciona los materiales necesarios para la formación de estas estructuras y, c) suministra las sustancias necesarias para regular el metabolismo.

Es sabido que una dieta variada suministra nutrientes suficientes para satisfacer los requerimientos metabólicos de un individuo, y que además la alimentación produce una serie de efectos fisiológicos beneficiosos, más allá de los beneficios nutricionales aceptados. Esto se debe a que se modulan funciones específicas, de forma que la dieta no sólo puede ayudar a alcanzar una salud óptima, sino que puede desempeñar una función importante reduciendo los riesgos de enfermedad.

En la actualidad, nuestra sociedad se caracteriza por una serie de factores que han hecho cambiar la actitud del consumidor frente a la alimentación. Estos factores podrían resumirse en los siguientes puntos:

- El creciente costo sanitario.
- El aumento paulatino de la esperanza de vida con el consiguiente aumento de la población mayor de 65 años.
- El deseo de una mejor calidad de vida.
- Un mayor conocimiento de la relación entre la dieta y la salud.

Estas realidades han provocado que el consumidor actual demande una alimentación no sólo equilibrada, sino que influya positivamente sobre su salud mejorando su estado

físico y disminuyendo el riesgo de sufrir enfermedades tales como diabetes, obesidad, enfermedades cardiovasculares, cáncer, hipertensión arterial, etc. Siendo en este punto donde entran a jugar un papel fundamental los *alimentos funcionales*. Por ello, las investigaciones han pasado a centrarse más en la identificación de componentes biológicamente activos en los alimentos, que ofrezcan la posibilidad de mejorar las condiciones físicas y mentales, así como de reducir el riesgo a contraer enfermedades [4,5].

2. Historia

La primera evidencia escrita sobre la existencia de alimentos funcionales, se sitúa en China en el año 1000 a.C. En Asia existe una larga tradición de atribuir propiedades curativas o terapéuticas a los alimentos y hierbas, pero éste tipo de creencias se han considerado anecdóticas y basadas en tradiciones populares. El término *alimento medicinal* fue usado con frecuencia en la literatura de la Dinastía Este Han, aproximadamente hacia el año 100 a.C. Otro término muy parecido, *alimentos especiales*, fue usado en trabajos médicos en la Dinastía Song en el año 1000, ya en nuestra era.

En Occidente, la creencia de que el alimento está íntimamente ligado a una salud óptima tampoco es un concepto nuevo. De hecho, Hipócrates médico griego del siglo V-VI a.C, dejó en su legado una frase mítica, "Que el alimento sea tu medicina y la medicina tu alimento" [6], sin ser consciente en absoluto de cómo esta afirmación seguiría manteniendo todo su significado después de 2500 años.

Situados ya en el siglo XXI, esta filosofía del "alimento como medicina" es la base del paradigma de los *alimentos funcionales*. El interés actual en los alimentos fisiológicamente funcionales comenzó en Japón, donde hace 28 años surgió por primera vez el término *"functional food"*, como un medio de mejorar la salud de su población bastante mermada como consecuencia de los efectos de la II Guerra Mundial y como forma de reducir los costes sanitarios. Japón fue pionero en establecer un sistema de

[4] Roberfroid, M. B., What is beneficial for health? The concept of functional food. *Food and Chemical Toxicology* **1999,** 37, (9-10), 1039-1041.
[5] http://www.eufic.org/article/es/page/BARCHIVE/expid/basics-alimentos-funcionales/
[6] Milner, J.A., Functional foods and health promotion, *Journal of Nutrition* **1999**, 129, 1395S–1397S.

aprobación para los alimentos funcionales, basado en resultados de investigaciones sobre los beneficios para la salud de productos concretos o de sus componentes. Así, en 1990 y como resultado del informe del Comité de Estudio de los Alimentos Funcionales, el Ministerio Japonés de Salud y Bienestar emitió un decreto por el cual se aprobaron los *"Alimentos de Uso Específico para la Salud"* (Foods for Specific Health Use, FOSHU), referidos a aquellos alimentos que contienen componentes ejercen un efecto positivo sobre las funciones fisiológicas del organismo humano, que van más allá de su aporte en macro y micronutrientes. En Europa y Norte América, el interés por el concepto de alimento funcional ha surgido recientemente debido a la evidencia científica de la relación existente entre Salud y Dieta. Hasta los primeros años de la década de los 80, los estudios se enfocaron principalmente hacia las enfermedades por déficit de nutrientes, mientras que a partir de ese momento los estudios se encaminaron ha descubrir el *potencial preventivo* de los alimentos. Precisamente el concepto de *prevención* de la nutrición es el que da lugar al nacimiento del concepto de alimento funcional.

Así, a mediados de los años 80 se crea un proyecto en Europa relativo a los alimentos funcionales por un grupo de expertos coordinado por ILSI para investigar estos aspectos.

Desde entonces se han celebrado varias reuniones en las que se han establecido las diferentes áreas de aplicación de los alimentos funcionales: crecimiento y desarrollo, metabolismo y biodisponibilidad de sustancias, defensa antioxidante, prevención y tratamiento de enfermedades o factores de riesgo cardiovascular, fisiología o función del tracto gastrointestinal, comportamiento y funciones psicológicas [7]. Hasta llegar a elaborar en 1999 el primer documento de consenso sobre conceptos científicos en relación a los alimentos funcionales.

Hoy día se continúa investigando acerca de los alimentos funcionales, con el fin de definir y obtener un mayor conocimiento sobre ellos, sus propiedades y efectos sobre las funciones fisiológicas del cuerpo humano.

[7] Diplock, A.T.; Aggett, P.J.; Ashwell, M.; Bornet, F.; Fern, E.B.; Roberfroid, M.B., Scientific concepts of functional foods in Europe: consensus document. *British Journal of Nutrition* **1999,** 81, (1), S1–S27.

3. Definición de alimento funcional

A pesar de que el término *alimento funcional* se ha intentado definir en diversas ocasiones [8], aún no existe una definición universalmente aceptada para este grupo de alimentos [9]. En la mayoría de los países todavía no hay una definición oficial para este concepto ya que poner una línea divisoria entre alimento convencional y funcional supone un reto incluso para expertos en alimentación y nutrición [10,11].

Japón fue el lugar de nacimiento del término alimento funcional. Como resultado de este largo proceso de investigación y con el fin de establecer una categoría de alimentos que poseyera la propiedad de mejorar los beneficios que aportan los alimentos a la salud y de este modo disminuir los elevados costes sanitarios, nació en 1991 el concepto de *"Alimentos de Uso Específico para la Salud"* (Foods of Specified Health Use o FOSHU). De acuerdo con el Ministerio de Sanidad japonés los alimentos FOSHU se definen como:

- Alimentos de los que se espera que tengan algún tipo de efecto específico sobre la salud o aquellos en los que las sustancias alérgenas hayan sido eliminadas.
- Alimentos en los que los efectos del enriquecimiento o la eliminación de determinados constituyentes debe ser evaluada científicamente y la atribución de efectos beneficiosos sobre la salud debido a su consumo haya sido permitida por las autoridades.

Para poder definir un alimento como FOSHU tienen que existir evidencias de que el alimento como tal, y no algún componente aislado del mismo, sea capaz de determinar un efecto positivo para la salud cuando se consume como parte normal de la dieta. Además la presentación los productos FOSHU tiene que ser como la de un alimento

[8] Roberfroid, M. B., Global view on functional foods: European perspectives. *British Journal of Nutrition* **2002,** 88, S133-S138.
[9] Alzamora, S. M.; Salvatori, D.; Tapia, M. S.; Lopez-Malo, A.; Welti-Chanes, J.; Fito, P., Novel functional foods from vegetable matrices impregnated with biologically active compounds. *Journal of Food Engineering* **2005,** 67, (1-2), 205-214.
[10] Mark-Herbert, C., Innovation of a new product category - functional foods. *Technovation* **2004,** 24, (9), 713-719.
[11] Niva, M., 'All foods affect health': Understandings of functional foods and healthy eating among health-oriented Finns. *Appetite* **2007,** 48, (3), 384-393.

convencional, sin modificar sus características. Nunca deben presentarse en forma de cápsulas o comprimidos. Esta última premisa fue modificada en 2001, aunque de todas formas los FOSHU siguen apareciendo mayoritariamente en forma de alimentos convencionales y no con forma de cápsulas y comprimidos [12].

A pesar de ello, al no existir consenso a nivel mundial sobre la definición de alimento funcional y sobre su legislación, han aparecido muchos términos que en algunos casos se utilizan como sinónimos, pero que realmente no lo son: *"alimentos de diseño"*, *"nutracéuticos"*, *"alicamentos"*, *"farmalimentos"*, etc.

A la hora de establecer normativas surgen dificultades porque es necesario establecer distinciones entre los productos que se venden como "alimentos" y los productos que contienen determinados componentes que han sido aislados de alimentos y que se venden en forma de en diferentes preparaciones y formulaciones farmacéuticas cápsulas, comprimidos, en polvo u otro tipo de producto concentrado. Así, cada uno de estos conceptos se podría definir de la siguiente forma:

- *Nutracéuticos:* Producto elaborado a partir de un alimento, pero que se vende en forma de píldoras, polvos, y otras presentaciones farmacéuticas no asociadas generalmente con los alimentos, y que ha demostrado tener propiedades fisiológicas beneficiosas o protege contra enfermedades crónicas.
- *Alicamentos*: El término "alicamento" no es sólo un concepto, ya que se refiere a productos mitad alimento mitad medicamento. El problema es que no es un término único aceptado universalmente. En España se conocen como alimentos funcionales. También se les denomina "alimentos frontera" porque pretenden tener cualidades preventivas y terapéuticas como algunos medicamentos.
- *"Novel Foods"*: son alimentos que de algún modo proceden de un organismo modificado genéticamente (alimentos transgénicos), que poseen una estructura molecular nueva o derivan de una fuente alimentaria inusual.

[12] Ohama, H.; Ikeda, H.; Moriyama, H., Health foods and foods with health claims in Japan. *Toxicology* **2006,** 221, (1), 95-111.

En 1999, la *European Commission's Concertad Action on Functional Food Science in Europe* (FUFOSE) coordinada por el *Internacional Life Science Institute* (ILSI) *Europe* definió alimento funcional como aquel que posee las siguientes características [13]:

- Es un alimento, no una cápsula, comprimido o cualquier otra forma de complemento dietético.
- Los beneficios nutricionales y saludables de estos alimentos, o de los ingredientes específicos, deben fundamentarse en una sólida base científica.
- Una vez ingerido, debe ejercer en el organismo una función específica, que permita la regulación de algún proceso fisiológico concreto:
  a) Prevenir una enfermedad específica
  b) Reforzar los mecanismos de defensa corporales.
- Desempeña sus funciones consumiéndose en las cantidades normalmente previstas por una dieta equilibrada.

Por otro lado, "The International Life Sciences Institute of North America" (ILSI) ha definido *alimento funcional* como "alimento que gracias a su contenido en componentes fisiológicamente activos proporciona beneficios para la salud al mismo tiempo que satisface las necesidades nutricionales básicas". Mientras que las autoridades sanitarias de Canadá lo definen como "alimento de apariencia similar a un alimento convencional, consumido normalmente como parte de la dieta, que ha demostrado aportar beneficios a nivel fisiológico y que además de satisfacer las necesidades nutricionales básicas es capaz de reducir el riesgo de sufrir enfermedades crónicas".

A continuación se encuentran resumidas las definiciones propuestas para estos tipos de alimentos en diferentes países [14]:

[13] Diplock, A. T.; Aggett, P. J.; Ashwell, M.; Bornet, F.; Fern, E. B.; Roberfroid, M. B., Scientific concepts of functional foods in Europe consensus document. *British Journal of Nutrition* **1999**, 81, (4), S1-S27.

[14] Arvanitoyannis, I.S.; Van Houwelingen-Koukaliaroglou, M., Functional foods: A survey of health claims, pros and cons, and current legislation. *Critical Reviews in Food Science and Nutrition* **2005**, 45, 385–404.

	Alimentos funcionales	Suplementos alimentarios	Nutraceúticos	Alicamentos	Alimentos de uso especial alimentario	Alimentos saludables	Novel foods
Unión Europea	A nivel europeo no existe una definición oficial, aunque algunos estados miembros tienen definiciones que usan a nivel industrial.	La directiva europea no trata este tipo de alimentos.	Este término no ha llegado a tener mucho éxito en Europa, por lo que no tiene un uso generalizado.	-	-	Estos alimentos son exclusivamente un producto del marketing, no un término legal. Normalmente se refiere a productos que se venden en tiendas especializadas.	
Estados Unidos	No hay una definición generalmente aceptada, aunque sí existe en el ámbito de la profesión nutricionista.	Existe una definición formal debida a "Nutrition Labeling and Education Act".	Existen definiciones en el campo de la nutrición/comunidad científica, pero este témino no se encuentra regulado oficialmente.	Se encuentra regulado por el FDA, refiréndose a alimentos enterales o alimentos para niños con algún tipo de enfermedad.	-	-	-
Japón	Definidos como *"Alimentos de Uso Específico para la Salud"* (Foods of Specified Health Use o FOSHU).	-	-	-	-	Este término está ampliamente aceptado y es usado con frecuencia tanto por los consumidores como por la industria.	-
Canadá	No existe una definición que regule este tipo de alimentos.	Alimento vendido o representado como suplemento a una dieta que puede ser inadecuada en cuanto a la energía o los nutrientes esenciales que aporta.	Este término es usado ampliamente por la comunidad científica y por los nutricionistas.	La normativa que regula los alimentos y las drogas debería ser revisada para dar una definición a este tipo de alimentos.	Existen 11 categorías diferentes para este tipo de alimentos.	No existe una definición clara para este término, pero se emplea frecuentemente por los miembros de esta comunidad	-

Como se puede observar, *alimento funcional* ha sido definido de infinidad de formas diferentes, dependiendo del autor que tratase el término en cada caso. Sin embargo, independientemente de cual sea la definición elegida, *alimento funcional* representa un concepto único y pertenece a una categoría diferente a la de los nutracéuticos, alicamentos, farmalimentos, alimentos de diseño… También hay que dejar claro que el concepto de *alimento funcional* está ligado a la nutrición y no a la farmacología. Los alimentos funcionales son alimentos, y no drogas ya que no presentan en absoluto efectos terapéuticos. Su única función en lo que se refiere a salud es la de ayudar a reducir el efecto de determinadas enfermedades y no la de prevenirlas.

Es importante resaltar que no obstante, y a pesar de todo lo dicho anteriormente, la legislación europea no considera los alimentos funcionales como una categoría específica de alimentos, sino como un concepto [15,16].

En resumen, se puede decir que los alimentos funcionales son aquellos dotados de beneficios específicos a nivel fisiológico que los distinguen de los alimentos tradicionales. La funcionalidad de estos alimentos viene determinada por una serie de *compuestos bioactivos* y su presencia y concentración va a estar en función de diversos factores entre los que se encuentran los factores climatológicos, agronómicos, tecnológicos y culinarios. Estos *compuestos bioactivos* confieren al alimento aquellas características específicas que lo convierten en funcional: ayudar a la prevención de enfermedades y mejorar el estado de bienestar del individuo; acciones que van más allá de satisfacer las necesidades nutricionales básicas. En definitiva, los *compuestos bioactivos* deben ser considerados como elementos saludables capaces de mejorar nuestra dieta.

4. Compuestos bioactivos

La existencia de compuestos bioactivos se ha visto evidenciada gracias a diversos estudios que han demostrado los efectos beneficiosos para la salud que aportan ciertos

[15] Stanton, C.; Ross, R. P.; Fitzgerald, G. F.; Van Sinderen, D., Fermented functional foods based on probiotics and their biogenic metabolites. *Current Opinion in Biotechnology* **2005,** 16, (2), 198-203.

[16] Coppens, P.; da Silva, M. F.; Pettman, S., European regulations on nutraceuticals, dietary supplements and functional foods: A framework based on safety. *Toxicology* **2006,** 221, (1), 59-74.

tipos de dieta, entre las que se incluyen la dieta vegetariana [17,18], la dieta de alto consumo de cereales integrales [19-21], la dieta "prudente" [22,23], la dieta mediterránea [24] y la dieta tradicional japonesa [25]. En general este tipo de dietas se caracterizan por un alto consumo de frutas, verduras, legumbres, aceite de oliva y cereales, y todas ellas han sido relacionadas con una reducción significativa del riesgo de padecer enfermedades cardiovasculares y otro tipo de enfermedades crónicas [26].

A pesar de los efectos beneficiosos para la salud que comportan los compuestos bioactivos, éstos no son compuestos esenciales para la vida, y por lo tanto no forman parte del grupo de los nutrientes. Sin embargo, los efectos producidos por ellos son mucho más sutiles que los de los nutrientes. Mientras que los compuestos bioactivos son capaces de influir en las actividades celulares modificando el riesgo de padecer enfermedades, los nutrientes simplemente previenen el riesgo de contraer una enfermedad debido a una carencia o déficit.

Hasta la fecha se han identificados infinidad de compuestos bioactivos y este número sigue creciendo de manera asombrosa. Entre las funciones que realizan estos compuestos encontramos que pueden actuar como antioxidantes, inhibidores e inductores de enzimas, de la expresión génica etc. etc. Pero estas actividades no son

[17] Fraser, G.E., Associations between diet and cancer, ischemic heart disease, and all-cause mortality in non-Hispanic white California Seventh-day Adventists. *Am. J. Clin. Nutr.***1999**, 70, (3), 532–538S.

[18] McEvoy, L.; Land, G., Life-style and death patterns of the Missouri RLDS church members. *Am. J. Public Health* **1981**, 71, (12), 1350–1357.

[19] Jacobs Jr., D.R.; Meyer, K.A.; Kushi, L.H.; Folsom, A.R., Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the IowaWomen's Health Study. *Am. J. Clin. Nutr.* **1998**, 68 (2) 248–257.

[20] Jacobs Jr., D.R.; Meyer, K.A.; Kushi, L.H.; Folsom, A.R., Is whole grain intake associated with reduced total and cause-specific death rates in older women? The IowaWomen's Health Study. *Am. J. Public Health* **1999**, 89,(3), 322–329.

[21] Jacobs Jr., D.R.; Meyer, H.E.; Solvoll, K., Reduced mortality among whole grain bread eaters in men andwomen in the Norwegian County Study. *Eur. J. Clin. Nutr.* **2001**, 55, (2), 137–143.

[22] Hu, F.B.; Rimm, E.B.; Stampfer, M.J.; Ascherio, A.; Spiegelman, D.; Willett, W.C., Prospective study of major dietary patterns and risk of coronary heart disease in men. *Am. J. Clin. Nutr.* **2000**, 72, (4) 912–921.

[23] Terry, P.; Hu, F.B.; Hansen, H.; Wolk, A., Prospective study of major dietary patterns and colorectal cancer risk in women. *Am. J. Epidemiol.* **2001**, 154, (12), 1143–1149.

[24] De Lorgeril, M.; Salne, P.J.; Martin, J.L.; Monjaud, I.; Delaye, J.; Mamelle, N., Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart Study. *Circulation* **1999**, 99, (6), 779–785.

[25] Kato, H.; Tillotson, J.; Nichaman, M.Z.; Rhoads, G.G.; Hamilton, H.B., Epidemiologic studies of coronary heart disease and stroke in Japanese men living in Japan, Hawaii and California. *Am. J. Epidemiol.* **1973**, 97, (6), 372–385.

[26] Kris-Etherton, P.M.; Lefevre, M.; Beecher, G.R.; Gross, M.D.; Keen, C.L.; Etherton, T.D., Bioactive compounds in nutrition and health-research methodologies for establishing biological function: The Antioxidant and Anti-inflammatory Effects of Flavonoids on Atherosclerosis. *Annual Review of Nutrition* **2004**, 24, 511–538.

suficientes para definir un compuesto bioactivo, además deben tener asociados algún tipo de efecto beneficioso para la salud.

El problema aparece cuando se trata de entender en profundidad cuales son los mecanismos exactos de estos compuestos y el por qué de sus efectos beneficiosos. Su descubrimiento, relativamente reciente, hace que los mecanismos de absorción, transporte en el organismo y metabolismo sean desconocidos para la gran mayoría de ellos. Por otro lado, la gran cantidad de compuestos, su diversidad y los numerosos factores que afectan a su actividad biológica (modo en que se consumen, efectos del procesado, factores ambientales, etc) hacen complicado este estudio. Además, también es de resaltar la diferente respuesta biológica de cada individuo frente al consumo de compuestos bioactivos, que variará dependiendo de sus características genéticas, su edad, su estado de salud, etc.

A continuación, en la figura, pueden observarse las principales familias de compuestos que forman parte del grupo de los compuestos bioactivos.

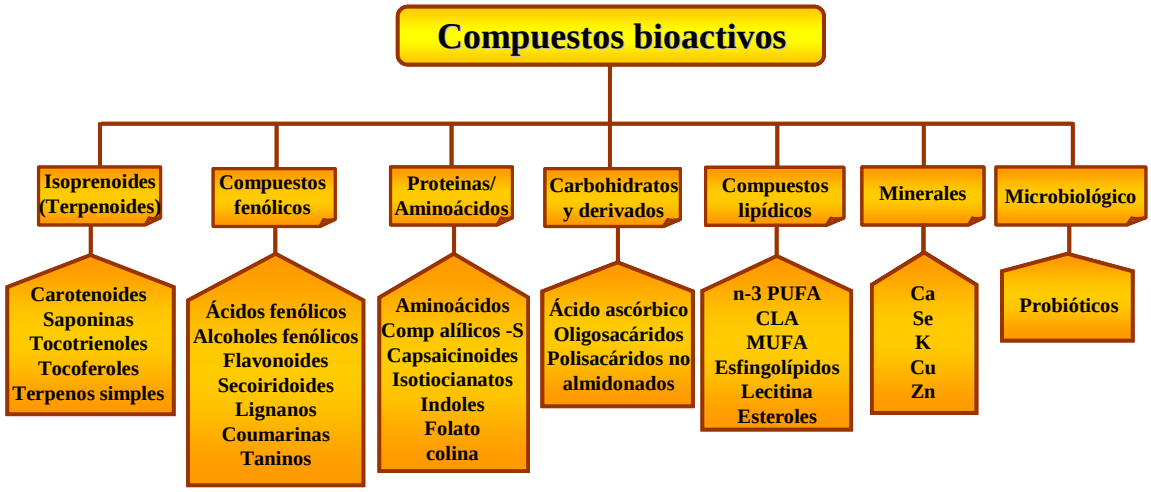

Figura. *Familias de compuestos bioactivos en alimentos.*

Los alimentos de origen vegetal contienen gran cantidad de compuestos bioactivos que son objeto de investigación debido al gran interés que despiertan por su alto potencial para combatir el riesgo de determinadas enfermedades.

De todos ellos nos centraremos en los compuestos fenólicos, cuyo estudio en diferentes matrices alimentarias ha sido el objetivo principal de la presente memoria de tesis.

4.1 Compuestos fenólicos

Los *compuestos fenólicos* son la fuente más importante de compuestos bioactivos de la dieta humana y se encuentran principalmente formando parte de las frutas, bebidas como el té, café, vino y zumos de fruta, chocolate, y aunque en menor proporción de verduras, legumbres y cereales. A lo largo de los años se han identificado más de 8000 compuestos fenólicos [27,28]. Esta familia de compuestos puede definirse como aquella en la que sus componentes poseen al menos un anillo aromático unido al menos a un grupo hidroxilo (–OH), y representa a una amplia variedad de sustancias que se puede dividir en diversos subgrupos: ácidos fenólicos (ácidos hidroxibenzoicos y ácidos hidroxicinámicos), flavonoides (antocianinas, prontocianidinas, flavonoles, flaconas, flavanonas, flavanoles, isoflavonas), estilbenos y lignanos.

De entre ellos, los grupos de compuestos de mayor importancia desde la perspectiva de la salud humana son los ácidos fenólicos, flavonoides (flavonas, flavonoles, 3-flavanoles, isoflavonas, flavanonas, antocianidinas) y lignanos [29].

La importancia de los compuestos fenólicos en la dieta se debe a las evidencias encontradas acerca de su capacidad antioxidante y del papel que juegan frente a la prevención de ciertas enfermedades (enfermedades cardiovasculares y cáncer, entre otras). Éstos pueden ayudar a limitar el daño producido por estas enfermedades actuando directamente sobre las especies reactivas de oxígeno o estimulando sistemas de defensa endógenos. Los grupos fenólicos de estos compuestos son capaces de aceptar un electrón para formar radicales fenoxilo relativamente estables deteniendo las reacciones oxidativas en cadena que se producen en las células [30,31].

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[27] Orzechowski, A.; Ostaszewski, P.; Jank, M.; Berwid, S.J., Bioactive substances of plant origin in food – impact on genomics, *Reproduction Nutrition Development* **2002**, 42, 461–477.

[28] Saura-Calixto, F.; Goñi, I., Antioxidant capacity of the Spanish Mediterranean diet. *Food Chemistry* **2006**, 94, 442-447.

[29] Hooper, L.; Cassidy, A., A review of the health care potential of bioactive compounds. *Journal of the Science of Food and Agriculture* **2006**, 86, 1805–1813.

[30] Kehrer, J.P.; Smith, C.V., Free radicals in biology: Sources, reactivities and roles in the etiology of human diseases. In: *Natural antioxidants*. Frei, B., ed. San Diego, Academic Press. **1994**, 25-62.

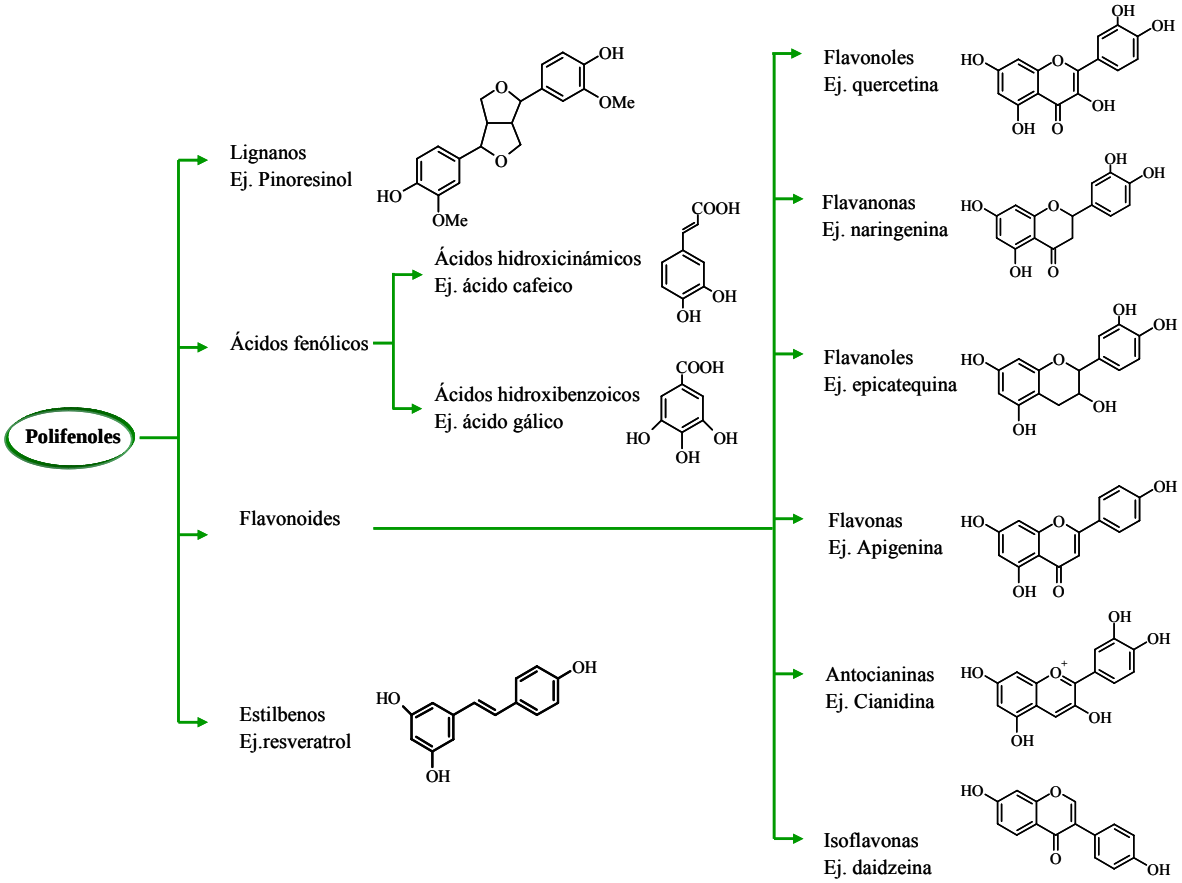

Figura. *Clasificación y estructura química de las familias de compuestos fenólicos principales presentes en la dieta.*

Los principales factores que van a condicionar sus propiedades biológicas, es decir su capacidad de acción frente a estas enfermedades, son su ingesta diaria y su biodisponibilidad.

Es difícil estimar la ingesta de compuestos fenólicos, ya que ésta se ve fuertemente influida por las preferencias y hábitos alimenticios de cada individuo. En 1976 Kuhnau [32] calculó que la ingesta diaria de compuestos fenólicos se encontraba alrededor de 1g/día, de los cuales los ácidos fenólicos constituyen aproximadamente un tercio del

[31] Scalbert, A.; Manach, C.; Morand, C.; Rèmèsy, C., Dietary polyphenols and the prevention of diseases. *Critical Reviews in Food Science and Nutrition* **2005**, 45, 287-306.

[32] Kuhnau, J., The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Review of Nutrition and Dietetics* **1976**, 24, 117-191.

total y los flavonoides los restantes dos tercios [33,34]. Estos datos se han continuado tomando como referencia hasta la actualidad ya que la información acerca del contenido y composición en compuestos fenólicos de los alimentos es todavía insuficiente para determinar con exactitud su ingesta diaria. A pesar de ello en los últimos años se han llevado a cabo diversos estudios acerca de la ingesta de determinado tipo de compuestos fenólicos: flavonoles [35], flavanonas [36], catequinas [37], ácidos fenólicos [33] y 3-flavanoles [38]; e incluso de la ingesta diaria de compuestos fenólicos en la dieta española, donde se ha estimado que se ingieren alrededor de 2590-3016 mg compuestos fenólicos/día [39].

No todos los compuestos fenólicos son iguales. Hoy en día, todavía es difícil discernir qué polifenol tiene mayor capacidad frente a determinadas enfermedades. Hay que tener en cuenta que las propiedades biológicas de los compuestos fenólicos dependen en gran medida de su biodisponibilidad, ya que los compuestos fenólicos presentes en mayor proporción en la dieta no son necesariamente aquellos que presentan mayor actividad en el ser humano. Las causas de esta menor actividad pueden ser debidas a una menor actividad intrínseca del compuesto, a su menor absorción por parte del intestino o a que se metabolicen o eliminen con mucha rapidez del organismo.

La estructura química de los compuestos fenólicos es un factor que determina el grado y la velocidad por la que estos compuestos van a ser capaces de ser absorbidos por el intestino, además de la naturaleza de los metabolitos que circulan en el plasma.

En numerosas ocasiones se han desarrollado trabajos en esta dirección con el fin de conocer la cinética y extensión con la que los compuestos fenólicos se absorben

[33] Scalbert, A.; Williamson, G., Dietary intake and bioavailability of polyphenols. *The Journal of Nutrition* **2000**, 130, 2073S–2085S.

[34] Manach, C.; Scalbert, A.; Morand, C.; Rèmèsy, C.; Jiménez, L., Polyphenols: food sources and bioavailability. *The American Journal of Clinical Nutrition* **2004**, 79, 727-747.

[35] Crozier, A.; Burns, J.; Aziz, A. A.; Stewart, A. J.; Rabiasz, H. S.; Jekins, G. I.; Edwards, C.A.; Lean, M.E.J., Antioxidant flavonols from fruits, vegetables and beverages: measurements and bioavailability. *Biological Research* **2000**, 3, (3), 79–88.

[36] Manach, C.; Morand, C.; Gil-Izquierdo, A.; Bouteloup-Demange, C.; Rèmèsy, C., Bioavailability in humans of the flavanones hesperidin and narirutin after the ingestion of two doses of orange juice. *European Journal of Clinical Nutrition* **2003**, 57, 235–242.

[37] Higdon, J. V.; Frei, B., Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions. *Critical Reviews in Food Science and Nutrition* **2003**, 43, 89–143.

[38] Pascual-Teresa, S.; Santos-Buelga, C.; Rivas-Gonzalo, J., Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. *Journal of Agricultural and Food Chemistry* **2000**, 48, 5331–5337.

[39] Saura-Calixto, F.; Serrano, J.; Goñi, I., Intake and bioaccessibility of total phenols in a whole diet. *Food Chemistry* **2007**, 101, 492-501.

midiendo para ello la concentración de estos compuestos en el plasma [40]. La concentración de metabolitos totales en el plasma se encuentra en un rango que va de 0 a 4 μmol/L después de una ingesta de 50 mg de equivalentes de aglicona. Los compuestos fenólicos que se absorben mejor por el cuerpo humano son las isoflavonas y el ácido gálico, seguidos por las catequinas, flavanonas y glucósidos de la quercetina, mientras que los peor absorbidos son las proantocianidinas, las galoil catequinas del té y las antocianinas. La cinética de cada una de los grupos de compuestos fenólicos en el plasma es diferente, alcanzando su concentración máxima en el plasma después de ≈1.5h o ≈5.5 h, dependiendo de por qué parte del intestino son absorbidos.

Las agliconas son fácilmente absorbibles por el intestino; sin embargo, la mayoría de los compuestos fenólicos se encuentran en forma de ésteres, glucósidos, u otro tipo de polímeros que no pueden ser absorbidos por el intestino en sus formas nativas. Para ello estas sustancias deben sufrir una hidrólisis que se va a dar lugar gracias a las enzimas que se encuentran en la mucosa del intestino o a la microflora presente en el colon [34].

Una vez absorbidos, los compuestos fenólicos son metabolizados en el hígado, que los secreta a través de la bilis y dirigidos de nuevo hacia el colon donde quedan expuestos a la acción de enzimas bacterianas (especialmente la β-glucuronidasa) que los hidrolizan y a continuación son reabsorbidos hacia la sangre. Esta recirculación enterohepática lleva a aumentar el tiempo de permanencia de los compuestos fenólicos en el organismo [34,41].

Se puede decir que la eliminación de los compuestos fenólicos del organismo se produce principalmente a través de la orina y de la bilis, y que una vez llevado a cabo el metabolismo, los compuestos fenólicos (aquellos absorbidos directamente por el intestino como los metabolizados en el hígado), pasan a los tejidos, entre ellos los riñones, que los eliminan mediante la orina. Hay estudios que demuestran que la cantidad de compuestos fenólicos que se encuentran intactos en la orina varía de un compuesto a otro, por lo tanto el estudio del porcentaje de compuestos fenólicos eliminado a través de la orina después de la ingestión de ciertas cantidades controladas de alimentos ricos en ellos, va a permitir la comparación de la diferente biodisponibilidad de cada uno de estos compuestos.

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[40] Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *The American Journal of Clinical Nutrition* **2005**, 81(supl), 230S–242S.

[41] Scalbert, A.; Morand, C.; Manach, C.; Rémésy, C., Absorption and metabolism of polyphenols in the gut and impact on health. *Biomededicine & Pharmacotherapy* **2002**, 56, 276–282.

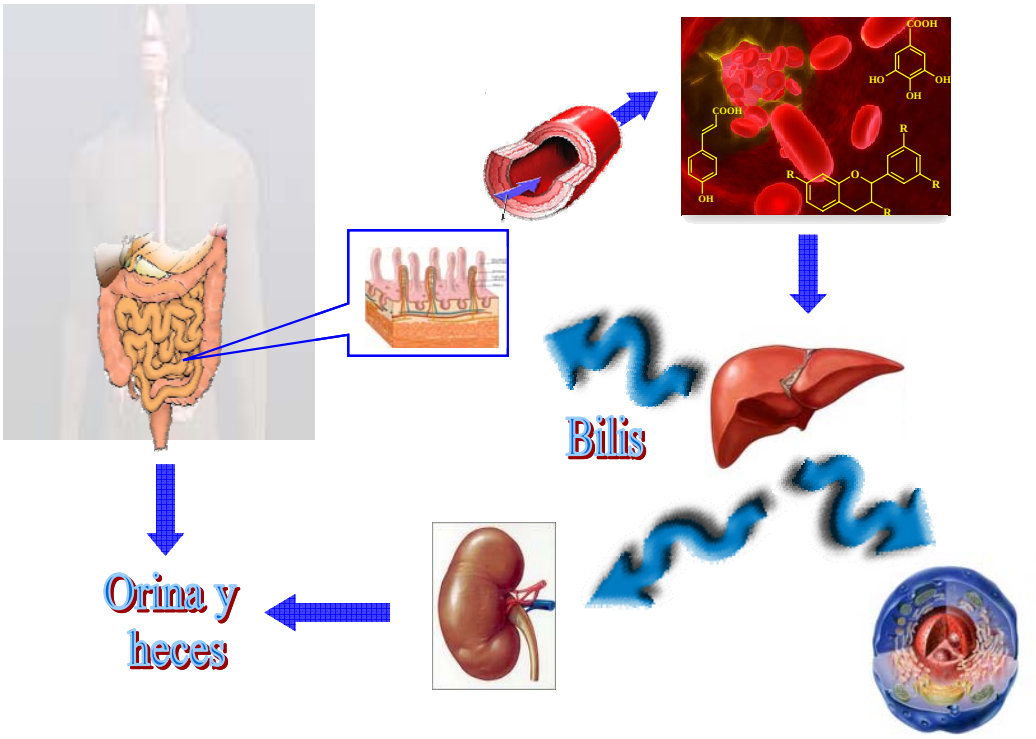

Figura. *Ruta de los compuestos fenólicos procedentes de la dieta y sus metabolitos en el cuerpo humano.*

El metabolismo de los compuestos fenólicos también va a alterar las propiedades específicas de éstos, así como su repuesta biológica a niveles celulares. Hoy en día poco se sabe acerca de cual es la fracción de compuestos fenólicos con mayor actividad de entre todos los que circulan en el organismo. El estudio en esta dirección tiene gran interés ya que estos datos permitirían conocer cual es la fuente principal de estos compuestos y poder desarrollar una dieta óptima para la salud.

B) Tratamiento de muestras alimentarias para el análisis de compuestos fenólicos

Tratamiento de muestras alimentarias para el análisis de compuestos fenólicos

Los cuatro pasos más comunes para poder desarrollar cualquier metodología analítica son los siguientes: muestreo, conservación de la muestra, preparación de la muestra y análisis de ésta (separación y detección) [42].

1. Muestreo

El muestreo es el primer paso a llevar a cabo cuando se quiere realizar un análisis. Debe ser coherente con el problema analítico planteado y, en particular con las características de la información analítica requerida. En él se recoge la parte del total de la muestra que será necesaria para poder hacer el análisis. Debe ser recolectada de modo que ésta represente realmente a la totalidad de la muestra.

En muchas ocasiones se la da poca importancia a este paso. Hay autores que lo pasan por alto no proporcionando información por ejemplo acerca de qué parte de la muestra están analizando. En el caso de alimentos (frutas, verduras,…), no es lo mismo tomar sólo el fruto o también la piel, cáscara, etc. El perfil fenólico obtenido para el análisis de un alimento puede variar sustancialmente dependiendo de qué zona de un alimento se esté analizando. A modo de ejemplo podemos decir, que no es lo mismo analizar los compuestos fenólicos en la cáscara de un cítrico o en sus semillas [43]. Por lo tanto para ser rigurosos en un análisis, es necesario especificar cómo se ha realizado la toma de muestra.

[42] Mitra, S.; Brukh, R., "Sample preparation: an analytical perspective" en "Sample preparation techniques in analytical chemistry". Ed. Mitra S. Wiley, New York **2003**, 1-36.

[43] Bocco, A.; Cuvelier, M. E.; Richard, H.; Berset, C., Antioxidant activity and phenolic composition of citrus peel and seed extracts. *Journal of Agricultural and Food Chemistry* **1998**, 46, 2123–2139.

2. Conservación de la muestra

Una buena conservación de la muestra tras su recogida también es de gran importancia, ya que en ocasiones transcurre un tiempo considerable entre la toma de muestra y el análisis. Una conservación adecuada de la muestra consiste en que la muestra conserve intactas tanto sus características físicas como químicas desde el momento en que se recoge hasta el momento en el que se realiza el análisis.

3. Preparación de la muestra

Este paso puede consistir en el conjunto de múltiples pasos tales como: secado de la muestra, homogeneización, tamizado, extracción, preconcentración, derivatización, hidrólisis, etc.

La preparación de la muestra es de suma importancia para cualquier analista digno de confianza, y los motivos por los cuales se lleva a cabo pueden ser muy diversos: desde incrementar la eficiencia de un procedimiento, hasta eliminar o reducir potenciales interferentes, mejorar la sensibilidad del método analítico incrementando la concentración del analito de interés en la matriz problema, pasando por convertir el analito en un derivado más susceptible de ser separado, detectado, y/o cuantificado [44].

Como ya se ha comentado anteriormente, los compuestos fenólicos pueden presentarse en la naturaleza en su forma libre o conjugados a moléculas de azúcar, ésteres, e incluso formando polímeros de varias unidades monoméricas. Además éstos no se disponen de manera uniforme dentro de las matrices de las que forman parte, sino que pueden estar asociados a las paredes celulares, a proteínas o carbohidratos. Por otra parte, su estabilidad varía considerablemente de unos a otros; algunos son relativamente estables, mientras que otros son térmicamente lábiles, inestables o sensibles a la oxidación [45-

[44] Luthria, D. L., Perspective: Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. *Journal of the Science of Food and Agricultura* **2006**, 86, 2266–2272.

[45] Naczk, M.; Shahidi, F., Extraction and analysis of phenolics in food. *Journal of Chromatography A* **2004**, 1054, 95–111.

47]. Otros factores a tener en cuenta debido a la gran variedad de compuestos fenólicos son su polaridad, acidez, número de grupos hidroxilo y anillos aromáticos, nivel de concentración, y complejidad de la matriz. Por ello, es prácticamente imposible desarrollar un método eficaz para la extracción de todos los compuestos fenólicos empleando un sistema único. Habrá que elegir el tratamiento de la muestra óptimo según el tipo de estructura química y propiedades de la muestra y compuestos analizados [48,49].

3.1. Hidrólisis

La hidrólisis preliminar de las muestras se ha usado como herramienta para la elucidación estructural y caracterización de compuestos glicosilados [50], para eliminar interferencias en futuros análisis por técnicas separativas, así como para simplificar los datos obtenidos de los análisis sobre todo en aquellos casos en los que no existen estándares comerciales [51,52].
Los tipos de hidrólisis empleadas son la hidrólisis enzimática, ácida y básica.

3.2. Filtración/dilución

La centrifugación, filtración y/o dilución forman parte del tratamiento de la muestra. En ocasiones las muestras líquidas son susceptibles de analizarse sin tener que ser

[46] Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D., Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, 125, 989–1009.
[47] Escarpa, A.; González, M. C., An overview of analytical chemistry of phenolic compounds in foods. *Critical Reviews in Analytical Chemistry* **2001**, 31, 57–139.
[48] Stalikas, C. D., Extraction, separation and detection methods for phenolic acids and flavonoids. *Journal of Separation Science* **2007**, 30, 3268-3295.
[49] Tura, D.; Robards, K., Sample handling strategies for the determination of biophenols in food and plants. *Journal of Chromatography A* **2002**, 975, 71-93.
[50] Markham, K.R., Techniques of Flavonoid Identification, Academic Press, London, 1982.
[51] Careri, M.; Elviri, L.; Mangia, A.; Musci, M., Spectrophotometric and coulometric detection in the high-performance liquid chromatography of flavonoids and optimization of sample treatment for the determination of quercetin in orange juice. *Journal of Chromatography A* **2000**, 881 (1-2), 449-460.
[52] Hertog, M. G.; Hollman, P. C. H.; Katan, M. B., Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *Journal of Agricultural and Food Chemistry* **1992**, 40 2379-2383.

sometidas a complejos tratamientos, simplemente mediante alguno de estos tratamientos físicos puede quedar lista para el análisis.

3.3. Extracción

La extracción líquido-líquido y sólido-líquido son los procedimientos de extracción más comunes empleados previo al análisis de compuestos fenólicos. Los disolventes más empleados para este tipo de extracciones son los alcoholes (metanol, etanol), acetona, éter dietílico y acetato de etilo. Sin embargo, hay casos en el que los ácidos fenólicos (de naturaleza muy polar) podrían no extraerse utilizando disolventes orgánicos puros, por lo que es recomendable emplear mezclas alcohol-agua o acetona-agua. La simplificación de la muestra conseguida mediante la extracción líquido-líquido ha demostrado mejorar los resultados obtenidos al realizar análisis mediante técnicas separativas [53].

Por otro lado la extracción en fase sólida (SPE) es otra técnica de preparación de muestra rápida y sensible [54]. Ha sido capaz de sustituir a métodos convencionales de aislamiento y extracción que resultaban tremendamente tediosos en su desarrollo. La SPE es capaz de llevar a cabo satisfactoriamente en un solo paso la preparación y concentración de la muestra.

La creciente demanda de nuevas técnicas de extracción, que se puedan automatizar y que permitan reducir los volúmenes de disolvente y el tiempo de análisis han hecho que se introduzcan otras metodologías de extracción como: extracción mediante fluidos supercríticos, microextracción en fase sólida, extracción asistida por microondas, extracción acelerada por disolvente,…[49].

[53] Rodríguez-Delgado, M. A.; Malovaná, S.; Pérez, J. P.; Borges, T.; García Montelongo, F. J., Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *Journal of Chromatography A* **2001**, 912 (2), 249-257.

[54] K. Coulibaly, I.J. Jeon, An overview of solid-phase extraction of food flavor compounds and chemical residues. *Food Reviews International* **1996**, 12 (1), 131-151.

3.4. Limpieza o "clean-up"

Dependiendo del tipo de muestra que se esté tratando, hay ocasiones en que el proceso de extracción no es totalmente selectivo, además de los compuestos de interés extrae otros compuestos que a la hora de realizar el análisis pueden interferir en la medida (principalmente carbohidratos y/o lípidos). Por ello resulta necesario llevar a cabo otra etapa de limpieza posterior a esta primera extracción [55,56].

El clean-up puede consistir en una segunda extracción líquido-líquido, una extracción en fase sólida, microextracción en fase sólida, etc.

4. Análisis de la muestra: Separación y detección

A continuación se enumeran a modo de resumen las principales técnicas empleadas para el análisis de compuestos fenólicos [57].

4.1. Cromatografía en capa fina

Aunque esta técnica se ha usado menos, desde los años 60, la cromatografía en capa fina ha jugado un papel importante en el análisis de compuestos fenólicos [58,59]. Es especialmente útil para el análisis rápido de extractos de plantas con el fin de emplearlas como sustancias farmacológicamente activas y previo a análisis detallados mediante técnicas instrumentales más complejas.

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[55] Ehala, S.; Vaher, M.; Kaljurand, M., Characterization of phenolic profiles of Northern European berries by capillary electrophoresis and determination of their antioxidant activity. *Journal of Agricultural and Food Chemistry* **2005**, 53 (16), 6484-6490.

[56] Michalkiewicz, A.; Biesaga, M.; Pyrzynska, K., Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey. *Journal of Chromatography A* **2008**, 1187 (1-2), 18-24.

[57] Robbins, R., Phenolic acids in foods: An overview of analytical methodology. *Journal of Agricultural and Food Chemistry* **2003**, 51, 2866-2887.

[58] Ragazzi, E.; Veronese, G.; Quantitative analysis of phenolic compounds after thin-layer chromatographic separation. *Journal of Chromatography A* **1973**, 77 (2), 369-375.

[59] Schulz, J. M.; Herrmann, K., Analysis of hydroxybenzoic and hydroxycinnamic acids in plant material : I. Sample preparation and thin-layer chromatography. *Journal of Chromatography A* **1980**, 195, 85–94.

Una de las principales desventajas de esta técnica es que la cuantificación de los compuestos resulta muy tediosa, por lo que rara vez se usa para tal fin.

4.2. Cromatografía de gases

La cromatografía de gases es una técnica que posee una inmejorable capacidad separativa, y es capaz de analizar gran cantidad de compuestos volátiles o susceptibles de serlo. Su sensibilidad y selectividad aumentan enormemente cuando ésta se combina con la espectrometría de masas.

Una de las características químicas de los grupos OH de los compuestos fenólicos es la capacidad de éstos de formar enlaces por puentes de hidrógeno. Este hecho dificulta el análisis de estos compuestos mediante cromatografía de gases porque disminuye su volatilidad. De todas formas existen numerosos trabajos donde se ha llevado a cabo el análisis de compuestos fenólicos (sobre todo en plantas) mediante esta técnica [60- 66].

Debido a las características de los compuestos fenólicos, para desarrollar su análisis en fase gas en muchas ocasiones es necesario un paso llamado derivatización (además de los pasos previos de preparación de la muestra). A pesar de ello existe algún trabajo sobre fenoles realizado sin derivatización [67].

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[65] Füzfai, Zs.; Molnár-Perl, I., Gas chromatographic-mass spectrometric fragmentation study of flavonoids as their trimethylsilyl derivatives: Analysis of flavonoids, sugars, carboxylic and amino acids in model systems and in citrus fruits. *Journal of Chromatography A* **2007**, 1149, 88–101.

[66] Chiou, A.; Karathanos, V. T.; Mylona, A.; Salta, F. N.; Preventi, F.; Andrikopoulos, N. K., Currants (Vitis vinifera L.) content of simple phenolics and antioxidant activity. *Food Chemistry* **2007**, 102, 516–522.

[67] Christov, R.; Bankova, V., Gas chromatographic analysis of underivatized phenolic constituents from propolis using an electron-capture detector. *Journal of Chromatography* **1992**, 623, 182-185.

4.3. Cromatografía líquida de alta resolución

En los últimos 20 años la técnica analítica que ha predominado a la hora de llevar a cabo la separación y caracterización de los compuestos fenólicos ha sido la cromatografía líquida de alta resolución (HPLC) con columna de fase reversa [57].

Muchos de los compuestos fenólicos, como las formas agliconas (hidrofóbicas) o las formas glucosiladas (hidrofílicas) de los flavonoides, tienen un elevado peso molecular, además de otras características, que hacen que dentro del mundo de las técnicas separativas el HPLC sea la que aporta mejores resultados en los análisis. Es una técnica única que permite realizar separaciones en las que se separan simultáneamente todos los compuestos analizados, además de sus posibles derivados o productos de degradación. Incluso es capaz de dar lugar a la determinación de cantidades muy bajas de analito en presencia de interferentes u otros compuestos que coeluyen.

Entre las ventajas que convierten al HPLC en la técnica más importante para el análisis de compuestos fenólicos en muestras sobre todo vegetales se encuentran: (1) la amplia variedad de columnas disponibles en el mercado y (2) la posibilidad de combinar 2 o más columnas en el mismo análisis.

Esta técnica permite el acoplamiento de numerosos sistemas de detección. La detección de compuestos fenólicos mediante HPLC se ha realizado principalmente empleando UV-Vis [53], batería de diodos (DAD) [68], espectrometría de masas (MS) [69- 72] y NMR [73].

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[68] Chen, L.; Wang, Q.; Liu J., Simultaneous analysis of nine active components in Gegen Qinlian preparations by high-performance liquid chromatography with diode array detection *Journal of Separation Science* **2006**, 29, 2203 – 2210.

[69] Dugo, P.; Presti, M. L.; Ohman, M.; Fazio, A.; Dugo, G.; Mondello, L., Determination of flavonoids in citrus juices by micro-HPLC-ESI/MS. *Journal of Separation Science* **2005**, 28, 1149 – 1156.

[70] Nicoletti, I.; Bello, C.; De Rossi, A.; Corradini, D., Identification and quantification of phenolic compounds in grapes by HPLC-PDA-ESI-MS on a semimicro separation scale. *Journal of Agricultural and Food Chemistry* **2008**, 56 (19), 8801-8808.

[71] Careri, M.; Mangia, A.; Musci, M., Overview of the applications of liquid chromatography-mass spectrometry interfacing systems in food analysis: Naturally occurring substances in food. *Journal of Chromatography A* **1998**, 794 (1-2), 263-297.

[72] Carrasco-Pancorbo, A.; Neusúß, C.; Pelsing, M.; Segura-Carretero, A.; Fernandez Gutiérrez, A., CE- and HPLC-TOF-MS for the characterization of phenolic compounds in olive oil. *Electrophoresis* **2007**, 28 (5), 806-821.

[73] Exarchou, V.; Godejohann, M.; Van Beek, T.A.; Gerothanassis, I.P.; Vervoort, J., LC-UV-Solid-Phase Extraction-NMR-MS Combined with a Cryogenic Flow Probe and Its Application to the Identification of Compounds Present in Greek Oregano. *Analytical Chemistry* **2003**, 75 (22), 6288-6294.

En los casos en los que los resultados obtenidos por UV y MS no son suficientes para identificar el compuesto de interés la espectrometría por NMR resulta ser una poderosa técnica complementaria para la determinación estructural. Los espectros de NMR de los compuestos fenólicos son en muchas ocasiones complejos, y la identificación de estos compuestos es difícil debido a la ausencia (en la mayoría de los casos) de patrones comerciales. Aunque la determinación estructural se puede realizar mediante espectros de RMN 2D sin necesidad de tener el patrón, para ello se requiere una concentración relativamente elevada de compuesto. La sensibilidad y la necesidad de aislar cantidades relativamente altas de compuesto son las limitaciones más importantes de la espectrometría NMR. De todas formas, en los últimos años se ha acoplado en línea a HPLC dando lugar a resultados muy interesantes.

4.4. Electroforesis capilar

En los últimos años la electroforesis capilar (CE) ha surgido como una técnica alternativa a la cromatografía líquida. Ésta es capaz de arrojar metodologías simples, económicas y precisas para el análisis de muchas sustancias, entre ellas los compuestos fenólicos de los alimentos [74- 77]. Sus principales ventajas con respecto a las técnicas cromatográficas son que consigue análisis en tiempos muy reducidos, consumiendo muy poca cantidad de muestra y disolventes.

Las modalidades de electroforesis más empleadas para el análisis de compuestos fenólicos son la electroforesis capilar en zona (CZE) y la cromatografía electrocinética micelar (MECK). La modalidad MECK ha demostrado ser potente y eficaz para determinación de procianidinas en alimentos [78]. La detección se ha hecho

[74] Frazier, R.A.; Papadopoulou, A., Recent advances in the application of capillary electrophoresis for food analysis. *Electrophoresis* **2003**, 24 (22-23), pp. 4095-4105.
[75] Frazier, R.A., Recent advances in capillary electrophoresis methods for food analysis. *Electrophoresis* **2001**, 22 (19), 4197-4206.
[76] Herrero, M.; Ibáñez, E.; Cifuentes, A.; Análisis of natural antioxidants by capillary electromigration methods. *Journal of Separation Science* **2005**, 28, 883-897.
[77] Cifuentes, A., Recent advances in the application of capillary electromigration methods for food analysis. *Electrophoresis* **2006**, 27, 283-303.
[78] Cifuentes, A.; Bartolome, B.; Gómez-Cordoves, C., Fast determination of procyanidins and other phenolic compounds in food samples by micellar electrokinetic chromatography using acidic buffers. *Electrophoresis* **2001**, 22, 1561–1567.

tradicionalmente mediante UV, aunque cada vez es más usual emplear otro tipo de detectores como los electroquímicos o la espectrometría de masas.

Las aplicaciones realizadas mediante EC se pueden dividir según la muestra analizada. Las fuentes de compuestos fenólicos más ampliamente analizadas mediante esta técnica son: uvas y vino [79,80], especias y plantas medicinales [81,82], diferentes tés [83], y aceite de oliva [84-86].

[79] Wang, S.P.; Huang, K.J., Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis. *Journal of Chromatography A* **2004**, 1032 (1-2), 273-279.

[80] Herrero-Martínez, J.M.; Ráfols, C.; Rosés, M.; Torres, J.L.; Bosch, E., Mixed micellar electrokinetic capillary chromatography separation of depolymerized grape procyanidins. *Electrophoresis* **2003**, 24 (4), 707-713.

[81] Herrero, M.; Arráez-Román, D.; Segura, A.; Kenndler, E.; Gius, B.; Raggi, M. A.; Ibáñez, E.; Cifuentes, A., Pressurized liquid extraction-capillary electrophoresis-mass spectrometry for the analysis of polar antioxidants in rosemary extracts. *Journal of Chromatography A* **2005**, 1084 (1-2), 54-62.

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[83] Weiss, D. J.; Anderton, C. R., Determination of catechins in matcha green tea by micellar electrokinetic chromatography. *Journal of Chromatography A* **2003**, 1011 (1-2), 173-180.

[84] Buiarelli, F.; Di Berardino, S. D. I.; Coccioli, F.; Jasionowska, R.; Russo, M. V., Determination of phenolic acids in olive oil by capillary electrophoresis. *Annali di Chimica* **2004**, 94 (9-10), 699-705.

[85] Bonoli, M.; Bendini, A.; Cerretani, L.; Lercker, G.; Toschi, T. G., Qualitative and semiquantitative analysis of phenolic compounds in extra virgin olive oils as a function of the ripening degree of olive fruits by different analytical techniques. *Journal of Agricultural and Food Chemistry* **2004**, 52 (23), 7026-7032.

[86] Carrasco Pancorbo, A.; Cruces-Blanco, C.; Segura Carretero, A.; Fernández Gutiérrez, A.Sensitive determination of phenolic acids in extra-virgin olive oil by capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry* **2004**, 52, 6687-6693.

C) Técnicas separativas y sistemas de detección empleados en esta memoria

Técnicas separativas y sistemas de detección empleados en esta memoria

1. Cromatografía Líquida de Alta Resolución (HPLC).

1.1. Introducción histórica y definición

Aunque en la segunda mitad del siglo XIX cabría asociar algunos experimentos como precursores de la cromatografía tal y como la conocemos hoy en día, la técnica como tal no fue desarrollada hasta principios del siglo XX por el botánico ruso M. S. Tswett [87-89]. Pero la cromatografía líquida propiamente dicha no fue descrita hasta 1930 por Kuhn y Lederer [90], momento a partir del cual ya no deja de desarrollarse de modo prácticamente continuo.

La modalidad clásica de la cromatografía clásica en columna consiste en hacer pasar por gravedad la fase líquida sobre el sólido soporte o activo retenido en una columna recta, generalmente de vidrio, de dimensiones considerables (varios centímetros de diámetro y una altura de 5 a 10 veces el diámetro), y recogida del eluido en fracciones. Esta cromatografía clásica o a baja presión presentaba importantes inconvenientes desde un punto de vista práctico [91]:

- 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

[87] Tswett, M., "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.
[88] Tswett, M., "Zur Kenntnis der Phaeophyceenfarbstoffe". *Ber. dtsch. botan. Ges*. **1906**, 24, 235–244.
[89] Tswett, M., Adsorptions analyse und chromatographische Methode. Anwendungen auf die Chemie des Chlorophylls. *Ber. Deutsch. Bot. Ges.* **1906**, 24, 384-385.
[90] Kuhn, R.; Lederer, E.; Zerlegung des Carotins in seine Komponenten. (Über das Vitamin des Wachstums, I. Mitteil.). Ber. dtsc. Chem. Ges. **1931**, 641, 349-1356.
[91] Valcárcel Cases, M.; Gómez Hens, A., "Cromatografía líquida en columna (I). Generalidades" en "Técnicas analíticas de separación". Ed. Reverté S. A. **1990**, 437-484.

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

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 [92-95]. 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 cualitativa y cuantitativa.

Así nació la cromatografía líquida de alta resolución (HPLC). 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

[92] Calvin Giddings, J.; "Dynamics of Chromatography. Principles and Theory". Ed. Marcel Dekker. **1965**.
[93] Zinder, L. R., Modern Practice of Liquid Chromatography. Before and after 1971. *Journal of Chemical Education* **1997**, 74, 37-44. (Waters symposium: high-performance liquid chromatography).
[94] Kirkland, J. J., "Modern Practice of Liquid Chromatography". Ed. Wiley-Interscience, New York. **1971**.
[95] Greibrokk, T., The contribution of Csaba Horváth to liquid chromatography. *Journal of Separation Science* **2004**, 27, 1249-1254.

inabordables o poco recomendables en GC (compuestos iónicos, muy polares, termolábiles, no volátiles, fases acuosas de muestra…). Su desarrollo comercial pleno empezó al comienzo de los años setenta.

1.2. Instrumentación

Un sistema moderno de HPLC está compuesto por cuatro elementos básicos [96]:

a) Un sistema capaz de gestionar e impulsar la fase móvil.
   - 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.).

b) La 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).

c) Un sistema que permita la inserción de las muestras.
   - Inyector: Permite la introducción de una cierta cantidad de muestra en el sistema.

d) Un sistema capaz de informar del resultado de la separación, es decir, un sistema de detección encargado de producir señales o respuestas analíticas ante la presencia de las especies que abandonan la columna (Puede utilizarse más de uno

[96] Cela, R.; Lorenzo, R. A.; Casais, M. C., "Cromatografía líquida en columna" en "Técnicas de separación en Química Analítica". Ed. Síntesis S. A. Madrid. **2002**, 399-498.

simultáneamente, colocándolos en serie), y un sistema de adquisición de datos, tarea de la cual se encargan los ordenadores, dotados con programas específicos para ello.

Evidentemente estos cuatro elementos básicos pueden adoptar diferentes configuraciones y/o especificaciones dependiendo del modo cromatográfico elegido y particularmente de las características de la fase móvil utilizada y de la propia muestra a separar.

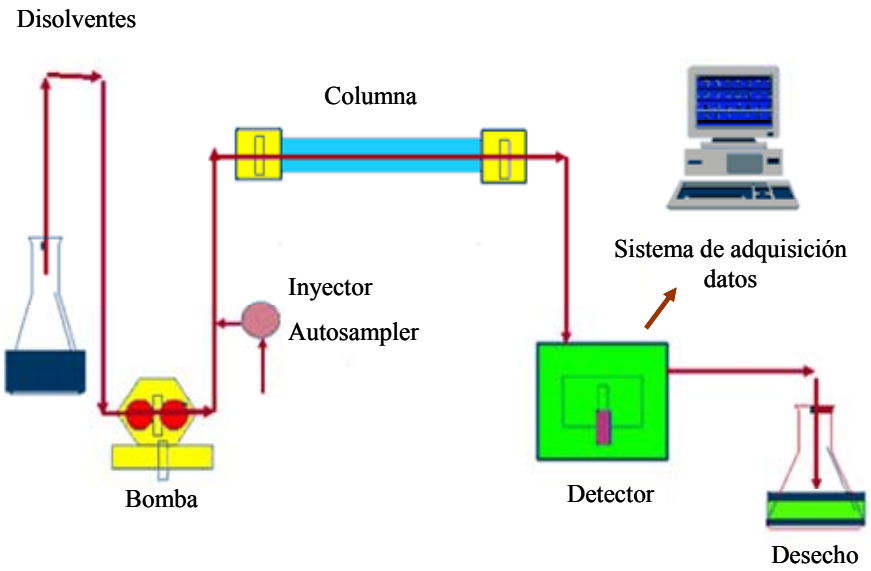

Figura. *Diagrama esquemático de un equipo de HPLC.*

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.

1.3. Tipos de HPLC

La cromatografía líquida en columna tiene una gran variedad de alternativas según la naturaleza de la fase estacionaria [97]:

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

***Tabla**. Tipos básicos de HPLC*

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:

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

[97] Valcárcel Cases, M.; Gómez Hens, A., "Cromatografía líquida en columna (II). Técnicas de adsorción y partición" en "Técnicas analíticas de separación". Ed. Reverté S. A. **1990**, 485-531.

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)
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.

2.3.1. Cromatografía de partición o de reparto

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.

Dentro de este tipo de cromatografía, deben hacerse dos distinciones básicas:
1. 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 de la alternativa más importante de este tipo de cromatografía, ya que una gran mayoría de muestras de interés en diversos ámbitos tienen naturaleza hidrofílica.

2. Dependiendo de 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.

Por ello, la cromatografía de partición en fase invertida con empleo de fases ligadas es la combinación más ventajosa, y por tanto la más empleada ocupando el 90% de las aplicaciones analíticas de interés.

2.4. 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 [96,98], que un esquema o diagrama de flujo a seguir en el desarrollo de un procedimiento de cromatografía líquida en fase invertida podría ser:

[98] Snyder, L. R.; Kirkland, J. J.; Glajch, J. L., "Systematic approach to the Reversed-Phase separation of regular samples" en "Practical HPLC method development". Ed. John Wiley & Sons, Inc. New York. **1997,** 403-437.

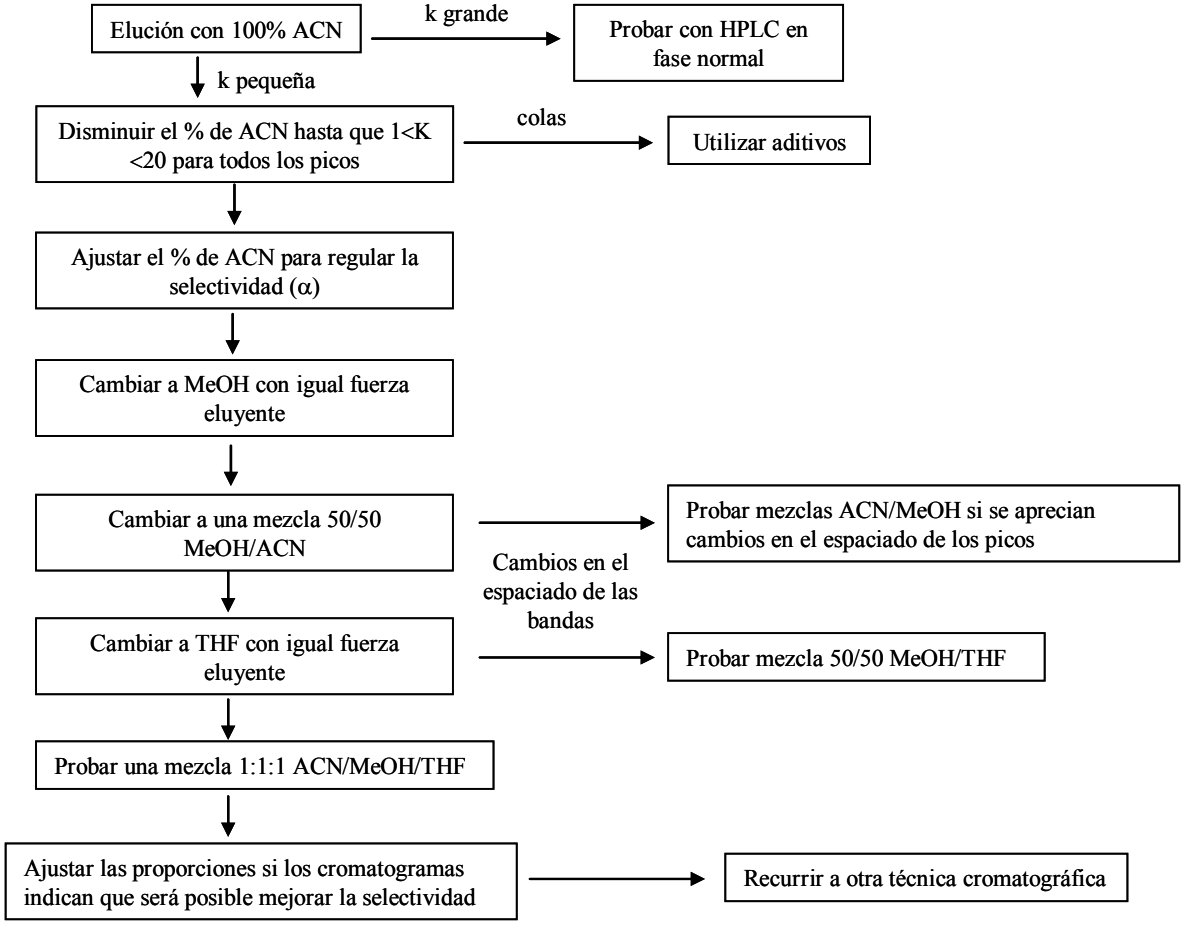

***Figura**. Diagrama de flujo en el desarrollo de un procedimiento de cromatografía líquida en fase invertida. (ACN: acetonitrilo; MeOH: metanol; THF: tetrahidrofurano).*

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.

1.4.1. 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 [99]. 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:

- 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

[99] Snyder, L. R.; Kirkland, J. J.; Glajch, J. L., "Non-ionic samples: Reversed- and Normal-Phase HPLC" en "Practical HPLC method development". Ed. John Wiley & Sons, Inc. New York. **1997,** 233-264.

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.

2. Electroforesis Capilar (CE)

2.1. 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 [100] 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:

[100] Tiselius. A., A new apparatus for electrophoretic analysis of colloidal mixtures. *Transactions of the. Faraday Society* **1937**, 33, 524-531.

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

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 [101] utilizó capilares de 3 mm de diámetro interno empleando campos eléctricos altos. Pero no fue hasta 1974 cuando Virtanen [102] 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. [103], 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 resolución (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.

[101] Hjertén. S., High performance electrophoresis. *Chromatograph. Reviews* **1967**, 9, 122-219.
[102] Virtanen. R., Zone electrophoresis in a narrow-bore tube employing potentiometric detection. *Acta Polytechnica Scandinavica* **1974**, 123, 1-67.
[103] Mikkers, F.; Everaerts, F.; Verheggen. T., High performance zone electrophoresis. *Journal of Chromatography* **1979**, 169, 11-20.

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.

2.2. Instrumentación

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

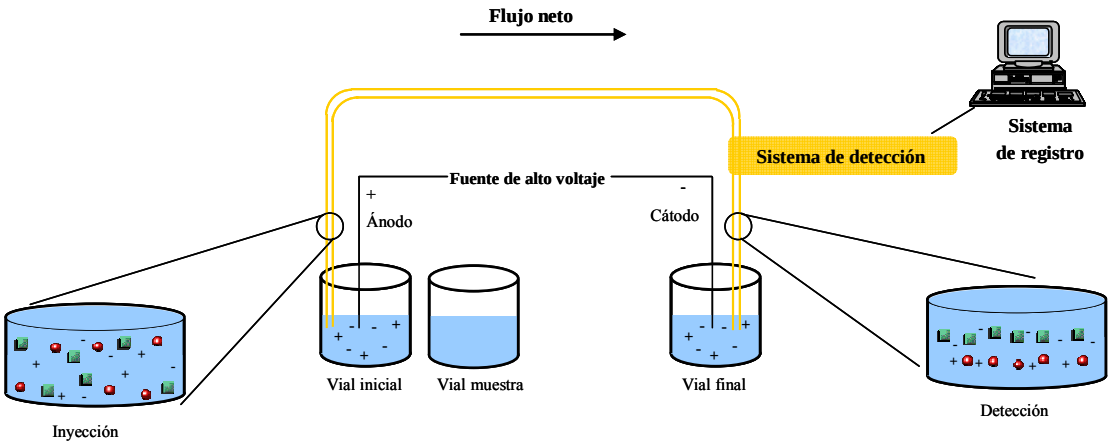

Figura. *Esquema básico de un instrumento de CE.*

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 [104].

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, 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 [105-107]:

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

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

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

[105] Oda, R. P.; Landers, J. P., "Introduction to Capillary Electrophoresis" en "Handbook of Capillary Electrophoresis". Ed. J. P. Landers. CRC Press. **1997**, 1-49.

[106] Cruces Blanco, C., "Electroforesis capilar". Ed. Universidad de Almería, Servicio Publicaciones Almería **1998**.

[107] Marina, M. L.; Ríos, A.; Valcárcel, M., "Fundamentals of Capillary Electrophoresis" en "Analysis and Detection by Capillary Electrophoresis". Ed. M. L. Marina, A. Ríos, M. Valcárcel. Elsevier **2005**, 1-28.

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 ν_e es la velocidad de migración de cada ion (m s^{-1}), μ_e es la movilidad electroforética ($m^2V^{-1}s^{-1}$) 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. 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.

1.3. 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.

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

Tabla. *Modos de separación en electroforesis capilar.*

2.3.1. 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 EOF, 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.

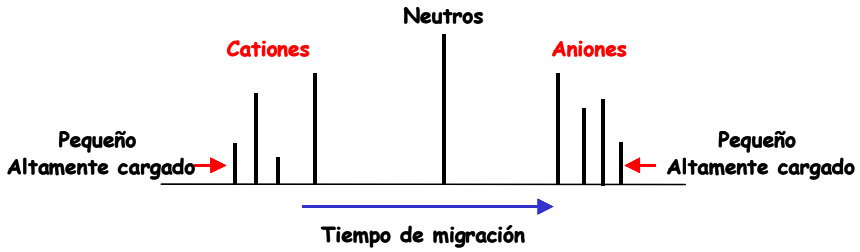

Figura. *Representación de un electroferograma por CZE indicando el orden de elución*

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

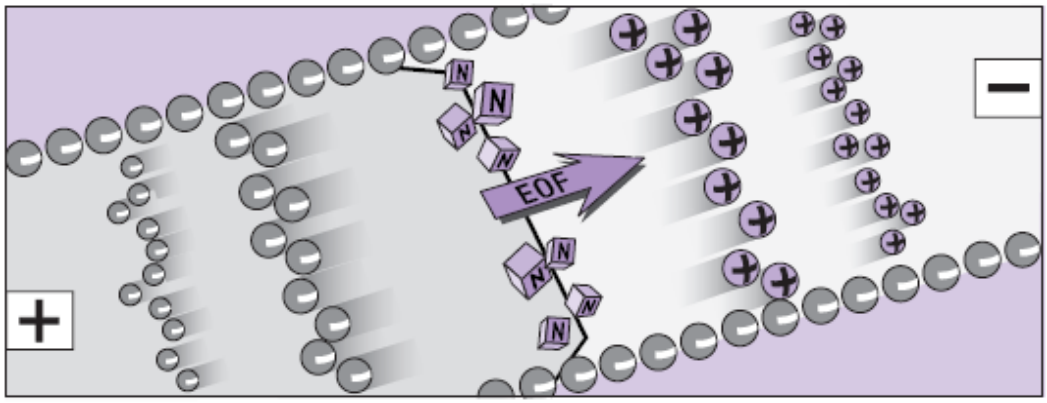

Figura. *Representación esquemática de una separación empleando electroforesis capilar en zona (CZE).*

Hay numerosas aplicaciones de este modo de separación, incluyendo aniones y cationes inorgánicos [108- 113], moléculas pequeñas contenidas en productos farmacéuticos o biomoléculas grandes [114- 120] y en otros campos de la Química Analítica [121- 127].

[108] Harrold, M. P.; Wajtusik, M. J.; Riviello, J.; Henson, R., Parameters influencing separation and detection of anions by capillary electrophoresis. *Journal of Chromatography* **1993**, 640, 463-471.
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[121] Klampfl, C. W., Analysis of organic acids and inorganic anions in different types of beer using capillary zone electrophoresis. *Journal of Agricultural and Food Chemistry* **1999**, 47, 987-990.
[122] Kaniansky, D.; Masár, M.; Madajová, V.; Marák, J., Determination of sorbic acid in food-products by capillary electrophoresis in a hydrodynamically closed separation compartment. *Journal of Chromatography A* **1994**, 677, 179-185.
[123] Recio, I.; Ramos, M.; Amigo L., Study of the polymorphism of ovine alpha(s1)- and alpha(s2)-caseins by capillary electrophoresis. *Journal of Dairy Research* **1997**, 64, 525-534.
[124] Soga, T.; Tajima, I.; Heiger, D. N.; Capillary electrophoresis for the determination of forensic anions in adulterated foods and beverages. *American Lab.* **2000** Agilent Tecnologies N. 5968-9463E.
[125] Pozdniakova, S.; Padarauskas, A.; Schwedt, G., Simultaneous determination of iron(II) and iron(III) in water by capillary electrophoresis. *Analytica Chimica Acta* **1997**, 351, 41-48.
[126] Dabek-Zlotorzynska, E.; Dlouhy, J. F.; Houle, N.; Piechowski, M.; Ritchie, S., Comparison of capillary zone electrophoresis with ion chromatography and standard photometric methods for the determination of inorganic anions in atmospheric aerosols. *Journal of Chromatography A* **1995**, 706, 469-478.
[127] Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Toschi, T. G., Liquid-liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. *Journal of Chromatography A* **2003**, 985, 425-433.

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 [128]. Alternativamente, los capilares pueden ser recubiertos con aditivos [129-131].

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.

2.3.1.1. Electroforesis capilar en zona con medios no acuosos

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 [132]. 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 [133]. 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.

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

[129] Razunguzwa, T. T.; Warrier, M.; Timperman, A. T., ESI-MS compatible permanent coating of glass surfaces using poly(ethylene glycol)-terminated alkoxysilanes for capillary zone electrophoretic protein separations. Analytical Chemistry **2006**, 78 (13), 4326-4333.

[130] Kato, M.; Imamura, E.; Sakai-Kato, K.; Nakajima, T.; Toyooka, T., Cationic amylopectin derivatives as additives for analysis of proteins in capillary electrophoresis. *Electrophoresis* **2006**, 27(10), 1895-1899.

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

[132] Miller, J. L.; Khaledi, M. G., "High Peformance Electrophoresis". Ed. Wiley, New York, **1998** (Chemical Analysis Series, vol. 146).

[133] Vaher, M.; Koel, M., Specific background electrolytes for nonaqueous capillary electrophoresis. *Journal of Chromatography A* **2005**, 1068, 83-88.

El modo de electroforesis capilar en zona descrito en este apartado (1.3.1) ha sido el que se ha utilizado en todos los métodos electroforéticos puestos a punto en durante el trabajo realizado durante el periodo de tesis doctoral. A continuación se exponen de manera muy resumida otros de los modos electroforéticos empleados con frecuencia.

2.4. Desarrollo de métodos en CE

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 [107,134-136]:

2.4.1. 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.

[134] McLaughlin, G. M.; Weston, A.; Hauffe, K. D., Capillary electrophoresis methods development and sensitivity enhancement strategies for the separation of industrial and enviromental chemicals. *Journal of Chromatography A* **1996**, 744, 123-134.

[135] Morris, V. M.; Hargreaves, C.; Overall, K.; Marriott, P. J.; Hughes, J. G., Optimization of the capillary electrophoresis separation of ranitidine and related compounds. *Journal of Chromatography A* **1997,** 766, 245-254.

[136] Dolník, V., Selectivity, differential mobility and resolution as parameters to optimize capillary electrophoretic separation. *Journal of Chromatography A* **1996**, 744, 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.

2.4.2. Optimización de la temperatura

La temperatura ejerce un papel importante en muchas separaciones ya que la movilidad de los analitos y el EOF 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.

2.4.3. 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).

2.4.4. 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.

2.4.5. Elección de la disolución de separación

2.4.5.1. 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 $Si\text{-}O^-$ 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.

2.4.5.2. 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.

2.4.5.3. 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.

2.4.5.4. 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 EOF 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 EOF 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.

2.4.5.5. 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 [137-140]. Se puede 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.

[137] Moseley, M. A.; Shabanowitz, J.; Hunt, D. F.; Tomer, K. B., Optimization of capillary zone electrophoresis/electrospray ionization parameters for the mass spectrometry and tandem mass spectrometry analysis of peptides. *Journal of The American Society for Mass Spectrometry* **1992**, 3, 289-300.

[138] Wahl, J. H.; Smith, R.D., Comparison of buffer systems and interface designs for capillary electrophoresis-mass spectrometry. *Journal of Capillary Electrophoresis* **1994**, 1, 62-71.

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

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

2.4.6. 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.

3. Detectores

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.

Las condiciones expuestas anteriormente influyen en gran medida a la hora de decidir los sistemas de detección que se acoplan. En el caso de CE el detector que se acople debe ser sensible a pequeñas cantidades de muestra y compatible con las dimensiones físicas del capilar, aún en detrimento de otras cualidades.

Los detectores que pueden acoplarse tanto a EC como HPLC son de naturaleza muy diversa (UV-Vis, índice de refracción, fluorescencia, electroquímicos, radioactivos, de dispersión de luz (ELS), espectrómetros de masas, resonancia magnética nuclear…).

Para la realización de la investigación llevada a cabo durante el periodo de tesis, se emplearon la detección espectrofotométrica UV-Vis, la espectrometría de masas (MS) y la resonancia magnética nuclear (NMR). Estos sistemas de detección se encuentran descritos de forma más detallada en los siguientes apartados.

3.1. Absorción UV-Visible

Los métodos basados en la absorbancia de la luz UV-Visible son los más utilizados en equipos comerciales de CE y HPLC, debido a su posibilidad para determinar un gran número de compuestos y grupos funcionales y su facilidad de manejo, a pesar de que su sensibilidad es mucho menor que la de otros sistemas de detección. 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 [141].

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 o fase móvil) 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

[141] Berzas Nevado, J. J.; Castañeda Peñalvo, G., "La detección espectrofotométrica UV-Visible en Electroforesis Capilar" en "Electroforesis capilar: aproximación según la técnica de detección". Ed. Univ. Granada. **2005,** 157-187.

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 [142].

Consecuentemente, la utilización del detector UV-Visible en CE y HPLC obliga a establecer métodos con un alto poder de resolución del analito respecto de todos los compuestos absorbentes contenidos en la muestra problema.

Para llevar a cabo la identificación de los compuestos 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

[142] Crego, A. L.; Marina, M. L., "UV-Vis absorbance detection in capillary electrophoresis" en "Analysis and Detection by Capillary Electrophoresis". Ed. M. L. Marina, A. Ríos, M. Valcárcel. Elsevier. **2005**, 225-296.

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.

Este tipo de detector 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 absorción UV-Vis nos aporta.

3.2. Espectrometría de masas

La espectrometría de masas es una técnica microanalítica que puede utilizarse para detectar y determinar cantidades dadas de un analito determinado. También puede emplearse para determinar la composición elemental y algunos aspectos de la estructura molecular de un analito. Estos objetivos se consiguen llevar a cabo a través de la medida experimental de la masa de los iones en fase gas producidos a partir de las moléculas de analito (es requisito fundamental que los iones estén en fase gas antes de que puedan ser separados según su relación carga/masa y posteriormente detectados) [143].

En los últimos años el acoplamiento entre CE y HPLC como técnicas analíticas de separación y la espectrometría de masas como sistema de detección (CE-MS y HPLC-MS) ha despertado un gran interés El acoplamiento de un detector tan selectivo como MS a técnicas tan versátiles dan como resultado unas potentísimas herramientas de análisis; se combinan así la rapidez del análisis, el alto poder de resolución, y un consumo pequeño de muestra, proporcionados por las técnicas separativas, con la selectividad del análisis y la información estructural que proporciona la MS. Una de las ventajas del acoplamiento de la MS a cualquier técnica separativa es que proporciona

[143] Watson, J. T.; Sparkman, O. D., "Introduction to Mass Spectrometry". Ed. John Wiley & Sons, Inc. New York. **2007**, 1-44.

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 [144].

3.2.1. Principios e instrumentación

Las tres partes básicas de un espectrómetro de masas son:

- Fuente de ionización: zona donde se introducen las moléculas, se evaporan, se ionizan y se aceleran.
- Analizador: separa a los iones de distinta masa, de modo que puedan llegar al detector a tiempos diferentes.
- Transductor/detector

A continuación puede observarse un esquema del acoplamiento entre una técnica separativa y MS.

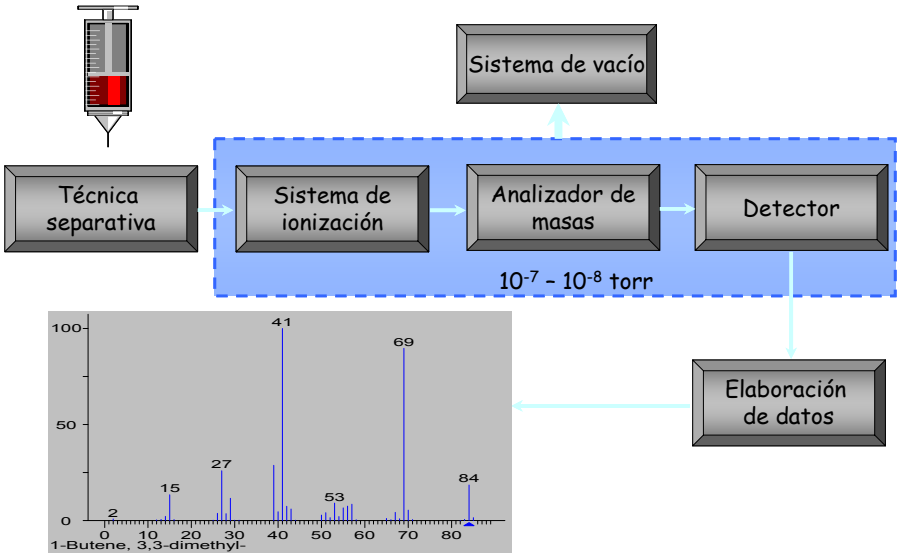
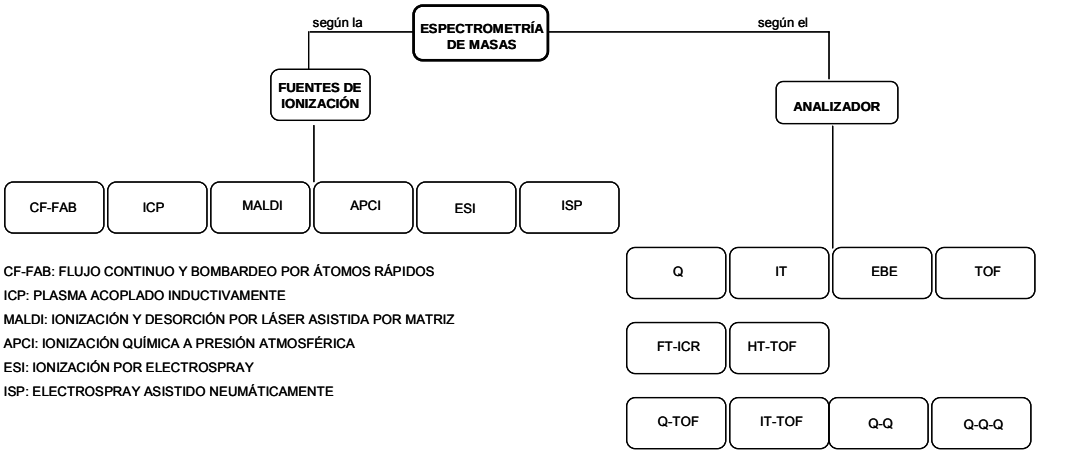


Figura. *Esquema de un acoplamiento entre una técnica separativa y MS.*

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

La técnica separativa se coloca al inicio del esquema, seguida de la fuente de ionización. 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

3.2.1.1 Interfase

Debido al volumen de muestra líquida procedente de CE o HPLC es necesaria la presencia de la interfase entre el capilar o columna cromatográfica y el espectrómetro de masas ya que la muestra debe entrar en fase gas. Además, el acoplamiento en el caso de la CE debe hacer frente a otra segunda adversidad como es el cierre del circuito eléctrico en el que trabaja este instrumento.

La misión de la interfase es eliminar al máximo la matriz o medio (buffer de separación o fase móvil) manteniendo los analitos. Para dicho fin, las interfases tienden a alcanzar

temperaturas elevadas. Por este motivo los modificadores o tampones empleados deben ser volátiles para minimizar las interferencias en MS [145].

Cualquiera que sea la fuente de ionización que se utilice en espectrometría de masas, una propiedad deseable es la estabilidad del ión producido. Los dos factores que afectan principalmente a la estabilidad de la fuente de ionización son :

a) Eficiencia de las especies. Si la muestra no es gas, la composición del material vaporizado puede no reflejar la composición de la muestra; la fragmentación puede ocurrir.
b) La eficacia de la ionización. La fracción ionizada por cada uno de los diversos métodos de ionización a menudo varía según la composición de la matriz.

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

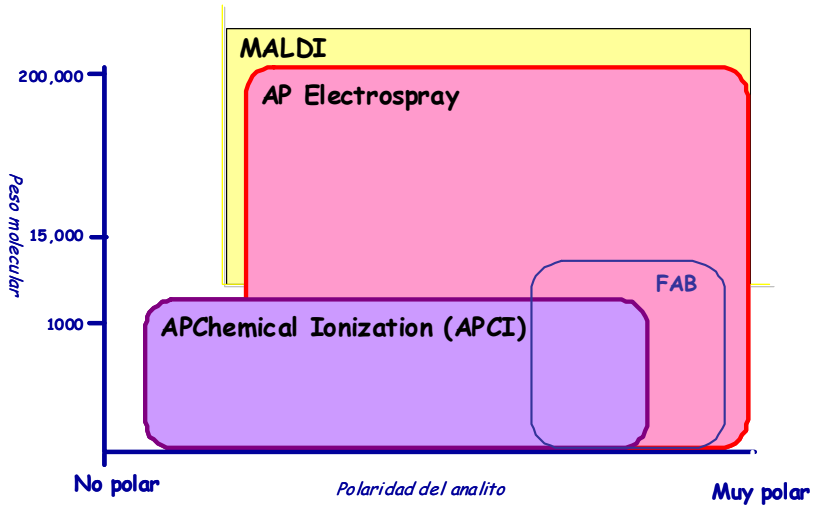


Figura. *Rango al que trabajan los sistemas de ionización más comunes.* (AP: Presión atmosférica)

[145] Rubinson, K. A.; Rubinson, J. F., "Contemporary Instrumental Analysis". Ed. Prentice Hall, **2000**, 522-583.

Ionización por Electrospray (ESI)

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

El mecanismo de ionización de esta interfase ha sido ampliamente debatido; aunque una cosa está clara, está basado en la desorción de iones de la superficie del líquido que llega, llamada "evaporación de iones" [146]. 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

[146] Watson, J. T.; Sparkman, O. D., "Introduction to Mass Spectrometry". Ed. John Wiley & Sons, Inc. New York. **2007**, 639-688.

atraídos hacia la entrada del espectrómetro de masas como consecuencia del voltaje aplicado [147,148].

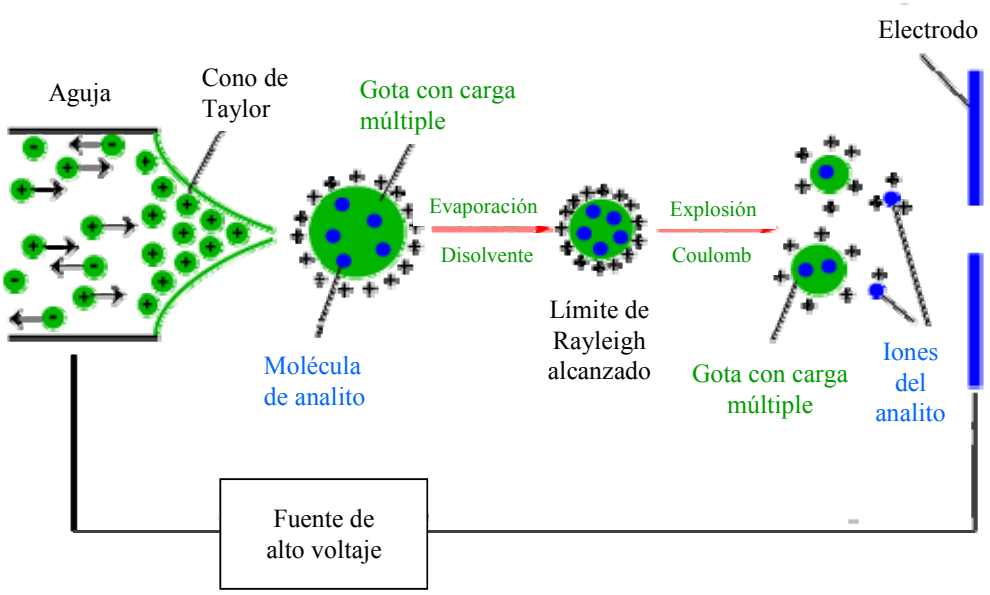


***Figura**. Proceso de formación del electrospray.*

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

[147] Simó, C.; Cifuentes, A., "Electroforesis Capilar: Detección mediante espectrometría de masas" en "Electroforesis capilar: aproximación según la técnica de detección". Ed. Univ. Granada. **2005,** 409-438.

[148] R. Martin Smith. "Instrumentation" en "Understanding Mass Spectra. A basic approach". Ed. K. L. Busch. John Wiley & Sons, Inc. **1999,** 1-40.

el modo negativo, se observa normalmente la desprotonación de las moléculas, pudiéndose formar también iones múltiplemente desprotonados $[M-nH]^{n-}$.

A) Ionización por Electrospray (ESI) en CE

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 [149- 156] teniendo como

[149] Schmitt-Kopplin, P.; Frommberger, M., Capillary electrophoresis-mass spectrometry: 15 years of developments and applications. *Electrophoresis* **2003**, 24, 3837-3867.
[150] Gelpi, E., Interfaces for coupled liquid-phase separation/mass spectrometry techniques. An update on recent developments. *Journal of Mass Spectrometry* **2002**, 37, 241-253.
[151] Chao, B. F.; Chen, C. J.; Li, F. A.; Her, G. R., Sheathless capillary electrophoresis-mass spectrometry using a pulsed electrospray ionization source *Electrophoresis* **2006**, 27 (11), 2083-2090.
[152] Huhn, C.; Neususs, C.; Pelzing, M.; Pyell, U.; Mannhardt, J.; Putz, M., Capillary electrophoresis-laser induced fluorescence-electrospray ionization-mass spectrometry: A case study. *Electrophoresis* **2005**, 26 (7-8), 1389-1397.
[153] Zamfir, A. D.; Dinca, N.; Sisu, E.; Peter-Katalinic, L., Copper-coated microsprayer interface for on-line sheathless capillary electrophoresis electrospray mass spectrometry of carbohydrates. *Journal of Separation Science* **2006**, 29 (3), 414-422.
[154] Ding, J.M.; Vouros, P., Advances in CE/MS. Recent developments in interfaces and applications. *Analytical Chemistry News & Features* **1999**, 71, 378A- 385A.

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.

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

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

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

B) 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.

[155] Cai, J.; Henion, J., Capillary electrophoresis-mass spectrometry. *Journal of Chromatography A* **1995**, 703, 667-692.
[156] Figeys, D.; Aebersold, R., High sensitivity analysis of proteins and peptides by capillary electrophoresis-tandem mass spectrometry: recent developments in technology and applications. *Electrophoresis* **1998**, 19, 885-892.

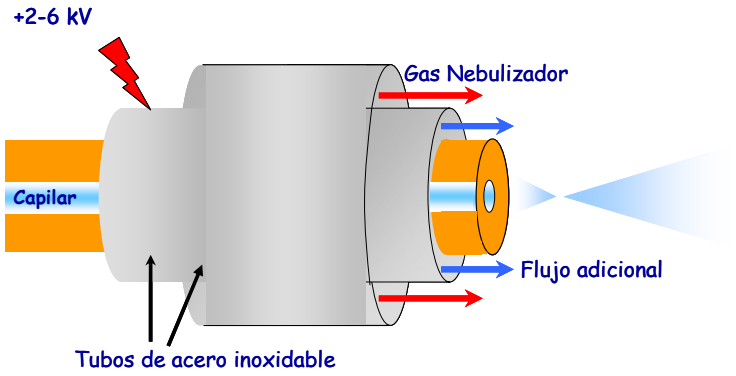


Figura. Esquema de una interfase ESI con flujo adicional.

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 [157], presión del gas nebulizador, situación del capilar con respecto al tubo concéntrico que lo rodea [158], 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 [159,160].

[157] Zheng, J.; Jann, M. W.; Hon, Y. Y.; Shamsi, S. A., Development of capillary zone electrophoresis-electrospray ionization-mass spectrometry for the determination of lamotrigine in human plasma. *Electrophoresis* **2004**, 25 (13), 2033-2043.

[158] Geiser, L.; Rudaz, S.; Veuthey, J. L., Validation of capillary electrophoresis - mass spectrometry methods for the analysis of a pharmaceutical formulation. *Electrophoresis* **2003**, 24 (17), 3049-3056.

[159] Samskog , J.; Wetterhall, M.; Jacobsson, S.; Markides, K., Optimization of capillary electrophoresis conditions for coupling to a mass spectrometer via a sheathless interface. *Journal of Mass Spectrometry* **2000**, 35 (7), 919-924.

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:

a) 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).
b) 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.
c) 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.
d) 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.

3.2.1.2. 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.

[160] Huikko, K.; Kotiaho, T.; Kostiainen, R., Effects of nebulizing and drying gas flow on capillary electrophoresis/mass spectrometry. *Rapid Communication of Mass Spectrometry* **2002**, 16, 1562-1568.

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/HPLC-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).

En los acoplamientos llevados a cabo entre CE/HPLC y MS en el desarrollo experimental de esta memoria, han sido utilizados dos analizadores: Trampa de iones y tiempo de vuelo; los cuales se describen a continuación.

Trampa de iones

El analizador de trampa de iones (IT) consiste fundamentalmente en un electrodo anular y dos electrodos laterales de geometría hiperbólica, que poseen una perforación que permite la entrada y la salida de los iones.

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

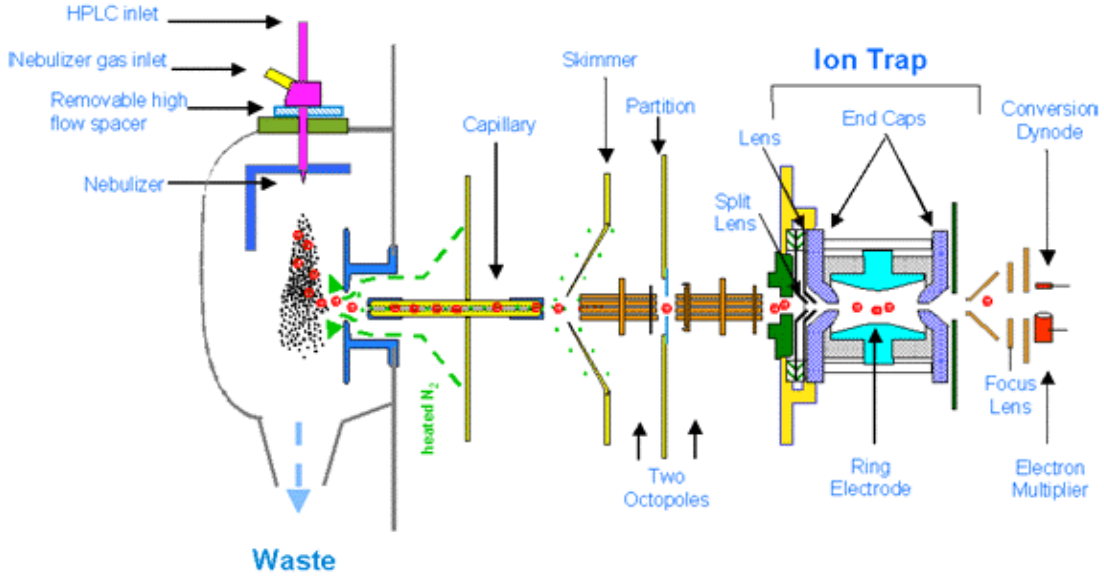


***Figura**. Esquema de un espectrómetro de masas ESI-IT.*

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:

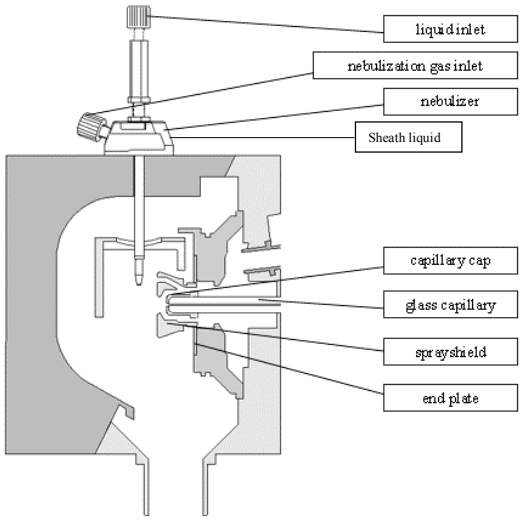


Figura. *"Spray chamber" o cámara de formación del spray para CE.*

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.

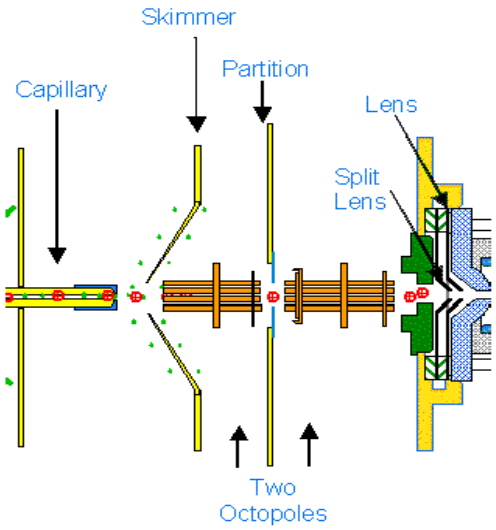


Figura. *Detalle del skimmer, optopolos y lentes.*

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.

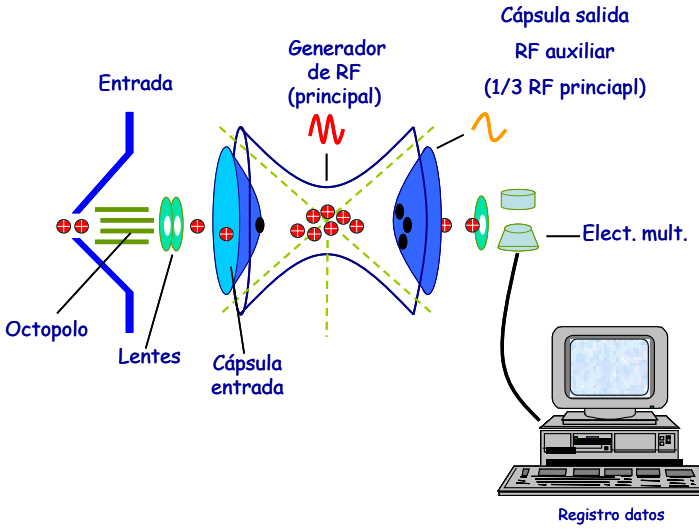


Figura. *Esquema de un analizador de trampa de iones (y del optopolo y lentes previas al analizador).*

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.

Tiempo de vuelo

El analizador de tiempo de vuelo (TOF) 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 mayor separación de los iones en el tiempo) y cuanto menor sea la dispersión en energías de los iones formados en la fuente [148].
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 [145].

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.

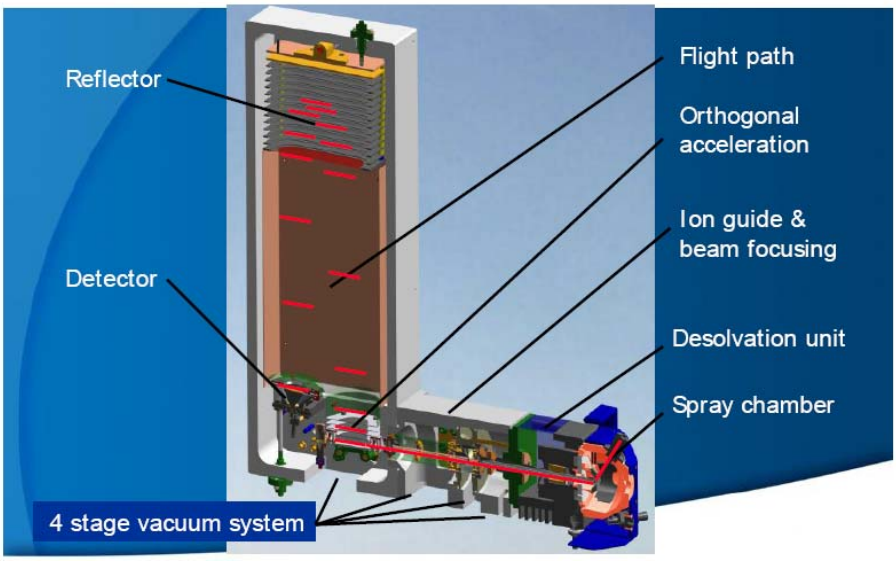


Figura. *Esquema del interior de un MicroTOF de Bruker Daltonics.*

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.

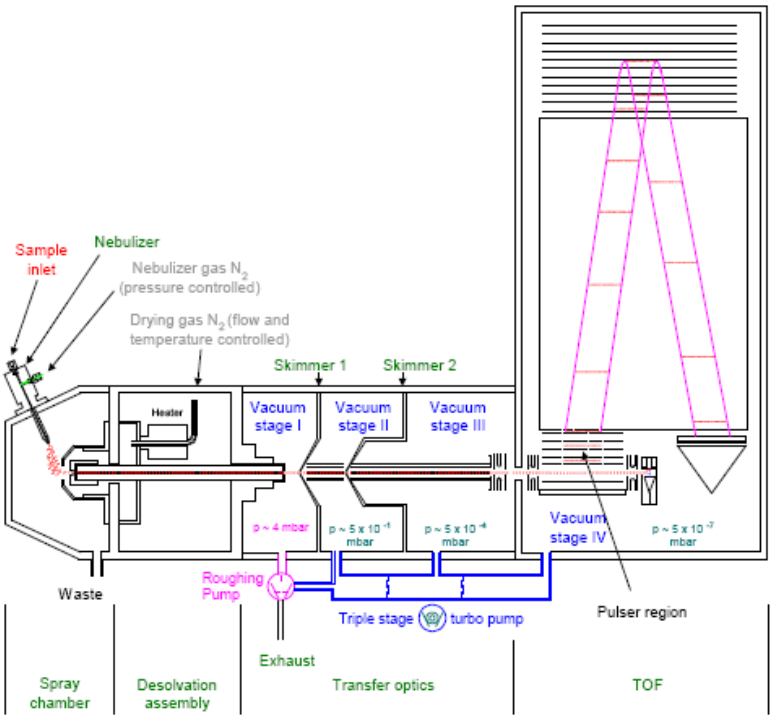


***Figura**. Esquema detallado de las partes de las que consta un ESI-MicroTOF.*

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.

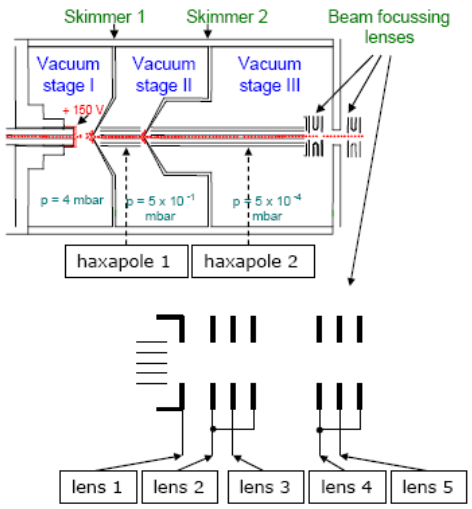


***Figura**. Esquema del módulo de transmisión con sus diferentes partes.*

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.

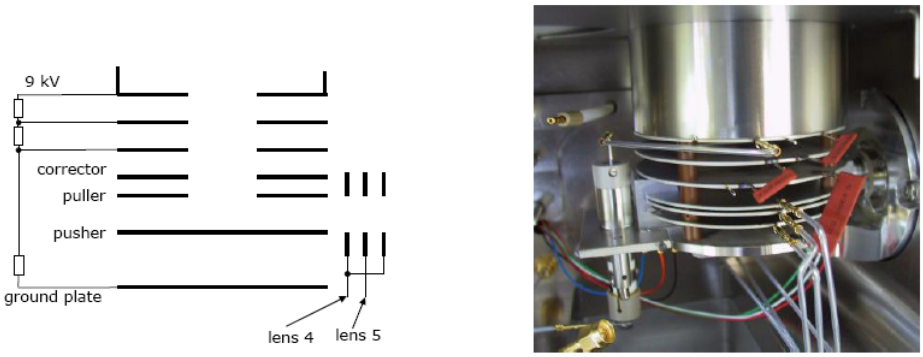


***Figura**. Imagen de la zona de aceleración ortogonal de un MicroTOF y esquema de sus componentes.*

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.

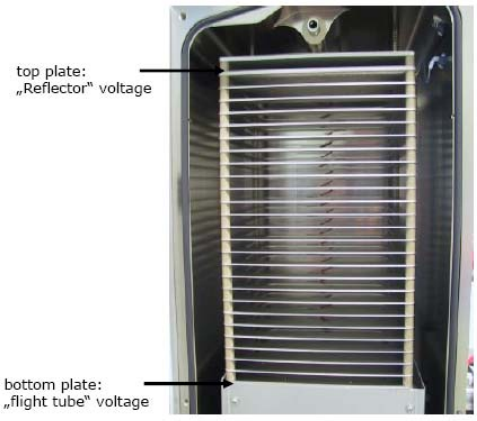


***Figura**. Imagen de de la parte final del tubo de vuelo donde se sitúa el reflector.*

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.

Entre las especificaciones más notables del analizador por tiempo de vuelo podemos citar:

- 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.

3.3. Resonancia Magnética Nuclear (NMR)

La espectrometría de resonancia magnética nuclear (NMR), junto con la espectrometría de masas, tiene mucha importancia en la determinación de la estructura molecular de los compuestos orgánicos y macromoléculas tanto bioquímicas como sintéticas.
La espectrometría de NMR consiste en medir la absorción de radiación de radiofrecuencia que experimenta una muestra situada en un campo magnético fuerte. La radiación que se utiliza está en el intervalo de 100 MHz a casi 1 GHz. Los campos magnéticos son altos; los instrumentos más sofisticados utilizan algunos de los campos magnéticos estables más altos que se pueden generar. C
Como en otras espectroscopias, la fracción de potencia absorbida es proporcional a la concentración de las especies absorbentes mientras que la energía de la transición se determina a partir de las propiedades atómicas y moleculares. No obstante, en NMR la energía también depende del campo magnético.
Un instrumento de NMR mide el espectro de una especie atómica cada vez. Los núcleos atómicos más comúnmente medidos son ^{1}H, ^{13}C, ^{19}F, ^{32}P. De todos ellos el más comun es, con diferencia, RMN-^{1}H, denominada frecuentemente RMN de protón. Ésta no es

sólo la más medida sino que inherentemente es también la más sensible por dos razones. En primer lugar el protón presenta el mayor núcleo magnético y, en segundo lugar, casi el 100% de la abundancia isotópica natural del hidrógeno es ^{1}H. El segundo núcleo de NMR más medido es el ^{13}C. No obstante, NMR-^{13}C es intrínsicamente menos sensible y además la abundancia natural de ^{13}C es sólo del 1.1%. Por estas razones, un buen espectro requiere medidas enriquecidas de ^{13}C, muestras más concentradas o medidas más largas (incluso días). Sin embargo, en la práctica NMR-^{13}C es realmente útil.

En RMN-^{1}H generalmente se emplean disolventes que no contienen hidrógeno o disolventes deuterados con el fin de que la absorción del posible protón del disolvente no solape a la señal de la muestra [161].

3.3.1. LC-NMR

En las últimas décadas se le ha prestado una especial atención al desarrollo de técnicas acopladas donde se combinen técnicas separativas con sistemas de detección tales como la espectrometría de masas (como se ha visto en el apartado anterior) y resonancia magnética nuclear. La idea del acoplamiento de una técnica como el HPLC capaz de separar de muestras complejas con NMR, que puede proporcionar información acerca de la estructura inequívoca de los compuestos separados ha despertado gran interés, pero no ha sido hasta los últimos años cuando se ha llevado a cabo este acoplamiento de manera satisfactoria.

Los primeros experimentos con LC-NMR se llevaron a cabo a finales de los 70 a manos de Watanabe y Niki [162], para ello transformaron el tubo convencional de NMR en un tubo a través del que la muestra podía entrar y salir, simplemente introduciendo un capilar de teflón en el tubo convencional. Más tarde Bayer *et al*. [163] continuaron haciendo pruebas en esta dirección, pero a pesar de ello los resultados obtenidos en cuanto a resolución seguían siendo peores que los de NMR convencional.

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[161] Rubinson, K. A.; Rubinson, J. F., "Contemporary Instrumental Analysis". Ed. Prentice Hall, **2000**, 484-521.

[162] Watanabe, N.; Niki, E., Direct-Coupling of FT-NMR to High Performance Liquid Chromatography. *Proceedings of the Japan Academy, Ser. B* **1978**; 54: 194-199.

[163] Bayer, E.; Albert, K.; Nieder, M.; Grom, E.; Keller, T., On-line coupling of high-performance liquid chromatography and nuclear magnetic resonance. *Journal of Chromatography A* **1979**; 186**,** 497- 507.

La primera muestra analizada por LC-NMR fue gasolina empleada en los aviones militares usando una columna de fase normal, cloroformo deuterado y freón 113 [164,165]. El uso exclusivo de columnas de fase normal limitaba enormemente el uso de esta técnica, pero el empleo de columnas de fase inversa creaba problemas adicionales por la necesidad de trabajar con disolventes protonados (mezclas de agua/acetonitrilo o metanol). Por ello y con el objetivo de superar estos problemas, se realizaron numerosos trabajos en la década de los 80 [166-169].

Poco a poco se consiguieron espectrómetros de NMR de campo magnético mayor, métodos que permitían la adecuada supresión de la señal producida por el disolvente, tubos de NMR que hacían posible el flujo continuo, etc., en general diferentes avances técnicos que mejoraron la conexión física entre HPLC y NMR de modo que convirtieron el acoplamiento en una herramienta práctica [170-172].

Hoy en día LC–NMR y LC–NMR/MS están consideradas como potentes técnicas para la elucidación estructural de compuestos desconocidos. Esto las convierte en herramientas de gran utilidad en el análisis de productos naturales, ya que los extractos de éstos contienen una gran cantidad de compuestos, en la mayoría de los casos de estructuras muy similares y por tanto muy difíciles de separar.

La adquisición de un espectro de NMR convencional requiere la disolución de la muestra a analizar en un disolvente deuterado, la introducción de ésta en el tubo

[164] Haw, J.F.; Glass, T.E.; Hausler, D.W.; Motell, E.; Dorn, H.C., Continuous flow high field nuclear magnetic resonance detector for liquid chromatographic analysis of fuel samples. *Analytical Chemistry* **1981**, 53 (14), 2327-2332.

[165] Haw, J. F.; Glass, T. E.; Dorn, H. C., Liquid chromatography/proton nuclear magnetic resonance spectrometry average composition analysis of fuels. *Analytical Chemistry* **1983**, 55 (1), 22-29.

[166] Bayer, E.; Albert, K.; Nieder, M.; Grom, E.; Wolff, G.; Rindlisbacher, M., On-line coupling of liquid chromatography and high-field nuclear magnetic resonance spectrometry. *Analytical Chemistry* **1982**, 54, 1747–1750.

[167] Bayer, E.; Albert, K., Continuous-flow carbon-13 nucler magnetic resonance spectroscopy. *Journal of Chromatography A* **1984**, 312, 91-97.

[168] Albert, K.; Nieder, M.; Bayer, E.; Spraul, M., Continuous-flow nuclear magnetic resonance. *Journal of Chromatography A* **1985**, 346, 17-24.

[169] Albert, K.; Bayer, E., High-performance liquid chromatography-nuclear magnetic resonance on-line coupling. *Trends in Analytical Chemistry* **1988**, 7 (8), 288-293.

[170] Wolfender, J. L.; Rodriguez, S.; Hostettmann, K., Liquid chromatography coupled to mass spectrometry and nuclear magnetic resonance spectroscopy for the screening of plant constituents. *Journal of Chromatography A* **1998**, 794 (1-2), 299-316.

[171] Albert, K., Liquid chromatography-nuclear magnetic resonance spectroscopy. *Journal of Chromatography A* **1999**, 856 (1-2), 199-211.

[172] Wilson, I.D., Multiple hyphenation of liquid chromatography with nuclear magnetic resonance spectroscopy, mass spectrometry and beyond. *Journal of Chromatography A* **2000**, 892 (1-2), 315-327.

cilíndrico convencional y posteriormente su colocación en el interior del imán. En el caso de LC-NMR, como se ha comentado anteriormente, se necesita una probeta que permita el flujo continuo de la disolución objeto de estudio. Además este acoplamiento debe tener en cuenta la sensibilidad del NMR, la supresión de la señal del disolvente, la compatibilidad de disolventes en NMR y HPLC y el volumen cromatográfico en relación con el volumen de la celda de flujo de NMR [173].

3.3.1.1. Modos en LC-NMR

Dependiendo del estado de la muestra durante la medida existen varios modos de operar en LC-NMR.

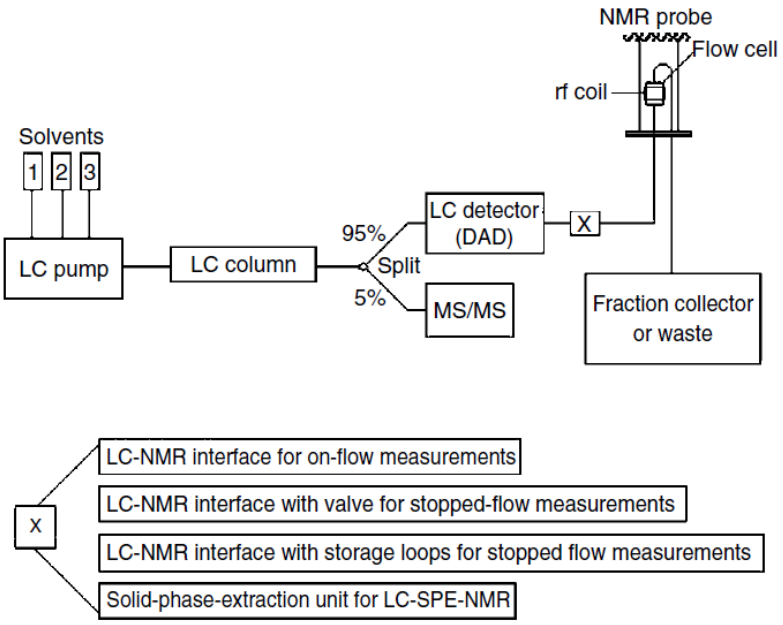

Figura. *Diferentes modos de trabajo en LC-NMR* [174].

[173] Elipe, M. V. S., Advantages and disadvantages of nuclear magnetic resonance spectroscopy as a hyphenated technique. *Analytica Chimica Acta* **2003**, 497 (1-2), 1-25.

[174] Exarchou,V.; Krucker, M.; Van Beek, T. A.; Vervoort, J.; Gerothanassis, I. P.; Albert, K., LC–NMR coupling technology: recent advancements and applications in natural products analysis. *Magnetic Resonance in Chemistry* **2005**, 43, 681–687.

LC-NMR con flujo continuo (On-flow)

En esta modalidad la muestra se mide sin parar el flujo, por lo que el espectrómetro de NMR actúa de manera similar a un detector UV o MS acoplado a un sistema cromatográfico. Los resultados se muestran normalmente en una figura bidimensional en la que en una dimensión aparece el espectro donde se muestran las frecuencias de las señales obtenidas y en la otra vemos los tiempos de retención [175]. El flujo óptimo de trabajo se elige como un compromiso entre el flujo requerido para obtener la mejor resolución en cromatografía y la mejor sensibilidad en NMR [176].

El modo continuo permite un análisis de ^{1}H-NMR rápido de la muestra a analizar, pero sólo permite observar las señales más intensas debido a poco tiempo de permanencia de cada analito dentro del imán. Por esta razón este tipo de experimentos se realiza como paso inicial para determinar los componentes mayoritarios de una muestra compleja. Otro de los principales problemas del modo en flujo continuo es que el corto tiempo de permanencia de los analitos en el imán impide además llevar a cabo experimentos de ^{13}C incluso en el caso de los componentes mayoritarios. En caso del uso de gradiente en HPLC surge de nuevo otra complicación; la señal de los analitos depende del disolvente empleado para la medida, por lo que la variación de la composición del disolvente en función del tiempo afecta a la señal [177].

LC-NMR en condiciones estáticas

Existen dos metodologías de trabajo en la que se llevan a cabo medidas en condiciones estáticas:

[175] Braumann, U; Spraul, M., "On-line LC–NMR and Related Techniques"..Ed. Albert, K.; John Wiley & Sons, Ltd. **2002**, 23-44.

[176] Korhammer, S. A.; Bernreuther, A., Hyphenation of high-performance liquid chromatography (HPLC) and other chromatographic techniques (SFC, GPC, GC, CE) with nuclear magnetic resonance (NMR): A review. *Fresenius'Journal of Analytical Chemistry* **1996**, 354 (2), 131-135.

[177] Griffiths, L., Optimization of NMR and HPLC Conditions for LC-NMR. *Analytical Chemistry* **1995**, 67 (22), 4091-4095.

a) Flujo parado (Stopped-flow): se hace uso de un sistema de válvulas para parar el flujo en el momento en que el analito llega a la celda de flujo donde se realizan los experimentos de NMR.
b) Empleo de "loops" donde se almacenan las distintas fracciones que contienen a los analitos individuales.

En ambos casos se pueden realizar experimentos más largos, incluso pudiendo realizarse experimentos en dos dimensiones.

En el modo "stopped-flow" después de la adquisición de datos de un analito, se restablece el análisis cromatográfico, repitiéndose el procedimiento para cada uno de los analitos a estudiar. Las diversas paradas sufridas para la toma de datos de cada analito pueden afectar a la calidad de la separación y, en el caso de analitos muy concentrados, éstos pueden contaminar la celda de medida (efecto memoria). Por ello esta modalidad de medida se recomienda en casos en los que los analitos a estudiar de la muestra no sean muy numerosos.
En el caso del almacenamiento de los analitos en "loops" debe tenerse en cuenta que estos sean estables hasta el momento en que se analizan en el imán. La ventaja de esta modalidad es entre análisis y análisis de NMR, la celda de medida puede lavarse.

3.3.1.2. LC-SPE-NMR

Con el objetivo de aumentar la sensibilidad de la detección en LC-NMR se trata de concentrar la muestra de interés. Así se podrá alcanzar la máxima concentración de un determinado analito en el menor volumen posible y proceder a su medida por NMR. Para ello se introdujo el uso de la extracción en fase sólida, una poderosa técnica capaz de concentrar la muestra de forma reproducible, rápida y selectiva [178].
Se han descrito numerosas aplicaciones en las que se ha usado la extracción en fase sólida acoplada en línea como método de concentración antes de la separación tanto en

[178] Thurman E. M.; Mills, M. S., "Solid-phase extraction" en "Chemical Analysis: A Series of Monographs on Analytical Chemistry and its Application". Ed. Wineforder, J. D., John Willey & Sons: New York, **1998**.

LC-UV [179,180], LC-MS [181] como en LC-NMR [182,183]. Además se han realizado trabajos conectando un dispositivo de extracción en fase sólida a la probeta de NMR [184].

El empleo del sistema acoplado LC-SPE-NMR permite el uso de disolventes protonados en cromatografía, por lo que la necesidad de la supresión de la señal del disolvente en NMR es mucho menor o incluso no necesaria, además proporciona un aumento de la señal relativa con respecto a LC-NMR convencional debido a la posibilidad de atrapar un determinado analito a lo largo de diferentes análisis de LC en un mismo cartucho.

En los últimos años LC-SPE-NMR se ha empleado para el estudio de metabolitos del paracetamol presentes en orina [185], identificación de antioxidantes en extractos de romero [186] y estractos de *Rhaponticum carthamoides* [187].

[179] Slobodnik, J.; Groenewegen, M. G. M.; Brouwer, E. R.; Lingeman, H.; Brinkman Th., U. A., Fully automated multi-residue method for trace level monitoring of polar pesticides by liquid chromatography. *Journal of Chromatography* **1993**, 642 (1-2), 359-370.

[180] Mattina, M. J. I.; MacEachern, G. J., Extraction, purification by solid-phase extraction and highperformance liquid chromatographic analysis of taxanes from ornamental Taxus needles. *Journal of Chromatography A* **1994**, 679 (2), 269-275.

[181] Slobodník, J.; Hogenboom, A. C.; Vreuls, J. J.; Rontree, J. A.; Van Baar, B. L. M.; Niessen, W. M. A.; Brinkman, U. A. Th., Trace-level determination of pesticide residues using on-line solid-phase extraction-column liquid chromatography with atmospheric pressure ionization mass spectrometric and tandem mass spectrometric detection. *Journal of Chromatography A* **1996**, 741 (1), 59-74.

[182] De Koning, J. A.; Hogenboom, A. C.; Lacker, T.; Strohschein, S.; Albert, K.; Brinkman, U. A. Th., On-line trace enrichment in hyphenated liquid chromatography-nuclear magnetic resonance spectroscopy. *Journal of Chromatography A* **1998**, 813 (1), 55-61.

[183] Griffiths, L.; Horton, R., Optimization of LC-NMR III - Increased signal-to-noise ratio through column trapping. *Magnetic Resonance in Chemistry* **1998**, 36 (2), 104-109.

[184] Nyberg, N. T.; Baumann, H.; Kenne, L., Solid-phase extraction NMR studies of chromatographic fractions of saponins from Quillaja saponaria. *Analytical Chemistry* **2003**, 75 (2), 268-274.

[185] Godejohann, M.; Tseng, L. H.; Braumann, U.; Fuchser, J.; Spraul, M., Characterization of a paracetamol metabolite using on-line LC-SPE-NMR-MS and a cryogenic NMR probe. *Journal of Chromatography A* **2004**, 1058 (1-2), 191-196.

[186] Pukalskas, A.; Van Beek, T. A.; De Waard, P., Development of a triple hyphenated HPLC-radical scavenging detection DAD-SPE-NMR system for the rapid identification of antioxidants in complex plant extracts. *Journal of Chromatography A* **2005**, 1074 (1-2), 81-88.

[187] Miliauskas, G.; Van Beek, T. A.; De Waard, P.; Venskutonis, R. P.; Sudhölter, E. J. R., Identification of radical scavenging compounds in Rhaponticum carthamoides by means of LC-DAD-SPE-NMR. *Journal of Natural Products* **2005**, 68 (2), 168-172.

***Figura**. Sistema LC-SPE-NMR.*

Parte experimental.
Resultados y discusión.

Bloque I: Aceite de oliva

La dieta mediterránea, en la que el aceite de oliva es la principal fuente de grasas ha estado y sigue estando asociada a una disminución de la mortalidad y un aumento de la esperanza de vida, así como a una menor incidencia de afecciones de tipo cardíaco, hipertensión, diversos tipos de cáncer (colon y mama principalmente) e incluso afecta positivamente en la repuesta inmunitaria [188- 191].

Las propiedades saludables del aceite de oliva han sido normalmente atribuidas a su alto contenido en ácidos grasos monoinsaturados (MUFA) y de entre ellos principalmente al ácido oleico, sobre todo debido a la influencia que éste tiene sobre el perfil lipídico del suero [192] y a su capacidad de disminuir la susceptibilidad a la oxidación del LDL tanto *in vivo* como *ex vivo* [193,194]. A pesar de ello, hay que tener en cuenta que el ácido oleico es también uno de los ácidos grasos predominantes en dietas como por ejemplo la de América del Norte. Esto lleva a pensar que el ácido oleico no es el único responsable de las propiedades saludables del aceite de oliva [195,196]. De hecho el aceite de oliva además de poseer un alto contenido en MUFA contiene multitud de componentes minoritarios con propiedades biológicas [197].

Visioli y Galli [195] han sugerido que las características que hacen único al aceite de oliva, radican en que éste no se produce mediante extracción con disolventes, sino

[188] Trichopoupou, A.; Vasilopoulou, E., Mediterranean diet and longevity. *British Journal of Nutrition* **2000**, 84 (2), S205-S209.

[189] Trichopoulou, A., Costacou, T.; Bamia, C.; Tricopoulos, D., Adherence to a Mediterranean diet and survival in a Greek population. *New England Journal of Medicine* **2003**, 348 (26), 2599-2608.

[190] Knoops, K.T.B.; De Groot, L.C.; Kromhout, D.; Perrin, A.-E.; Moreiras-Varela, O.; Menotti, A.; Van Staveren, W.A., Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: The HALE project. *Journal of the American Medical Association* **2004**, 292 (12), 1433-1439.

[191] Stark, A. H.; Madar, Z., Olive oil as a functional food: Epidemiology and nutricional approaches. *Nutrition Reviews* **2002**, 60 (6), 170-176.

[192] Riccardi, G.; Rivellese, A., An update on monounsaturated fatty acids. *Current Opinion in Lipidology* **1993**, 4, 13-16.

[193] Scaccini, C.; Nardini, M.; D'Aquino, M.; Gentili, V.; Di Felice, M.; Tomassi, G., Effect of dietary oils on lipid peroxidation and on antioxidant parameters of rat plasma and lipoprotein fractions. *Journal of Lipid Research* **1992**, 33, 627-633.

[194] Wiseman, S. A.; Mathot, J. N.; de Fouw, N. J.; Tijburg, L. B. Dietary non-tocopherol antioxidants present in extra virgin olive oil increase the resistance of low density lipoproteins to oxidation in rabbits. *Atherosclerosis* **1996**, 120, 15-23.

[195] Visioli, F.; Galli, C., Olive oil: more than just oleic acid. *American Journal of Clinical Nutrition* **2000**. 72, 853-853.

[196] Visioli, F.; Bogani, P.; Grande, S.; Galli, C., Mediterranean food and health: building human evidence. *Journal of Physiology and Pharmacology* **2005**, 56 (1), 37-49.

[197] Covas, M. I.; Ruiz-Gutiérrez, V.; De la Torre, R.; Kafatos, A.; Lamuela-Raventós, R.M.; Osada, J.; Owen, R.W.; Visioli, F., Minor components of olive oil: Evidence to date of health benefits in humans. *Nutrition Reviews* **2006**, 64 (10 supl 1), S20-S30.

simplemente con el empleo de métodos mecánicos que preservan intactos tanto la naturaleza química del aceite y como los antioxidantes naturales que éste produce a modo de respuesta frente al estrés ambiental.

Entre los componentes minoritarios del aceite de oliva se encuentran: hidrocarburos saturados, hidrocarburos insaturados (escualeno), alcoholes alifáticos superiores, alcoholes di-triterpenicos, esteroles y metilesteroles, tocoferoles y tocotrienoles, carotenoides (luteína y beta-caroteno), clorofilas y compuestos fenólicos. Éstos últimos han demostrado contribuir significativamente sobre los beneficios que el aceite de oliva reporta a la salud [198-200].

Los compuestos fenólicos del aceite de oliva han manifestado tener las siguientes propiedades: 1) poseen capacidad antioxidante incluso superior a la demostrada por la vitamina E cuando actúan sobre la oxidación de los lípidos y el ADN [201,202]; 2) previenen la disfunción endotelial (responsable de numerosas enfermedades como la arteriosclerosis, la hipertensión arterial, la sepsis, la trombosis, la vasculitis, hemorragias, etc) [203]; 3) inhiben la agregación plaquetaria inducida [204]; y 4) mejoran la transcripción del ARNm de la enzima antioxidante glutatión peroxidasa [205]. Además hay que destacar la potencial actividad quimiopreventiva de estos

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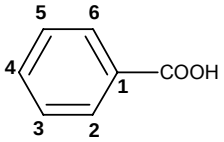
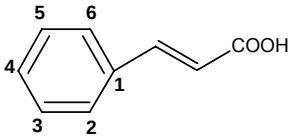
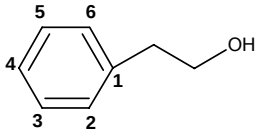
analitos y los efectos anti-inflamatorios, similares a los del ibuprofeno, que exhibe uno de ellos, el oleocantal (forma dialdehídica del deacetoxi ligustrósido aglicona) [206].

Por otro lado los compuestos fenólicos contribuyen a las propiedades organolépticas del aceite de oliva [207,208] y son un sistema de defensa del aceite frente a la oxidación de los ácidos grasos insaturados, convirtiéndose en uno de los factores más importantes en cuanto a la estabilidad oxidativa (shelf-life) se refiere [209,210].

Los compuestos fenólicos que componen el aceite de oliva pueden clasificarse en las siguientes categorías [211]:

Nombre del compuesto	Sustituyente (Pm)	Estructura
Ácido benzoico y derivados		
Ácido 3-hidroxibenzoico	3-OH (138)	
Ácido *p*- hidroxibenzoico	4-OH (138)	
Ácido 3,4-dihidroxibenzoico	3,4-OH (154)	
Ácido gentísico	2,5-OH (154)	
Ácido vanílico	3-OCH_3, 4-OH (168)	
Ácido gálico	3,4,5-OH (170)	
Ácido siríngico	3,5-OCH_3, 4-OH (198)	
Ácido cinámico y derivados		
Ácido *o*-cumárico	2-OH (164)	
Ácido *p*- cumárico	4-OH (164)	
Ácido cafeico	3,4-OH (180)	
Ácido ferúlico	3-OCH_3, 4-OH (194)	
Ácido sinápico	3,5-OCH_3, 4-OH (224)	
Fenil etil alcoholes		
Tirosol [(*p*-hidroxifenil)etanol] o *p*-HPEA	4-OH (138)	
Hidroxitirosol [(*3,4*-dihidroxifenil)etanol] o 3,4-DHPEA	3,4-OH (154)	

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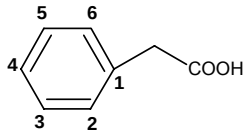
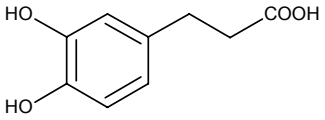
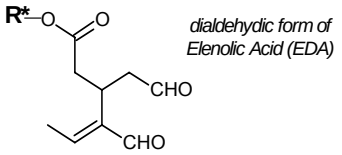
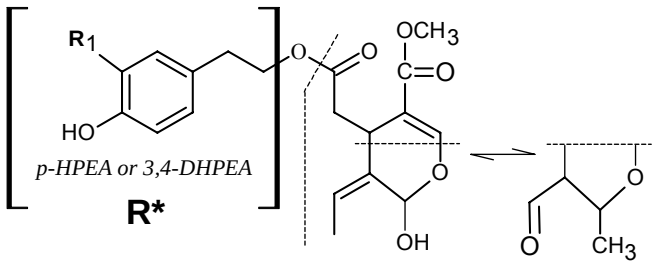
[210] Velasco, J.; Dobarganes, C., Oxidative stability of virgin olive oil. *European Journal of Lipid Science and Technology* 2002, 104 (9-10), 661-676.

[211] Tabla tomada de una de las publicaciones derivadas del trabajo desarrollado en esta tesis (Review que constituye el capítulo 1 de la presente memoria).

Otros ácidos fenólicos y derivados		
Ácido *p*- hidroxifenil acético	4-OH (152)	
Ácido 3,4- dihidroxifenil acético	3,4-OH (168)	
Ácido 4-hidroxi-3-metoxifenilacético	3-OCH_3, 4-OH (182)	
Ácido 3-(3,4-dihidroxifenil) propanoico	(182)	

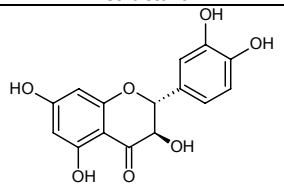
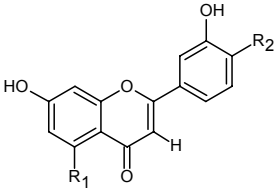
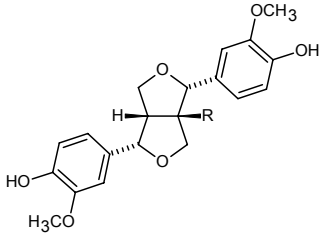
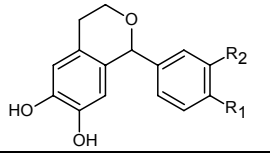
Nombre del compuesto	**Sustituyente (Pm)**	**Estructura**
Formas dialdehídicas de los secoiridoides		
Decarboximetil oleuropeina aglicona (3,4-DHPEA-EDA)	R_1-OH (304)	
Decarboximetil ligustrósido aglicona (*p*-HPEA-EDA)	R_1-H (320)	

Nombre del compuesto	**Sustituyente (Pm)**
Agliconas de los secoiridoides	
Oleuropeína aglicona o 3,4-DHPEA-EA	R_1-OH (378)
Ligustrósido aglicona o *p*-HPEA-EA	R_1-H (362)
Forma aldehídica de la oleuropeína aglicona	R_1-OH (378)
Forma aldehídica del ligustrósido aglicona	R_1-H (362)



p-HPEA or 3,4-DHPEA

Elenolic Acid (EA) *aldehydic form of Elenolic Acid (EA)*

Nombre del compuesto	**Sustituyente (Pm)**	**Estructura**
Flavonoles		
(+)-taxifolín	(304)	
Flavonas		
Apigenina	R_1-OH, R_2-H (270)	
Luteolina	R_1-OH, R_2-OH (286)	
Lignanos		
(+)-pinoresinol	R-H (358)	
(+)-1-acetoxipinoresinol	R-$OCOCH_3$ (416)	
(+)-1-hidroxipinoresinol	R-OH (374)	
Hidroxiisocromano		
1-fenil-6,7-dihidroxi-isocromano	R_1,R_2-H (242)	
1-(3'-metoxi-4'-hidroxi)fenil-6,7-dihidroxi-isocromano	R_1-OH,R_2-OCH_3 (288)	

De todos ellos, los compuestos fenólicos que se encuentran en mayor proporción en el aceite de oliva son los secoiridoides, oleuropeína y ligustrósido agliconas y sus formas dialdehídicas, aunque tirosol e hidroxitirosol también se hallan en concentraciones importantes. Entre ellos constituyen el 90 % del total de los compuestos fenólicos del aceite de oliva [212].

Alrededor del 80 % de los compuestos fenólicos que puede contener un aceite de oliva se pierden cuando el aceite es sometido al refinado, por ello serán los aceites de oliva vírgenes aquellos que presentan mayor contenido en compuestos fenólicos [213].

Es muy importante tener en cuenta que la composición fenólica de un aceite de oliva es el resultado de la interacción de numerosos factores entre los que se encuentran: la variedad de aceituna, las condiciones climáticas, el grado de maduración, el sistema de riego y la tecnología empleada para producir el aceite [214,215].

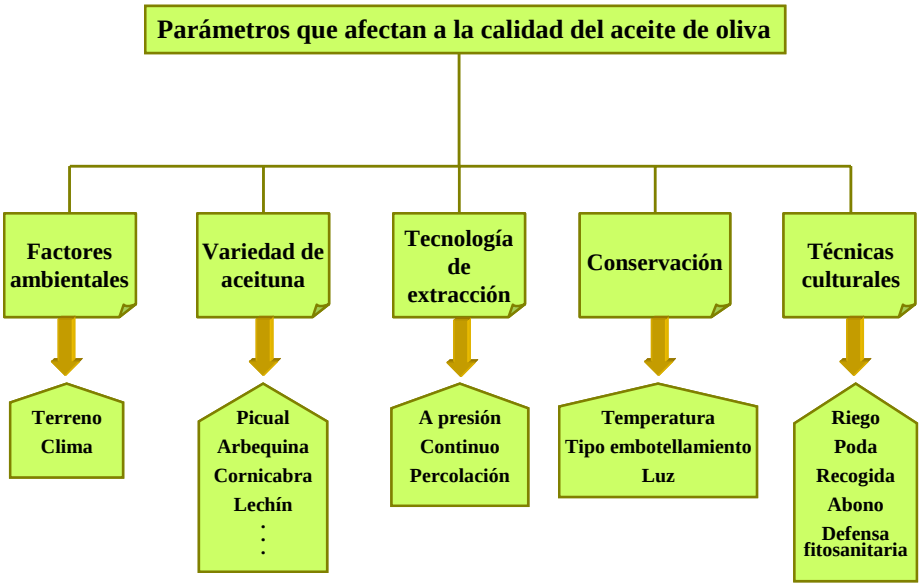


Figura. *Parámetros que afectan a la calidad del aceite de oliva.*

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Capítulo 1

Compuestos fenólicos en aceites de oliva virgen: propiedades sensoriales, beneficios para la salud, actividad antioxidante y métodos analíticos. Una revisión de la última década.

Publicación incluída en este capítulo:

Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. (*Molecules*, **2007**, 12, 1679-1719)

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Cuando nos planteamos por primera vez un problema científico, el primer paso, y quizás el más importante, es realizar una profunda revisión bibliográfica que nos ayude a adentrarnos en el tema, obteniendo información acerca de lo que ya ha sido estudiado y cómo fue llevado a cabo de manera experimental. De este modo nos podemos plantear el interés de un trabajo futuro en esa línea y sopesar cuál sería el mejor camino para llevarlo a cabo. Por ello se creyó oportuna la realización de un trabajo de revisión que constituyera este primer capítulo de la memoria.

Los antepasados que vivían en el área mediterránea conocían de forma empírica los beneficios de tomar aceite de oliva. En la actualidad estos beneficios han sido corroborados por diversas investigaciones conducidas bajo las directrices del Consejo Oleícola Internacional y continúan financiándose infinidad de estudios internacionales enfocados en las siguientes direcciones:

- Papel del aceite de oliva en la epidemiología de las enfermedades cardiovasculares.
- Papel del aceite de oliva en la prevención de la aterosclerosis y sus funciones metabólicas.
- Papel del aceite de oliva en el desarrollo óseo y del sistema nervioso.
- El aceite de oliva y su papel en las funciones digestivas.
- El aceite de oliva en nutrición y dietética.
- Aspectos químicos del aceite de oliva.
- La acción antioxidante del aceite de oliva y sus propiedades anti-envejecimiento en los niveles celulares y mitocondriales.
- El papel protector del aceite de oliva en los tumores de mama.
- El papel del aceite de oliva en la diabetes.
- Los componentes minoritarios del aceite de oliva.

En cada una de estas líneas los compuestos fenólicos del aceite de oliva juegan un papel fundamental.

Este trabajo de revisión, que se presenta como primer capítulo de la memoria, representa una visión amplia y clara de la importancia de los compuestos fenólicos del aceite de oliva, exponiendo las propiedades que les aportan al aceite, así como las metodologías puestas a punto para llevar a cabo su determinación que han sido publicados en la última década.

Los grandes bloques en los que se divide este review se podrían resumir de la siguiente forma:

a) En primer lugar se recopilan las evidencias, encontradas hasta el momento de su publicación, acerca de las propiedades beneficiosas para la salud que reporta el consumo de aceite de oliva y que se atribuyen a alguno de los diferentes compuestos fenólicos que contiene.

b) Otra de las características más destacadas del aceite de oliva es su elevada estabilidad oxidativa, que se ha comprobado se debe en gran parte a la acción antioxidante de los compuestos fenólicos. Por eso hemos querido resumir los diferentes trabajos realizados encaminados a explicar la capacidad antioxidante de estos compuestos, pasando desde el efecto que producen cada uno de ellos y por qué, hasta las distintas metodologías empleadas para determinarla.

c) A nivel sensorial el aceite de oliva posee unos rasgos característicos de aroma y sabor que lo convierten en único. Aquí de nuevo los compuestos fenólicos entran en acción, ya que muchos de ellos son los responsables de características tales como el amargor o la astringencia. En este trabajo se describen desde la importancia del proceso tecnológico en las características sensoriales del aceite (y por ello en el contenido fenólico), pasando por las diferentes formas de analizar estas características (panel de catadores o métodos analíticos), hasta llegar a las relaciones establecidas directamente entre un compuesto y la característica que aporta al aceite.

d) Por último, nos ocupamos del aspecto más puramente analítico, las técnicas y metodologías analíticas empleadas para la determinación de la fracción fenólica en los últimos años. HPLC (ampliamente aceptada como la técnica por excelencia para

el análisis de plifenoles) y EC (capaz de reducir considerablemente los tiempos de análisis manteniendo una buena separación) han sido las dos técnicas separativas empleadas principalmente para este tipo de análisis. Para poder resumir todas las metodologías estudiadas, éstas se han recogido en diversas tablas dedicadas a HPLC acoplada a MS, HPLC acoplada a RMN y EC acoplada tanto a UV como MS. Por último se presenta otra tabla resumen en la que podemos ver los diferentes métodos de extracción empleados en los métodos de EC, así como las aplicaciones de cada uno de ellos.

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Review

Phenolic Molecules in Virgin Olive Oils: a Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical Methods. An Overview of the Last Decade

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Abstract: Among vegetable oils, virgin olive oil (VOO) has nutritional and sensory characteristics that to make it unique and a basic component of the Mediterranean diet. The importance of VOO is mainly attributed both to its high content of oleic acid a balanced contribution quantity of polyunsaturated fatty acids and its richness in phenolic compounds, which act as natural antioxidants and may contribute to the prevention of several human diseases. The polar phenolic compounds of VOO belong to different classes: phenolic acids, phenyl ethyl alcohols, hydroxy-isochromans, flavonoids, lignans and secoiridoids. This latter family of compounds is characteristic of Oleaceae plants and secoiridoids are the main compounds of the phenolic fraction. Many agronomical and technological factors can affect the presence of phenols in VOO. Its shelf life is higher than other vegetable oils, mainly due to the presence of phenolic molecules having a catechol group, such as hydroxytyrosol and its secoiridoid derivatives. Several assays have been used to establish the antioxidant activity of these isolated phenolic compounds. Typical sensory gustative properties of VOO, such as bitterness and pungency, have been attributed to secoiridoid molecules. Considering the importance of the phenolic fraction of VOO,

high performance analytical methods have been developed to characterize its complex phenolic pattern. The aim of this review is to realize a survey on phenolic compounds of virgin olive oils bearing in mind their chemical-analytical, healthy and sensory aspects. In particular, starting from the basic studies, the results of researches developed in the last ten years will be focused.

Keywords: Phenols; Virgin olive oil; Sensory properties; Antioxidant activity; Analytical techniques.

Phenolic molecules in virgin olive oil

Oleuropein belongs to a specific group of coumarin-like compounds, the secoiridoids, which are abundant in *Oleaceae*. Secoiridoids are compounds that are usually glycosidically bound and produced from the secondary metabolism of terpenes. The secoiridoids, found only in plants belonging to the family of *Olearaceae* that includes *Olea europaea* L., are characterised by the presence of elenolic acid in its glucosidic or aglyconic form, in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol and tyrosol), elenolic acid and, eventually, a glucosidic residue. Oleuropein is an ester of hydroxytyrosol (3,4-DHPEA) and the elenolic acid (EA) glucoside (oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*) [1-5]. Secoiridoids of VOO in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation. These newly formed substances, having amphiphilic characteristics, are partitioned between the oily layer and the vegetation water, and are more concentrated in the latter fraction because of their polar functional groups. During storage of VOO hydrolytic mechanisms that lead to release of simple phenols, such as hydroxytyrosol and tyrosol, from complex phenols as secoiridoids may be involved [6-8]. The most abundant secoiridoids of VOO, identified for the first time by Montedoro *et al*. [1-3, 9] and confirmed also by other authors [10-13], are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol (p-HPEA) respectively termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, and an isomer of the oleuropein aglycon (3,4-DHPEA-EA) (Table 1). In 1999 another hydroxytyrosol derivative, hydroxytyrosol acetate (3,4-DHPEA-AC) was found in virgin olive oil [14].

Phenolic acids are secondary aromatic plant metabolites that are widely spread throughout the plant kingdom [15-17]. These naturally occurring phenolic acids contains two distinguishing constitutive carbon frameworks, namely the hydroxycinnamic and hydroxybenzoic structures. Elucidation of their roles in plant life is only one of the many ongoing investigations regarding phenolic acids: one vast area of interest lies in food quality [18-20]. Phenolic acids have been associated with color and sensory qualities, as well as with the health-related and antioxidant properties of foods [21-22]. One impetus for analytical investigations has been the role of phenolics in the organoleptic properties (flavor, astringency, and hardness) of foods [23-24]. Additionally, the content and profile of phenolic acids, their effect on fruit maturation, prevention of enzymatic browning, and their roles as food preservatives has been evaluated [25]. Recent interest in phenolic acids stems from their potential protective role, through ingestion of fruit and vegetables, against diseases that may be related to oxidative damage (coronary heart disease, stroke, and cancers) [26-28].

In particular, several phenolic acids such as gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*- and *o*-coumaric, ferulic and cinnamic acid have been identified and quantified in VOO (in quantities lower than 1 mg of analyte kg^{-1} of olive oil). In this regard two research groups have extensively analyzed samples of VOO for these types of compounds [29-32]. In one of these mentioned articles, for instance, the authors found that trans-cinnamic acid, sinapinic acid, caffeic acid and 3,4-dihydroxyphenylacetic acid were present in several monovarietal VOO of the six Spanish olive cultivars analyzed [31]; therefore, these compounds might be potential markers of geographical origin or the olive fruit variety.

(+)-Pinoresinol is a common component of the lignan fraction of several plants such as *Forsythia* species [33] and *Sesamum indicum* seeds, whereas (+)-1-acetoxypinoresinol and (+)-1-hydroxy-pinoresinol and their respective glucosides have been detected in the bark of the olive tree (*Olea europaea L.*). According to Owen *et al.* [34], the quantity of lignans in VOO may be up to 100 mg kg^{-1}, but as with the simple phenols and SIDs, considerable inter-oil variation exists. As suggested by Brenes *et al.* [35], the amount of lignans may be used as varietal marker, and they reported a method to authenticate VOO produced by Picual olives based on the very low content of the lignan (+)-1-acetoxypinoresinol in these oils.

A few years ago, Bianco *et al.* [36] investigated the presence of hydroxy-isochromans in VOO. In fact, during the malaxation step of VOO extraction, hydrolytic processes through the activity of glycosidases and esterases augment the quantity of hydroxytyrosol and carbonylic compounds, thus favouring the presence of all compounds necessary for the formation of isochroman derivatives. Two hydroxy-isochromans, formed by the reaction between hydroxytyrosol and benzaldehyde or vanillin, have been identified by HPLC-MS/MS technique and quantified in commercial VOOs.

Flavonoids are widespread secondary plant metabolites. During the past decade, an increasing number of publications on the health beneficial effects of flavonoids have appeared, such those related to cancer and coronary heart diseases [37-40]. Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation, or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones, and flavanols depending upon the presence of a carbonyl carbon at C-4, an OH group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 with an OH group at C-3, respectively. Several authors have reported that flavonoids such as luteolin and apigenin are also phenolic components of VOO [41-46]. Luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. There are also several interesting studies in which several flavonoids have been found in olive leaves and fruits [47-50].

Table 1. Phenolic compounds in virgin olive oil: compounds name, general chemical structure and molecular weight.

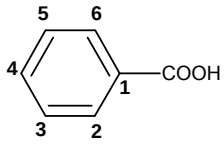
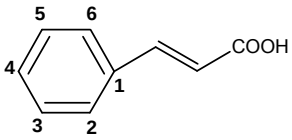
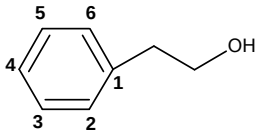
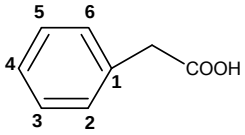
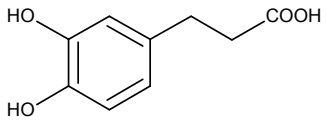
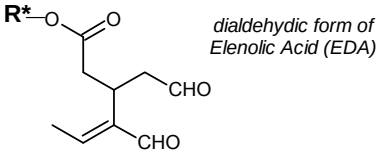
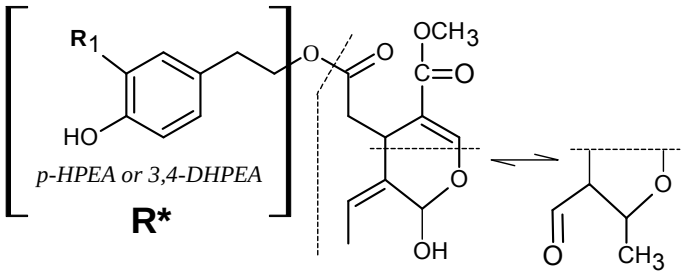
Compound	Substituent (MW)	Structure
Benzoic and derivatives acids		
3-Hydroxybenzoic acid	3-OH (138)	
p- Hydroxybenzoic acid	4-OH (138)	
3,4-Dihydroxybenzoic acid	3,4-OH (154)	
Gentisic acid	2,5-OH (154)	
Vanillic acid	3-OCH_3, 4-OH (168)	
Gallic acid	3,4,5-OH (170)	
Syringic acid	3,5-OCH_3, 4-OH (198)	
Cinnamic acids and derivatives		
o-Coumaric acid	2-OH (164)	
p-Coumaric acid	4-OH (164)	
Caffeic Acid	3,4-OH (180)	
Ferulic Acid	3-OCH_3, 4-OH (194)	
Sinapinic Acid	3,5-OCH_3, 4-OH (224)	
Phenyl ethyl alcohols		
Tyrosol [(*p*-hydroxyphenyl)ethanol] or *p*-HPEA	4-OH (138)	
Hydroxytyrosol [(*3,4*-dihydroxyphenyl)ethanol] or 3,4-DHPEA	3,4-OH (154)	
Other phenolic acids and derivatives		
p-Hydroxyphenylacetic acid	4-OH (152)	
3,4-Dihydroxyphenylacetic acid	3,4-OH (168)	
4-Hydroxy-3-methoxyphenylacetic acid	3-OCH_3, 4-OH (182)	
3-(3,4-Dihydroxyphenyl)propanoic acid	(182)	
Dialdehydic forms of secoiridoids		
Decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA)	R_1-OH (304)	 *dialdehydic form of Elenolic Acid (EDA)*
Decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA)	R_1-H (320)	

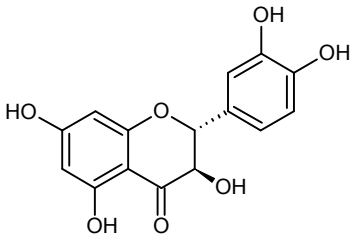
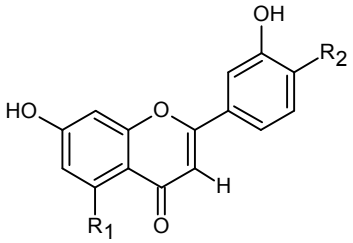
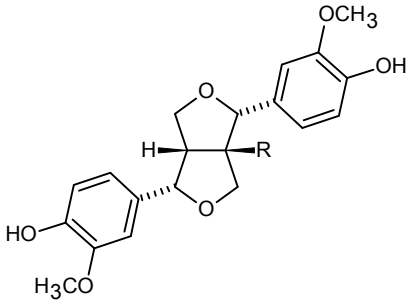
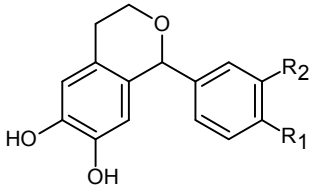
Table 1. Cont.

Compound	Substituent (MW)
Secoiridoid Aglycons	
Oleuropein aglycon or 3,4-DHPEA-EA	R_1-OH (378)
Ligstroside aglycon or *p*-HPEA-EA	R_1-H (362)
Aldehydic form of oleuropein aglycon	R_1-OH (378)
Aldehydic form ligstroside aglycon	R_1-H (362)

Structure



Elenolic Acid (EA) *aldehydic form of Elenolic Acid (EA)*

Compound	Substituent (MW)	Structure
Flavonols		
(+)-Taxifolin	(304)	
Flavones		
Apigenin	R_1-OH, R_2-H (270)	
Luteolin	R_1-OH, R_2-OH (286)	
Lignans		
(+)-Pinoresinol	R-H (358)	
(+)-1-Acetoxypinoresinol	R-$OCOCH_3$ (416)	
(+)-1-Hydroxypinoresinol	R-OH (374)	
Hydroxyisochromans		
1-Phenyl-6,7-dihydroxyisochroman	R_1,R_2-H (242)	
1-(3'-Methoxy-4'-hydroxy)phenyl-6,7-dihydroxyisochroman	R_1-OH,R_2-OCH_3 (288)	

Why are the phenolic compounds present in virgin olive oil so important? Why is their determination so interesting and difficult?

Last year, Boskou published an interesting review [51] wherein the sources of natural phenolic antioxidants were discussed, and the following idea was highlighted: "Widely distributed in the plant kingdom and abundant in our diet, plant phenols are today among the most talked about classes of phytochemicals". To answer to the question of "why are phenolic compounds so interesting?", the author of the review summarized several issues which have been studied in depth during the last decade:

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

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

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

– The ability of certain classes of plant phenols such as flavonoids (also called polyphenols) to bind to proteins. Flavonol–protein binding, such as binding to cellular receptors and transporters, involves mechanisms which are not related to their direct activity as antioxidants.

– The stabilization of edible oils, protection from formation of off-flavors and stabilization of flavours.

– The preparation of food supplements.

Focusing on phenolic compounds of virgin olive oil and bearing in mind the reasons for being so important, attention must be paid to the fact that this class of compounds has not been completely characterized due to the complexity of their chemical nature and the complexity of the matrix in which they are found. Moreover, one of the current problems for developing rapid and reproducible analysis of phenolic compounds is the absence of suitable pure standards, in particular secoiridoid molecules and lignans.

Health aspects linked to phenols in VOO

VOO is an integral ingredient of the Mediterranean diet and accumulating evidence suggests that it may have health benefits which include reduction of risk factors of coronary heart disease, prevention of several types of cancers, and modification of immune and inflammatory responses. VOO can be considered as example of a functional food, with a variety of components that may contribute to its overall therapeutic characteristics [52]. Its nutritional and healthy values and pleasant flavour have contributed to an increase in consumption of VOO which has fostered cultivation of olives outside the traditional olive oil producing region of the Mediterranean basin into newer areas such as Australia, Argentina and South Africa. The nutritional value of VOO arises from high levels of oleic acid, and from minor components such as phytosterols, carotenoids, tocopherols and hydrophilic phenols [53].

VOO contains at least 30 phenolic compounds. The major phenolic compounds are oleuropein derivatives, based on hydroxytyrosol which are strong antioxidants and radical scavengers. Recently there has been a surge in the number of publications that has investigated their biological properties. Bisignano *et al*. [54] found that hydroxytyrosol and oleuropein have antimicrobial activity against

several bacterial strains that are causal agents of intestinal or respiratory tract infections in humans. In a more recent *in-vivo* study, Glatzle and co-workers [55] demonstrated that enteral immunonutrition with VOO more effectively reduced septic pulmonary dysfunction compared to a fish oil-enriched lipid formula at the same concentration.

It has recently been found that hydroxytyrosol is renally excrete: while some of hydroxytyrosol is unchanged, some is also metabolized to the following metabolites: glucuronide conjugate, sulphate conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylacetaldehyde [56-57]. The radical scavenging potencies of these metabolites of hydroxytyrosol have also been investigated using the radical assay DPPH. The glucuronide conjugate was more potent than hydroxytyrosol while the sulphate conjugate was nearly devoid of radical scavenging activity. When phenol-rich VOO characterized by increasing concentrations of catecholic compounds were administered to human volunteers, Visioli and co-authors [58] observed a dose-dependent urinary excretion of hydroxytyrosol and its metabolite homovanillic alcohol. In a later study, the same authors [59] noticed that the urinary levels of unconjugated tyrosol and hydroxytyrosol correlated with their intake, except at the highest dose, which increased the quantity of glucuronide conjugate. Tuck *et al.* investigated the *in vivo* fate of hydroxytyrosol and tyrosol after intravenous and oral dosing of these tritium labelled compound to rats [60]. No significant differences in the amount of phenolic compounds eliminated in urine between the intravenous dosing method and the oral oil-based dosing method for either tyrosol or hydroxytyrosol were found.

Phytochemical compounds such as oleuropein and oleuropein aglycon have been intensively studied for some promising results with respect to their effects on human health and their potential medicinal properties. It has been found that diets containing olive oil phenols may increase *in vivo* resistance of LDLs to oxidation; the effectiveness of oleuropein has been explained in part through its ability to act as an antioxidant and in part through a hypocholesterolaemic effect [61]. In an investigation by Coni *et al.* [62] it was found that when fed a diet rich in oleuropein to rabbits, the ability of LDL to resist to oxidation increased, thanks to its antioxidant capacity; moreover, they found a significant reduction of the plasmatic levels of total, free and ester-derivatives of cholesterol. Oleuropein aglycon, the bitter component of olives and olive oil, is among the first example of how selected nutrients from an VOO-rich "Mediterranean diet" can directly regulate HER2-driven breast cancer disease [63]. As oleuropein aglycon exhibits synergistic anti-tumor effects when concurrently given to breast cancer cells chronically exposed to trastuzumab (Tzb; Herceptin™) for several months, this further underscores the potential clinical relevance of these findings.

On the basis of their shared throat irritant properties (pungency), Beauchamp *et al*. [64-65] examined whether *p*-HPEA-EDA, now referred to as "oleocanthal", might mimic the pharmacological effects of ibuprofen, a potent modulator of inflammation and analgesia. It was found that, like ibuprofen, both enantiomers of *p*-HPEA-EDA caused dose-dependent inhibition of COX-1 and COX-2 activities (cyclo-oxygenase enzymes that catalyze key steps in the biochemical inflammation pathways derived from arachidonic acid) but had no effect on lipoxygenase *in vitro*. These authors hypothesized that long-term consumption of oleocanthal may help to protect against some diseases by virtue of its ibuprofen-like COX-inhibiting activity (by reducing the risk of developing some cancers and by lowering platelet aggregation in the blood). However, as noted by Fogliano and Sacchi [66], no data is available about the concentration of the various aglycons, including oleocanthal in plasma and urine after VOO consumption; absorption and bioavailability studies indicate however that tyrosol and

hydroxytyrosol are likely to be bio-available. It is worth mentioning that acid hydrolysis of oleocanthal would produce the elenolic acid, a dialdehyde compound even more similar to ibuprofen than oleocanthal itself.

In the context of the Mediterranean diet and coronary heart diseases, it has also been shown that VOO rich in phenols increases the resistance of LDL to oxidation, both *in vitro* and *ex vivo* [67-69]. The study carried out by Bogani *et al.* [70] confirmed the anti-thrombotic and anti-inflammatory effects of VOO phenolic components, in a postprandial setting; in fact, the results showed significant reductions in serum concentration of inflammatory markers (TXB_2 and LTB_4) at 2 and 6 h after consumption of VOO, but not after consumption of either olive oil or corn oil. They also evaluated the effects of these different oils on *in vivo* indexes of oxidative stress (plasma antioxidant capacity and urinary hydrogen peroxide levels) and showed the antioxidant activity of VOO phenolics after ingestion (increased plasma antioxidant capacity after 2 h of VOO consumption).

Foods containing high amounts of lignans such as flaxseed have been found to be protective against breast cancer, and in particular, to exert an anti-estrogenic effect; this latter observation might be explained by considering the structural similarities between the lignans and the synthetic antiestrogen tamoxifen [71].

Two hydroxy-isochromans, 1-(3'-methoxy-4'-hydroxyphenyl)-6,7-dihydroxyisochroman and 1-phenyl-6,7-dihydroxyisochroman, are formed by reaction between hydroxytyrosol and vanillin and benzaldehyde, respectively, (under very mild conditions). They have only recently been discovered in VOO [36] and are active in inhibited platelet aggregation and thromboxane release evoked by agonists (sodium arachidonate and collagen) that induce reactive oxygen species-mediated platelet activation [72].

A large number of studies, mainly experimental models, have been performed on certain minor components of olive oil. However, as commented in an excellent review by Covas *et al.* [73], the precepts of evidence-based medicine require high-level scientific evidence to be provided before nutritional recommendations for the general public can be formulated. Scientific evidence required is provided by randomized, controlled, double-blind clinical trials (level I evidence), and to some extent by large cohort studies (level II evidence). Basic research, despite its usefulness in permitting adoption on a mechanistic approach, does not provide evidence for nutritional recommendations. Of course, the level of evidence of a particular study depends not only on its design, but also on its quality (external and internal validity, homogeneity of the sample, and statistical power). Finally, evidence is built by the agreement of the results of several similar studies. In the same review, the authors highlighted that in experimental studies, olive oil phenols have been shown to:

1) have antioxidant effects, greater than those of vitamin E, on lipid and DNA oxidation [74-77];

2) prevent endothelial dysfunction by decreasing the expression of cell adhesion molecules [78], increasing nitric oxide (NO) production and inducible NO synthesis [79] and quenching vascular endothelium intracellular free radicals [80];

3) inhibit platelet-induced aggregation [81];

4) enhance the mRNA transcription of the antioxidant enzyme glutathione peroxidase (GSH-Px). It should be mentioned however that to regard this last point controversial results have been obtained depending on the tissue in which the gene expression was evaluated [75, 82].

Other potential activities of VOO phenolic compounds include chemopreventive activity [77]. The anticarcinogenic activity of phenols may be due not only to their antioxidant properties, but also to

their ability to reduce the bioavailability of food carcinogens and to inhibit their metabolic activation [83-84]. There are several mechanistic considerations of the role of phenolic compounds as anticarcinogens, as reviewed by Yang *et al.* [85].

Phenols as related to oxidative stability of VOO

Oxidation is an inevitable process that starts after the VOO has been extracted and leads to deterioration that becomes more pronounced during oil storage. Initially lipids are radically oxidised to hydroperoxides, which are odourless and tasteless [86] and do not account for sensory changes. However, decomposition occurs through homolytic cleavage of the hydroperoxide group with production of various volatile compounds, known as secondary oxidation products, which are responsible for typical unpleasant sensory characteristics. Oxygen, light, temperature, metals, pigments, unsaturated fatty acid composition, as well as the quantity and kind of natural antioxidants, are all factors that can influence the free radical mechanism of the autoxidation process in a different manner [87-88].

Natural antioxidants exhibit complex properties between air-oil and oil-water interfaces that significantly affect their relative activities in different lipidic systems. The presence of hydrophilic phenolic compounds in VOO and their high antioxidant activity can be explained by the so-called "polar paradox" [89] which dictates that "polar antioxidants are more effective in non polar lipids, whereas non-polar antioxidants are more active in polar lipid emulsions". According to Frankel [90] in a bulk oil system the hydrophilic antioxidants, such as polar phenols, are oriented in the air-oil interface (a low quantity of air is always trapped in the oil) and become more protective against oxidation than the lipophilic antioxidants, like tocopherols, which remain in solution in the oil.

In a study carried out by Paiva-Martins and co-workers [91] it was found that when food is processed with VOO in the presence of water, olive phenolic extracts with higher quantities of 3,4-DHPEA-EA and 3,4-DHPEA-EDA would be better than VOO extracts with higher quantities of hydroxytyrosol, despite the higher antioxidant activity of hydroxytyrosol in bulk oil.

Moreover, the orientation of phenolic compounds in the oil-water interface and the active surface of water droplets influence the protection against the oxidation of oil. Recently, some researchers [92-95] have determined that VOO contains a low quantity of water (ranging from 450 mg kg^{-1} to 3,000 mg kg^{-1} depending on the extraction technology), that increases when samples were not filtered. Part of the total water content presents in VOO is free and available for chemical and enzymatic reactions and also keeps hydrophilic phenols in solution. This can explain the hydrolytic process that occurs both to phenols (by esterases) and triacylglycerols (by lipase) during prolonged VOO storage. Thus, more rapid oxidation of the unfiltered oil could be expected. Instead, according to Gomez-Caravaca *et al.* [95] and Tsimidou *et al*. [96], stability of unfiltered samples, when measured in terms of resistance to accelerated oxidation (value by OSI or Rancimat instruments) was in all cases significantly higher than that of the corresponding filtered oils. This coincided with a higher total phenolic content in unfiltered VOO. Undoubtedly, a loss of a significant fraction of phenols during filtration is related to the reduction of oxidative stability.

Chain-breaking antioxidants, such as phenolic compounds, react with lipid radicals to form non-reactive radicals, interrupting the propagation chain. In fact, these compounds are able to donate an electron or a hydrogen atom to the lipid radical formed during the propagation phase of lipid oxidation

and stabilize the resulting phenoxyl radical by delocalizing the unpaired electron [97-100]. Phenolic compounds exert their antioxidant abilities in VOO by scavenging peroxyl and alkoxyl radicals, and by chelation of transition metals ions present in trace quantities [101]. Paiva-Martins and Gordon [102-103] have studied the antioxidant effects of pure phenolic compounds (hydroxytyrosol, hydroxytyrosol acetate, oleuropein, 3,4-DHPEA-EA, and 3,4-DHPEA-EDA) by both the diphenylpicrylhydrazyl (DPPH) assay and the ferric reducing antioxidant potential (FRAP) assay in bulk oil and in emulsions (both with and without ferric ions). The compounds showing the best antioxidant activity in oil in water emulsions in the presence of iron were 3,4-DHPEA-EA and 3,4-DHPEA-EDA, which in contrast to hydroxytyrosol and oleuropein did not show pro-oxidant activity. However, when the radical scavenging activity was measured for these compounds, 3,4-DHPEA-EA showed a much higher activity than 3,4-DHPEA-EDA, suggesting that chelation of iron was of major significance in determining the antioxidant activity of these compounds in the presence of iron and water. According to this latter behaviour, Bendini *et al.* [104] showed evidence that 3,4-DHPEA-EDA has the ability to chelate copper in bulk oil.

The stability of VOO is improved by synergistic interactions between various antioxidants present (both phenolic and non-phenolic) and the lipid composition. Such cooperative activity seems to explain the antioxidant synergism observed when α-tocopherol and ascorbic acid or ubiquinol are used in combination. Recent investigations have also demonstrated an antioxidant synergism between α-tocopherol and some phenolics (green tea catechins and quercetin) [105-106]. Bendini *et al.* [104] hypothesized that phenols having an ortho-dihydroxyl structure, and in particular an isomer of 3,4-DHPEA-EDA, were able to reduce the oxidized forms of tocopherols (tocopheryl radicals and quinones). This was substantiated by Pazos *et al.* [107] who demonstrated that phenols, as well as several benzoic acids and epicatechin gallates, were potentially active in the regeneration of α-tocopherol via reduction of α-tocopheroxyl radical. Moreover this capacity was found to be directly proportional to the ability of phenolic compounds to transfer a single H atom.

There have been numerous studies on the relative antioxidant potency of the individual olive oil phenols, although it may vary depending on the methods used for evaluation. Many authors have frequently studied the ability of antioxidant molecules or extracts to scavenge some free radicals, and in this regard, several stable, coloured free radicals (DPPH and ABTS) are widely used due to their intense absorbance in the visible region. In this case, the hydrogen-donating activity can be determined.

However, as a general guide to their potency, oleuropein and hydroxytyrosol have been shown to be more effective than vitamin E [108] and butylated hydroxytoluene (BHT) or other synthetic antioxidants approved for use in foods [109-111].

From comparison with the principal phenolic constituents of VOO, it has been claimed that hydroxytyrosol is the most active antioxidant compound [112]. Both hydroxytyrosol and oleuropein have been shown to be scavengers of superoxide anions, and inhibitors of the hypochlorous acid-derived radicals, but hydroxytyrosol was more effective than oleuropein in this regard [113]. Both compounds also scavenged hydroxyl radicals, but in this case oleuropein showed greater activity [114]. Gordon *et al.* [115] investigated the antioxidant activity of hydroxytyrosol acetate by scavenging of DPPH radicals in comparison with that of the phenolic extract from VOO and the pure components hydroxytyrosol, oleuropein, 3,4-DHPEA-EA, and α-tocopherol in bulk oil and oil-in-water emulsions. In this study the authors showed that hydroxytyrosol acetate had a weaker DPPH radical scavenging

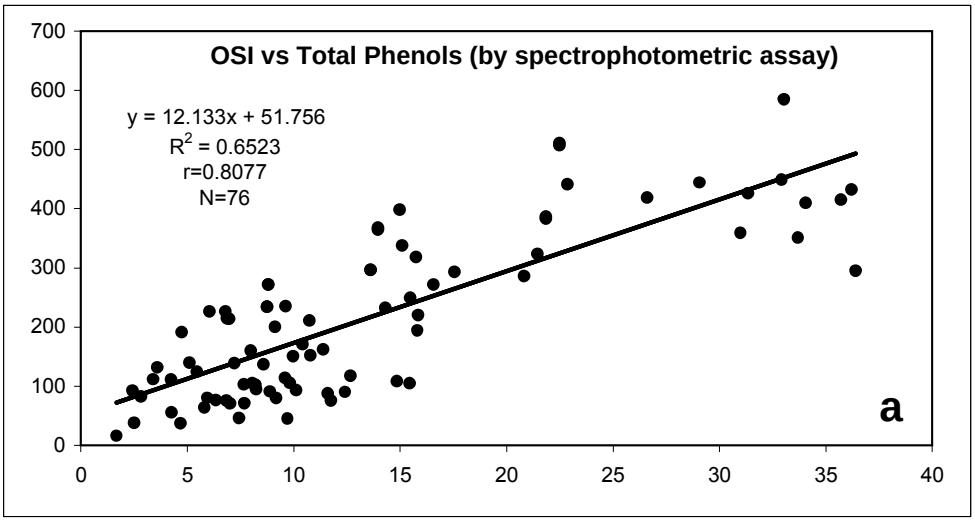
activity than hydroxytyrosol and 3,4-DHPEA-EA was slightly less effective than hydroxytyrosol acetate in oil but was the most effective hydroxytyrosol derivative in an emulsion oil in water.

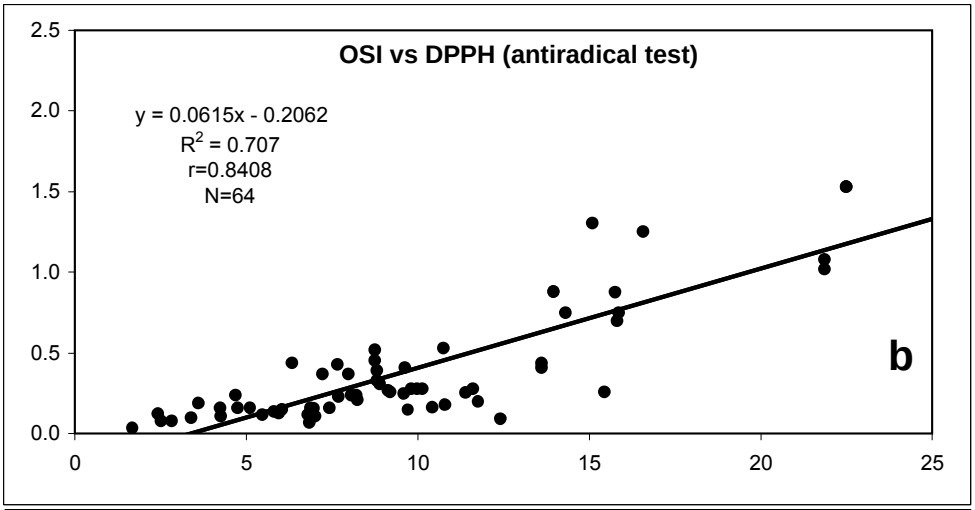
It is well known that the high oxidative stability of VOO is primarily due to *o*-diphenols such as hydroxytyrosol and its oleosidic forms [108]. Aparicio *et al.* [116], using statistical analysis of data relative to 79 VOO of olives *cv*. Hojiblanca and Picual, measured correlations between oxidative stability (valued by Rancimat) and several compositional variables. The phenols (R^2=0.87), o-diphenols (R^2=0.77), and the oleic/linoleic ratio (R^2=0.71) had the highest values, followed by chlorophylls (R^2=0.68), total tocopherols (R^2=0.65) and carotenoids (R^2=0.59). Principal components analysis confirmed that phenols, oleic/linoleic ratio, and tocopherols had the maximum correlation with oxidative stability. From these results, the phenolic content would contribute around 51% of the stability of VOO, and particularly 30% for phenols and 21% for *o*-diphenols whereas, the oleic/linoleic ratio would account for only 27%. Since a hypothetical synergy effect was detected between these chemical variables, it is more prudent to conclude that 78% of the stability is due to the combined effect of both variables. The authors surmised that the contribution of total tocopherols was around 9%, whereas the remaining percentage of 13% could be attributed to chlorophylls and carotenoids.

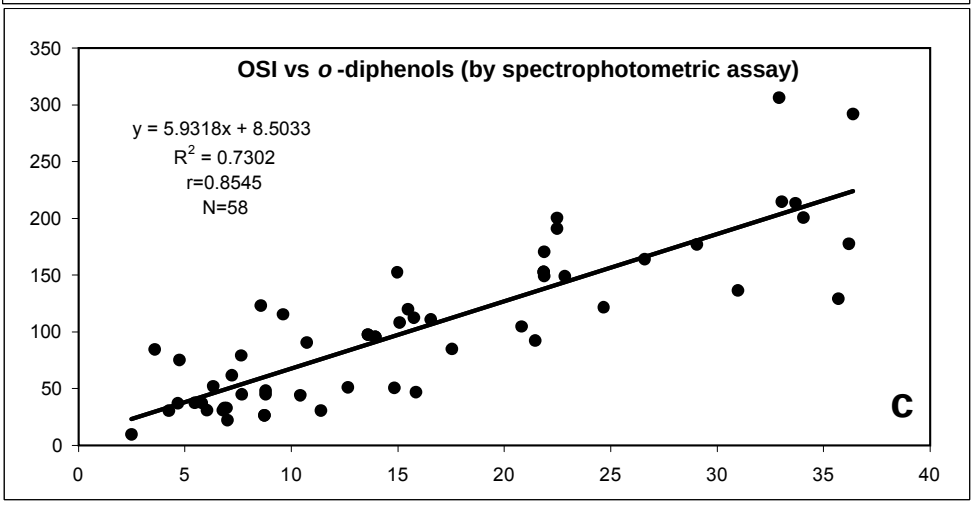
In experiments carried out by Carrasco-Pancorbo and co-authors [117], the antioxidant activity of several single phenolic compounds of VOO (hydroxytyrosol, tyrosol, elenolic acid, 3,4-DHPEA-EDA, (+)-pinoresinol, (+)-1-acetoxypinoresinol, oleuropein aglycon and ligstroside aglycon) was evaluated by different chemical approaches: radical assay (DPPH), accelerated oxidation in a lipid model system (OSI, oxidative stability index), and an electrochemical method (flow injection analysis FIA-amperometry and cyclic voltammetry). These authors verified that, as is generally assumed, the presence of a single hydroxyl group on benzenic ring conferred only limited antioxidant activity. On the other hand, the presence of a catechol moiety enhances the ability of the phenolic compounds to act as antioxidants. The results obtained in all three tests showed that hydroxytyrosol, 3,4-DHPEA-EDA and oleuropein aglycon were the strongest in terms of antioxidant power. Elenolic acid, which does not have a phenolic ring, was one of the compounds that presented the weakest antioxidant activity, as also reported by Briante *et al.* [118]; this compound together with (+)-pinoresinol, tyrosol, ligstroside aglycon and (+)-1-acetoxypinoresinol showed pro-oxidant effect when tested by OSI. Similar results were found by Nenadis *et al.* [119]: tyrosol, hydroxytyrosol and their secoiridoid derivatives were examined calculating the bond dissociation enthalpy (BDE) of phenolic hydroxyl groups and the ionization potential (IP) as descriptors to predict the H-atom-donating and electron-donating abilities of antioxidants, respectively. Catechol derivatives had the lowest BDE values (77.7-80.1 kcal mol^{-1}), whereas the lignans, pinoresinol and 1-acetoxypinoresinol, and other monophenols had much higher BDE values (85.1-88.0 kcal mol^{-1}), which suggested a lower potential for radical scavenging.

In a recent work, Lorenz *et al.* [120] investigated the antioxidant and radical scavenging properties of several phenolic isochromans. All hydroxy-isochromans tested exceeded the scavenging effect of trolox (an hydrophilic analogue of α-tocopherol). They found excellent ROS/RNS (reactive species of oxygen/nitrogen) scavenging features of the hydroxy-isochromans and also concluded that their simple synthesis added to their interest as candidates for pharmaceutical interventions that protect against the deleterious action of ROS/RNS.

Figure 1 Correlations among OSI values (in hours), phenolic amounts and antioxidant activity (DPPH test) by spectrophotometric assays. a, OSI vs Total Phenols (mg gallic acid kg^{-1} VOO); b, OSI vs DPPH (mmol trolox kg^{-1} VOO); c, OSI vs *o*-diphenols (mg gallic acid kg^{-1} VOO). Analyses were carried out over three years; in each figure the number of samples is reported (N). Three replicates were prepared and analyzed for each sample.







Franconi *et al.* [121] tested the antioxidant activity of two VOO (Seggianese and Taggiasca characterized by different quali-quantitative phenolic profiles) on human LDL by measuring malondialdehyde and conjugate diene generation induced by copper ions. In both tests antioxidant potency correlated with total phenols; moreover, reduction of malondialdehyde and generation of conjugate diene was dependent on the amount of total phenols. High levels of secoiridoids enhance the antioxidant activity, suggesting that VOO rich in these compounds could have health-protecting properties consistent with a low extent of LDL oxidation.

Recently, the interest in oxidized forms of VOO phenols has significantly increased, especially in relation to determination of freshness/ageing status [122-123]. Moreover, characterization of these oxidized phenolics could represent an analytical instrument to investigate the thermal processes of the oils during refinement [124]; this could also provide the means to verify fraudulent practices such as "gentle deodorization" (under soft refining conditions) or blending of VOO with other oils. In 2005 Rios *et al.* [122] compared the performance of HPLC-APCI-MS and GC-IT-MS analytical techniques to evaluate the oxidation products of elenolic acid, oleuropein and ligstroside aglycons. Five oxidation phenols were identified with gas-chromatography. Armaforte *et al.* [125] showed that SPE procedure (usually used to extract the phenolic fraction from VOO) may be a not appropriate analytical step when VOO contains significant polar oxidation products (from phenols or lipids); in fact, these latter compounds could interfere with the retention mechanism of phenols during their extraction. These authors also proposed an index to establish the degree of freshness of VOO. This value, or TPAR (ratio between total peak area of reduced and oxidized forms of phenols) is close to 1 for fresh samples whereas it decreases rapidly in VOO with an increasing content of oxidized phenols.

Carrasco-Pancorbo and co-authors [124] by studying the phenolic profiles of the oils after a drastic heating treatment (at 180°C) found several “unknown” compounds, (by using HPLC-UV, HPLC-MS and CE-UV) that were probably linked to phenol oxidation. In particular, seven peaks significantly increased when the thermal treatment was longer (from 1 to 3 h) and their presence was also confirmed in refined olive oils. The concentration of hydroxytyrosol, elenolic acid, 3,4-DHPEA-EDA and 3,4-DHPEA-EA decreased more quickly with the thermal treatment than other phenolic compounds present in olive oil, confirming their high antioxidant power; moreover 3,4-DHPEA-AC and *p*-HPEA-EA were more resistant to heat treatment, whereas the amount of (+)-pinoresinol and (+)-1-acetoxypinoresinol were almost unchanged.

Sensory properties elicited by phenols in VOO

Virgin olive oil is a natural fruit juice obtained directly from olives without any further refining process. Its flavour is characteristic and is markedly different from those of other edible fats and oils. The combined effect of the taste, odor (directly via the nose or indirectly through the retronasal path via the mouth) and chemical responses (pungency, astringency, metallic, cooling or burning) gives rise to the sensation generally perceived as “flavor” [126]. VOO, when extracted from fresh and healthy olive fruits (*Olea europaea* L.) and properly processed and stored, is characterized by an unique combination of aroma and taste that is highly appreciated [127-128]. The sensory aspect, due to the use of VOO as a seasoning on cooked and especially raw foods, has great repercussions on its acceptability. Thus, since sensory quality plays an important role in directing the preference of consumers, many attempts have been made to clarify the relationships between the sensory attributes

in a VOO as perceived by assessors and its volatile and phenol profiles, which are responsible for aroma and taste, respectively [128].

Few individuals, except for trained assessors of VOO, know that bitterness and pungency perceived by taste are positive attributes for a VOO. These two sensory characteristics are strictly connected by the quali-quantitative phenolic profile of the product. An example of the positive correlations between amount of phenols and bitter and pungent intensities is shown in Figures 2a and 2b.

Some phenols mainly elicit the tasting perception of bitterness; however, other phenolic molecules can stimulate the free endings of the trigeminal nerve located in the palate and also in the gustative buds giving rise to the chemesthetic perceptions of pungency, astringency and metallic attributes. Thus, the intensity of bitterness and pungency is mainly related to the olives cultivar and the ripening stage and, as reported by many authors, are especially abundant in oils obtained from unripe fruits. For instance, Caponio *et al.* [129] showed that in *Coratina* and *Oliarola Salentina* VOO, oleuropein and its aglycon form both decrease as ripening of the olives progressed. From this data, the bitter to pungent taste would appear to be mainly ascribable to oleuropein aglycon since greater amounts of this phenolic compound are present in the *Coratina* oils with respect to *O. Salentina* oils, which are known to have a sweet taste. In order to attenuate such these taste sensations, the authors suggested the need to postpone harvesting of *Coratina* olives.

Figure 2 Sensory profile and phenolic content of two different VOO (HPh, high phenols oil and LPh, low phenols oil). **a**, sensory profiles of samples by Quantitative Descriptive Analysis (QDA); the intensity of each descriptor is evaluated on a 0-5 points scale; different perception routes: (1) orthonasal, (2) retronasal. **b**, single and total phenolic content of samples; A, hydroxytyrosol; B, tyrosol; C, vanillic acid; D, unknown phenolic compound with a retention time of 30.69 min; E, unknown phenolic compound with a retention time of 36.27 min; F, 3,4-DHPEA-EDA; G, (+)-pinoresinol; H, (+)-1-acetoxypinoresinol + *p*-HPEA-EDA; I, 3,4-DHPEA-EA; L, *p*-HPEA-EA.

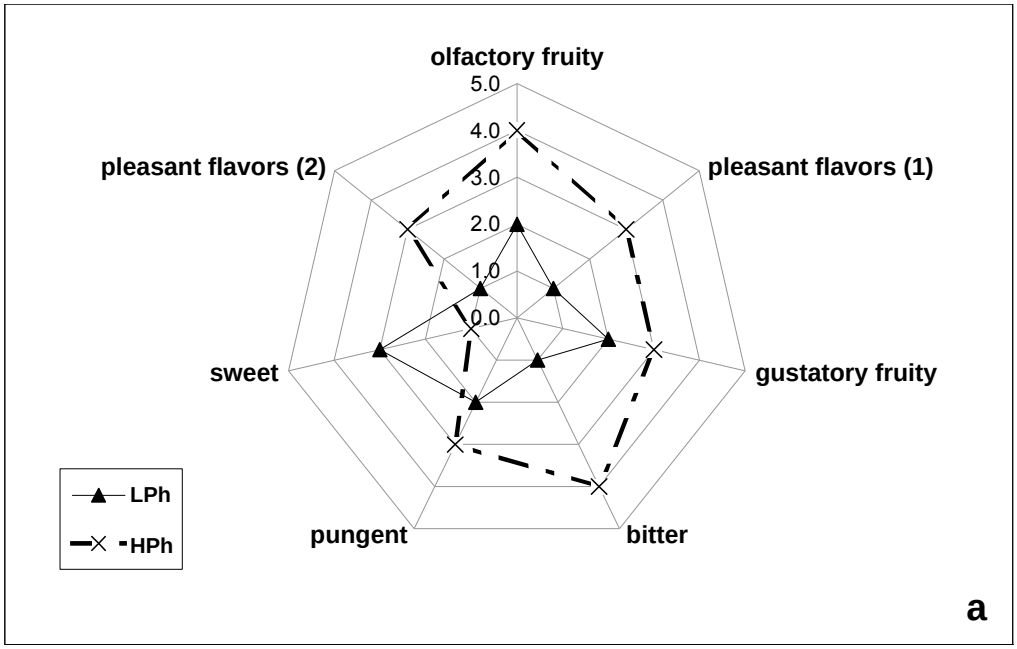
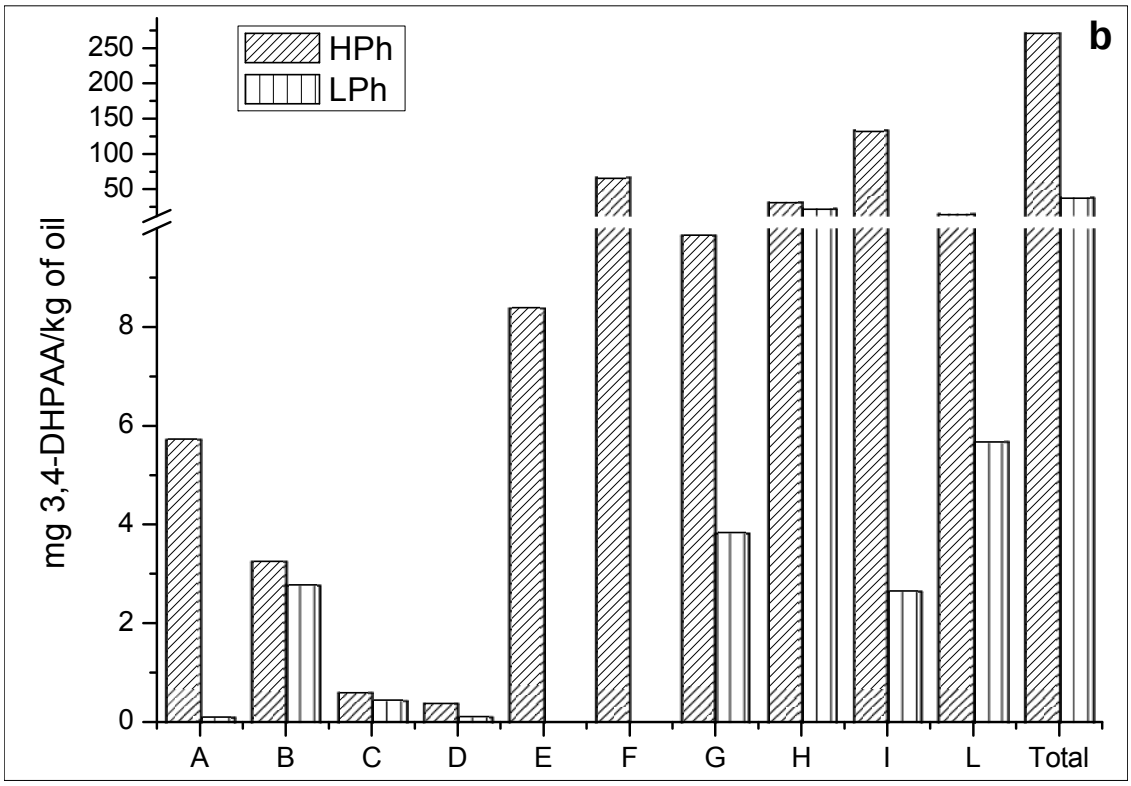


Figure 2. Cont.



A Quantitative Descriptive sensory Analysis (QDA) carried out by Rotondi *et al*. [130] confirmed a decreasing trend of the positive olive oil descriptors, such as bitterness and pungency, when *Nostrana di Brisighella* olives ripened. The highest statistically significant intensity was at the beginning of fruit skin pigmentation. The decrease in bitterness and pungency was also related to a reduction in total phenols and *o*-diphenols levels. In particular, a positive correlation between the secoiridoids content and bitterness and pungency was observed.

With the aim of reducing the bitterness intensity in VOOs, disfavored by many consumers when present at high intensity, some authors [131] developed postharvest technology based on hot-water treatments of olive fruits (cultivars Manzanilla, Picual, and Verdial) in the temperature range of 60-68°C or [132] with air-heating (40°C during 24, 48, and 72 h). These treatments promote a reduction in bitterness that is directly related to the time and temperature of treatment, probably due to a partial inhibition of glycosidases and esterases; in fact, these enzymes are involved in the release of secoiridoid derivatives from oleuropein during the crushing malaxation process. However, this heat treatment also affected other quality traits such as oxidative stability and color and could produce a change in the aroma profile of the VOO as well.

The standard method of analyzing the bitter taste of olive oil is by sensory analysis using a panel of tasters [133]. However, an analytical panel is often not likely to be available, since a permanent staff of trained tasters and a highly specialized panel chief is necessary. Many consumers from extra-European countries are not accustomed to the typical high intensity of bitterness or pungency of fresh VOO and, consequently, must be blended with less bitter VOO. For this reason, methods for the evaluation of the bitterness level based on physical-chemical determinations would be very useful for the industry. Several authors have found a strong relationship between these sensory attributes and the content of phenolic compounds in the olive oils. In 1992 Gutiérrez *et al.* [134] proposed an analytical method for

measurement of bitterness, based on solid phase extraction (SPE) of phenols and their spectrophotometric detection at 225 nm; this parameter termed IB or index of bitterness, was highly correlated to the sensory intensity of bitterness and is still the most widely used for its determination. Some years later, Mateos and co-workers [135] showed that several non-bitter phenolic compounds could also absorb at 225 nm; consequently, they asserted that this index was not appropriate for comparing bitterness of VOO obtained from blend of olive varieties characterized by very different phenolic profiles (e.g. Picual and Arbequina). Moreover, Mateos suggested that evaluation of the bitterness level of a VOO could be described by the experimental equation obtained from the regressions between intensity of bitterness and the concentrations of oleuropein aglycon using a Panel test and chromatographic analysis, respectively.

Some researchers suggest that secoiridoid derivatives of hydroxytyrosol are the main contributors to olive oil bitterness. Recently, a procedure called taste dilution analysis (TDA) was reported by Frank and co-authors to underlie the sensory threshold of bitter for oleuropein derivatives [136]. Bitterness was assessed by preparing serial dilutions of samples in water and then tasting in order of increasing concentration until the concentration at which the diluted sample can be differentiated from water as judged in a triangle test is found. When an isomer (or isomers) of oleuropein aglycon was prepared by β-glucosidase hydrolysis of oleuropein isolated from olives and evaluated by assessors, it was found to be bitter with a threshold of 50 μmol. Using the same evaluation technique, no bitterness was observed for hydroxytyrosol or elenolic acid.

Andrewes *et al.* [137] assessed the relationship between polyphenols and olive oil pungency. *p*-HPEA-EDA was the key source of the burning sensation found in many olive oils. In contrast, 3,4-DHPEA-EDA, tasted at an equivalent concentration, produced very little burning sensation. This is a clear example of the different sensory properties of a secoiridoid derivative of hydroxytyrosol and tyrosol. In 2005, Beauchamp and co-authors [64] measured the pungent intensity of *p*-HPEA-EDA isolated from different VOO confirming this molecule is the principal agent in VOO responsible for throat irritation. These researchers also tested the throat-irritant properties of its synthetic form (named "oleocanthal", with oleo- for olive,-canth- for sting, and -al for aldehyde) dissolved in non-irritating corn oil. They found an effect comparable to that of the purified compound from VOO and a dose-dependent activity.

In 2003, Gutierrez-Rosales and co-authors [138] isolated the major peaks found in the phenolic profile of VOO using preparative HPLC; after dissolving in water these molecules purified were then tasted to evaluate the intensity of bitterness. It was concluded that the peaks corresponding to the 3,4-DHPEA-EDA, 3,4-DHPEA-EA and p-HPEA-EDA were those mainly responsible for the bitter taste of VOO. As previously reported, Mateos *et al.* [135] verified the better correlation between the aldehydic form of oleuropein aglycon and bitterness.

Recently some researchers [139] have studied the temporal perception of bitterness and pungency in monovarietal VOOs; analyses were performed by a trained sensory panel utilizing a time–intensity (TI) evaluation technique; bitterness curves had a faster rate of rising and declining than pungency curves: the curves for bitterness reached a maximum after approximately 16–20 s, whereas the maximum of the perception of pungency is registered between 26 and 29 s and is independent of the maximum intensity of the perception.

As already discussed, several authors have associated some phenols with bitterness, thus obtaining models and relationships between individual phenols separated by HPLC and bitterness intensity [135,

138,140]. In these reports, bitterness was measured by a panel test or calculated from K_{225} values. Moreover tyrosinase- and peroxidase-based biosensors are being developed for the bitterness assessment [141], and are showing interesting possibilities. However, HPLC is often not available in many olive oil mill laboratories because of economic reasons, as well as specialized technical staff and biosensors. As an alternative, Beltrán and co-authors [142] proposed that measurement of phenol content can be used. This is a simple analytical method that involves liquid-liquid extraction and colorimetric measurement using Folin-Ciocalteau reagent [143]. In their experimental work, the authors analyzed the relationship between phenol content and K_{225} for oils from four of the most important olive cultivars worldwide (Frantoio, Hojiblanca, Picual, Arbequina); 360 samples were used to develop the model. As a practical application, bitterness intensities were evaluated by sensory analysis of 25 VOO samples, and were then estimated by applying the prediction model. In order to provide an easy tool for bitterness estimation, VOO bitterness was classified by its phenol content into four categories (results expressed as mg of caffeic acid per kg of oil and intensity of bitterness between 0 and 5 values): phenol contents equal or lower than 220 mg kg^{-1} corresponded to non-bitter oils or oils with almost imperceptible bitterness (intensities 0–1.5); slight bitterness corresponded to 220–340 mg kg^{-1} (intensities 1.6–2.5); bitter oils have a phenol contents ranging from 340 to 410 mg kg^{-1} (intensities 2.5–2.99); and a phenol contents higher than 410 mg kg^{-1} corresponds to quite bitter or very bitter oils (intensities higher than 3). In general, the authors determined that the oils were classified correctly into the same bitterness categories by both methods at 92%, achieving 100% of correct classification for the lowest and highest bitterness categories.

New analytical approaches to characterization of the phenolic profile and applied studies during the last decade

In order to utilize VOO as a source of phenolic compounds, to develop complete compositional databases and to obtain more accurate data about the intake of antioxidants further chemical characterization is needed. Identification and quantitation, based traditionally on HPLC (with different detectors, such as UV, fluorescence, coulometric electrode array detection, amperometric detector) [144-150], GC-FID [151-154] and, more recently CE-UV, can be aided today by MS and NMR, which is a focus of the present review.

Liquid chromatography/mass spectrometry (LC-MS) has been widely accepted as the main tool in identification, structural characterization and quantitative analysis of phenolic compounds in olive oil. Using a mass spectrometer for detection offers some undoubted advantages, such as independence of a chromo- or fluorophore, lower LOD than UV in most cases [155], the possibility to obtain structural information and easy separation of coeluting peaks using the information about mass as a second dimension.

The sensitivity of response in MS is clearly dependent on the interface technology employed. In LC-MS analysis of phenolic compounds, atmospheric pressure ionization interfaces, i.e. APCI and electrospray ionization (ESI), are used almost exclusively today, and both positive and negative ionization are applied. In general, phenolic compounds are detected with a greater sensitivity in the negative ion mode, but the results from positive and negative ion modes are complementary, and the positive ion mode shows structurally significant fragments [156].

On the other hand, optimal ionization depends not only on the interface parameters, but also on the mobile phase of the liquid chromatography. As a first rule, the use of non-volatile salts in the mobile phase (common in other chromatographic methods) should be avoided, as they would interfere with the ionization source. The mobile-phase composition and its pH need also careful optimization as they may influence the ionization efficiency of the analytes.

The selection of the analyzer, apart from its accessibility, is determined by the required sensitivity and selectivity and the general objectives. LC-atmospheric pressure ionization (API)-MS typically only yields a single strong ion, which reduces its ability to make analyte accurate identifications. In the most cases, single-stage MS is used in combination with UV detection to facilitate the identification of phenolic compounds in olive oil samples with the help of standards and/or reference data. Ion Trap or QqQ provide the possibility of doing MS/MS or MS^n, which can be used for structure elucidation or for additional selectivity to gain sensitivity by reducing the chemical noise [157]. MS/MS and MS^n involve two (or more) stages of mass analysis, separated by a fragmentation step. TOF MS, which is one of the most advanced MS analyzers, provides excellent mass accuracy [158] over a wide dynamic range if a modern detector technology is chosen. The latter, moreover, allows measurements of the correct isotopic pattern [159], providing important additional information for the determination of elemental composition [160].

Table 2 provides an overview of methodologies based on LC-tandem mass spectrometry used for the analysis of phenolic compounds in olive oil. The table does not include several publications in which the analysis of olive fruit, leaves, pulp and pomace, olive tree wood, as well as olive oil waste waters were carried out by using HPLC-MS [14, 49, 156, 161-168]. Other important issues are the presence of phenolic metabolites of VOO in the human low density lipoprotein fraction [169-170].

High-resolution spectroscopic techniques, and particularly NMR spectroscopy, are finding interesting applications in the analysis of complex mixtures of various food extracts that contain phenols.

During the past decade proton nuclear magnetic resonance spectroscopy (NMR) has been successfully used in olive oil analysis [171-172]. Currently available high-resolution spectroscopic techniques, coupled with the facilities of computerized mathematical or other treatment of data have found interesting applications in the field of agricultural and food science without the necessity for a separative technique coupled with NMR, as commented by Gerothanassis [173]. Additionally the usefulness of ^{1}H NMR spectroscopy has been increasingly recognized for its non-invasiveness, rapidity, and sensitivity for a wide range of compounds in a single measurement. However, difficulties may arise in relation to the information obtained from spectra of multicomponent mixtures such as olive oil. Strong signal overlap, dynamic range problems, diversity of intensities due to various concentrations of the food constituents, and the inherent lack of scalar coupling information between different moieties lead to ambiguous or incomplete assignments, thus hindering detection even with the use of multidimensional NMR [174]. One possible approach to these problems involves the combination of the advantages of NMR spectroscopy with those of chromatography. Coupled techniques such as LC-NMR or LC-NMR/MS may provide information on overall composition and enable the identification of individual phenols in complex matrices. Moreover, on-line solid phase extraction (SPE) in LC-NMR for peak storage after the liquid chromatography separation prior to NMR analysis or similar techniques have been recently applied.

Table 2. Summary of separation of phenolic compounds in the polar fraction of VOO using HPLC-MS methods.

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
120 min	**A:** H_2O **B:** MeOH	Spherisorb ODS 25 cm x 4.6 mm i.d.; 10μm	Gradient	Combination between LLE [42] and SPE	UV in HPLC. MS off-line	Separation of the polar fraction of VOO in two parts. Antioxidant activity assessment	[175]
93 min	**A**: H_2O + CH_3COOH 0.5% **B**: MeCN	Spherisorb ODS 2, 25 cm x 4.6 mm i.d.	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	[41]
25 min	H_2O:CH_3CN (82:18 v/v) + CH_3COOH 0.02%	Nucleosil ODS, 25 cm x 2.1 mm or 25 cm x 1.1 mm i.d. 5 μm	Isocratic	LLE with buffer; SPE with phenyl cartridges (acidification)	UV, fluorescence, MS, MS/MS HPLC-APCI (negative ion mode)	Phenolic acids	[30]
60 min approx.	**A**: H_2O + HCOOH 0.045% **B**: MeOH + HCOOH 0.045%	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 μm	Gradient	-Phenolic acids as Cartoni [30] -HYTY and TY: 3 g oil across cartridge phenylic	MS; MS/MS	Olives and VOOs. MS/MS using Multiple Reaction Monitoring (MRM) (high specificity and sensitivity in MS spectra)	[176]
HPLC method and conditions of Cortesi et al. [177]		C18 column (RP) Alltech 25 cm x 4.6 mm i.d.	Gradient	LLE: Montedoro et al. [1] using butylated hydroxytoluene (BHT)	MS; MS/MS	Analysis of oleuropein aglycon by APCI-MS. Phenolic compound profile	[178]
HPLC method of Romani et al. [179]		Lichrosorb RP18, 25 cm x 4.6 mm i.d. 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	[180]
60 min	**A**: H_2O + CH_3COOH 2mM **B**: MeOH + CH_3COOH 2mM	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 μm	Gradient	LLE with methanol/water (80:20 v/v), acidification and passed through a C18 cartridge	MS and MS/MS (API/MS in negative ion mode)	Identification of a new class of phenolic compounds in olive oils: hydroxy-isochromans	[35]

Table 2. Cont.

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
HPLC method and conditions of Brenes et al. [44]					UV; electrochemical, fluorescence, MS.	Use of a lignan (1-acetoxypinoresinol) to authenticate Picual VOOs. Use of GC too.	[35]
50 min or 70 min	**A**: H_2O + H_3PO_4 0.5% **B**: MeOH/ MeCN (50:50 v/v)	Lichrospher 100 RP18, 25 cm x 4.0 mm i.d. 5 μm	Gradient	SPE (diol-bound phase)	UV, HPLC-MS in ESI(positive ion mode)	Dialdehydic and aldehydic forms of oleuropein aglycon and ligstroside aglycon	[181]
65 min	**A**: H_2O + CH_3COOH 2% **B**: MeOH/ MeCN (50:50 v/v)	C18 Luna column, 25 cm x 3.0 mm i.d. 5 μm	Gradient	Comparative study of 5 extraction methods (LLE and SPE)	UV, DAD; MS	HPLC and CE methods. (HYTY, TY, oleuropein, ligstroside aglycon and decarboxymethyl oleuropein aglycon)	[182]
65 min	**A**: H_2O + CH_3COOH 2% **B**: EtOH	Phenomenex Luna (phenyl-hexyl)phase; 25 cm x 4.6 mm i.d. 5 μm	Isocratic	LLE with methanol/water (80:20 v/v) Montedoro et al. [1]	UV; MS (ESI in negative ion mode)	Isolation of individual polyphenols to study sensory properties	[137]
60 min approx.	**A**: H_2O + HCOOH 0.09% **B**: MeOH + HCOOH 0.09%	Nucleosil ODS, 25 cm x 2.1 mm i.d. 5 μm	Gradient	Separation of phenolic compounds in two fractions after C18 cartridge. Group A: 12 g oil Group B. 3 g oil	UV; fluorescence; MS; MS/MS	Improve of extraction system of [176]. Determination of isomer of dihydroxy- and dimethoxybenzoic acids. Comparison among LOD in HPLC-UV, HPLC-FL and HPLC-MS/MS	[183]
75 min	**A**: H_2O + CH_3COOH 0.5% **B**: MeCN	C18 Luna column, 25 cm x 3.0 mm i.d. 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 stability and organoleptic properties of olive oil	[184]
HPLC method of Rotondi et al. [184]			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	[185]

Table 2. Cont

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
60 min	**A**: H_2O + 0.2% acetic acid **B**: MeCN	Lichrospher 100, 12.5 cm x 4.0 mm i.d. 5 μm	Gradient	LLE with methanol (500 mg of oil)	Refractive index detector; MS	TY, Vanillic acid, Lut and Apig. Squalene (with Refractive Index detector). Quantitation in 7 samples.	[45]
45 min	**A**: H_2O + 0.2% acetic acid **B**: MeCN	Inertsil ODS-3, 15 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (80:20, v:v) (45 g of oil)	UV; ESI-MS	Antioxidant activity of olive pulp and olive of Arbeq. cv	[46]
40 min	**A**: H_2O + 0.2% acetic acid **B**: MeCN	C18 Luna column, 15 cm x 2.0 mm i.d. 5 μm	Gradient	Diol cartridge (3 g of oil)	UV (DAD); MS; MS/MS (QqQ)	Quantification of 23 compounds in 3 olive oils. Possible models of derived secoiridoids (nine basic models of Lig and Ol aglycons found in bibliography)	[186]
70 min	**A**: H_2O + 0.5% acetic acid **B**: MeCN	C18 Luna column, 25 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (60:40, v:v) (60 g of oil)	UV (DAD); MS	Isolation of several phenolic compounds and study of their antioxidants properties (DPPH, OSI and electrochemical method)	[117]
50 min	**A**: H_2O + HCOOH (pH 3.2) **B**: MeCN	C18 Luna column, 25 cm x 3.0 mm i.d. 5 μm	Gradient	LLE with ethanol/water (7:3, v:v) (25 ml of oil)	UV (DAD); ESI-MS	Evaluation of lignans free and linked HYTY and TY in VOO. TLC to determine the presence of lignans.	[187]
30 min	**A**: H_2O + 0.1% acetic acid **B**: MeCN	RP C18 2.1 x 100 mm, 3.5 mm particle size; XTerra MS	Gradient	SPE-Diol (60 g of oil) diluted 1:10	ESI-TOF (TOF)	Determination of all the well-known phenolic compounds of oil and more than 25 “new” compounds	[188]

One of the pioneers in this field was Montedoro [3] who identified four new phenolic compounds in olive oil in 1993. This paper reported the NMR, UV and IR characterization of the compounds under study, and finally, concluded that the newly identified compounds were an isomer of oleuropein aglycon, the dialdehydic from of elenolic acid linked to hydroxytyrosol, and the dialdehydic from of elenolic acid linked to tyrosol. The results obtained by Limiroli [189] and Bariboldi [190] were useful in contributing to a more in-depth understanding of the secoiridoid fraction of VOO. Following these results, several authors have used NMR to analyze phenolic compounds in olive oils, which summarize the reports which include methodologies combining HPLC and NMR (Table 3).

Table 3. Summary of separation of phenolic compounds in the polar fraction of VOO using HPLC-NMR (as coupled techniques or by NMR as off- line technique after HPLC).

Time of analysis	Mobile phase	Stationary phase	Type of elution	Extraction System	Detection System	Observations	References
45 min	**A**: H_2O + CH_3COOH 2% (pH 3.1) **B**: MeOH	Erbasil C_{18}, 15 cm x 4.6 mm i.d.	Gradient	LLE with methanol/water	UV; NMR and IR	Spectroscopic characterization of secoiridoid derivatives	[3]
60 min	**A**: H_2O + CH_3COOH 0.2% **B**: MeOH	Spherisorb ODS 2, 25 cm x 4.6 mm i.d.	Gradient	Same as Montedoro et al. [1]	Photodiode array; MS; NMR	Simple phenols, flavonoids, secoiridoids	[44]
HPLC method of Montedoro et al. [1]		Column RP18 Latex; 25 cm x 4.0 mm i.d. 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).	[34]
HPLC method of Montedoro et al. [1-3]		Column RP18 Latex; 25 cm x 4.0 mm i.d. 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	[191]
50 min	**A**: H_2O + CH_3COOH 3% **B**: MeCN:MeOH (50:50 v/v)	Lichrosphere 100 RP18, 25 cm x 4.0 mm i.d. 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 aglycon)	Phenols, flavones and lignans. Colorimetric determination of *o*-diphenols. GC-MS	[192]

Table 3. Cont,

60 min	**A**: H_2O + CH_3COOH 0.5% **B**: MeOH/ MeCN (50:50 v/v)	Lichrospher 100 RP_{18}, 25 cm x 4.0 mm i.d. 5 μm	Gradient	Comparative study of LLE and SPE (diol and C_{18}-phase)	Photodiode array detector; MS, NMR.	Simple phenols, secoiridoids and lignans	[193]
30 min	**A**: H_2O + 0.1% trifluoroacetic (TFA-*d*) **B**: MeCN+ 0.1% (TFA-*d*)	Phenomenex RP-C18, 25 cm x 4.6 mm i.d. 5 μm	Gradient	LLE with methanol/water (80:20 v/v) (50 g of oil)	LC-SPE-NMR system	Complete characterization of 27 phenolic compounds in olive oil. 7 compounds not detected in the past	[174]

Recently, Christophoridou *et al.* [174] reported the first application of the hyphenated LC-SPE-NMR technique using postcolumn solid-phase extraction to direct identification of new phenolic compounds in the polar fraction of VOO. The addition of a post-column SPE system to replace of the loop system of the LC-NMR, resulted in higher sensitivity (significant increase of the signal to noise [S/N] ratio); in fact, S/N improvements by up to a factor of 4 could be demonstrated with this new technology [16]. The spectra recorded were one dimensional (1D) ^{1}H-NMR and two dimensional (2D) NMR. The presence of phenols was confirmed from the respective LC-SPE-NMR spectra, which were assigned on the basis of existing ^{1}H-NMR databases and with total correlation spectroscopy (TOCSY). The most interesting findings of this study were the verification of the presence of the lignan syringaresinol, the presence of two stereochemical isomers of the aldehydic form of oleuropein and the detection of homovanillyl alcohol.

As commented above the researches that studied olive mill waste, brines olive drupes, tissues of olive cultivars, alperujo, olives, olive leaves were not included in Table 3 [49, 161, 194-199]. Servili *et al.* [200] in a HPLC investigation of the phenols present in olive fruit, VOO, vegetation waters and pomace, and subsequently by 1D- and 2D-NMR achieved the complete spectroscopic characterization of demethyloleuropein and verbasoside extracted from olive fruit.

There are also several interesting reports describing the analysis of olive oil by ionspray ionization tandem mass spectrometry (IS-MS/MS) and ESI-MS/MS with NMR, without the use of a previous separative technique [201-203]. For the purposes of this review it is important to include a recent publication by Christophoridou *et al.* [204], where the authors demonstrate the potential of ^{31}P-NMR spectroscopy to detect and quantify a large number of phenols in VOO extracts. This novel analytical method is based on derivatization of the hydroxyl and carboxyl groups of phenolic compounds with 2-chloro-4,4,5,5 tetramethyldioxaphospholane and the identification of the phosphitylated compounds on the basis of the 31P chemical shifts.

Even if the characterization and quantification of phenolic compounds have been successfully carried out by GC and HPLC, the use of faster analytical techniques and screening tools, allowing a rapid screening of phenolic compounds from VOOs, is strongly recommended. Although compared with GC or HPLC, CE is a relatively new technique in food analysis. A large variety of foods have

already analyzed by this technique, as CE can represent a good compromise between analysis time and satisfactory characterization for same classes of phenolic compounds in VOO.

CE is characterized by high separation efficiency, small sample and electrolyte consumption, and the separation requires only several minutes. This last characteristic is the main advantage versus chromatographic methods, which makes CE useful for routine analysis as well as for controlling and monitoring processes in a number of industrial fields [205-213]. Moreover, CE is relatively well suited to analysis of samples with complex matrices, like VOO.

CE technique can be coupled with different detectors (UV, FIL, electrochemical detectors, MS…). To date, for the analysis of phenolic compounds in VOO, there are several papers reporting the use of CE with ultraviolet detection; it is possible to study results obtained by using CE-MS in only two papers (Table 4).

Along these lines, the use of CE as an analytical separation technique coupled with mass spectrometry as a detection method can provide important advantages in the analysis of phenolic compounds of olive oil because of the combination of the high separation capabilities of CE and the power of MS for identification and confirmation method.

Using mass spectrometric detection, differences in optical detection must be considered. First, the separation electrolyte has to be volatile, reducing the choice of buffering system primarily to ammonia, acetate, or formate. While there are reports documenting nonvolatile buffers from UV-CE, only low buffer concentrations can be used and thus lower sensitivity must be accepted. Generally, nonaqueous solvents are well-suited for hyphenation with MS and add another parameter to modify selectivity.

As commented before for HPLC coupled with MS, CE can also be coupled with different MS analyzers (i.e., with quadrupole, ion trap, time-of-flight, etc.) and use several ionization methods (APCI, ESI, MALDI). 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. Regarding the analyzers, ion trap (IT) and TOF systems are the two analyzers more common in the lab of food analysis [214], although single-quadrupole MS is still often used as an easy and affordable detector.

Of particular interest is the coupling of CZE to ESI-TOF-MS. This coupling combines the abovementioned benefits of CZE separation with the high selectivity due to mass accuracy of 5 ppm, which opens the possibility of determining elemental compositions. The analysis of the true isotopic pattern by ESI-TOF-MS has recently been shown to provide an additional analytical dimension for identification [160].

During the last decade, concerning phenolic compounds present in VOO, it is possible to find reports in which applicative work is carried out, as well as other where a new analytical method is developed. Herein, the publications including CE-UV and CE-MS are summarized (see Tables 4 and 5).

Table 4. Summary of optimized conditions of capillary electrophoresis methods where VOO samples are analyzed. V, voltage; T, temperature, i. d., internal diameter of capillary; L_{ef}, effective length of capillary; [Buffer]; buffer concentration.

	Instrumental Variables						**Experimental Variables**			
References	**λd [nm]**	**V [kV]**	**T [ºC]**	**i. d. [μm]**	**Lef [cm]**	**t_{inj} [s]**	**Type of Buffer**	**[Buffer] [mM]**	**pH**	**Organic modifiers and other variables**
[182]	200	27	30	50	40	3 s (0.5 p.s.i)	Sodium Tetraborate	45	9.6	-
[215]	CZE method of Bendini et al.[182]									
[185]	CZE method of Bendini et al.[182]									
[31]	210	25	25	75	50	8 s (0.5 p.s.i)	Sodium Tetraborate	25	9.6	-
[29]	200	18	25	50	36	2 s (1.5 p.s.i)	Sodium Tetraborate	40	9.2	-
[32]	210	-25	25	75	50	8 s (0.5 p.s.i)	Sodium Tetraborate	50	9.6	20% 2-propanol
[216]	214/250	25	25	75	100	8 s (0.5 p.s.i)	Sodium Tetraborate	30	9.3	
[217]	214/MS (ESI-IT)	25	25	50	100	10 s (0.5 p.s.i)	NH_4OAc	60	9.5	5% 2-propanol Sheath liquid (60:40 v/v 2-propanol/ water and 0.1% v/v of TEA at a flow rate of 0.28 mL/h)
[218]	200/240/ 280/330	28	22	50	40	8 s (0.5 p.s.i)	Sodium Tetraborate	45	9.3	
[219]	CZE method of Carrasco-Pancorbo [218]									
[188]	MS (ESI-TOF)	30	25	50	85	10 s (50 mBar)	Ammonium hydrogen carbonate	25	9.0	Sheath liquid (2-propanol/ water 50:50 v/v at a flow rate of 4 μL/min)

Table 5. Summary of extraction systems used and compounds detected in VOO samples with the application of each method. HYTY, hydroxytyrosol; TY, tyrosol; DHPE, 2,3-dihydroxyphenylethanol; VA, vanillic acid; DOA, decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA); Ac Pin, (+)-1-acetoxypinoresinol; Lig Agl, ligstroside aglycone; Ol Agl, oleuropein algycone; EA, elenolic acid

References	Initial quantity of oil→Final quantity of solvent ($MeOH/H_2O$ (50:50 v/v)) in the extraction process (kind of extraction)	Detected compounds in olive oil	Other relevant aspects
[182]	2 g → 1 mL (LLE [220])	HYTY, TY, unidentified secoiridoids compounds	1st paper where CE is used for the analysis of phenolic compounds from oils
[215]	2 g → 1 mL (LLE [220])	HYTY, TY, DHPE, unidentified oleuropein aglycone derivatives	
[185]	2 g → 0.5 mL (LLE [220], as modified in [184])	HYTY, TY, VA, DOA, Ac Pin	
[31]	60 g → 0.5 mL (LLE [31])	13 phenolic acids + taxifolin (flavanonol)	Potent extraction system which permits detection of small quantities of phenolic acids
[29]	10 g → non specified (Combination of LLE-SPE [29])	5 phenolic acids	
[32]	60 g → 0.5 mL (LLE [31])	13 phenolic acids + taxifolin (flavanonol)	Co-electroosmotic CE
[216]	60 g → 2 mL (SPE-Diol [216])	TY, Pin, Ac Pin, DOA, Lig Agl, HYTY, Ol Agl, EA	Use of standards obtained by semipreparative-HPLC
[217]	60 g → 2 mL (SPE-Diol [216])	11 phenols (simple phenols, lignans, complex phenols and EA)	1st paper in which CZE-ESI-IT MS is used for the analysis of phenolic compounds from oils
[218]	60 g → 2 mL (SPE-Diol [216])	26 compounds belonging to all the different families of phenolic compounds present in olive oil	26 compounds in less than 10 min. 1st paper in which flavonoids are detected by CE, and 1st "multicomponent" method for the determination of olive oil phenols
[219]	60 g → 2 mL (SPE-Diol [216])	Applicative work using a previously method [218]	Interesting from a quantitative and applicative point of view
[188]	60 g → 2 mL (SPE-Diol [216]) and diluted 1:10	All the "well-known" phenolic compounds and 28 other analytes	1st paper in which CZE-ESI-TOF MS is used for the analysis of phenolic compounds from oils. TOF permits the "identification" of new compounds in the oil's profile

Concluding remarks and future outlook

The amount of phenolic compounds is a very important parameter when evaluating the quality of VOOs. Phenols are closely related with both the resistance of the oil to oxidation and the typical bitter and pungent tastes. Furthermore, some studies have shown that the amount of phenols, particularly those with a catecholic structure, together with a favorable monounsaturated to polyunsaturated fatty acid ratio, is related to several healthy attributes. These different aspects make VOO a very valuable and appreciated dietary lipidic condiment, and add importance to the determination of its phenolic compounds, both qualitative and quantitatively. The most commonly methods used for phenolic determination in VOO are based on GC and HPLC, and more recently on CE, coupled with different detector systems (UV, FLD, amperometric or coulometric). If the literature regarding phenolic compounds of VOO is analyzed in detail, it is evident that this class of compounds has not been completely studied, because of the complexity of their chemical nature and the complexity of the matrix in which they are found. During the last ten years, MS and NMR have become indispensable to study the quali-quantitative profiles of phenols and their oxidative forms, and detectors with the power to identify compounds and provide the analyst with information about the molecular structure are essential.

Apart from the interest on knowing in composition of the polar fraction of VOO, the determination of these compounds also helps to understand their health benefits that include reduction of risk factors of coronary heart disease, prevention of several varieties of cancer and modification of immune and inflammatory responses. It is also of interest to distinguish what phenolic molecules are responsible for bitterness, pungency, astringency and metallic sensations and to evaluate the antioxidant activities of the polar fraction.

Although excellent progress has already been made, it is expected that the use of different methodologies of potent techniques coupled with rapid, reliable and sophisticated detectors will become more common in the near future; there are still many “unknown” compounds present in the polar fraction of olive oil and it is very important to carry out collaborative studies to join the efforts of the scientific community.

Abbreviations: 3,4-dihydroxyphenyl-ethanol or hydroxytyrosol (3,4-DHPEA); 3,4-dihydroxyphenyl-ethanol acetate or hydroxytyrosol acetate (3,4-DHPEA-AC); 3,4-dihydroxyphenyl-ethanol linked to elenolic acid (3,4-DHPEA-EA); 3,4-dihydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (3,4-DHPEA-EDA); 1-acetoxypinoresinol (Ac Pin); buthylated hydroxytoluene (BHT); Capillary Electrophoresis (CE); cyclooxygenase (COX); diphenylpicrylhydrazyl (DPPH); elenolic acid (EA); electrospray ionization mass spectrometry (ESI-MS); Flame Ionization Detector (FID); gas chromatography (GC); High Performance Liquid Chromatography (HPLC); HPLC-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS); ion-trap mass spectrometry (IT-MS); low-density lipoprotein (LDL); Liquid-Liquid- extraction (LLE); limit of detection (LOD); mass spectrometry (MS); tandem mass spectrometry (MS/MS); multiple-stage mass spectrometry (MS^n); nuclear magnetic resonance (NMR); Oxidative Stability Instrument (OSI); *p*-hydroxyphenyl-ethanol or tyrosol (*p*-HPEA); *p*-hydroxyphenyl-ethanol linked to elenolic acid (*p*-HPEA-EA); *p*-hydroxyphenyl-ethanol linked to dialdehydic form of elenolic acid (*p*-HPEA-EDA); pinoresinol (Pin); triple

quadrupole (QqQ); Solid-Phase Extraction (SPE); virgin olive oil (VOO); secoiridoid (SID); time of flight mass spectrometry (TOF-MS); Total Peak Area Ratio (TPAR); ultraviolet (UV).

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Capítulo 2

Identificación y cuantificación mediante electroforesis capilar de los compuestos de la fracción fenólica del aceite de oliva virgen extra.

Publicación incluída en este capítulo:

Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil.
(*Electrophoresis*, **2005**, 26, 3538–3551)

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Uno de los objetivos iniciales de la tesis doctoral fue el estudio de la fracción fenólica del aceite de oliva, en concreto de aquellos compuestos que no habían podido determinarse previamente mediante electroforesis capilar y que constituyen gran parte de esta fracción.

Aunque ya había sido estudiada previamente utilizando otras técnicas como HPLC e incluso GC, ésta era la primera vez que se afrontaba su estudio mediante el uso de electroforesis capilar. Hasta la fecha se habían publicado varios trabajos para el análisis de este tipo de compuestos, pero en ellos se centraban exclusivamente en el estudio de los ácidos fenólicos o la identificación realizada sobre muestras reales no daba resultados muy satisfactorios [216,217,84,86]. El fuerte interés por el empleo de una técnica nueva era, al mismo tiempo que demostrar la potencialidad de la electroforesis capilar para este tipo de determinaciones, conseguir disminuir los tiempos de análisis manteniendo una buena separación.

Por ello llevamos a cabo la puesta a punto de un método electroforético en zona capaz de determinar una gran parte de los compuestos pertenecientes a la fracción fenólica.

Como paso previo a la puesta a punto del método se realizaron una serie de trabajos preliminares:

- Puesta a punto del método de extracción de los compuestos de interés.
- Aislamiento de compuestos fenólicos no comerciales mediante HPLC.

[216] Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Gallina-Toschi, T.; Lercker, G., Liquid-liquid and solid-phase extraction from phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. *Journal of Chromatography A* **2003**, 985, 425-433.

[217] Bonoli, M.; Montanucci, M.; Gallina-Toschi, T.; Lercker, G., Fast separation and determination of tyrosol, hydroxytyrosol and other phenolic compounds in extra-virgin olive oil by capillary zone electrophoresis with ultraviolet-diode array detection. *Journal of Chromatography A* **2003**, 1011, 163-172.

Se pretendía, por una parte, extraer el mayor número de compuestos posible con un porcentaje de recuperación elevado. Y por otra parte, obtener patrones de aquellos compuestos de los cuales no existían patrones comerciales con el fin de poder llevar a cabo la cuantificación.

Este método permitió por primera vez la identificación de compuestos como el pinoresinol, ligustrósido aglicona, oleuropeína aglicona y ácido elenólico mediante electroforesis capilar, dándose una alternativa a la ampliamente usada HPLC y demostrando la versatilidad de la EC.

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Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil

A capillary zone electrophoresis method has been carried out to determine and quantitate some compounds of the polyphenolic fraction of virgin olive oil which have never previously been determined before using capillary electrophoresis, such as elenolic acid, ligstroside aglycon, oleuropein aglycon, and (+)-pinoresinol. The compounds were identified using standards obtained by semipreparative high-performance liquid chromatography (HPLC). A detailed method optimization was performed to separate the phenolic compounds present in olive oil using a methanol–water extract of Picual extra-virgin olive oil, and different extraction systems were compared (C18-solid phase extraction (SPE), Diol-SPE, Sax-SPE and liquid-liquid extraction). The optimized parameters were 30 mM sodium tetraborate buffer (pH 9.3) at 25 kV with 8 s hydrodynamic injection, and the quantitation was carried out by the use of two reference compounds at two different wavelengths.

Keywords: Antioxidant; Capillary zone electrophoresis; Olive oil; Polyphenols
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1 Introduction

Extra-virgin olive oil is unique among other vegetable oils because of the high level of phenolic compounds [1]. These levels are possible because it is obtained from the olive fruit (*Olea Europea* 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.

Among the various components of the unsaponificable fraction of the olive oil, phenol compounds are the most important for their contribution to flavor, stability, and nutritional value of oil [2, 3]. Besides, it has been commented that the consumption of olive oil contributes to lower the incidence of coronary heart disease and some cancers [4].

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Abbreviations: DAD, diode array detector; **LLE**, liquid-liquid extraction

Some of the most important phenolic compounds in virgin olive oil are the ligstroside and oleuropein aglicons and their respective decarboxylated derivatives; the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol; simple phenols such as tyrosol and hydroxytyrosol; phenolic acids, namely, cinnamic or benzoic acids derivatives; and lignans, (+)-pinoresinol, and 1-(+)-acetoxypinoresinol [5].

In recent years, capillary electrophoresis (CE) has proved to be a fast technique allowing the combination of short analysis times and high separation efficiency for the analysis of food components [6, 7], particularly for phenolic compounds of extra-virgin olive oil [8–12], olive mill wastewater [13], and alperujo [14], but the most important polyphenols have not so far been identified and specially quantitated by CE, although these polyphenolic compounds have been determined by HPLC in a wide number of papers [15–21].

To determine the amount of polyphenols in virgin olive oil, it is very important to completely extract this fraction. Traditionally, the phenolic fraction of olive oil has been isolated by extraction of an oil solution in a lipophilic solvent (usually hexane) with several portions of methanol or methanol/water (with different levels of water ranging between 0 and 40%), followed by solvent evaporation of the aqueous extract and a cleanup of the residue by solvent partition obtaining good recoveries [22–25]. Despite the common assumption that C_{18}-phase is less suitable for the isolation of polar components from a nonpolar matrix

than normal-phase SPE, C_{18}-cartridges have often been tested for isolation of phenolics from virgin olive oil [26–28]. Anionic exchange cartridges have also been used to isolate the phenolic fraction from various seed oils [29].

The aim of this work is to identify several compounds of the polyphenolic fraction in seven Spanish varieties of extra-virgin olive oils using standards obtained by semi-preparative high-performance liquid chromatography (HPLC), and their quantitation using two reference compounds at two different wavelengths.

2 Materials and methods

2.1 Apparatus

Experiments were performed with a Beckman P/ACE™ System MDQ CE instrument connected to a diode array detector (DAD). A GOLD software (Beckman Coulter, Fullerton, CA, USA) installed in a PC 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. The ID of the capillary cartridge was 75 μm with a total length of 110 cm and an effective separation length of 100 cm. The temperature was controlled by the use of a fluorocarbon-based cooling fluid. HPLC analyses were performed with an HP 1100 series (Agilent Technologies, Palo Alto, CA, USA), equipped with a binary pump delivery system, a degasser, an autosampler, a diode array UV-VIS detector, and a mass spectrometer detector (MSD). The semipreparative HPLC column used was a Phenomenex Luna (C_{18}) column (Torrance, CA, USA), 10 μm ID, 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 ID, 25 cm × 3.0 mm (Phenomenex), with a C_{18} precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL/min.

2.2 Reagents, stock solutions, and reference compounds

Dopac (3,4-dihydroxyphenylacetic acid) was acquired from Sigma Aldrich (St. Louis, MO, USA), 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 dopac and 6500 μg/mL for oleuropein glucoside. Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma; and ammonium chloride and ammonium acetate from Panreac (Montcada I Reixac; Barcelona, Spain), which were all used as running buffers at different concentrations and pHs. Methanol and *n*-hexane were acquired from Panreac 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). Solid phase extraction (SPE) cartridges DSC-Diol and DSC-Sax were obtained from Supelco (Bellefonte, PA, USA), and C18-SPE was purchased from Varian Iberica S. L. (Madrid, Spain).

2.3 Samples

Extra-virgin olive oil samples were obtained from several Spanish geographic zones. Each of them was from a unique variety of olive fruit called Picual, Picudo, Hojiblanca, Lechín de Sevilla, Lechín de Granada, Arbequina, and Cornicabra. The concentration of polyphenols in oils changes during the storage, and this process is heavily influenced by oil acidity and variety. For this reason, all the olive oil samples used in this study were obtained at the same time of the year (November 2004). Refined sunflower oil was used as glyceridic matrix for recovery studies.

2.4 Preparation of oil spiked with phenolic extract

Virgin olive oil (120 g) dissolved in hexane (120 mL) was extracted with 120 mL of methanol/water (60/40 v/v) under nitrogen atmosphere. The methanolic phase was re-extracted with hexane (60 mL) to remove residual neutral lipids. Both phases were separated by centrifugation (4000 rpm, 15 min), and the methanolic phase was collected and evaporated (35°C) to dryness, and then the residue was dissolved in methanol (2 mL). Two refined sunflower oil samples spiked with the obtained phenolic extract were prepared. Aliquots (1 or 0.5 mL) of the methanolic extract were added to 60 g refined sunflower oil. After homogenization by intensive shaking, the solvent was evaporated using nitrogen.

2.5 SPE and liquid-liquid extraction (LLE) procedure

To determine the real amount of phenolic compounds in olive oil, it is necessary to completely extract this fraction from the extra-virgin olive oil. In order to isolate the phenolic fraction, two types of extraction system were used: SPE (C_{18}-SPE(a), Diol-SPE(b), Sax-SPE(c)) and LLE(d). The phenolic profiles were determined, and the results obtained using SPE methods and the liquid-liquid extraction method compared. The extraction protocol for (a)–(c) was the following: the cartridge was placed in a vacuum elution apparatus and conditioned by passing

10 mL methanol and then 10 mL hexane. Extra-virgin oil (60 g) was dissolved in 60 mL 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 at a temperature of 35°C. The residue was dissolved with 2 mL methanol/water (50/50 v/v) and filtered through a 0.25 μm filter before the CE analysis. The LLE was carried out with the extraction conditions and amounts of oil that are described by Carrasco Pancorbo *et al.* [11].

2.6 HPLC isolation and analysis of polyphenolic compounds

2.6.1 Isolation of polyphenols using semipreparative HPLC analysis

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, and the run was ended. 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. In this way, the following compounds were isolated: hydroxytyrosol, tyrosol, elenolic acid, deacetoxy oleuropein aglycon, (+)-pinoresinol, (+)-1-acetoxypinoresinol, oleuropein aglycon, and ligstroside aglycon. The structures of the isolated compounds can be seen in Table 1.

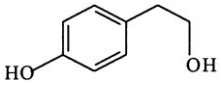
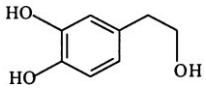
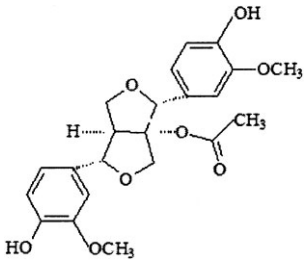
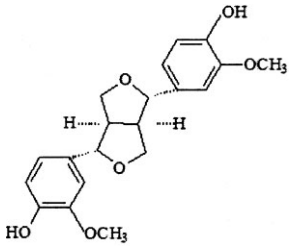
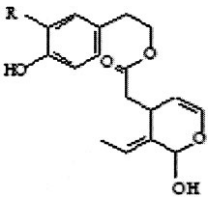
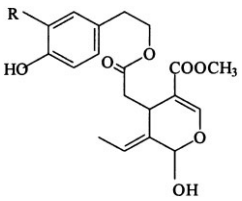
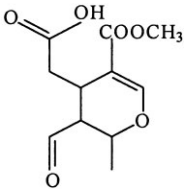
2.6.2 Analysis of the isolated compounds using analytical HPLC

After isolation, the analysis of these compounds was done with the analytical column to check the purity of the isolated compounds and confirm their identity, using the same gradient employed in the semipreparative HPLC method. The injection volume was 10 μL. The wavelengths were set at 240, 280, and 330 nm. The detection was made using MS as well, and the analyses were carried out using an electrospray interface operating in positive mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 35 psi; gas drying temperature, 350°C; 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°C; vaporizer temperature, 300°C; capillary voltage, 3000 V; fragmentor voltage, 60 V. The polarity of the electrospray interface and APCI and all the parameters of MS detector were optimized using the height of the MS signal for the main compounds detected in the methanol–water extracts of extra-virgin olive oil as an analytical parameter. Using the same mobile phases, another gradient was used to check 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). This last value was maintained for 5 min, and the run was ended.

2.7 Electrophoretic procedure

CE separation was carried out on a fused-silica capillary (75 μm ID, 375 μm OD, total length 110 cm, a detection window was created at 100 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of 100 μm × 800 μm). Every time a new capillary was used. It was preconditioned by rinsing with 0.1 M NaOH for 10 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 (30 mM sodium tetraborate adjusted to pH 9.3) for 5 min before each sample injection. Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 psi) for 8 s (1 psi = 6895 Pa). Electrophoretic separations were performed at 25 kV for 60 min, resulting in a current of ~40 μA. The temperature was maintained at 25°C. After each analysis, the capillary tubing was rinsed for 5 min with Milli-Q water. All solutions, buffers, and samples were filtered through a 0.25 μm syringe filter. The running buffer was changed after four runs. UV detection was performed at 214 and 250 nm simultaneously. DAD was used over the range of 190–600 nm to achieve spectral data. Peak identification was done by comparing both

Table 1. Chemical structures of the phenolic compounds studied

Simple phenols	 Tyrosol	 Hydroxytyrosol
Lignans	 1-(+)-Acetoxypinoresinol	 (+)-Pinoresinol
Complex phenols	 R = OH: Deacetoxy oleuropein aglycon	 R = H: Ligstroside aglycon R=OH: Oleuropein aglycon
Other phenolic compounds	 Elenolic acid	

migration time and spectral data obtained from real samples and standards, and also with spiked methanol–water extracts of olive oil with HPLC-collected compounds at several concentration levels. Peak areas were used for the quantitation of the analytes *versus* two reference compounds (oleuropein glucoside and dopac).

3 Results and discussion

3.1 Effect of experimental variables in the CE method

The effect of experimental variables was studied on an extract of a Picual extra-virgin olive-oil sample. The effect of pH was the first experimental variable tested by adjusting the buffer (sodium tetraborate) pH between 7.5 and 10.5 by adding a proper amount of 1.0 M HCl or 1.0 M NaOH. A more detailed study between pH 8.4 and 10.3 was carried out (in steps of 0.2) and is shown in Fig. 1. We observed that at pH values lower than 9.3, the analysis times were shorter, but the resolution for the compounds studied was worse, especially in the area located from deacetoxy oleuropein aglycon to oleuropein aglycon. For instance, it was impossible to determine the lignans at pH 8.6. However, at pH 9.9 the resolution between peaks was slightly better, but the analysis time was longer. Furthermore, at pH values higher than pH 10 (see Fig. 1e), the separation of the peaks migrating at shorter analysis times was worse, while the separation of the analytes migrating at higher times was good. Therefore, the optimum pH value of 9.3 is a compromise between the resolution of all the phenolic compounds and the analysis time.

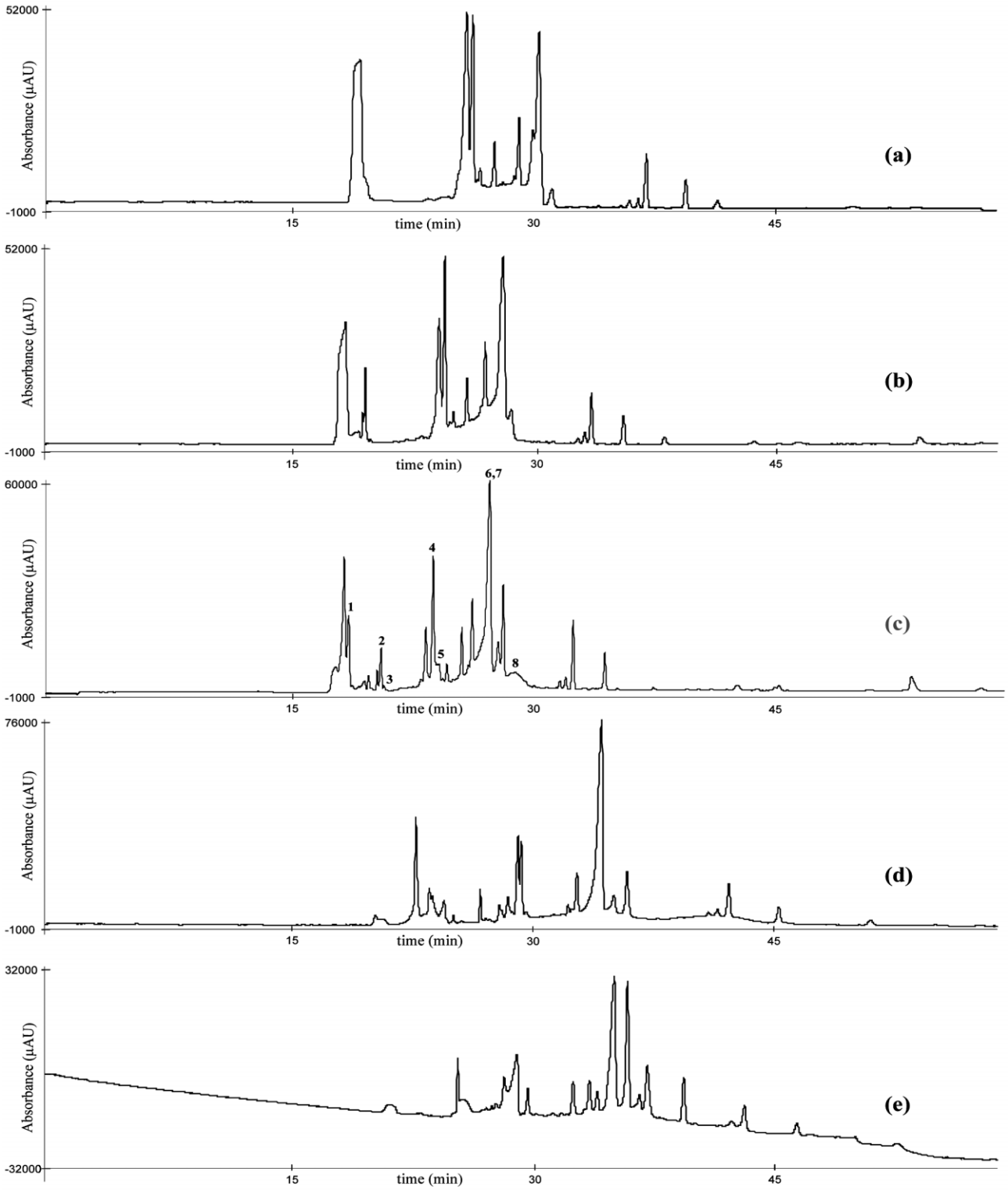


Figure 1. CZE electropherograms of a methanol/water extract obtained from an extra-virgin olive oil of Picual variety at different pH values. (a) pH 8.6, (b) pH 8.9, (c) pH 9.3, (d) pH 9.9, (e) pH 10.3. Separation conditions: capillary, 110 cm × 75 μm; applied voltage, 25 kV; applied temperature, 25°C; buffer, 30 mM sodium tetraborate; hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 214 nm. Peak identification numbers: 1, tyrosol; 2, (+)-pinoresinol; 3, 1-(+)-acetoxypinoresinol; 4, deacetoxy oleuropein aglycon (DAOA); 5, ligstroside aglycon; 6, hydroxytyrosol; 7, oleuropein aglycon; and 8, elenolic acid.

Three different buffers were tested: ammonium chloride, ammonium acetate, and sodium tetraborate. All of them were prepared in a 9–10 pH range, and sodium tetraborate was the buffer that gave the best resolution for phenolic compounds in a satisfactory time (see Fig. 2). The buffer concentration was investigated in a range between 10 and 100 mM (in steps of 10). The results of this study are shown in Fig. 3. When the tetraborate concentration was increased, it led to longer analysis times and improved peak resolution, due to its specific complexing effect on the polyhydroxylated species (phenols and polyphenols) [30, 31]. In fact, tetraborate complexes came close to groups on the polyphenol ring resulting in a new charged species, which will be electrophoresed by

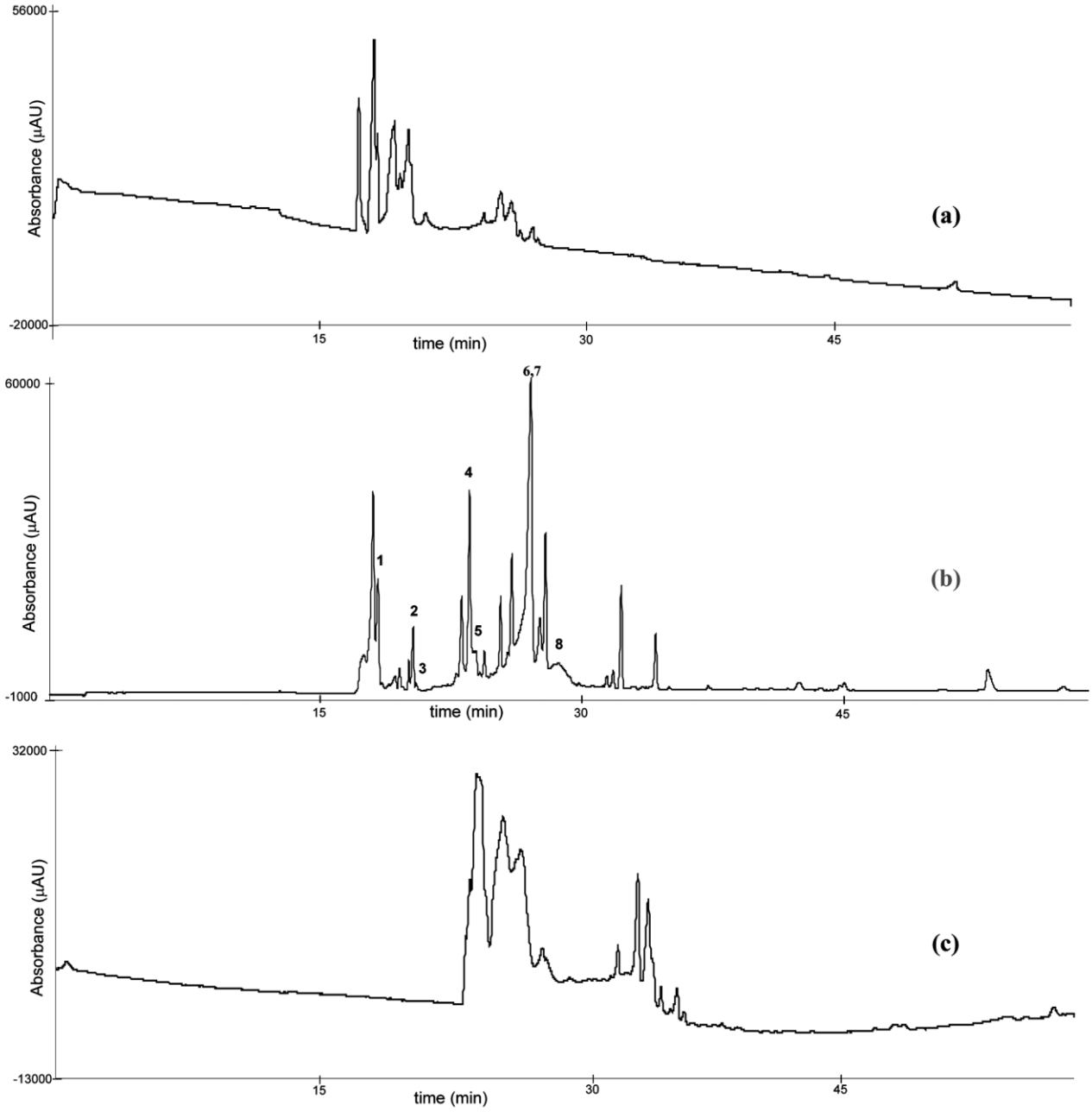


Figure 2. CZE electropherograms of an methanolic extract of Picual extra-virgin olive oil using different types of buffers. (a) Ammonium chloride, (b) sodium tetraborate, (c) ammonium acetate. Separation conditions: capillary, 110 cm × 75 μm; applied voltage, 25 kV; applied temperature, 25°C; buffer, 30 mM for ammonium chloride and sodium tetraborate and 40 mM for ammonium acetate (in order to provide a similar current value); hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 214 nm. For the identification of the peaks see Fig. 1.

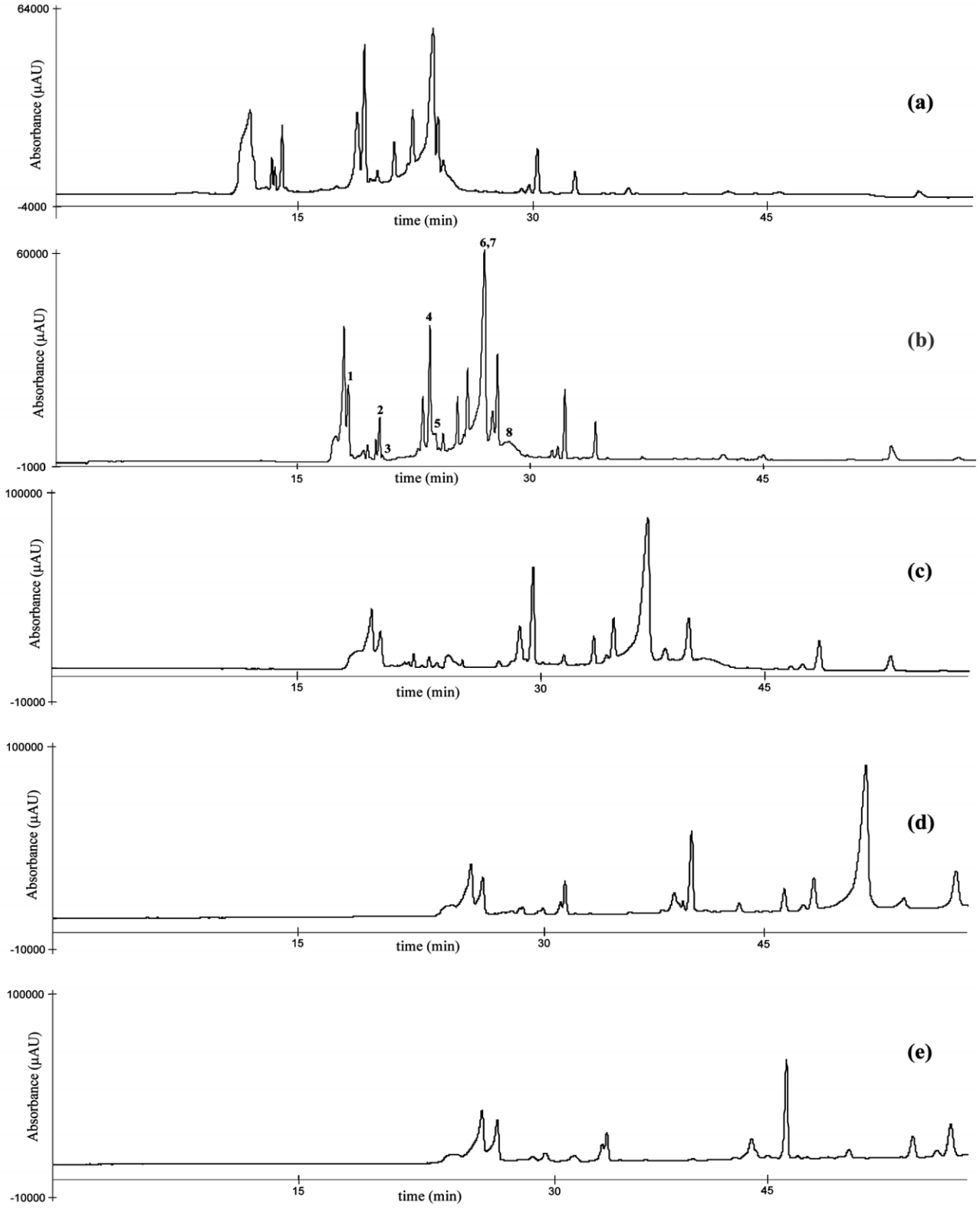


Figure 3. Opimization of the separation of phenolic compounds present in an extract of Picual extra-virgin olive oil using different concentrations of sodium tetraborate buffer. (a) 20 mM, (b) 30 mM, (c) 40 mM, (d) 60 mM, (e) 80 mM. Separation conditions: capillary, 110 cm × 75 μm; applied voltage, 25 kV; applied temperature, 25°C; hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 214 nm. For the identification of the peaks see Fig. 1.

its difference in the charge-to-mass ratio. However, over a concentration of 30 mM of sodium tetraborate analysis time was considerably increased, but the resolution between compounds was similar. We found that 30 mM of sodium tetraborate buffer at pH 9.3 represented the best compromise for the resolution of the compounds studied and a reasonable analysis time.

3.2 Effect of instrumental variables in the CE method

The applied voltage was varied from 15 to 30 kV. The voltage used to obtain the shorter analysis time and maintain a good resolution was 25 kV. The Joule heat generated by the capillary was well dissipated by the equipment at this voltage. The experimental work was carried out at a temperature of 25°C. Finally, an injection time of 8 s was applied by hydrodynamic injection.

The optimum electropherogram obtained for an extract of extra-virgin olive oil of Picual variety under optimized conditions is presented in Fig. 4. In the first 30 min the elution order observed is as follows: tyrosol, (+)-pinoresinol, (+)-1-acetoxypinoresinol, deacetoxy oleuropein aglycon, ligstroside aglycon, oleuropein aglycon, and hydroxytyrosol (overlap), and elenolic acid. After 30 min the phenolic acid zone is observed. The determination of the phenolic acids with this method is possible, but this family has recently been studied in depth by other research groups [11, 12, 32].

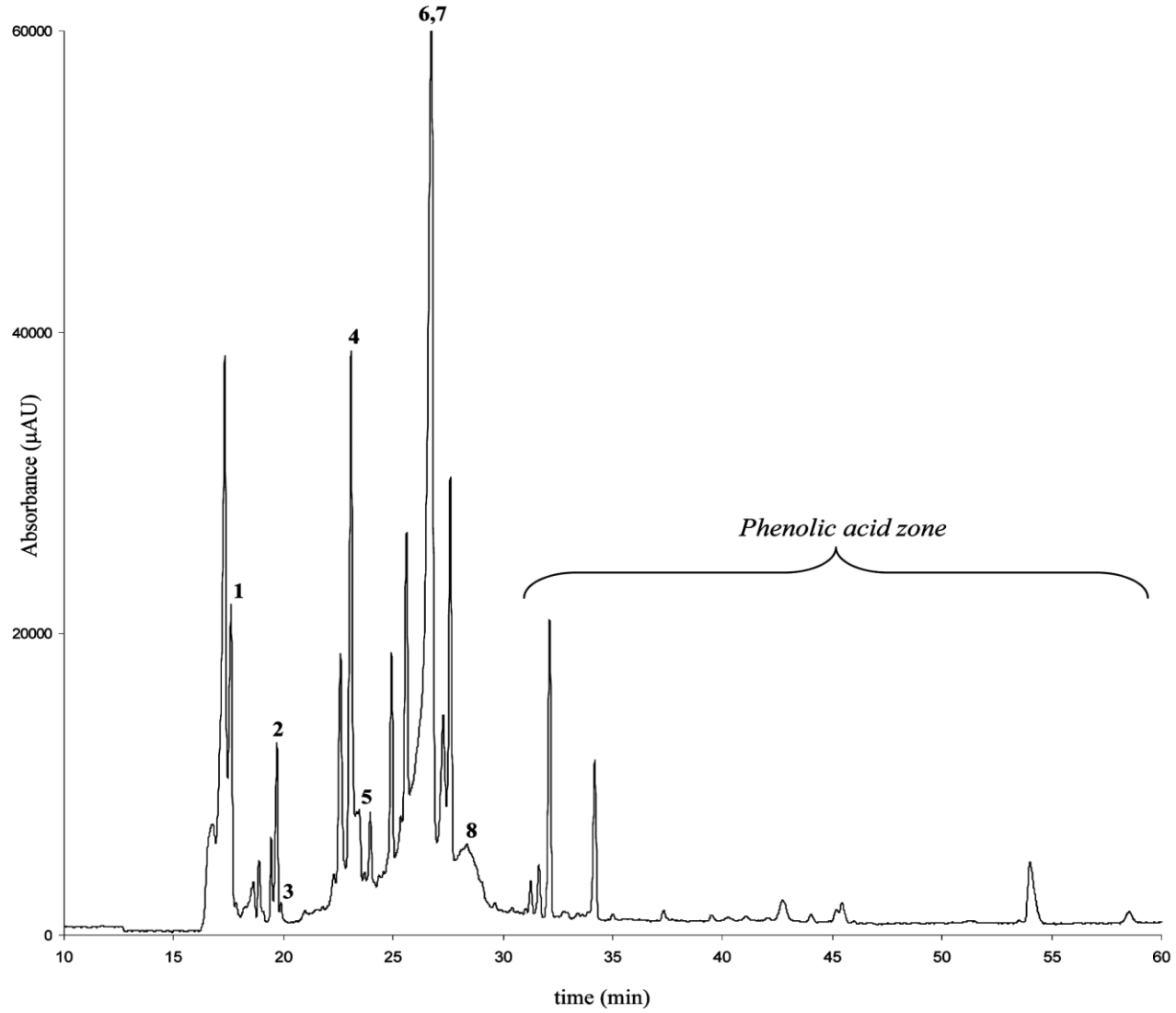


Figure 4. CZE of extra-virgin olive oil sample under optimized conditions. Separation conditions: capillary, 110 cm (100 cm effective length) × 75 μm; applied voltage, 25 kV; applied temperature, 25°C; buffer, 30 mM sodium tetraborate (pH 9.30); hydrodynamic injection, 0.5 psi for 8 s. Detection was performed at 214 nm. Peak identification numbers as in Fig. 1.

3.3 Extraction of polyphenols of virgin olive oil

To isolate the phenolic compounds of olive oil, the two types of extraction systems described before were compared: SPE (C_{18}-SPE(a), Diol-SPE(b), Sax-SPE(c)) and LLE(d). The differences among the phenolic profiles obtained using these extraction systems are shown in Fig. 5. These differences were more significant when the relative intensities of the phenolic compound peaks were observed than when the profiles of the electropherograms were studied by checking what analytes were extracted with each system. It is possible to say that the results obtained with C_{18}-SPE(a) and Sax-SPE(c) were similar, and those obtained using Diol-SPE(b) and LLE(d) seemed significant as well.

We chose Diol-SPE because, as several authors stated before [33], it seems to be very appropriate for the extraction of polar fraction from nonpolar matrices. Although LLE seems to be slightly more appropriate in terms of the intensity of the signals of some compounds (as can be seen in Figs. 5b and d), we decided to use Diol-SPE for its ease of use because of SPE automated workstations and the extraction time is shorter.

Several authors have quantitated before [8, 33, 34] the recoveries obtained using this system for the compounds studied. The problem for doing these studies is that the phenolic fraction of virgin olive oil consists of a heterogeneous mixture of compounds, which are in most cases not commercially available. Therefore, similar phenolic compounds have been frequently used in the past. To overcome this obstacle, we spiked a refined sunflower (phenolic-free) oil with an exactly specified dose of a phenolic extract that was prepared by a previous LLE of a virgin olive oil. Thus, the optimized SPE (diol-bound cartridges) was used for the extraction of the phenolic compounds of the spiked sunflower oil samples to calculate the recovery of this isolation technique. The results are summarized in Table 2. To calculate the recovery of oleuropein aglycon, we used the information obtained at 214 and 250 nm. The reason for this fact is that hydroxytyrosol has a minimum absorbance at 250 nm, and the oleuropein aglycon has its maximum absorbance near this value and we can consider that the contribution of hydroxytyrosol at this wavelength is lower than the contribution of oleuropein aglycon. In Fig. 6, it is possible to observe the fact that the most appropriate wavelength for the determination of oleuropein aglycon and elenolic acid is 250 nm.

Table 2. Recovery (%) of phenolic compounds isolated by Diol-SPE from reference sunflower-oil samples (n = 3)

Analyte	Diol-SPE[a)]	SD (a)	Diol-SPE[b)]	SD (b)
Tyrosol	69.50	1.72	71.29	2.21
Pinoresinol	75.27	2.45	76.54	3.21
1-Acetoxypinoresinol	88.71	2.12	89.87	4.01
Deacetoxy oleuropein aglycon	80.15	1.98	81.27	1.98
Ligstroside aglycon	84.58	2.56	85.35	1.67
Oleuropein aglycon + hydroxytyrosol	73.33	3.81	75.86	1.91
Oleuropein aglycon[c)]	74.62	3.24	74.85	2.31
Elenolic acid[c)]	74.95	4.51	77.58	2.45

a) Refined sunflower oil spiked with 1 mL of extract of virgin olive-oil phenolics
b) Refined sunflower oil spiked with 0.5 mL of extract of virgin olive-oil phenolics
c) Calculated using the information at 214 and 250 nm

3.4 Identification and quantitation of polyphenols in several Spanish extra-virgin olive oils

Extracts of seven monovarietal extra-virgin olive oils of different varieties (Picual, Hojiblanca, Lechín de Sevilla, Cornicabra, Picudo, Lechín de Granada y Arbequina) were analyzed. An extract of sunflower oil was also analyzed to confirm that it was appropriate as a glyceridic matrix without phenolic compounds for recovery studies. All samples were injected in the CE instrument seven times (n = 7).

As we commented before, peak identification was done by comparing both migration time and spectral data obtained from real samples and standards, and also with spiked methanol–water extracts of olive oil with HPLC-collected compounds at several concentration levels.

Standard calibration graphs were prepared for two reference compounds, *i.e.*, oleuropein (oleuropein glucoside) and dopac (3,4-dihydroxyphenylacetic acid), at two different wavelengths.

The detection limit (LOD), quantitation limit (LOQ), 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 [35]. Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity between different concentrations depending on the analytes studied. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.990 for dopac and oleuropein at the two wavelengths.

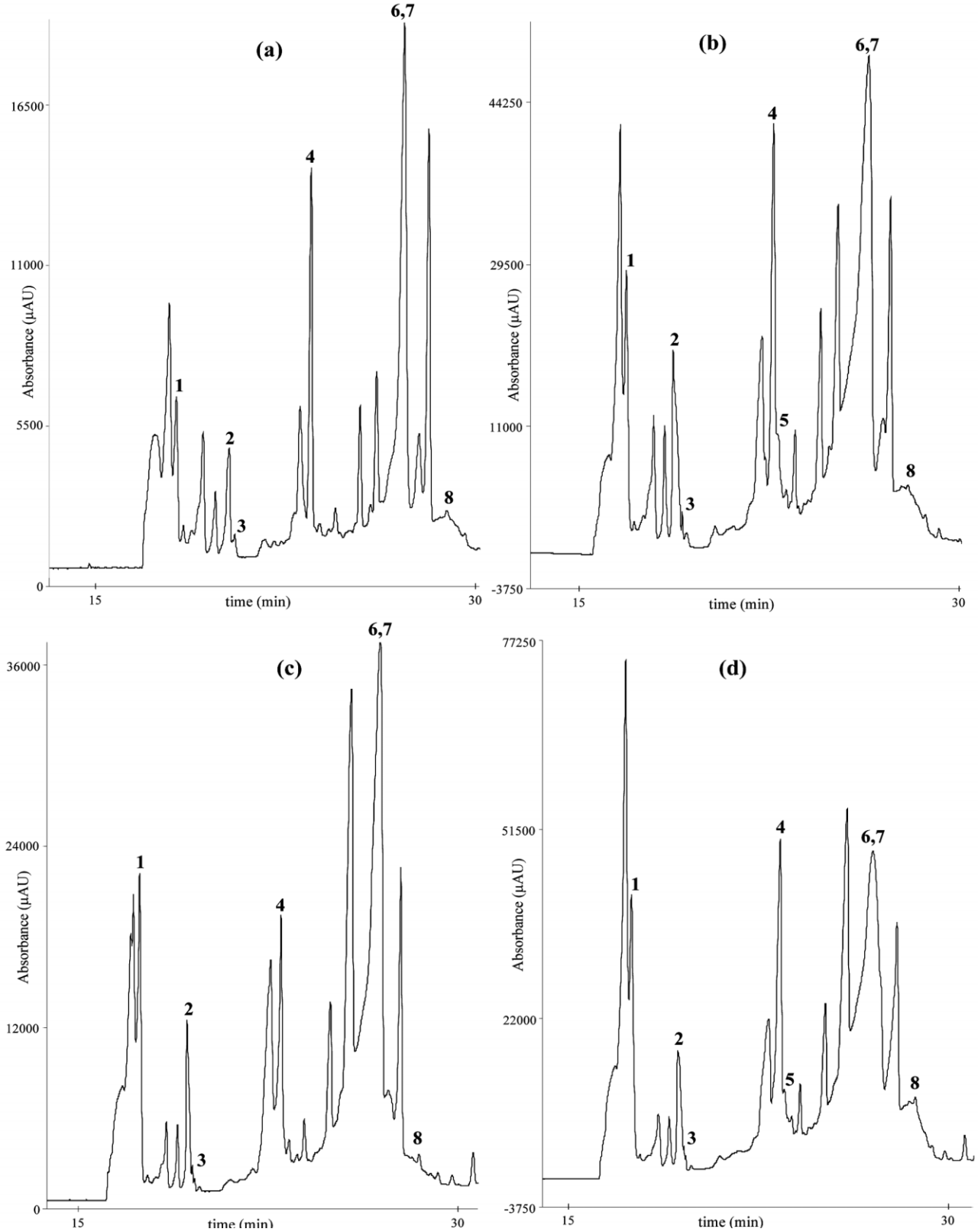


Figure 5. CZE electropherogram of the phenolic fraction extracted from an extra-virgin olive oil of Picual variety using four different extraction systems: (a) SPE-C_{18}, (b) SPE-Diol, (c) SPE-Sax (d) LLE. The absorbance scales (μAU) in the four electropherograms are different. (Identification numbers as in Fig. 4; instrumental and experimental parameters as in Fig. 4).

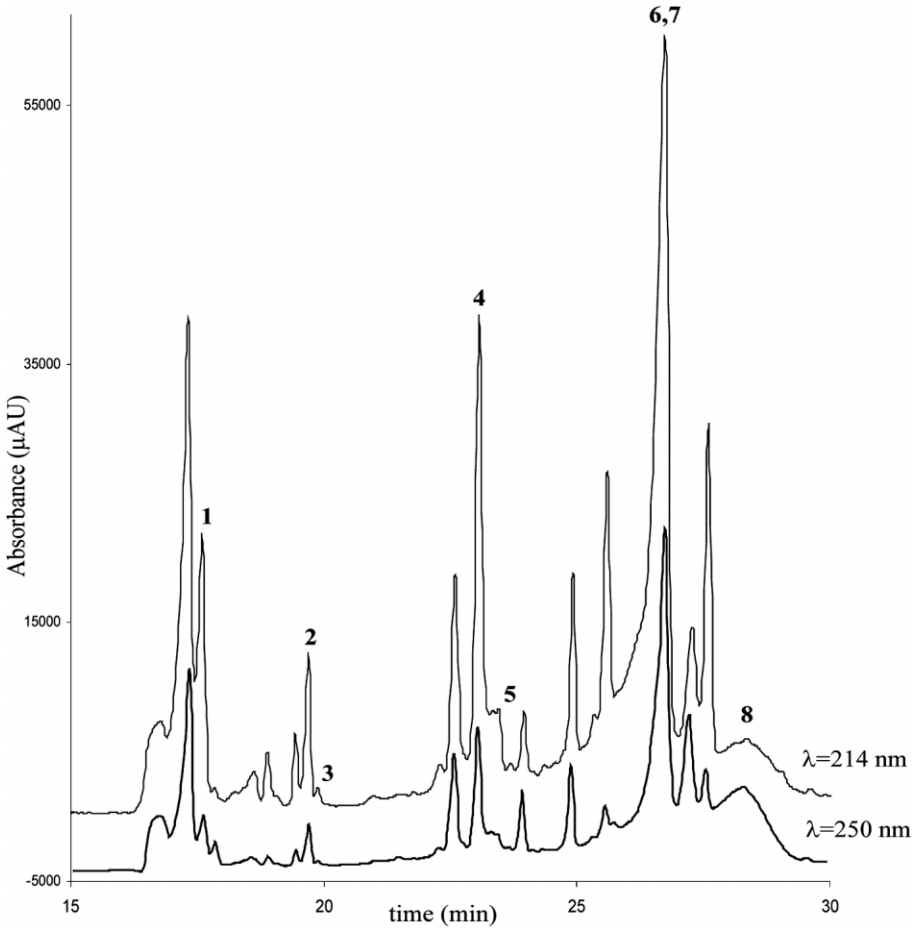


Figure 6. CZE electropherogram of the phenolic fraction obtained from an extra-virgin olive oil of Picual cultivar at two different wavelengths. (Identification numbers as in Fig. 4; instrumental and experimental parameters as in Fig. 4).

All the features of the proposed method are summarized in Table 3. Tyrosol was quantitated using the calibration curve of dopac at 214 nm; (+)-pinoresinol, (+)-1-acetoxy-pinoresinol, deacetoxy oleuropein aglycon, and ligstroside aglycon were quantitated with the calibration curve of oleuropein obtained at 214 nm. However, for the quantitation of elenolic acid and oleuropein aglycon the curve of oleuropein at 250 nm was used. Although oleuropein aglycon and hydroxytyrosol have the same retention time (overlap), it was possible to calculate (approximately) the concentration of oleuropein in the virgin olive-oil extracts using the curve of calibration of oleuropein at 250 nm, since hydroxytyrosol has a minimum of absorbance at this wavelength.

Using the described Diol-SPE system and CZE method, the seven virgin olive oil varieties and the refined sunflower oil were analyzed. The differences in the polyphenolic profiles are shown in Fig. 7. The absorbance scales in the eight electropherograms are different, due to the considerable differences in concentration between the compounds studied in each variety. The quantitative results are presented in Table 4, where the units are μg analyte/kg olive oil.

This is the first time that oleuropein aglycon, elenolic acid, and (+)-pinoresinol have been determined by CE in olive oil and they were found in all the Spanish extra-virgin olive oils studied. However, ligstroside aglycon was only detected in the Picual variety.

(+)-1-Acetoxypinoresinol was very abundant in Arbequina oils, while in the Picual variety the quantity of this compound was very low. Therefore, (+)-1-acetoxypinoresinol could be considered as a potential marker able to authenticate the oils obtained from olives of this variety. This fact has been used in a recent paper to distinguish this variety [36].

In Lechín de Sevilla extracts it is possible to see that deacetoxy oleuropein aglycon is the highest peak and the most representative compound of this oil.

It is important to highlight that Picual olive oil is the richest in terms of concentration of tyrosol and oleuropein aglycon, despite what can be seen in Fig. 7, where the peak of oleuropein aglycon in Lechín de Granada is higher than the same peak in Picual. This fact can be explained in the

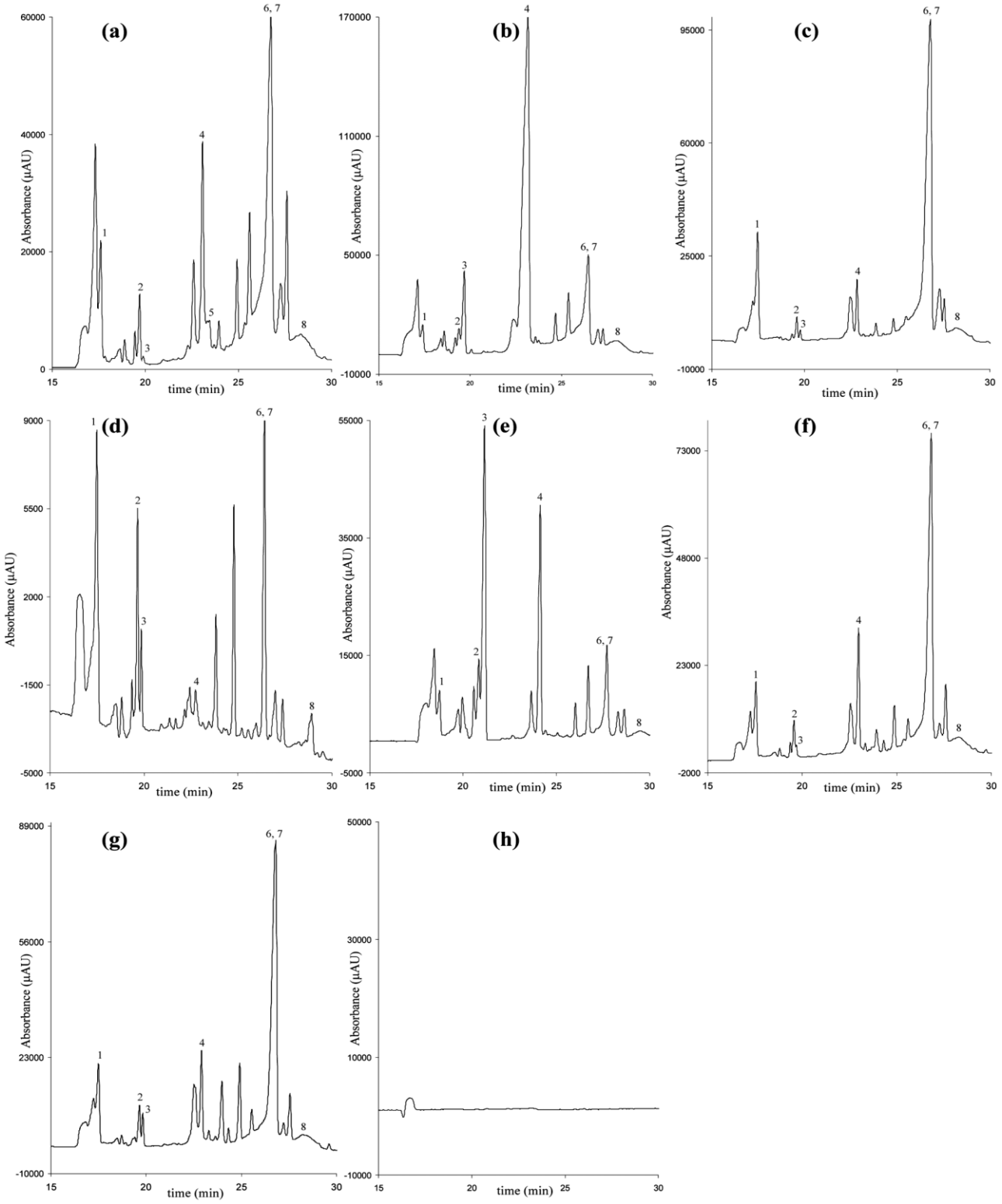


Figure 7. CZE electropherogram of the phenolic fraction extracted from extra-virgin olive oil samples by SPE-Diol. (a) Picual, (b) Lechín de Sevilla, (c) Lechín de Granada, (d) Cornicabra, (e) Arbequina, (f) Picudo, (g) Hojiblanca and (h) Sunflower. For identification of the compounds see Fig. 1. Detection was performed at 214 nm. The absorbance scales (μAU) in the eight electropherograms are different, due to considerable concentration differences between the compounds under study in each variety (instrumental and experimental parameters as in Fig. 4).

Table 3. Analytical parameters of proposed method

Analyte	RSD, % (intermediate value)	LOD, μg/mL	LOQ, μg/mL	Calibration-range, μg/mL	Calibration equations	r^2
3,4-Dihydroxyphenylacetic acid	3.65	0.032	0.108	0.108–500	$y = 6952x - 250\,362$	0.9918
Oleuropein glucoside λ = 214 nm	3.37	0.372	1.241	1.241–6500	$y = 607.65x + 5856.6$	0.9926
Oleuropein glucoside λ = 250 nm	4.93	0.824	2.748	2.748–2000	$y = 274.46x - 4889.5$	0.9905

Table 4. Results of the analysis of real samples ($n = 7$) (value = $X \pm$ SD) (μg/kg = μg analyte/kg olive oil)

Analyte	Arbequina	Cornicabra	Hojiblanca	Lechín de Sevilla	Lechín de Granada	Picudo	Picual
Tyrosol[a)]	2616.13± 86.06	2736.08± 94.93	3775.34± 104.94	3001.09± 92.46	4252.09± 145.45	3227.09± 95.77	6663.01± 136.47
Pinoresinol[b)]	9997.94± 319.53	4370.48± 1326.62	6967.51± 253.58	620.82± 17.32	3480.25± 128.77	4487.30± 161.60	6938.68± 284.49
1-(+)-Acetoxypino-resinol[b)]	36 200.89± 1448.87	1203.15± 45.12	3419.22± 141.22	26 785.41± 996.37	856.72± 31.79	582.27± 22.28	167.16± 6.15
Deacetoxy oleuropein aglycon[b)]	24 164.07± 823.29	851.60± 32.18	15 665.59± 558.75	291 521.53± 10 159.93	9289.91± 343.58	17 656.74± 674.45	28 814.59± 1051.26
Ligstroside aglycon[b)]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6598.95± 245.42
Oleuropein aglycon[c)]	14 535.26± 516.90	1790.39± 59.45	54 599.09± 2045.38	74 556.00± 2413.43	69 259.11± 2036.72	38 171.50± 1400.97	83 727.38± 2786.66
Elenolic acid[c)]	6721.49± 194.92	1038.75± 37.39	20 836.93± 645.94	31 552.98± 788.82	20 453.23± 695.41	16 689.31± 534.06	23 188.30± 672.46

n.d., not detectable
a) Quantitated with a calibration curve of 3,4-dihydroxyphenylacetic acid at λ = 214 nm
b) Quantitated with a calibration curve of oleuropein glucoside at λ = 214 nm
c) Quantitated with a calibration curve of oleuropein glucoside at λ = 250 nm

following way: oleuropein aglycon and hydroxytyrosol have the same migration time (overlap), and although the highest quantity of oleuropein aglycon corresponds to Picual variety, the peak is higher in Lechín de Granada because the contribution of hydroxytyrosol is higher in this variety.

(+)-Pinoresinol has been detected in all the studied samples, but is especially significant in virgin olive oil of Lechín de Granada cultivars. Finally, elenolic acid was found in a similar concentration in almost all oils studied, but in Lechín de Sevilla it was significantly more abundant.

4 Concluding remarks

This work reports a qualitative and quantitative CE determination of extra-virgin olive-oil phenolic compounds. The Diol-SPE and LLE methods were more effective for the extraction of tyrosol, hydroxytyrosol, secoiridoids, and lignans than the other extraction procedures. This is apparently the first paper to show the identification of (+)-pinoresinol, ligstroside aglycon, oleuropein aglycon, and elenolic acid in virgin olive oil samples using a CE method.

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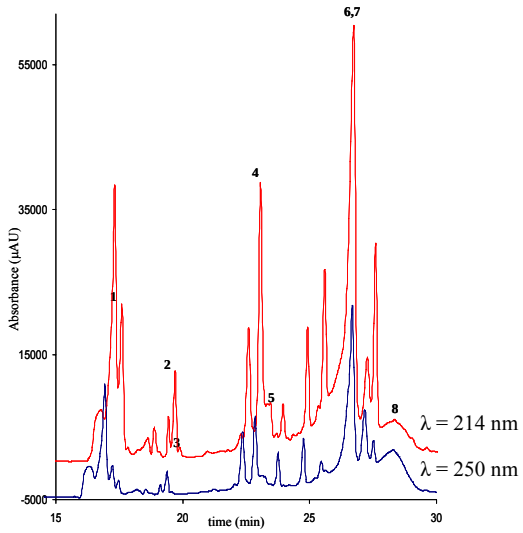
La puesta a punto de este método fue realizada empleando para ello muestras reales de aceite de oliva después de haberlas sometido al proceso de extracción. Una vez optimizado se aplicó sobre 7 variedades diferentes de aceite de oliva monovarietal obteniéndose buenos resultados para todas ellas.

Se consiguieron identificar 8 de los compuestos más importantes de la fracción fenólica, de los cuales la oleuropeína aglicona, el ligustrósido aglicona y sus derivados son los compuestos mayoritarios de esta fracción.

El tiempo de análisis no se consiguió reducir en gran medida, manteniéndose entorno a los 30 minutos. A pesar de todo, este método se presentaba como una alternativa a los métodos de HPLC que estaban alrededor de los 45-120 minutos en el momento de su publicación.



Electroferogramas obtenidos con las condiciones óptimas del método expuesto en este capítulo a dos longitudes de onda diferente. 1, tirosol; 2, (+)-pinoresinol; 3, 1-(+)-acetoxipinoresinol; 4, decarboxioleuropeína aglicona; 5, ligustrósido aglicona; 6, hidroxitirosol; 7, oleuropeína aglicona; y 8, ácido elenólico.

Como puede verse en la figura anterior la separación de los compuestos fue bastante satisfactoria. De todas formas los compuestos 6 y 7 tenían el mismo tiempo de migración. El problema que esto suponía a la hora de su identificación y cuantificación se resolvió adquiriendo los electroferogramas a distintas longitudes de onda, que coincidían con los máximos y mínimos de absorción de cada uno de ellos.

Cronológicamente este trabajo fue el primero que se realizó a lo largo del período de tesis doctoral, y permitió abrir el camino a futuras investigaciones en las que se trató de estudiar más en profundidad la fracción fenólica al completo y disminuir los tiempos de análisis. Así, en 2006 se publicó otra metodología en la que se conseguían determinar 26 compuestos fenólicos en menos de 10 minutos y en cuyo desarrollo también tomé parte [218].

[218] Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A., A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extravirgin olive oil. *Journal of Separation Science* **2006**, 29, 2221-2233.

Capítulo 3

Electroforesis capilar en medio no acuoso acoplada a espectrometría de masas de tiempo de vuelo para la caracterización de la fracción fenólica del aceite de oliva: inyección directa de aceite de oliva enriquecido.

Publicación incluída en este capítulo:

Non-aqueous capillary electrophoresis-electrospray-time of flight mass spectrometry to reveal phenolic compounds from olive oil: introducing *enriched* olive oil directly inside capillary.

(*Electrophoresis*, aceptado bajo revisión)

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Hasta el momento la gran mayoría de los estudios para el análisis de los compuestos fenólicos mediante electroforesis capilar se han realizado en medio acuoso, pero la naturaleza apolar de la matriz aceite de oliva y el interés por simplificar el paso de preparación de muestra llevó a plantearse el desarrollo de una metodología de electroforesis capilar acoplada a espectrometría de masas empleando disolventes orgánicos (electroforesis capilar en medio no acuoso o NACE) para el análisis de la fracción objeto de estudio en esta memoria, la fracción fenólica.

Las principales ventajas de este método se podrían resumir como se indica a continuación:

- La posibilidad de realizar inyección directa de aceite de oliva dentro del capilar. Esto es viable gracias al empleo de disolventes orgánicos y abre expectativas a una futura eliminación del proceso de extracción previo al análisis del aceite de oliva.
- Además el uso de disolventes orgánicos en electroforesis capilar presenta una serie de ventajas ya que sus propiedades fisicoquímicas permiten manipular con relativa facilidad el tiempo de análisis, la selectividad de la separación y la resolución. Simplemente cambiando la composición del buffer de separación (combinando diferentes disolventes en distintas proporciones) es posible alcanzar buenos resultados en los análisis.
- Es también de destacar que disolventes orgánicos muy empleados en electroforesis capilar (y también en esta metodología) como son el metanol y acetonitrilo, son altamente compatibles con EC-MS debido a su elevada volatilidad.

Este método se aplicó sobre muestras de cinco variedades diferentes de aceituna: Arbequina, Picual, Hojiblanca, Lechín de Sevilla y Cornicabra; demostrándose su potencial en diferentes variedades de aceite de oliva.

La fiabilidad y también la potencialidad del método NACE desarrollado se comprobaron comparándolo con los métodos CZE previamente puesto a punto en nuestro grupo de investigación. En términos generales ésta metodología es comparable a la desarrollada en CZE, con la particularidad, ya mencionada anteriormente, de que el método NACE es capaz de admitir inyección directa de aceite proporcionando a esta metodología un interés añadido.

Non-aqueous capillary electrophoresis-electrospray-time of flight mass spectrometry to reveal phenolic compounds from olive oil: introducing *enriched* olive oil directly inside capillary

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Abbreviations

BGE, background electrolyte; EVOO, Extra virgin olive oil; CD, ciclodextrins; SDS, sodiumdodecyl sulphate; MeOH, methanol; EtOH, ethanol; NMF, N-methylformamide; DMF, N,N-dimethylformamide; FA, formamide; RSD, relative standard deviation.

Keywords: Capillary electrophoresis / Electrospray-time of flight-mass spectrometry /Non-aqueous capillary electrophoresis / Olive oil / Phenolic compounds

Abstract

Most CE methods for the analysis of phenols from olive oil use an aqueous electrolyte separation medium, although the importance of non-aqueous CE (NACE) is obvious, as this kind of CE seems to be more compatible with the hydrophobic olive oil matrix and could facilitate its direct injection.

In the current work we develop a method involving SPE and NACE coupled to ESI-TOF MS. All the CE and ESI-TOF MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity in their determination. Electrophoretic separation was carried out using a CE buffer system consisting of 25 mM NH_4OAc/AcH in Methanol/ACN (1/1, v/v) at an apparent pH value of 5.0. We studied in depth the effect of the nature and concentration of different electrolytes dissolved in different organic solvents and other experimental and instrumental CE variables. The results were compared with those obtained by CZE (with aqueous buffers) coupled to ESI-TOF MS; both methods offered to the analyst the chance to study phenolic compounds of different families (such as phenolic alcohols, lignans, complex phenols, flavonoids…) from virgin olive oil by injecting methanolic extracts with efficient and fast CE separations. In the case of NACE method, we also studied the direct injection of the investigated matrix introducing a plug of olive oil directly into the capillary.

1 Introduction

The need to obtain better separation efficiencies, automation and reliable quantitation led to the development of techniques that allow the evolution of conventional electrophoresis to other electrophoresis which could be carried out under high electric field. In this way capillary electrophoresis emerged as a powerful and versatile analytical separation technique at the beginning of the 80's [1]. Some years later, in 1984, Walbroehl and Jorgensen used background electrolytes based on pure organic solvents for the first time [2]. Since then CZE using non-aqueous background electrolytes (BGEs) has been an interesting alternative to traditional aqueous or aqueous-organic BGEs, although it had attracted no attention until the early 1990s. NACE exploits the vastly different physicochemical properties of organic solvents. The introduction of nonaqueous electrolyte solutions in CE has expanded the range of solvent parameters such as the dielectric constant, viscosity, polarity and autoprotolysis, offering new possibilities for changes in separation selectivity [3- 9]. With mere adjustment of BGE composition, parameters like resolution, analysis time and selectivity can be fine-tuned or even drastically altered. Nonaqueous media offer the potential of separation mechanisms based on interactions that are too weak or cannot take place in aqueous background electrolytes. Electrostatic and donor-acceptor interactions are the ones which dominate in NACE [10].
As far as detection in NACE is concerned, several works have been published [7, 9, 11- 16]. Among the different forms of detection (UV, mass spectrometry, electrochemical and fluorescence), UV has been the most common. Nevertheless nonaqueous medium has been proved to be ideal in ESI-MS, since it provides stable spray and high sensitivity. NACE buffer fulfills all requirements regarding an effective sample introduction approach to MS in terms of volatility, electrical current generation, flow rate and ionization. MS sensitivity is maximized and stability is optimal in nonaqueous CE–ESI–MS system.

The analytical applications of NACE have been principally focused on pharmaceutical and environmentally important analytes, although NACE methodologies have been also used successfully to determine compounds like phenols [17- 21], theaflavins [22], benzoic and sorbic acids [23].

Extra virgin olive oil (EVOO) and specially its phenolic fraction have been deeply studied by techniques such as HPLC-UV/HPLC-MS [24- 29] and also CZE-UV/CZE-MS [30- 33]. The study of this fraction of EVOO has a big interest because of the properties that phenolic compounds provide to olive oil: nutritional properties, sensory characteristics and shelf life [34, 35]. They also play an important role in human nutrition as preventive agents against several diseases [36, 37].

The aim of the present work was to study in depth the profile of phenolic fraction of EVOO by NACE using the physicochemical properties of different organic solvents to get a proper separation detecting as many phenolic compounds as possible. The results obtained by NACE-ESI-TOF MS were compared with those got by aqueous CE-ESI-TOF MS in terms of selectivity, analysis time, separation efficiency and sensitivity. Moreover, to take advantage of NACE, olive oil was dissolved in several organic solvents and analyses were carried out introducing a plug of olive oil directly into the capillary. The direct injection of olive oil can open up great expectations, since it could give to the analyst the possibility to analyze in the future olive oil without previous extraction.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogen carbonate, ammonium dihydrogen carbonate, ammonium borate, ammonium formate,

93 ammonium salts (cethyltrimethylammonium chloride, dodecyltrimethylammonium bromide and
94 dodecyltrimethylammonium chloride), ammonium dihydrogen phosphate and ammonium
95 hydroxide were from Sigma (St Louis, MO), whilst ammonium acetate was purchased from
96 Merck (Darmstadt, Germany). Formic acid and acetic acid were from Merck (Darmstadt,
97 Germany).The organic solvents, methanol (MeOH), ACN, ethanol (EtOH), N-methylformamide
98 (NMF), N,N-dimethylformamide (DMF), DMSO, formamide (FA) and 2-propanol, all from
99 Sigma-Aldrich (St. Louis, MO), were used for the CE running buffers and sheath liquids.
100 Double-deionized water was obtained with a Milli-Q water purification system (Millipore,
101 Bedford, MA, USA).
102 CE buffers were prepared by weighting the proper amount of electrolyte (to get the desired
103 concentrations) and adjusting the apparent pH when necessary by adding ammonium hydroxide
104 or acetic acid. The buffers were stored at 4ºC and warmed to room temperature before use.
105 Sodium hydroxide solution (1.0 N) from Agilent technologies was used for capillary cleaning
106 procedures and activation of the capillary wall. All solutions and buffers were degassed by
107 ultrasonication before use.
108 Additives like ciclodextrins (CDs) were also used in some buffers. β-CD was purchased from
109 Sigma Aldrich.
110
111 **2.2 Samples**
112
113 EVOO samples used in the current study were from 5 varieties of olive fruit called Arbequina,
114 Picual, Hojiblanca, Lechín de Sevilla and Cornicabra (January 2007). For simplicity we present
115 in this paper the electrophoretic profiles of extracts of Picual extra-virgin olive oil, although we
116 used the other varieties during the optimization of the separation conditions of our method.
117 Using more than one variety, we could ensure the potential of the presented methodology for the
118 analysis of these compounds in any kind of olive oil.

Commercial refined olive oil samples were used to check ion suppression phenomena and the specificity of the method.

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 [32]. EVOO (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 non-polar fraction of the oil. Finally, the sample was recovered by passing through eight portions (of 5 mL) of MeOH and brought to dryness in a rotary evaporator under reduced pressure and a temperature of 35ºC.

After this step, two different procedures to prepare extracts, for NACE and aqueous CE, were followed. Aqueous CE extracts were prepared by dissolving the residue from the rotary evaporator with 2 mL of MeOH/Water (1:1, v/v) and filtered through a 0.25 μm filter before the CE analysis. The extract of EVOO was diluted 1:10 with MeOH/Water before injection into the CE system. The same was made in order to prepare the extracts for the preliminary NACE studies, but using only MeOH as solvent. Once the potencial of NACE method was tested, enriched EVOO samples were prepared by redissolving the residue from the rotary evaporator in a mixture of 10 g of olive oil and 10 mL of 1-propanol.

2.3 Capillary electrophoresis analyses

CE was performed using a P/ACE System MDQ (Beckman Instruments, Fullerton, CA, USA). 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.

NACE separation was evaluated based on a wide range of different background electrolytes. Different apparent pHs, buffer concentrations, as well as different organic modifiers used to dissolve the buffer were tested (see Results and Discussion Section). After thorough optimization

145 (see below), we chose as a running buffer 25 mM NH_4OAc/AcH in MeOH/ACN (1/1, v/v) at an
146 apparent pH value of 5.0. The separation voltage was set to 30 kV at the inlet of the capillary.
147 Injection was performed hydrodynamically, typically 50 mbar were applied for 10 s,
148 corresponding to about 10 nL injected (0.6 % of the capillary). For CE-MS coupling, a coaxial
149 sheath-liquid sprayer was used (Agilent Technologies). Isopropanol/water (1:1) was applied as
150 sheath-liquid at a flow rate of 4 μL/min delivered by a 5 mL gas-tight syringe (Hamilton, Reno,
151 NV, USA) using a syringe pump of Cole-Parmer (Vernon Hill, IL, USA). An electrospray
152 potential of + 4.5 kV was applied at the inlet of the MS (negative mode). A nebulizer gas
153 pressure of 0.15 bar was applied to assist the spraying. Drying gas temperature was set at 180ºC;
154 and drying gas flow at 5 L/min.
155 Analysis in aqueous CE were made by using 25 mM ammonium hydrogen carbonate at pH 10.4
156 as buffer, considering the previous results published by Carrasco-Pancorbo *et al*. [38]. In that
157 paper, the authors found that 25 mM ammonium hydrogen carbonate at pH 9.0 and 10.4 were the
158 best background electrolytes depending on the olive oil variety analyzed. In the case of Picual
159 EVOO, the second pH value gave better results. The rest of CE instrumental variables and ESI-
160 TOF MS conditions were the same to compare the results more satisfactorily.
161
162 All new capillaries were conditioned before their first use by flushing with 1 M NaOH for 10
163 min followed by a rinse with water for 20 min. Initially the capillary washing routine between
164 runs consisted of 3 min with 1-propanol followed by 3 min with running buffer (all rinses done
165 using N_2 at a pressure of 20 psi). However, we observed that resolution and reproducibility of
166 separation were lost after ten injections. Therefore, conditioning was completed by flushing the
167 capillary with NaOH 0.1M during 2 min every three analysis before rinsing with 3 min of 1-
168 propanol and 3 min of running buffer.
169 Using this protocol, the % relative standard deviation (RSD) values for migration times of
170 analytes were lower than 1.0% for five consecutive runs, indicating an adequate capillary

171 reconditioning between runs. Therefore, this latter protocol was used (see Repeatability study
172 section).
173
174 **2.4 Mass Spectrometry**
175
176 MS was performed using the microTOF™ (Bruker Daltonik, Bremen, Germany), an orthogonal-
177 accelerated TOF mass spectrometer (oaTOF-MS). Transfer parameters were optimized by direct
178 infusion experiments with EVOO extracts, as well as with several of the most important
179 compounds belonging to this polar fraction of the olive oil which are commercially available
180 (tyrosol and hydroxytyrosol (phenyl alcohols); luteolin and apigenin (flavonoids); dopac,
181 vanillic, caffeic and *o*-coumaric acid (phenolic acids); and oleuropein glucoside (secoiridoid)).
182 Thus, good sensitivity at a reasonable resolution was obtained (5,000–10,000 at 250 *m/z*). The
183 trigger time was set to 50µs, corresponding to a mass range of 50–800 *m/z*. Spectra were
184 acquired by summarizing 30,000 single spectra, defining the time resolution to 1.5 s.
185
186 The accurate mass data of the molecular ions were processed through the software DataAnalysis
187 4.0 (Bruker Daltonik GmbH), which provided a list of possible elemental formula by using the
188 Smart Formula 3D™ editor. The editor uses a CHNO algorithm, which provides standard
189 functionalities such as minimum/maximum elemental range, electron configuration and ring-plus
190 double bonds equivalents, as well as a sophisticated comparison of the theoretical with the
191 measured isotope pattern (SigmaValue™) for increased confidence in the suggested molecular
192 formula [39].
193 During the development of the CE method, external calibration was performed using sodium
194 formate cluster by switching the sheath liquid to a solution containing 5 mM sodium hydroxide
195 in the sheath liquid of 0.2% formic acid in water:isopropanol 1:1 v/v. By using this method, an
196 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 NACE-ESI-TOF MS analyses

3.1.1 Optimization of the separation conditions

The methanol extracts of the mentioned EVOOs were used in the preliminary studies. Once we were sure about the potential of a NACE methodology to study phenols in olive oil, direct injection of enriched olive oil was 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. Parameters such as ionic strength and type of buffer, solvents used for the running buffer, additives and voltage were studied during the optimization.

Separation was evaluated using different background electrolytes that have been proved to give successful results in NACE. After checking in depth the results we got during the preliminary studies, we decided to test Ammonium dihydrogen carbonate, ammonium borate, ammonium formate, cuaternarium ammonium salts, ammonium dihydrogen phosphate, ammonium acetate and sodium hydroxide as running buffer at a concentration of 20 mM, apparent pH of 6.5 and MeOH/ACN (50/50) as solvent. The best results taking into account the number of peaks, resolution among them and analysis time were obtained using ammonium acetate as running buffer.

Ammonium borate and ammonium dihydrogen phosphate did not provide good results, analysis time was really long and resolution was not better than the one obtained with the other solvents used. In Fig. 1 the influence of different background electrolytes on migration time of most representative phenolic compounds studied can be seen. Only the BGEs that produced acceptable results are shown. It can be observed that ammonium acetate was the BGE that presented the best resolution keeping analysis time not too long. Ammonium dihydrogen carbonate and sodium hydroxide presented shorter analysis time but separation was worse than the one obtained using ammonium acetate; whilst ammonium formate provided bad separation and long analysis time. The concentration of the selected buffer was changed between 10 to 50 mM, and the apparent pH was studied in a range from 4.0 to 7.0 by adding different amounts of acetic acid, being 25 mM of ammonium acetate pH 5 the best conditions in terms of efficiency, selectivity and migration time.

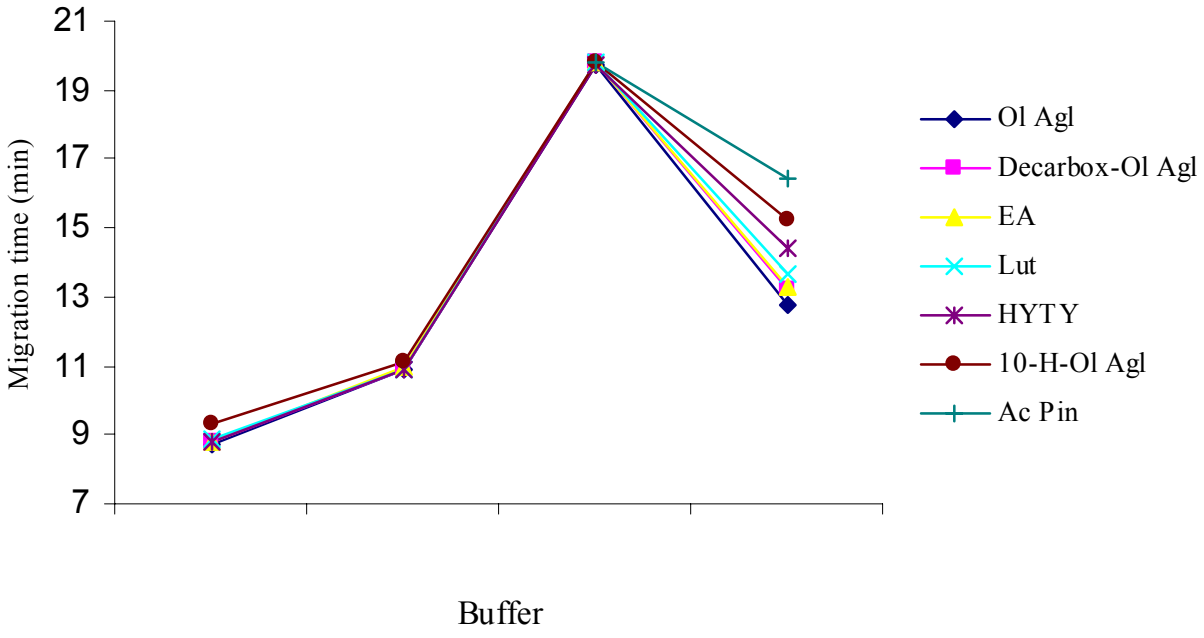


Figure 1. Effect of different background electrolytes (buffers) in the migration time of Ol Agl, Decarbox-Ol Agl, EA, Lut, HYTY, 10-H-Ol Agl and Ac Pin.

Different organic solvents and mixtures of them were used in order to improve electrophoretic selectivity. The ion mobility can be correlated with solvent properties like the ratio of solvent permittivity and viscosity [40], as well as for solvent mixtures when the Smolukowski equation [41] is applied. MeOH, ACN, NMF, DMF, FA, 2-propanol, EtOH and DMSO were tested at 25 mM of ammonium acetate pH 5. Table 1 shows the physicochemical properties of these solvents. The best results were obtained for MeOH, ACN, DMF and DMSO.

Table 1. Properties of organic solvents at 25ºC.

Solvent	T_{boil} (ºC)	η (mPa*s)	ε	pK_{auto}	γ (10^{-2} Nm^{-1})
Acetonitrile	81.6	0.341	37.6	≥ 33.3	2.760
MeOH	64.7	0.545	32.7	17.2	2.212
N-Methyformamide	182	1.65	182.4	10.7	3.87
N,N-Dimethylformamide	153	0.802	36.71	29.4	3.52
Dimethyl sulphoxide	189	1.996	46.68	33.3	4.286
Formamide	210.5	3.30	111.0	16.8	5.791
2-propanol	82.2	2.044	19.92	21.08	2.124
Ethanol	78.3	1.078	24.55	18.88	2.190
H_2O	100	0.890	78.36	14	7.181

T_{boil} = boiling point
η = coefficient of viscosity
ε =dielectric constant (relative permittivity)
pK_{auto}= autoprotolysis constant
γ = coefficient of surface tension

Fig. 2 presents a comparison of the influence of the four mentioned solvents on (a) migration time and (b) peak intensity of most representative phenolic compounds studied. It can be seen that the solvents behaved according to what it was described before by Grob *et al.* [42]. In Fig. 2(a) it is shown that mobility was higher in the case of ACN and DMF and it had its lower values when DMSO and MeOH were used. Concerning resolution power, DMSO and MeOH were the best solvents. Moreover, in Fig. 2(b) it can be observed that peak intensity was the highest with MeOH followed by ACN. These results evidenced that MeOH was the pure solvent that provided the best compromise in terms of separation and intensity of the peaks. Indeed, it has been already described in literature that MeOH (and ACN or even their mixtures) [14, 43] are

the most commonly used solvents for background electrolytes in NACE. MeOH is a solvent rather similar to water (considering its pK_{auto} and viscosity); ACN is a very different type of solvent from both water and MeOH, although its dielectric constant (36.01 at 25ºC) is similar to that of MeOH.

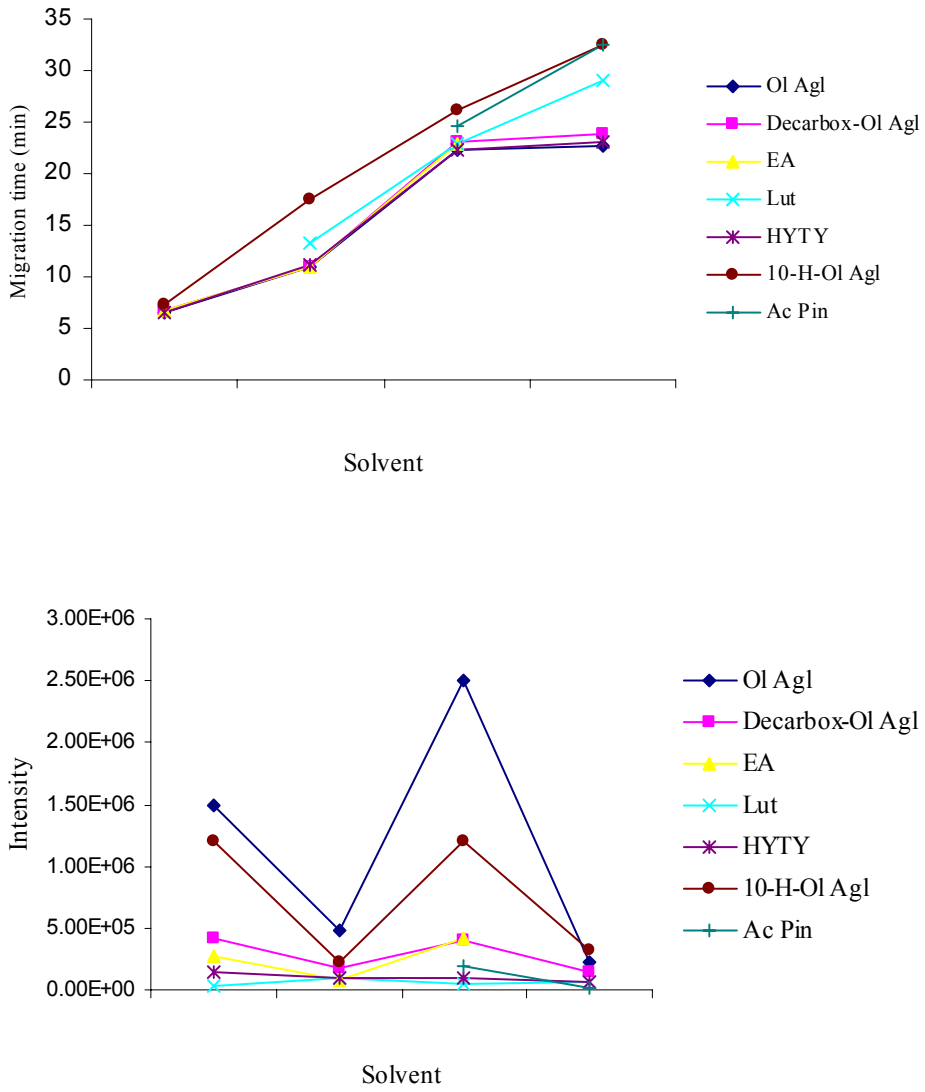


Figure 2. Influence of the different solvents used to dissolve ammonium acetate on **(A)** the migration time of Ol Agl, Decarbox-Ol Agl, EA, Lut, HYTY, 10-H-Ol Agl and Ac Pin, and **(B)** the intensity of their signals.

Because of these reasons and the results commented above, the behaviour of the analysis time was studied using different mixtures of MeOH and ACN (Fig. 3).

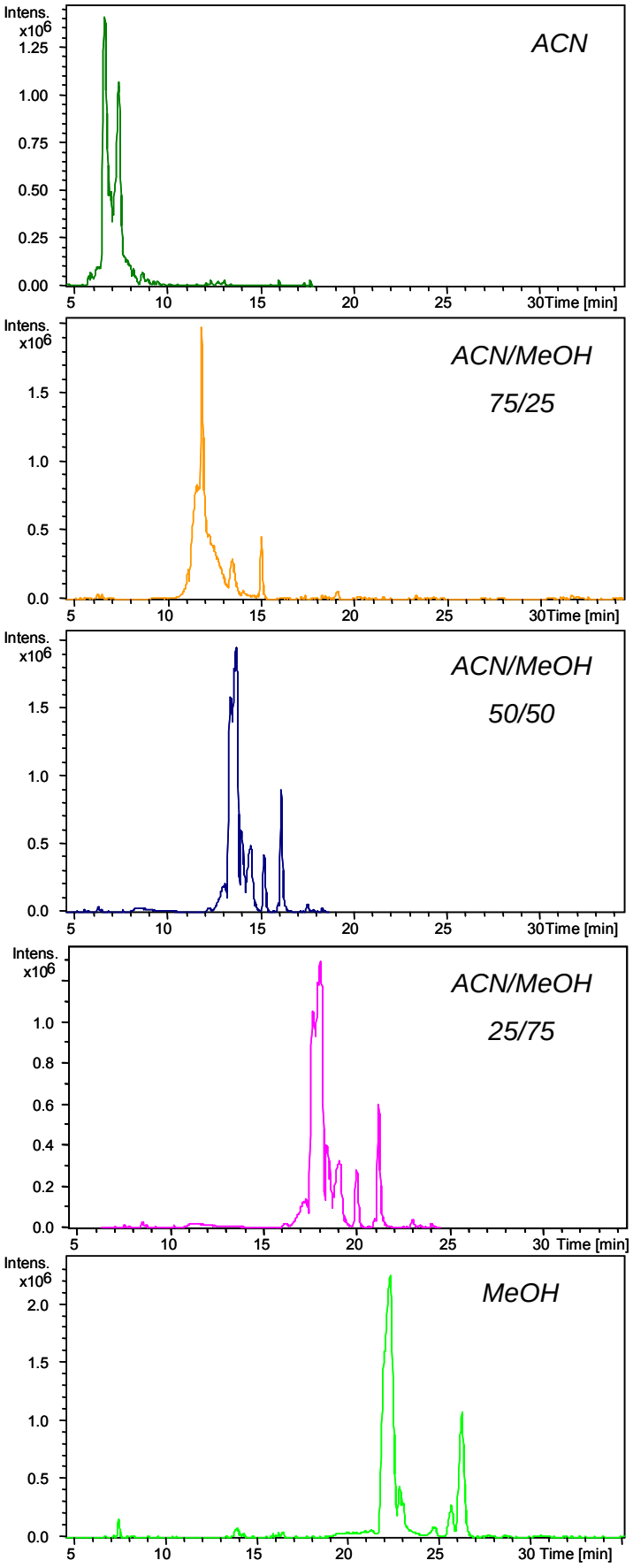


Figure 3. Effect of the presence of ACN, MeOH and their mixtures as solvent used to dissolve the background electrolyte in the BPE of a Picual extra-virgin olive enriched oil.

From Fig. 3, it is clear that the separation selectivity in CE is considerable different in mixtures of MeOH and ACN than in pure solvents. We carried out the separation of a Picual extra-virgin olive enriched oil by using just MeOH or ACN and also by using mixtures of them (ACN/MeOH 75/25, ACN/MeOH 50/50, ACN/MeOH 25/75). The mobilities of the compounds under study were much lower in pure MeOH and increased with ACN content. In Fig. 3 we can also observe that the best separation was achieved with MeOH/ACN 50/50, since higher content of MeOH did not improve the separation and lengthened the analysis time.

Looking for another alternative, we tried the analysis of enriched EVOO adding CDs in the electrophoretic medium. Indeed, the use of CDs in NACE has also been successful, since Wang and Khaledi [44] and Valkó *et al.* [45,46] have reported powerful CE separations in solvents like formamide, NMF and DMF using β-CD as an additive. 100mM of β-CD were added to the running buffer in DMF, but it did not lead to better results. DMF was chosen due to the fact that a wide number of publications claim it as one of the best for this kind of analysis.

3.1.2. Direct injection of olive oil

To carry out the direct injection of olive oil, oil samples were diluted in several organic solvents (1:1 v/v). DMSO, 1-pentanol, acetone, 1-propanol, THF and 1,4-dioxane were found to be miscible with olive oil; while MeOH, 2-propanol, EtOH, ACN, FA and DMF were not miscible with it. These results are in agreement with those given by Mendonça *et al.* [21]. Among the solvents that could dissolve olive oil, 1-propanol has been described to present the best physical properties that make it a good choice to use it with CE. Because of this reason, 1-propanol was chosen to perform the direct injection in NACE analysis.

3.1.3. Optimization of cleaning procedure

Special attention was paid to the cleaning procedure of the capillary after each analysis. In the beginning the capillary was flushed only with buffer solution during 3 min between the analyses, but resolution and reproducibility of separation were not good after three injections. This fact led us to use another solvent to clean the capillary before rinsing with buffer solution. Solvents that were proved to be miscible with olive oil were tested, since these solvents should be able to solve the residues of olive oil. 1-propanol was found out to be the best one to clean the capillary. Cleaning after each analysis with 3 min 1-propanol and 3 min buffer solution, the resolution and reproducibility of separation remained good for ten injections. As last step, and to achieve the better resolution and reproducibility of separation possible, a rinse with NaOH 0.1M during 2 min before 1-propanol was added every 3 analysis.

3.1.4. Analysis of extracts of extra-virgin olive oil by NACE-ESI-TOF MS

Analyses of EVOO by NACE-ESI-TOF MS were performed in negative ion mode as well as in positive ion mode. In positive ion mode; in general, the profiles were satisfactory, although signal intensity was lower than in negative ion mode. As far as the different families of phenolic compounds are concerned, we can say that flavonoids and lignans were ionized quite properly in positive polarity, whilst secoiridoids, simple phenols and phenolic acids (or very related compounds) can be detected better in negative polarity. Even though we decided to use negative polarity for the rest of the analyses, it is important to highlight that the results concerning "unknown" peaks were in good agreement working in both polarities.

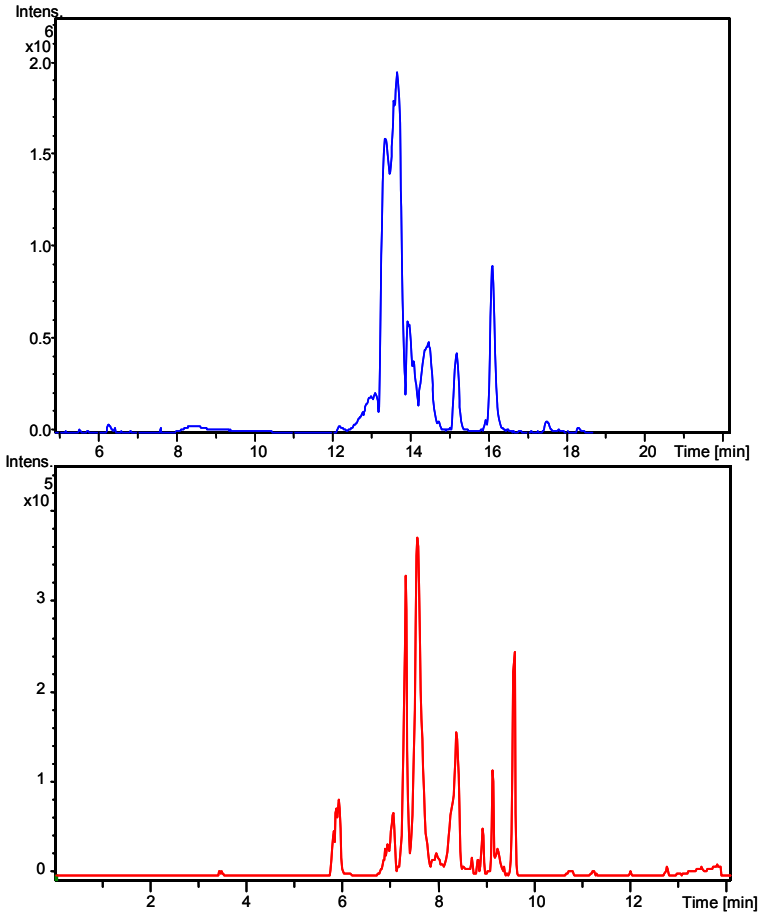


Figure 4. (A) BPE as obtained by NACE-ESI-TOF-MS of a Picual extra-virgin olive enriched oil at the optima electrophoretic and MS conditions.
(B) BPE as obtained by aqueous CE-ESI-TOF-MS of a Picual extra-virgin olive oil extract at the optima electrophoretic and MS conditions.
(ESI and MS conditions were exactly the same in both cases. We just identify in the profiles the major peaks in each case)

Fig. 4 shows the optimum profile for a Picual enriched EVOO by the described NACE method and Table 2 and Table 2b summarize well-known and "unknown" compounds determined by the NACE method in the negative ion mode. Table 2 includes molecular formula, calculated and experimental *m/z*, error, sigma value, classification order (number of possibilities), tolerance (ppm) in Smart 3D editor and first compound in the list of possibilities; while Table 2b comprises experimental *m/z,* selected ion, tolerance (ppm) in Smart 3D, list of possibilities in Smart 3D editor (in increasing order of sigma), error (ppm) for the first compound and sigma

341 value for the first compound. For further discussion about the detected phenolic substances, see

342 section 3.4).

343 **Table 2.** Well-known phenolic compounds determined by NACE-ESI-TOF MS in enriched
344 Picual extra-virgin olive oil.

Compound	*Formula*	*m/z experimental*	*m/z calculated*	*Error (ppm)*	*Sigma Value*	*Classification order*	*Tolerance (ppm)*	*First compound*
HYTY	$C_8H_{10}O_3$	153.0563	153.0557	-3.483	0.0171	1st (1)	15	
TY	$C_8H_{10}O_2$	137.0614	137.0608	-4.497	0.0032	1st (1)	15	
Lig Agl	$C_{19}H_{22}O_7$	361.1289	361.1293	0.965	0.0543	2nd (10)	15	$C_{20}H_{17}N_4O_3$
Decarbox-Lig Agl	$C_{17}H_{20}O_5$	303.1232	303.1238	1.851	0.0321	1st (4)	15	
H-Pin	$C_{20}H_{22}O_7$	373.1283	373.1293	2.680	0.0254	1st (7)	15	
Pin	$C_{20}H_{22}O_6$	357.1344	357.1344	-0.206	0.0132	2nd (9)	15	$C_6H_{13}N_{16}O_3$
Ac Pin	$C_{22}H_{24}O_8$	415.1397	415.1398	0.342	0.0394	2nd (19)	15	$C_{17}H_{23}N_2O_{10}$
10-H-Ol Agl	$C_{19}H_{22}O_9$	393.1199	393.1191	-2.079	0.0099	1st (16)	15	
Ol Agl	$C_{19}H_{22}O_8$	377.1242	377.1242	-0.019	0.0158	1st (13)	15	$C_{11}H_{13}N_{12}O_4$
DOA	$C_{17}H_{20}O_6$	319.1199	319.1187	-3.781	0.0107	1st (8)	15	$C_{13}H_{15}N_6O_4$
HYTY-Ac	$C_{10}H_{12}O_4$	195.0662	195.0663	0.479	0.0079	1st (3)	15	
EA	$C_{11}H_{14}O_6$	241.0709	241.0718	3.731	0.0077	2nd (4)	15	$C_8H_5N_{10}$
Apig	$C_{15}H_{10}O_5$	269.0446	269.0455	3.358	0.0242	1st (5)	15	
Lut	$C_{15}H_{10}O_6$	285.0397	285.0405	2.764	0.0064	1st (6)	15	
Syring	$C_{22}H_{26}O_8$	417.1545	417.1555	2.260	0.0345	2nd (16)	15	$C_7H_{21}N_{12}O_9$
Vanillin	$C_8H_8O_3$	151.0406	151.0401	-3.744	0.0476	1st (1)	20	

345

346 **Table 2b.** "Unknown" phenolic compounds determined by NACE-ESI-TOF MS in enriched
347 Picual extra-virgin olive oil.
348

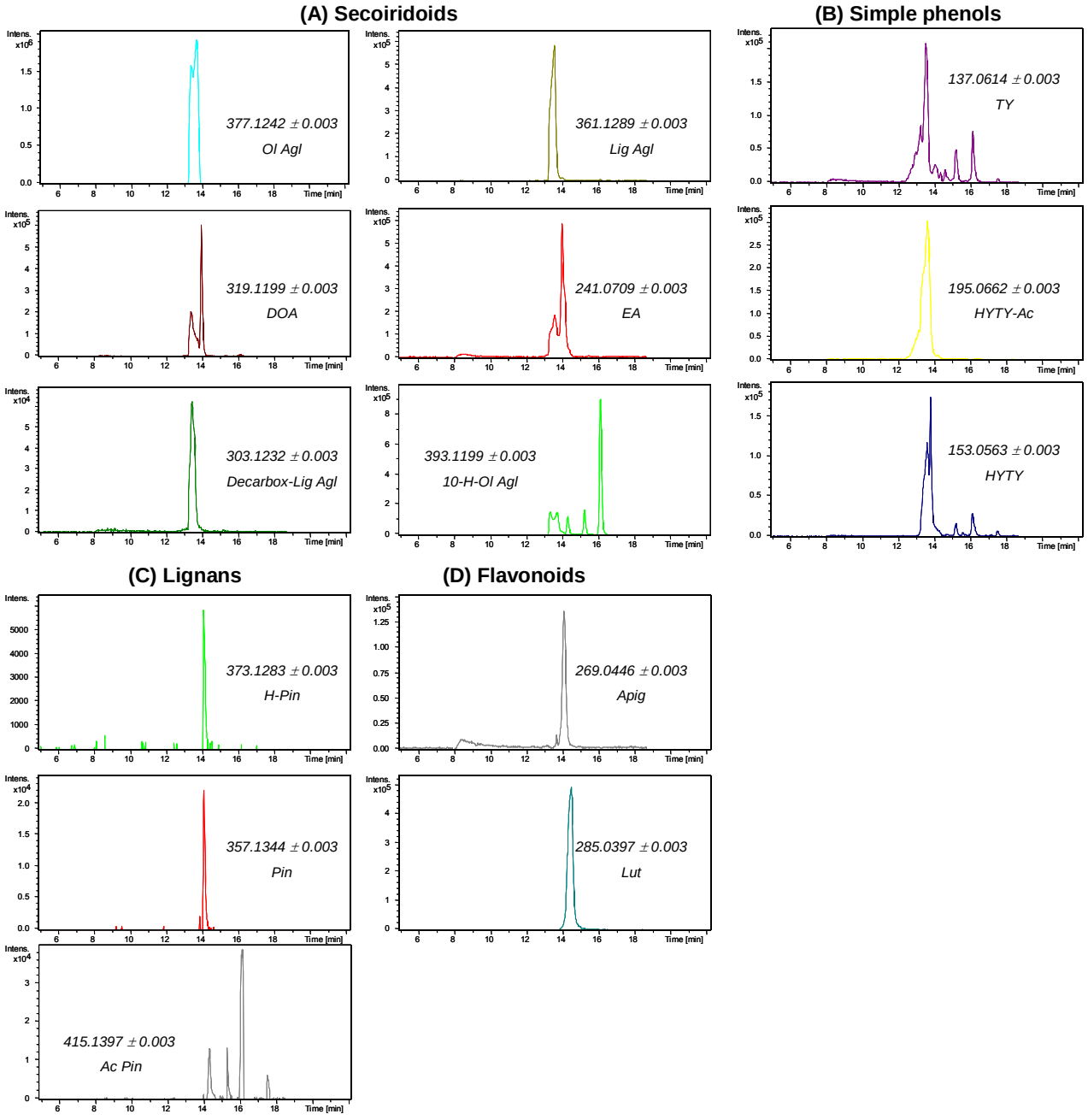
m/z experimental	*Tolerance (ppm)*	*List of possibilities in Smart Formula3D editor (in increasing order of sigma)*	*Error (ppm)*	*Sigma Value (for the first compound)*
201.0248	10	$C_4H_9O_9$ / $C_8H_9O_6$ / $C_1H_1N_{10}O_3$ / $C_5H_5N_4O_5$	1.824 2nd (77.716)	0.0543 2nd (0.0098)
257.0664	10	$C_{11}H_{13}O_7$ / $C_8H_5N_{10}O_1$ / $C_7H_9N_6O_5$ / $C_{12}H_9N_4O_3$	0.993	0.123
183.0668	20	$C_9H_{11}O_4$ / $C_{10}H_7N_4$ / $C_5H_7N_60_2$	-2.669	0.0229
213.0758	15	$C_6H_9N_6O_3$ / $C_{10}H_{13}O_5$ / $C_{11}H_9N_4O_1$ / $C_5H_{13}N_2O_7$	-7.829 2nd (4.774)	0.0090 2nd (0.0109)
471.3496	10	$C_{30}H_{47}O_5$ / $C_{22}H_{39}N_{12}$ / $C_{21}H_{43}N_8O_4$ / $C_{25}H_{47}N_2O_6$	-3.366	0.0201
281.2496	10	$C_{18}H_{33}O_2$ / $C_{14}H_{29}N_6$	3.698	0.0056
407.1366	10	$C_{20}H_{23}O_9$/ $C_{17}H_{15}N_{10}O_3$ / $C_{21}H_{19}N_4O_5$/ $C_{13}H_{27}O_4$	-4.567	0.0155
157.1227	15	$C_9H_{17}O_2$ / $C_5H_{13}N_6$	4.326	0.0136
315.2531	10	$C_{18}H_{35}O_4$ / $C_{14}H_{31}N_6O_2$ / $C_{13}H_{15}N_2O_6$ / $C_{19}H_{31}N_4$	3.119	0.0221
155.0709	20	$C_8H_{11}O_3$ / $C_4H_7N_6O_1$	3.031	0.0132
335.1138	10	$C_{17}H_{19}O_7$ / $C_{18}H_{15}N_4O_3$ / $C_{14}H_{11}N_{10}O_1$ / $C_4H_7N_{20}$	-0.664	0.0156
315.1229	10	$C_{18}H_{19}O_5$ / $C_{19}H_{15}N_4O_1$ / $C_{14}H_{15}N_6O_3$ / $C_4H_{11}N_{16}O_2$	2.700	0.0234
297.2422	10	$C_{18}H_{33}O_3$ / $C_{14}H_{29}N_6O_1$ / $C_{13}H_{33}N_2O_5$	4.272	0.0331
295.2269	15	$C_{18}H_{31}O_3$ / $C_{13}H_{31}N_2O_5$ / $C_{14}H_{27}N_6O_1$	3.252	0.0287
199.0608	20	$C_9H_{11}O_5$/ $C_{10}H_7N_4O_1$ / $C_5H_7N_6O_3$	1.909	0.0112
333.1351	10	$C_{18}H_{21}O_6$ / $C_{19}H_{17}N_4O_2$ / $C_{15}H_{13}N_{10}$	-2.227	0.0118
317.1030	10	$C_{17}H_{17}O_6$ / $C_{18}H_{13}N_4O_2$ / $C_{14}H_9N_{10}$ / $C_{13}H_{13}N_6O_4$	0.273	0.0094

349

350 Fig. 5 contains extracted ion electropherograms (EIEs) of the well-known phenolic compounds

351 detected in a Picual extra-virgin olive enriched oil (containing information about the m/z

352 experimental, molecular formula and the name of the compound).



353

354 **Figure 5**. EIEs of the well-known phenolic compounds detected in a Picual extra-virgin olive
355 enriched oil (containing information about the m/z experimental, molecular formula and the
356 name of the compound).

357 We studied the effect of other two phenomena that can affect several analytical figures of merit,
358 such as detection capability, precision, and accuracy: ion suppression and specificity.

359 The ion suppression effect may be caused by a number of factors such as the matrix or
360 interference co-eluting compounds. We used a similar strategy to the one described by Nelson
361 and Dolan [47] for systematic investigation of ion suppression in HPLC-MS analysis. So, we

produced a continuous flow of analyte solution (using the syringe pump for sheath-liquid) resulting in a constant but elevated baseline in MS detection. We dissolved a similar amount of phenolic compounds to the one found in our samples in isopropanol/water (1:1) and we used it as sheath-liquid at a flow rate of 4 μL/min. This flow was then continuously mixed with eluate from an NACE-separated blank sample. Consequently, ion-suppressing components will be detectable as a drop in the baseline over the complete CE run. Such investigations could enable optimization of CE conditions to prevent coelution of analyte and interfering substances. Moreover, we spiked extracts of Picual enriched extra-virgin olive oil samples with different amount of phenolic compounds making sure that even if the resolution of our method was not extremely high, the method could be able to quantify them avoiding ionization suppression phenomena.

The specificity of the method was tested by screening analysis of blank (in terms of phenols) olive oil samples. There were no impurity peaks or contamination at the retention times corresponding to the analytes.

3.2. Aqueous CE-ESI-TOF MS analyses

The analyses were performed by using the aqueous CE conditions previously described in Materials and Methods section.

Determined compounds have also been summarized in two tables (Table 3 and 3b). Well-known compounds table contains information about molecular formula, calculated and experimental *m/z*, error, sigma value, classification order (number of possibilities), tolerance (ppm) in Smart 3D and first compound in the list of possibilities, while “unknown” compounds table includes experimental *m/z,* selected ion, tolerance (ppm) in Smart 3D, list of possibilities in Smart 3D editor (in increasing order of sigma), error (ppm) for the first compound and sigma value for the first compound.

Table 3. Well-known phenolic compounds determined by CE-ESI-TOF-MS in an extract of Picual extra-virgin olive oil.

Compound	*Formula*	*m/z experimental*	*m/z calculated*	*Error (ppm)*	*Sigma Value*	*Classification order*	*Tolerance (ppm)*	*First compound in the list of possibilities*
HYTY	$C_8H_{10}O_3$	153.0550	153.0557	-4.573	0.0071	1st (1)	15	
TY	$C_8H_{10}O_2$	137.0613	137.0608	-3.703	0.0063	1st (1)	15	
Lig Agl	$C_{19}H_{22}O_7$	361.1286	361.1293	1.938	0.0543	2nd (10)	15	$C_{20}H_{17}N_4O_3$
Decarbox-Lig Agl	$C_{17}H_{20}O_5$	303.1238	303.1238	0.039	0.0321	1st (4)	15	
H-Pin	$C_{20}H_{22}O_7$	373.1283	373.1293	2.680	0.0154	1st (7)	15	
Pin	$C_{20}H_{22}O_6$	357.1337	357.1344	1.899	0.0419	2nd (9)	15	$C_6H_{13}N_{16}O_3$
Ac Pin	$C_{22}H_{24}O_8$	415.1388	415.1398	2.395	0.0231	2nd (17)	15	$C_{17}H_{23}N_2O_{10}$
10-H-Ol Agl	$C_{19}H_{22}O_9$	393.1179	393.1191	2.971	0.0395	2nd (16)	15	$C_{16}H_{13}N_{10}O_3$
Ol Agl	$C_{19}H_{22}O_8$	377.1242	377.1242	-0.071	0.0390	2nd(13)	15	$C_{11}H_{13}N_{12}O_4$
DOA	$C_{17}H_{20}O_6$	319.1171	319.1187	5.066	0.0349	1st (7)	15	$C_{13}H_{15}N_6O_4$
HYTY-Ac	$C_{10}H_{12}O_4$	195.0661	195.0663	1.025	0.0097	1st (3)	15	
EA	$C_{11}H_{14}O_6$	241.0707	241.0718	4.404	0.0076	1st (4)	15	
Apig	$C_{15}H_{10}O_5$	269.0447	269.0455	3.075	0.0423	1st (5)	15	
Lut	$C_{15}H_{10}O_6$	285.0411	285.0405	-2.105	0.0234	1st (6)	15	
Syring	$C_{22}H_{26}O_8$	417.1548	417.1555	1.678	0.0435	2nd (16)	15	$C_7H_{21}N_{12}O_9$
Vanillin	$C_8H_8O_3$	151.0406	151.0401	-3.744	0.0212	1st (1)	20	
Ferulic acid	$C_{10}H_{10}O_4$	193.0512	193.0506	-2940	0.0344	1st (1)	20	

Table 3b. "Unknown" phenolic compounds determined by CE-ESI-TOF-MS in an extract of Picual extra-virgin olive oil.

m/z experimental	*Tolerance (ppm)*	*List of possibilities in Smart Formula3D editor (in increasing order of sigma)*	*Error (ppm)*	*Sigma Value (for the first compound)*
281.2482	20	$C_{18}H_{33}O_2$/ $C_{13}H_{33}N_2O_4$ / $C_{14}H_{29}N_6$	1.345	0.0043
143.1082	20	$C_8H_{15}O_2$	-2.886	0.0321
182.0821	20	$C_9H_{12}N_1O_3$ / $C_5H_8N_7O_1$	0.738	0.0076
471.3458	10	$C_{30}H_{47}O_5$ / $C_{31}H_{43}N_4$ / $C_{26}H_{43}N_6O_2$ / $C_{23}H_{51}O_9$	4.612	0.0176
315.2572	10	$C_{18}H_{35}O_4$/ $C_{14}H_{31}N_6O_2$ / $C_{19}H_{31}N_4$ / $C_{13}H_{35}N_2O_6$	3.387	0.0076
299.2584	10	$C_{18}H_{35}O_3$ / $C_{13}H_{35}N_2O_5$/ $C_{14}H_{31}N_6O_1$	2.673	0.0042
295.2269	10	$C_{18}H_{35}O_4$/ $C_{14}H_{31}N_6O_2$ / $C_{19}H_{31}N_4$	3.387	0.0144
297.2378	15	$C_{18}H_{33}O_3$ / $C_{13}H_{33}N_2O_5$ / $C_{14}H_{29}N_6O_1$	3.941	0.0032
257.0666	15	$C_{11}H_{13}O_7$ / $C_8H_5N_{10}O_1$ / $C_{12}H_9N_4O_3$ / $C_7H_9N_6O_5$	0.373	0.0123
315.1226	15	$C_{18}H_{19}O_5$/ $C_{14}H_{15}N_6O_3$ / $C_{10}H_{11}N_{12}O_1$	3.808	0.0188
333.1345	10	$C_{18}H_{21}O_6$/ $C_{14}H_{17}N_6O_4$ / $C_{15}H_{13}N_{10}$	-0.562	0.0230
166.0509	20	$C_8H_8N_1O_3$ / $C_4H_4N_7O_1$	0.293	0.0121
150.0563	20	$C_8H_8N_1O_2$ / $C_4H_4N_7$	-1.783	0.0492
157.1235	20	$C_9H_{17}O_2$	0.787	0.0050
183.0664	20	$C_9H_{11}O_4$ / $C_{10}H_7N_4$	-0.455	0.0123
199.0607	20	$C_9H_{11}O_5$ / $C_5H_7N_6O_3$ / $C_{10}H_7N_4O_1$	2.455	0.0243

3.3. Repeatability and reproducibility in aqueous and non-aqueous CE

Repeatability was studied by carrying out the analysis using the optimized method of one of the samples (MeOH/Water extract or enriched EVOO in aqueous CE or NACE respectively), repeating it twelve times in the same day (intraday precision, n=12) and doing the same process

three consecutive days (interday precision, *n*=36). The RSDs of peak areas/migration time and migration times were determined considering eight of the compounds present in the extracts: five of the well-known compounds (hydroxytyrosol acetate, 10-hydroxy oleuropein aglycon, oleuropein aglycon, decarboxilated ligstroside aglycon, apigenin) and three of the "unknown" compounds (471.3496, 315.2531, 199.0608**).** Data obtained in this study are summarized in Table 4. Both intraday and interday repeatabilities calculated on the migration time for these analytes (expressed as RSD) and the intraday and interday repeatability values on the total peak area/migration time (expressed as RSD) were acceptable.

Table 4. R.S.D. values of the peak area and migration time obtained in the study of repeatability and reproducibility.

	Repeatability (AQUEOUS)				**Repeatability (NON-AQUEOUS)**			
	Intraday (n=12)		*Interday* (n=36)		*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	Peak area/migration time	Migration time
HYTY-Ac	2.00	0.52	4.99	1.23	1.89	0.23	3.99	1.12
10-H-Ol Agl	2.26	0.76	3.26	1.67	2.03	0.46	3.06	1.70
Ol Agl	3.03	0.62	3.25	1.09	3.23	0.56	2.25	1.03
Decarbox-Lig Agl	3.32	0.78	4.12	1.09	2.32	0.87	3.12	1.19
Apig	1.25	0.91	2.41	1.78	1.15	0.99	2.01	1.56
471.3496	1.99	0.65	3.06	1.89	2.00	0.45	3.32	1.49
315.2531	1.32	0.91	2.09	1.01	1.12	0.81	3.19	1.00
199.0608	1.45	1.25	2.45	2.03	1.25	0.90	4.09	1.83

The intraday repeatability values on the peak area/migration time (expressed as RSD) were between 1.25 and 3.32 for the CE method and between 1.12 and 3.23 for NACE, whereas the interday repeatability values on the peak area/migration time were between 2.09 and 4.99 for the CE method and between 2.01 and 4.09 for NACE.

The intraday repeatability values on the migration time (expressed as RSD) were between 0.52 and 1.25 for the CE method and between 0.23 and 0.99 for NACE, whereas the interday repeatability values on the migration time were between 1.01 and 2.03 for the CE method and between 1.00 and 1.83 for NACE.

3.4. Comparison between the results obtained by NACE-ESI-TOF MS and aqueous CE-ESI-TOF MS

BPE showing the profile obtained for non-aqueous and aqueous CE can be observed in Fig. 4. The intensity of the peaks in the BPE of NACE method is higher, which means that a non aqueous media and the injection of an extract of enriched olive oil led to a better sensitivity. The concentration in phenolics of aqueous and non aqueous extracts was approximately the same (See preparation of extracts in Section 2.2), but the intensity obtained with NACE was around ten times higher.

Tables 2 and 2b and Tables 3 and 3b evidence that both methods are able to detect and identify compounds belonging to the most important families of phenols present in EVOO. Moreover, as it can be seen in those Tables, aqueous and non-aqueous CE methods are in a good agreement regarding to the "unknown" phenolic compounds.

As far as analysis time is concerned, aqueous CE method produces faster results. However, NACE method allows making direct injections of EVOO obtaining satisfactory results.

NACE method was able to determine 16 well-known phenolic compounds and 16 "unknown" analytes. On the other hand, aqueous CE method could give to the analyst information about 17 compounds previously described in extra-virgin olive oil and the same number of "unknown" compounds. Regarding "new" analytes found in oil, the results achieved by both methods are in good agreement. Eleven "unknown" compounds found by NACE and aqueous CE methods were exactly the same, and also the molecular formula found by Smart formula 3D editor were coincident. The results were very similar to those obtained by Carrasco-Pancorbo *et al.* [38]. However, NACE method detected 5 compounds that aqueous CE was not able to see (143.1082, 182.0821, 299.2584, 166.0509 and 150.0563), whilst aqueous CE method found 6 analytes that NACE conditions did not observe (201.0248, 213.0758, 407.1366, 155.0709, 335.1138 and 317.1030).

It is worth to highlight that Lig Agl, Decarbox-Lig Agl and specially 10-H-Ol Agl were detected with a very low intensity by CE method while the intensities found for these compounds with the NACE method were similar to the intensities of the other compounds. Because of that it can be said that the relative intensities of those compounds were higher with the NACE method.

Aqueous and non-aqueous capillary electrophoresis have demonstrated to be reliable methodologies for the analysis of the phenolic fraction of EVOO. Both of them are able to determined most important compounds of this fraction as well as a wide group of still "unknown" phenolic compounds.

The advantage that NACE presents is the possibility to carry out the determination of phenols using direct injection of oil and the higher sensibility of this method.

4 Conclusions

The analysis of the phenolic fraction of EVOO by non-aqueous CE coupled to ESI-TOF MS has been studied for the first time. NACE has proven to be a reliable technique to characterize this fraction in a short time of analysis and, because of the characteristics of organic solvents, NACE also allows the direct injection of olive oil in capillary. The fact of doing direct injection of olive oil in NACE is one of the greatest advantages that can be mentioned.

Despite that analysis time is still better for aqueous CE, both aqueous and non-aqueous CE are able to determine most of well-known phenolic compounds present in EVOO and they are also in agreement about the presence of the "unknown" phenolic compounds.

In order to improve the detection limits and the identification powerfulness of the method, sample stacking techniques will be applied in the future and MS/MS analyses will be performed as well. These future steps could help to obtain higher sensibility and supplementary information about the "unknown" compounds.

476 **5 References**

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El método NACE consiguió reducir un tiempo de análisis de alrededor de 18 minutos para determinar todos los analitos. A pesar de los estudios realizados acerca del comportamiento de los distintos disolventes orgánicos y del buffer con respecto al tiempo de migración e intensidad de la señal (Figuras 1 y 2), las condiciones escogidas como óptimas fruto de un compromiso entre resolución, tiempo de análisis e intensidad de la señal condujeron al electroferograma óptimo que puede verse en la Figura 4 A.

Merece especial mención el hecho de que el método NACE en el que se utiliza inyección directa de aceite de oliva enriquecido en compuestos fenólicos sea capaz de determinar prácticamente los mismos compuestos determinados mediante otras metodologías CZE. Mediante NACE se identifican 16 de los 17 analitos determinados normalmente por CZE y entre los compuestos desconocidos se llegan a identificar 5 masas pertenecientes a compuestos que no habían podido ser detectados con el método CZE (hay que tener en cuenta que al estar realizando inyección directa de aceite, estas masas pueden corresponder a otro tipo de compuestos, no necesariamente a compuestos fenólicos).

El orden de elución de los compuestos se ve alterado de una metodología a otra debido a las diferentes propiedades fisicoquímicas de los disolventes orgánicos empleados con respecto al agua. Los valores de pH, que están perfectamente definidos para los medios acuosos, deben ser considerados muy cuidadosamente en disolventes orgánicos porque suelen ser sustancialmente diferentes de los encontrados en agua y por ello influyen considerablemente en la separación.

Capítulo 4

Efecto de diferentes sistemas de filtración en el contenido fenólico de aceite de oliva virgen mediante HPLC-DAD-MS.

Publicación incluída en este capítulo:

Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD.

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El siguiente capítulo tiene un enfoque más tecnológico. A pesar de ello encaja perfectamente en el tema de estudio de esta tesis y en este bloque, ya que empleando la cromatografía líquida de alta resolución se estudian, entre otros, los efectos que se producen a nivel de los compuestos fenólicos en el aceite de oliva cuando éste se ve sometido a diferentes sistemas de filtración.

Los procesos de filtración empleados en este estudio fueron el algodón y el filtro de papel acompañado de sulfato sódico anhidro. Estos métodos de filtración son los más empleados en las pequeñas almazaras, por lo que este estudio es perfectamente aplicable a las situaciones reales que pueden darse en éstas.

Los aceites utilizados fueron aceites provenientes de diferentes regiones de Italia (ya que este trabajo fue realizado durante la estancia de investigación realizada en la Universidad de Bolonia, Italia), producidos en años diferentes (por lo que se ve afectado el estado oxidativo), obtenidos mediante tecnologías diferentes y almacenados de forma distinta. Esto permitió obtener una visión amplia y extrapolable a una gran variedad de aceites.

A la hora de realizar esta investigación se llevaron a cabo una serie de pasos que detallamos a continuación:

- Elección de la muestras de aceite objeto de estudio.
- Filtración de los aceites.
- Análisis del contenido en agua.
- Evaluación de la estabilidad oxidativa.
- Análisis colorimétrico.
- Extracción y análisis del contenido en compuestos fenólicos.

En este trabajo quedan reflejadas las discusiones acerca de los resultados obtenidos para cada uno de estos parámetros tratados, subrayándose el estudio realizado mediante HPLC sobre el efecto de la filtración en la variación del contenido fenólico del aceite de oliva antes y después de ésta. Este punto cobra mayor importancia debido al hecho de que, hasta el momento en el que se desarrolló, no existían referencias bibliográficas acerca de trabajos de este tipo llevados a cabo determinando la variación del contenido fenólico del aceite de oliva empleando para ellos técnicas separativas avanzadas como es el caso del HPLC.

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Effect of Filtration Systems on the Phenolic Content in Virgin Olive Oil by HPLC-DAD-MSD

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Abstract: This research has been carried out to determine the filtration effect on the phenolic content and on the visual characteristics of olive oil. The influence of the filtration system on the phenolic and water content of virgin olive oil that differed in the year of production, production system and the olive variety was measured. Samples were filtered in the laboratory using two different systems (cotton or filter paper plus sodium sulphate anhydrous). Qualitative and quantitative variation of the phenolic fraction of virgin olive oils was evaluated by HPLC-DAD-MS and correlated with their water content (by Karl Fischer titration) and oxidative stability under forced conditions (by OSI). Colorimetric assays were also carried out in order to calculate the effect of filtration on the visual characteristics of virgin olive oil. After filtration the oxidative stability index decreased and in particular, filtration with cotton showed a significant loss of hydroxytyrosol, a phenol endowed with high antioxidant activity. One interesting behaviour was highlighted: Filtration with either cotton or paper plus anhydrous sodium sulphate led to an apparent increase in the phenolic content. These apparently contradictory data can be explained by considering that the reduction of the water content permits a higher availability of phenolic compounds that remain in oil and are extracted with the methanol-water mixture. Lastly, the filtration of virgin olive oil produced a loss in the intensity of green color and an increase in its lightness.

Key words: Virgin olive oil, phenols, water, filtration, HPLC-DAD-MSD

INTRODUCTION

Polyphenols is a term widely used to designate substances that possess a benzene ring bearing one or more hydroxyl groups, including functional derivatives (Harborne *et al*., 1989). Polyphenols are polar compounds that can be found in the olive fruit; however many of these compounds are modified or lost during the production process of virgin olive oil (Brenes *et al*., 1995). The final quantity of polyphenols is also influenced by the cultivar, climatic conditions during growth and degree of ripening (Di Giovacchino *et al*., 2002; Cerretani *et al*., 2005). Virgin olive oil is dominated by secoiridoid derivatives, followed by flavonoids and phenolic alcohols. The presence of secoiridoid derivatives provides an indication of the degradation pathways for the phenolic oleosides present in olive paste and wet pomace (Artajo *et al*., 2007). These derived compounds appear in virgin olive oil and possess antioxidant activity and a lower polarity compared to those in olive fruits (as glycosidic compounds). The partition coefficients between olive oil and water depend on the structure of these compounds and the number of hydroxyl groups.

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The most abundant secoiridoids in virgin olive oil are the dialdehydic forms of elenolic acid linked to hydroxytyrosol or tyrosol (3,4-DHPEA-EDA or *p*-DHPEA-EDA) and an isomer of the oleuropein aglycone (3,4-DHPEA-EA). This can be explained by the fact that DHPEA-EA and 3,4-DHPEA-EDA are the compounds with the highest partition coefficients, as reported by Servili *et al.* (2005).

It is known that olive oil has a low quantity of water (Fregapane *et al.*, 2006; Mendez *et al.*, 2007) and for this reason olive oil can be considered as a water-in-oil emulsion. The presence of phenolic compounds in virgin olive oil and their high antioxidant activity can be explained by the so-called polar paradox (Porter *et al.*, 1989) dictating that: Polar antioxidants are more effective in non polar lipids, whereas non-polar antioxidants are more active in polar lipid emulsions.

Frankel *et al.* (1994) demonstrated that interfacial phenomena are key to a better understanding of antioxidant action in heterogeneous foods and biological systems. The orientation of phenolic compounds in the oil-water interface and the active surface of water droplets influence protection against the oxidation of oil. This study concluded that lipophilic antioxidants are more effective in an oil-in-water emulsion system than in bulk oil, while an opposite trend has been found for hydrophilic antioxidants.

An important factor to consider is the visual appearance of virgin olive oil, as it will strongly influence consumer preference. Color is an intrinsic characteristic of each food product and helps to identify it, to the extent that the consumer is disconcerted if the color changes. From a hedonistic point of view, the color of olive oil can be considered an important organoleptic attribute that is a basic criterion in assessing quality, according to consumer preferences (McEwan, 1994; Pagliarini *et al.*, 1994).

The compounds responsible for the color of virgin olive oil are chlorophylls, carotenoids and flavones (as apigenin and luteolin). Chlorophylls give olive oil its yellow-green color, carotenoids contribute in the yellow-red range (Mínguez-Mosquera *et al.*, 1991) and flavones, having an absorbance maximum at around 330-350 nm, provide a yellow color.

Usually, the color of food is measured in L*a*b*. The L*a*b*, or CIELab, color space is an international standard for color measurements, adopted by the Commission Internationale d'Eclairage (CIE) in 1976. L* is the luminance or lightness component, which ranges from 0 to 100 and parameters a* (from green to red) and b* (from blue to yellow) are the two chromatic components, which range from -120 to 120 (Papadakis *et al.*, 2000; Segnini *et al.*, 1999; Yam *et al.*, 2004). Nevertheless, the measurement of color is not currently required by regulations established by the European Economic Community (European Union Commission, 1991) to assess the quality of olive oil.

The aim of this report was to evaluate how different filtration processes (normally carried out during virgin olive oil production) affect the characteristics of virgin olive oil. In particular, oxidative stability, water content, the presence of each phenolic compound and color changes of virgin olive oil have been investigated. Eight types of virgin olive oil have been examined that were filtered using two different filtration systems (cotton or filter paper plus anhydrous sodium sulphate).

In our knowledge this is the first study in which olive oils with different origins have been analyzed in order to determine the effects of filtration focusing on the phenolic profile by using a separative technique as HPLC. Furthermore, the filtration systems used have been those that are traditionally applied in small mills.

MATERIALS AND METHODS

Apparatus

All HPLC analyses were performed using a HP 1100 Series instrument (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump delivery system, degasser, autosampler, diode-

array UV-VIS Detector (DAD) and Mass-Spectrometer Detector (MSD). The analytical HPLC column used was a C_{18} Luna column, 5 μm, 25 cm×3.0 mm (Phenomenex, Torrance, CA, USA), with a C_{18} pre-column (Phenomenex) filter. The mobile phase flow rate was 0.5 mL min^{-1}. All analyses were carried out at room temperature.

The CIELab color space analyses were carried out using a ColorFlex instrument (HunterLab, Reston, VA, USA). To evaluate oxidative stability an eight-channels Oxidative Stability Instrument (OSI) (Omnion, Decatur, IL, USA) was used. The water content of virgin olive oils was obtained using a TitroMatic 1S instrument (Crison Instruments, S.A.; Alella, Barcelona, Spain).

Reagents and Standards

The standard used for HPLC quantification (3,4-dihydroxyphenylacetic acid) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Methanol, acetic acid, acetonitrile and *n*-hexane were from Merck and Co. Inc. (Darmstadt, Germany). All solvents were HPLC-grade and filtered through a 0.45 μm nylon filter disk (Lida Manufacturing Corp., Kenosha, WI, USA) prior to use. Double-deionized water with a conductivity less than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Hydranal-Titran 2 and Hydranal-solvent oil (solvents used to measure the water content with the volumetric titration of Karl Fischer) were from Riedel-deHaën (Seelze, Germany).

Samples

Eight samples of virgin olive oil were obtained from different geographic zones in Italy. They differed in the production year (oxidative state), production plant (traditional and continuous) and storage conditions.

The analysis were carried out on July-September 2006 in the laboratories of the Department of Food Science of the University of Bologna, Cesena (Italy).

Filtration Processes

Two different filtration systems were utilized to reduce the water content of virgin olive oils: (a) cotton and (b) paper and sodium sulphate anhydrous.

- Cotton: Fifty gram of virgin olive oil was passed through 0.5 g of cotton.
- Paper and sodium sulphate anhydrous: Fifty gram of virgin olive oil were passed through filter paper. Next, 100 g of anhydrous sodium sulphate per L of oil was added to the sample and the oil was shaken in order to eliminate the water.

Extraction of Polar Phenolic Fraction

Phenolic compounds were extracted from virgin olive oil by a liquid-liquid extraction method according to Pirisi *et al.* (2000). The dry extracts were dissolved in 0.5 mL of a methanol/water (50/50, v/v) solution and filtered through a 0.2 μm syringe filter (Whatman Inc., Clinton, NJ, USA). The extracts were frozen and stored at -43°C.

Determination of Phenolic Compounds by HPLC-DAD-MSD

Determination of the phenolic fraction was performed using an HPLC-DAD/ESI-MSD equipped with a reverse phase C_{18} Luna™ column according to Rotondi *et al.* (2004). Phenolic compounds detected at 280 nm were quantified using a 3,4-dihydroxyphenylacetic acid standard calibration curve ($r^2 = 0.9987$). Phenolic compounds were tentatively identified based on their UV-vis and mass spectra (Table 1) obtained by HPLC-DAD/ESI-MSD.

Table 1: Absorption maxima and fragmentation patterns using the ESI interface of the compounds under study

		Major fragments ESI positive			
Phenolic compounds	λ_{max} (nm)	m/z $[M + 1]^+$	m/z $[M + Na]^+$	m/z $[M + K]^+$	Other fragments
Hydroxytyrosol	232/280	-	-	-	137.1
Tyrosol	230/276	-	-	-	121.0
Vanillic acid	228/260/294	169.1	191.1	-	-
Decarboxymethyl-oleuropein aglycon	232/282	-	343.1	359.1	137.1
Pinoresinol	234/280	359.1	381.2	397	398.7
Decarboxymethyl-ligstroside aglycon+ Acetoxypinoresinol	234/280	-/417.1	327.1/439	343.1/455	121.0/385.1
Oleuropein aglycon	232/280	379.1	401.1	417.1	137.1
Ligstroside aglycon	230/276	363.1	385.1	401.1	121.0

Evaluation of Oxidative Stability under Forced Conditions

These analyses were carried out in an eight-channel Oxidative Stability Instrument (OSI) following Carrasco-Pancorbo *et al*. (2005) analytical protocol.

Determination of Water Content in Virgin Olive Oil

The water content was analyzed with a TitroMatic 1S instrument. This measurement uses a Karl-Fischer titration based on a bivoltametric indication (2-electrode potentiometry). A solution of chloroform: Hydranal-solvent oil (a methanolic solvent) 2:1 (v/v) was used to dissolve the sample and Hydranal-Titran 2 was used as a titrating reagent. Each sample was introduced three times and the quantity of sample was measured with the back weighing technique. The sample was dissolved in the solution of chloroform: Hydranal-solvent (2/1, v/v) oil and the titrating reagent was added until the equivalence point. The quantity of water was expressed as mg of water per kg of oil (n = 3).

Statistical Analysis

Data were analyzed using Statistica 6.0 (Statsoft, Tulsa OK, USA) statistical software. The significance of differences at a 5% level between averages was determined by a one-way ANOVA using Tukey's test.

RESULTS AND DISCUSSION

Effect of Filtration on the Water Content

In most olive oil samples the water content decreased significantly after filtration (Table 2). With the exception of sample 7, the control sample always had a higher water content, whereas samples that had been filtered with cotton showed a significant decrease in the quantity of water. However, samples that had been filtered using paper and anhydrous sodium sulphate presented a significant decrease in only four of the eight samples (samples 2, 3, 4 and 8).

Filtration with cotton, termed filtration Bari style, is especially widespread in the olive oil industry plants located in the South of Italy and as is shown from experimental data in Table 2, can be considered effective in reducing the water content.

Evaluation of Oxidative Stability under Forced Conditions

The Oxidative Stability Index (OSI) decreased after the filtration with cotton and paper plus anhydrous sodium sulphate (Table 2). This effect was more pronounced in samples that showed a higher OSI value. For example, the OSI of sample 2 decreased about 4.4% with cotton filtration and by 18.0% with anhydrous sodium sulphate plus paper; sample 6, another control sample with a high oxidative stability, showed a reduction of 15.0% with cotton filtration and 19.0% with anhydrous sodium sulphate plus paper. However samples 4 and 5 that had a low OSI value compared to the

Table 2: Analytic results of virgin olive oils: Oxidative stability (OSI, RSD% = 2.3), Water content (mg of water per kg of olive oil) and Phenolic compounds (mg of analyte as 3,4-dihydroxyphenylacetic acid per kg of olive oil). Quantification of the Individual Components (n = 7) (±SD)

		OSI (h)	H_2O	HYTY	TY	VA
Sample 1	Control sample	18.35	1070.0±92.4 (a)	44.7±1.9 (a,b)	50.1±1.6 (a)	1.6±0.1 (b)
	Cotton	16.65	699.9±34.2 (b)	37.5±3.4 (b)	57.6±3.8 (a)	0.7±0.2 (c)
	Paper+SSA	16.25	935.4±9.7 (a)	51.5±4.7 (a)	58.9±5.5 (a)	5.1±0.8 (a)
Sample 2	Control sample	27.25	1089.8±104.8 (a)	8.7±0.6 (a)	4.2±0.3 (a)	0.8±0.4 (b)
	Cotton	26.05	668.9±12.1 (b)	5.2±0.2 (a)	3.7±0.2 (a)	0.7±0.3 (b)
	Paper+SSA	22.35	806.4±8.2 (b)	8.5±0.9 (a)	4.2±0.4 (a)	1.6±0.5 (a)
Sample 3	Control sample	14.45	1584.8±16.3 (a)	13.0±0.7 (a)	40.0±2.1 (a)	0.8±0.2 (a)
	Cotton	13.30	780.1±49.8 (b)	5.9±0.3 (c)	23.9±2.1 (b)	1.0±0.3 (a)
	Paper+SSA	12.95	702.8±50.4 (b)	8.7±0.3 (b)	25.2±0.7 (b)	1.0±0.1 (a)
Sample 4	Control sample	8.65	1312.4±51.4 (a)	1.9±0.1 (b)	36.0±1.7 (a)	0.5±0.2 (c)
	Cotton	8.40	740.1±39.1 (b)	1.0±0.0 (c)	25.5±1.0 (c)	0.8±0.1 (b)
	Paper+SSA	8.45	796.5±18.2 (b)	3.8±0.1 (a)	30.6±1.1 (b)	1.2±0.1 (a)
Sample 5	Control sample	7.45	1136.0±120.3 (a)	2.0±0.5 (a,b)	5.8±0.9 (a)	1.8±1.3 (b)
	Cotton	6.85	786.4±30.2 (b)	1.5±0.2 (b)	7.1±1.3 (a)	2.4±0.5 (b)
	Paper+SSA	6.35	1033.9±34.7 (a)	2.4±0.3 (a)	6.9±1.1 (a)	5.3±1.2 (a)
Sample 6	Control sample	20.70	790.0±46.9 (a,b)	6.9±0.6 (a)	5.0±0.7 (a)	3.6±0.4 (c)
	Cotton	17.60	727.4±19.6 (b)	5.0±0.3 (b)	5.7±0.5 (a)	5.7±0.1 (b)
	Paper+SSA	16.70	859.4±17 (a)	7.6±0.7 (a)	6.2±0.6 (a)	10.2±0.9 (a)
Sample 7	Control sample	19.65	975.3±110.6 (a)	23.3±0.9 (a)	39.7±1.3 (a,b)	1.1±0.1 (b)
	Cotton	19.45	810.1±17.2 (a)	18.7±1.1 (b)	41.7±2.2 (a)	3.5±0.3 (a)
	Paper+SSA	16.30	861.2±57.4 (a)	22.2±1.1 (a)	37.6±2.4 (b)	3.4±0.2 (a)
Sample 8	Control sample	17.20	1369.5±17.6 (a)	3.1±1.5 (a,b)	7.6±1.0 (b)	3.5±1.4 (a)
	Cotton	16.55	768.5±82.2 (b)	7.9±1.9 (a)	19.2±3.0 (a)	nd
	Paper+SSA	14.70	720.6±29.3 (b)	2.9±0.9 (b)	7.9±1.0 (b)	4.7±1.2 (a)

Table 2: Continued

		DMOA	Pin	DLA+AcPin	Ol Agl	LA
Sample 1	Control sample	12.8±8.7 (a)	nd	21.7±3.9 (b)	35.6±9.6 (a)	10.9±2.5 (b)
	Cotton	9.1±2.1 (a)	5.2±0.7 (a)	26.2±2.3 (a,b)	37.5±1.9 (a)	14.9±1.0 (a)
	Paper+SSA	9.3±0.7 (a)	4.2±2.8 (a)	30.5±2.3 (a)	33.3±1.4 (a)	14.3±0.9 (a)
Sample 2	Control sample	18.7±2.1 (a)	12.0±2.1 (a)	16.7±1.5 (c)	22.8±2.2 (a)	4.9±1.1 (a,b)
	Cotton	13.5±1.2 (b)	nd	29.8±2.8 (a)	15.7±1.2 (b)	3.5±0.7 (b)
	Paper+SSA	14.6±1.7 (b)	12.6±1.1 (a)	21.7±2.7 (b)	20.4±2.4 (b)	5.8±0.9 (a)
Sample 3	Control sample	3.4±0.9 (a)	5.8±0.9 (a)	24.6±2.5 (a)	10.7±2.9 (a)	2.8±0.9 (a)
	Cotton	2.2±0.5 (a)	2.9±2.5 (a)	22.2±8.4 (a)	8.6±4.4 (a)	3.5±2.1 (a)
	Paper+SSA	3.1±0.6 (a)	4.7±0.3 (a)	26.2±1.2 (a)	8.8±1.0 (a)	5.1±0.9 (a)
Sample 4	Control sample	3.6±0.4 (a)	4.9±0.7 (a)	25.8±3.1 (a)	6.8±0.6 (a)	4.6±1.4 (b)
	Cotton	1.5±0.3 (c)	4.3±0.4 (a)	28.5±2.6 (a)	7.1±0.4 (a)	5.9±0.7 (a,b)
	Paper+SSA	2.6±0.4 (b)	4.4±0.3 (a)	26.6±0.9 (a)	8.0±0.8 (a)	7.0±0.3 (a)
Sample 5	Control sample	4.3±1.3 (a,b)	4.00±0.94 (a)	11.4±2.2 (b)	3.9±0.8 (b)	0.4±0.1 (b)
	Cotton	3.4±0.3 (b)	4.89±0.77 (a)	18.3±2.6 (a)	6.6±1.4 (a)	9.9±1.8 (a)
	Paper+SSA	5.6±0.9 (a)	5.70±0.92 (a)	22.2±3.8 (a)	6.9±0.8 (a)	1.5±0.1 (b)
Sample 6	Control sample	6.3±2.0 (a)	1.16±1.35 (b)	27.6±1.1 (a)	10.2±0.3 (a)	1.2±0.0 (b)
	Cotton	7.1±0.5 (a)	3.11±0.84 (a,b)	30.9±4.3 (a)	11.0±1.6 (a)	2.4±1.0 (a)
	Paper+SSA	5.6±0.5 (a)	3.54±0.27 (a)	32.5±2.7 (a)	10.4±0.4 (a)	2.3±0.2 (a,b)
Sample 7	Control sample	0.9±1.1 (c)	5.65±0.29 (a)	7.9±0.4 (b)	32.1±0.8 (a)	18.8±0.3 (a)
	Cotton	2.4±0.4 (b)	4.61±0.51 (b)	10.4±0.9 (a)	28.2±2.1 (b)	15.7±1.5 (b)
	Paper+SSA	4.3±0.3 (a)	4.25±0.18 (b)	9.8±0.5 (a)	22.4±1.2 (c)	12.6±0.6 (c)
Sample 8	Control sample	16.2±2.2 (b)	nd	66.4±9.2 (b)	23.0±3.6 (b)	17.6±3.9 (b)
	Cotton	25.4±4.8 (a)	nd	141.0±15.3 (a)	36.2±3.4 (a)	31.0±4.5 (a)
	Paper+SSA	11.9±1.2 (b)	nd	59.1±1.8 (b)	15.8±1.1 (c)	13.0±3.0 (b)

Paper+SAA, Filtration by paper and sodium sulphate anhydrous; HYTY, Hydroxytyrosol; TY, tyrosol; VA, Vanillic acid; DMOA, Decarboximethyl oleuropein aglycon; Pin, pinoresinol; DLA+AcPin, Decarboxymethyl ligstroside aglycon+acetoxypinoresinol; Ol Agl, Oleuropein aglycon; LA, Ligstroside aglycon, Letter(s) a-c in brackets indicate statistically significant differences (HSD Tukey $p<0.05$)

control sample, demonstrated a decrease of 2.9 and 8.1%, respectively, with cotton filtration and a reduction of 2.3 and 14.8%, respectively, with anhydrous sodium sulphate plus paper filtration.

Considering these results, it can be surmised that the oxidative stability of virgin olive oils is lower when the water content is decreased (after filtration), which is related either to a loss of phenolic compounds or a reduction in their antioxidant activity. The decrease of antioxidant activity, as

mentioned before, can be explained by the polar paradox. In fact, phenolic compounds, being polar molecules, have a higher activity in a water-in-oil emulsion. However after filtration the water content is reduced. As a consequence, the antioxidant capacity of these compounds diminishes, probably due to their particular orientation around small droplets of water.

Evaluation of Behavior of Individual Phenolic Compounds after Filtration

Hydroxytyrosol, decarboxymethyl oleuropein aglycon and oleuropein aglycon are, in that order, the phenolic molecules of virgin olive oil having the highest antioxidant activity (Carrasco-Pancorbo *et al*., 2005). In general, hydroxytyrosol showed a significant decrease after filtration with cotton with respect to the control sample. This behavior can be explained considering the partition coefficient between olive oil and water of this compound (Kp = 0.01 as reported Servili, 2005), which makes it more soluble in water than other phenols.

As reported in Table 2, the concentration of several phenolic compounds seemed to increase after filtration, but is related to the fact that filtration reduces the water content even though the loss of phenolic compounds is not proportional. In fact, it is assumed that the majority of phenolic compounds located around water droplets remain in olive oil.

It is also possible to hypothesize that extraction of phenolic compounds in control samples does not allow for complete recovery of these analytes; indeed, when the analytes are in a more polar matrix the affinity of phenolic extraction to the solvent (methanol/water, 60/40, v/v) is lower and their separation is more difficult. On the other hand, if the extraction with a hydroalcoholic solution is done after the partial elimination of water, phenols are more available to the solvent mixture.

This study represents a novelty in the fact that this apparently increase of phenolic content has been explained by the performance of a study about the variation in water content of virgin olive oil. This type of study has never been considered before by other investigations carried out about filtration effect (Fregapane *et al*., 2006).

Filtration Effect on the Colorimetric Parameters

A large amount of the particles in suspension was retained by the filtration system. As shown in Table 3, the luminosity of olive oil (L* value) increased after filtration. Furthermore, when the control

Table 3: Values of L*a*b* coordinates of the eight virgin olive oils studied: unfiltered samples (control sample) and filtered samples (cotton and paper plus anhydrous sodium sulphate) (±SD)

		L*	a*	b*	Sample description
Sample 1	Control sample	63.2±0.0	6.7±0.0	99.0±0.2	Clean and deep green
	Cotton	65.6±0.0	6.5±0.0	97.8±0.1	
	Paper+SSA	65.5±0.1	6.8±0.0	101.8±0.2	
Sample 2	Control sample	63.2±0.2	3.8±0.0	88.1±0.6	Veiled and light green
	Cotton	70.7±0.1	1.9±0.1	93.4±0.2	
	Paper+SSA	70.1±.03	1.8±0.0	92.7±0.4	
Sample 3	Control sample	61.1±0.1	1.1±0.0	59.7±0.0	Clean and light green
	Cotton	71.3±0.3	-1.5±0.1	62.9±0.1	
	Paper+SSA	74.0±0.1	-0.9±0.0	69.4±0.1	
Sample 4	Control sample	61.6±0.1	3.5±0.0	79.9±0.3	Veiled and light green
	Cotton	71.6±0.1	0.9±0.0	89.3±0.7	
	Paper+SSA	69.8±0.0	3.1±0.0	93.4±0.4	
Sample 5	Control sample	62.3±0.2	6.9±0.0	96.2±0.2	Very veiled and deep green
	Cotton	63.6±0.1	7.4±0.0	98.9±0.4	
	Paper+SSA	65.3±0.1	7.0±0.0	103.8±0.4	
Sample 6	Control sample	52.2±0.0	9.1±0.0	85.1±0.2	Very veiled and light green
	Cotton	54.3±0.0	8.4±0.0	86.1±0.1	
	Paper+SSA	54.2±0.1	8.5±0.0	87.8±0.2	
Sample 7	Control sample	67.2±0.4	6.2±0.1	101.5±0.4	Very veiled and deep green
	Cotton	67.8±0.1	6.9±0.0	103.8±0.5	
	Paper+SSA	68.9±0.0	7.0±0.0	104.8±0.5	
Sample 8	Control sample	60.3±0.2	4.1±0.1	85.5±0.7	Very veiled and light green
	Cotton	70.6±0.1	2.3±0.0	99.0±0.3	
	Paper+SSA	71.1±0.0	2.0±0.0	99.3±0.2	

Paper+SAA, filtration by paper and sodium sulphate anhydrous

sample had a deep green color, the a* value increased after filtration and the intensity of green color was minimized; whereas if the control sample was light green, the a* value decreased and the contribution of green color was more apparent. The b* value had a tendency to increase because the yellow color was more evident when the olive oil had been filtered.

CONCLUSIONS

By study of two filtration systems it can be concluded that the oxidation stability decreases after filtration due to elimination of water. This could be due both to the decrease of the concentration of phenols with a higher antioxidant activity, particularly hydroxytyrosol and to the decrease of antioxidant activity of phenolic compounds when the water content is lowered. Furthermore, filtrated olive oils had a higher component of yellow color, luminosity and in some cases, the intensity of green color diminished. Presently, consumers have more knowledge about olive oil and would choose a veiled and deep green oil over one that is transparent and light green oil (filtered). However there are also consumers that prefer transparent oils. Thus filtration may reduce the quality of virgin olive oils (oxidative stability decrease) and many consumers may not prefer these products due to their unfavorable visual characteristics.

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Como complemento a la discusión desarrollada en el artículo, cabe destacar que después del proceso de filtración:

- El contenido en agua del aceite disminuye.
- La concentración de la mayoría de los compuestos fenólicos entre los que se encuentran hidroxitirosol, decarboximetil oleuropeína aglicona y oleuropeína aglicona (descritos como los compuestos fenólicos que poseen mayor capacidad antioxidante) aumenta.

A pesar de estas realidades, la capacidad antioxidante del aceite de oliva disminuye después de la filtración, lo que en principio nos llevó a pensar que se producía una incongruencia, ¿cómo era posible que al disminuir el contenido en agua aumentara el contenido en compuestos fenólicos siendo que éstos son compuestos polares? y ¿cómo podía disminuir la actividad antioxidante del aceite de manera tan notable si la concentración en fenoles aumentaba?. La primera respuesta se halló en la mayor afinidad de los compuestos de interés por el disolvente de extracción (metanol/agua) cuando estos se encuentran en una matriz pobre en agua. Y la segunda respuesta, y complementaria a la primera es la paradoja polar, es decir, que moléculas polares como los compuestos fenólicos van a tener mayor actividad en emulsiones no polares (p.ej. agua en aceite). Además estas moléculas van a tener una especial orientación alrededor de las pequeñas gotas de agua que potencian su actividad, perdiéndose cuando el contenido en agua disminuye por cualquier causa.

Capítulo 5

Efectos del ataque de la mosca del olivo (*Bactrocera oleae*) en el perfil fenólico y otros parámetros químicos del aceite de oliva.

Publicación incluída en este capítulo:

Effects of fly attack (*Bactrocera oleae*) on the phenolic profile and selected chemical parameters of olive oil.
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El capítulo 5, al igual que el anterior, es un trabajo analítico dirigido a estudiar aspectos tecnológicos del aceite de oliva. Muchas investigaciones han sido llevadas a cabo enfocadas a ver cómo la calidad de un aceite de oliva puede verse influida por la acción de insectos como la mosca del olivo (*Bactrocera oleae*). Evidentemente, la calidad del aceite depende en gran medida del estado sanitario en que se encuentre el fruto. Se ha demostrado que el ataque por la mosca del olivo afecta a diferentes parámetros cualitativos (acidez, número de peróxidos, características sensoriales,…) e incluso altera la composición química del aceite; sin embargo, no se han desarrollado investigaciones en las que se estudie en profundidad el perfil fenólico frente al ataque de la mosca. Hoy en día, cada vez se propone con más fuerza la inclusión del contenido fenólico como parámetro de calidad del aceite de oliva ya que obviamente influye en la estabilidad del aceite, por ello se vio interesante la realización de este trabajo.

En este caso el objetivo ha sido estudiar la influencia del ataque de la mosca del olivo sobre el perfil fenólico y otros parámetros del aceite de oliva tales como: acidez libre e índice de peróxidos (PV), composición en ácidos grasos, índice de estabilidad oxidativa (OSI) y determinación del poder antioxidante mediante una técnica electroquímica; con el fin de revelar si alguno de estos parámetros es suficientemente robusto e independiente de otras variables como para ser capaz de determinar si un aceite proviene o no de aceitunas infectadas por la mosca del olivo.

La composición en compuestos fenólicos de un aceite depende de muchos factores tales como la variedad de aceituna, las condiciones climatológicas y ambientales, aspectos tecnológicos,…Por ello se escogieron muestras de aceite pertenecientes a diversas variedades de aceituna, obtenidas mediante procesos tecnológicos diferentes, y con distinto porcentaje de ataque de la mosca, pero todas ellas cultivadas en la misma región (Abruzzo, Italia).

El análisis del perfil fenólico fue llevado a cabo de manera exhaustiva mediante electroforesis capilar y la tendencia de los resultados fueron estudiados estadísticamente y comparados con los resultados de otro tipo de análisis ampliamente descritos en bibliografía (acidez libre e índice de peróxidos (PV), composición en ácidos grasos, índice de estabilidad oxidativa (OSI,…). De este modo se podría comprobar si esta influencia era significativa o por el contrario hay otros parámetros más adecuados para determinar el ataque de la aceituna por *Bactrocera oleae.*

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Effects of Fly Attack (*Bactrocera oleae*) on the Phenolic Profile and Selected Chemical Parameters of Olive Oil

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The phenolic fraction of virgin olive oil influences both its quality and oxidative stability. One of the principal threats of the quality of olive fruit is the olive fly (*Bactrocera oleae*) as it alters the chemical composition. The attack of this olive pest has been studied in order to evaluate its influence on the quality of virgin olive oil (free acidity, peroxide value, fatty acid composition, water content, oxidative stability, phenols, and antioxidant power of phenolic fraction). The study was performed using several virgin olive oils obtained from olives with different degrees of fly infestation. They were acquired in different Italian industrial mills from the Abruzzo region. Qualitative and quantitative analyses of phenolic profiles were performed by capillary electrophoresis−diode array detection, and electrochemical evaluation of the antioxidant power of the phenolic fraction was also carried out. These analyses demonstrated that the degree of fly attack was positively correlated with free acidity ($r = 0.77$, $p < 0.05$) and oxidized products ($r = 0.58$, $p < 0.05$), and negatively related to the oxidative stability index ($r = -0.54$, $p < 0.05$) and phenolic content ($r = -0.50$, $p < 0.05$), mainly with secoiridoid compounds. However, it has been confirmed that the phenolic fraction of olive oil depends on several parameters and that a clear correlation does not exist between the percentages of fly attack and phenolic content.

KEYWORDS: Virgin olive oil; phenols; qualitative parameters; oxidative stability; capillary electrophoresis; olive soundness

INTRODUCTION

Virgin olive oil is obtained from the fruit of the olive tree (*Olea europaea* L.) solely by mechanical or other physical means under conditions that do not alter its properties and must not undergo any treatments other than washing, decantation, centrifugation, or filtration (*1*). These processes maintain volatile and other minor compounds such as phenols that enhance the characteristic flavor of virgin olive oil (*2*).

Stability is not a standard parameter used to measure quality. However, it provides information about the hypothetical shelf life of the oil. In particular, lower stability indicates a poorer quality (e.g., greater acidity, higher peroxide values and extinction coefficients, and lower sensorial score). It has been shown that 78% of the stability, evaluated by Rancimat, is due to the combined effect of two variables, namely, phenolic compounds and the oleic/linoleic (O/L) ratio.

Phenolic compounds can be active as antioxidants and also can inhibit the free radical chain reaction (*3*). Their antioxidant properties and in particular their hydrogen-donating capacities are modulated by the presence of different chemical groups in the phenol backbones. Mainly, phenolic compounds having an *o*-catechol group in their structure such as those found in virgin olive oil (**Figure 1**), such as hydroxytyrosol and its oleosidic forms, are powerful antioxidants (*4, 5*). Using different assays, Carrasco-Pancorbo et al. (*3*) evaluated the antioxidant capacity of different phenolic compounds and concluded that among them, hydroxytyrosol, oleuropein, and decarboxy-methyl oleuropein aglycons with an o-catecholic structure exhibited the

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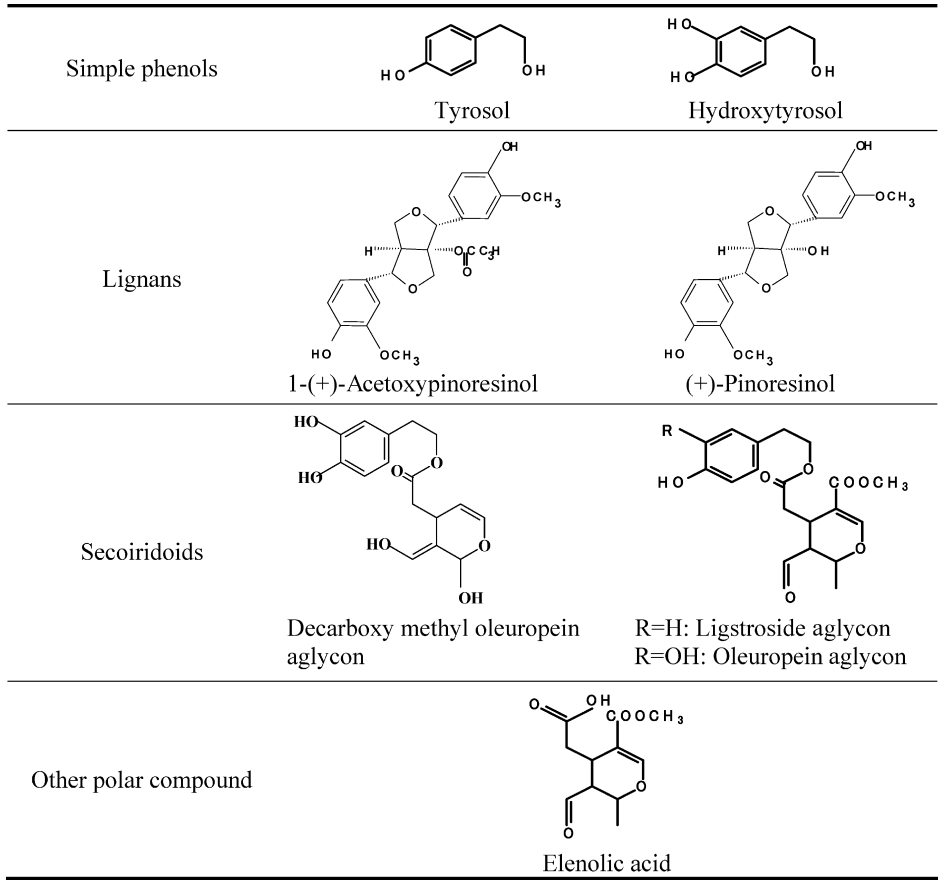

Figure 1. Structures of the phenolic compounds under study.

strongest antioxidant activity. In contrast, monohydroxylated phenols as tyrosol and ligstroside aglycon had very poor radical-scavenging activity.

Phenolic compounds have a positive effect on the health, sensory properties, and oxidative stability of olive oil (*2, 6–9*). But despite that it is well known that the phenolic fraction is influenced not only by the olive cv. but also by climatic and environmental conditions (*10, 11*), agronomic practices, and technological process (*10–14*).

Plants are subject to attacks from different organisms and as a result have evolved a complex, integrated defense system against potential pathogenic organisms to ensure survival, growth, and development. It has been shown that plants respond to pathogenic attack by synthesizing compounds that activate the defense system in fruits (*15*).

The quality of virgin olive oil is strongly related to the health status of the fruit from which it is extracted. One of the most detrimental enemies of the quality of olive oil is the olive fruit fly (*Bactrocera oleae*). This insect can reduce oil yield, affect quality parameters (acidity, peroxide value, ultraviolet (UV) absorbance, and organoleptic quality), and negatively alter the chemical composition (sterols, phenols, fatty acid, and volatile fraction) (*12, 16–24*). The severity of the negative effects depends on the stage of the development of the olive fly, the intensity of the attack, and olive variety. It has been shown that olive oils produced from fruits that have been attacked by the olive fly present an increase in acidity, peroxide values, and UV absorbance. Moreover, it has been demonstrated that the phenolic content and total amount of volatile compounds decrease, and no significant variations can be observed in fatty acid composition (*24, 25*). While there are several publications about the influence of *Bactrocera oleae* on the qualitative parameters of olive oil, potential variations in the phenolic profile have not been considered in depth.

Traditionally, free acidity and peroxide values have been considered the qualitative chemical parameters of virgin olive oil (*1*). Nevertheless, in recent years the evaluation of phenolic compounds as a qualitative parameter has been proposed (*26*).

The first aim of this investigation was to assess different qualitative parameters of olive oil in various commercial olive oil samples depending on the percentage of fly attack. The second aim was related to the study of changes in the quality and oxidative stability of these olive oils, with particular emphasis to correlations with the phenolic profile and antioxidant power of the phenolic fraction. This statistical treatment of data allowed us to determine which qualitative parameter of olive oil was more robust and less influenced by all the variables.

MATERIALS AND METHODS

Reagents, Stock Solutions, and Reference Compounds. 3,4-Dihydroxyphenylacetic acid (dopac) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), and oleuropein (oleuropein glucoside) was obtained from Extrasynthèse (Genay, France). The stock solutions of these two analytes were prepared in methanol/water (50/50, v/v) at a concentration of 500 μg/mL in the case of dopac and 6000 μg/mL for oleuropein glucoside. Dopac was used for the quantification of simple phenols 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.

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 pH values.

Double-deionized water with a conductivity less than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Hydranal-Titran 2 and Hydranal-solvent oil (solvents used to measure

Table 1. Information Relative to Olives (Cultivars, Area of Production, Healthy State) and Corresponding Oil Samples (Code and Technological System of Their Production)

code	olive varieties	town of production	tech. system[a]	% fly attack
S1	Dritta, Leccino	Loreto Aprutino (PE)	C	2%
S2	Dritta, Intosso	Città S. Angelo (PE)	C	2.5%
S3	Intosso	Città S. Angelo (PE)	C	2.5%
S4	Leccino	Loreto Aprutino (PE)	C	2.5%
S5	Dritta, Leccino	Loreto Aprutino (PE)	C	4%
S6	Dritta	Loreto Aprutino (PE)	P	5%
S7	Dritta	Loreto Aprutino (PE)	P	5%
S8	Dritta	Loreto Aprutino (PE)	P	5%
S9	Dritta	Loreto Aprutino (PE)	P	5%
S10	Carpinetina	Farindola (PE)	P	5%
S11	Leccino	Rocca S. Giovanni (CH)	C	5%
S12	Gentile	Rocca S. Giovanni (CH)	C	5%
S13	Gentile	Rocca S. Giovanni (CH)	C	5%
S14	Dritta, Leccino	Morro d'oro (TE)	D+C	5%
S15	Tortiglione	Cologna (TE)	D+C	7.5%
S16	Mix	Ortona (CH)	C	7.5%
S17	Leccino	Casoli (CH)	C	7.5%
S18	Gentile	Casoli (CH)	C	10%
S19	Mix	Orsogna (CH)	C	10%
S20	Mix	Morro d'oro (TE)	C	10%
S21	Gentile	Crecchio (CH)	C	15%
S22	Intosso	Casoli (CH)	C	15%
S23	Mix	Guardiagrele (CH)	P	25%
S24	Dritta	Loreto Aprutino (PE)	C	25%
S25	Leccino	Crecchio (CH)	C	30%
S26	Mix	Crecchio (CH)	P	35%
S27	Dritta	Cappelle (PE)	P	35%
S28	Mix	Orsogna (CH)	C	35%
S29	Mix	Cepagatti (PE)	C	45%
S30	Gentile, Leccino	Rocca S. Giovanni (CH)	C	60%
S31	Mix	Orsogna (CH)	C	60%
S32	Mix	Cologna (TE)	C	85%

[a] P, pressure system; C, continuous system; D+C, destoner plus continuous system.

the water content with the volumetric titration of Karl Fisher) were from Riedel-deHaën (Seelze, Germany).

All solvents used were analytical or HPLC grade (Merck & Co. Inc., Darmstadt, Germany).

Samples. Thirty-two virgin olive oils produced from different industrial mills located in the Abruzzo region (Italy, December 2006) were analyzed. Samples differed in the percentage of fly attack, variety of olive cultivars, and technological system used (pressure or centrifugation, with or without a destoning phase) as reported in **Table 1**. The degree of infestation was calculated as the number of damaged olives per 100 fruits, considering both the presence of exit holes and grubs.

Free Acidity and Peroxide Value (PV). These parameters were determined according to the official methods described in European Regulation EEC 2568/91 and amendments (*27*). PV was expressed as mequiv O_2 kg^{-1} of oil. The samples were stored in the absence of light and at room temperature in order to measure PV after three months of storage. Relative standard deviation (RSD) of the free acidity method was 1.7, and RSD of the PV method was 1.4.

Determination of Water Content in Virgin Olive Oil. The water content was analyzed with a TitroMatic 1S instrument (Crison Instruments, S.A.; Alella, Barcelona, Spain). This measurement uses a Karl Fischer titration based on a bivoltametric indication (2-electrode potentiometry). A solution of chloroform/Hydranal-solvent oil (a methanolic solvent) 2:1 (v/v) was used to dissolve the sample, and Hydranal-Titran 2 was used as a titrating reagent. Each sample was introduced three times, and the quantity of the sample was measured with the back weighting technique. The sample was dissolved in a solution of chloroform/Hydranal-solvent oil, and the titrating reagent was added until the equivalence point was reached. The quantity of water was expressed as mg of water/kg of oil ($n = 3$). RSD of the water method was 3.5.

Fatty Acid Composition. The fatty acid composition of oil samples was determined as methyl esters by capillary gas chromatography (GC) (Clarus 500 GC Perkin-Elmer Inc., Shelton, CT) analysis after alkaline treatment, according to Bendini et al. (*28*). Alkaline treatment was carried out by mixing 0.05 g of oil dissolved in 2 mL of *n*-hexane with 1 mL of 2 N potassium hydroxide in methanol according to Christie (*29*).

Oxidation Stability Index (OSI) Time. These analyses were carried out in an eight-channel OSI instrument (Omnion, Decatur, IL, USA). Virgin olive oil samples (5.0 ± 0.1 g) were heated at 110 °C under atmospheric pressure, and air (150 mL min^{-1} of flow rate) was allowed to bubble through the oil. Under these conditions, the oxidative process reaches its final steps, and the short-chain volatile acids produced are recovered and measured conductimetrically in distilled water. The time required to produce a sudden increase in conductivity (due to volatile acid formation) determines an induction period (OSI time), expressed in hours and hundredths of hours, which can be used to measure the stability of oil.

Extraction of Polar Phenolic Fraction. Phenolic compounds were extracted from virgin olive oil by a liquid−liquid extraction method according to Pirisi et al. (*30*). The dry extracts were dissolved in 0.5 mL of a methanol/water (50/50, v/v) solution and filtered through a 0.2 μm syringe filter (Whatman Inc., Clinton, NJ, USA). Extracts were frozen and stored at −43 °C.

Electrophoretic Procedure. Capillary electrophoretic separation was performed by the capillary zone electrophoresis method proposed by Carrasco-Pancorbo et al. (*31*). A Beckman 5500 capillary electrophoresis instrument connected to a diode array detector was used. This method uses a capillary with 50 μm i.d. and a total length of 47 cm (40 cm to the detector) with a detection window of 100 × 200 μm, and a buffer solution containing 45 mM sodium tetraborate pH 9.3

Antioxidant Power (AOP) Determination. Phenolic extracts were measured in a FIA apparatus at a potential set at 0 mV vs Ag/AgCl. The apparatus consists of a Minipuls II peristaltic pump (Gilson, France), a high pressure injection valve model 7125 (Rheodine, USA) equipped with a 20 μL loop, an electrochemical cell model UniJet (BAS, West Lafayette, USA) mounted with a glassy carbon working electrode (3 mm diameter), and an amperometric detector AMEL 559 HPLC detector (AMEL, Milan, Italy) linked to a chart recorder (RC 102; Pharmacia, Sweden). The flow rate of phosphate buffer (pH 7.4) was 150 μL min^{-1}. All extracts were injected in triplicate. The current produced during the electrochemical oxidation of the phenolic compounds was recorded. Quercetin was used as the reference compound, and the concentration of phenolic compounds was expressed as μg/mL quercetin equivalent (QE); AOP was expressed as QE_0, corresponding to QE.

Statistical Analysis. Data were analyzed using Statistica 6.0 (Statsoft, Tulsa OK, USA) statistical software. The values reported 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 are both at $p < 0.05$.

RESULTS AND DISCUSSION

Free Acidity and PV. The free acidity values of the oils studied ranged from 0.14% and 3.81%. Taking into account the acidity of the oil samples (*32*), there were 25 oils with very low acidity values (≤0.8%) that could be classified as extra virgin olive oils; five samples (S22, S25, S26, S27, and S32) with an acidity between 0.8−2% that were defined as virgin olive oils, and finally two oils (S30 and S31) with an acidity higher than 2% that were considered as lampante olive oils. We demonstrated that the majority of oils with a fly attack more than 30% had an acidity higher than 0.8%, which means that these oils belonged to the category of virgin or lampante instead of extra virgin. Furthermore, the two samples that had suffered a fly attack higher than 50% had a very high acidity value and because of this belonged to the category of lampante olive oils.

Regarding PV, the freshly pressed samples showed values from 5.3 to 19 mequiv O_2 kg^{-1} oil and an average value of 9.90. These values are slightly higher than those usually obtained

Table 2. Chemical Characteristics of Olive Oil Samples[a]

sample	FA	PV	PV 3	H_2O	OSI	O/L	AOP
S1	0.5	8.4	10.0	1557	28.5	10.1	96.6
S2	0.3	6.6	9.1	1121	40.6	10.8	104.0
S3	0.2	5.9	9.2	904	35.4	9.7	71.4
S4	0.3	9.9	8.2	1342	42.1	13.7	54.8
S5	0.4	9.3	9.9	1585	28.8	10.7	45.7
S6	0.5	10.2	11.0	1946	22.0	10.2	82.5
S7	0.6	8.8	10.0	2053	23.6	10.7	103.3
S8	0.6	8.4	13.2	1938	17.5	11.4	31.9
S9	0.7	10.9	10.2	2095	17.5	10.8	52.4
S10	0.4	7.9	11.1	2389	29.8	10.3	34.8
S11	0.3	11.3	9.0	1407	23.7	11.7	20.9
S12	0.4	7.9	12.0	1421	14.0	4.5	37.0
S13	0.6	12.2	13.0	1479	12.2	4.8	19.1
S14	0.7	7.1	10.0	927	18.2	10.6	12.7
S15	0.2	8.8	7.2	951	20.6	4.2	167.0
S16	0.4	9.7	10.7	974	20.8	7.7	42.2
S17	0.3	6.1	8.2	1463	38.5	14.3	5.2
S18	0.1	5.3	6.4	1002	27.1	12.2	30.3
S19	0.6	13.8	11.0	1079	20.5	12.3	11.4
S20	0.3	7.8	10.7	820	23.9	8.1	52.7
S21	0.6	9.8	16.8	1365	8.3	4.4	10.5
S22	0.9	9.1	15.0	1517	20.6	11.3	42.9
S23	0.3	7.5	8.2	3332	28.5	12.8	51.7
S24	0.8	11.2	12.0	1863	31.7	11.4	42.8
S25	1.0	12.1	15.5	1366	17.2	11.5	18.5
S26	0.9	11.3	12.2	2068	16.9	12.6	20.8
S27	1.2	19.0	20.2	1573	12.5	11.0	13.2
S28	0.7	8.3	10.0	1084	24.8	12.3	26.3
S29	0.5	10.4	14.8	1257	18.7	9.5	41.9
S30	3.8	14.8	17.4	1146	7.5	4.9	9.3
S31	2.3	11.9	17.4	1176	12.5	11.3	13.9
S32	1.9	14.9	15.2	1192	9.1	8.2	10.6

[a] FA, free acidity percentage (g oleic acid on 100 g of oil); PV and PV3, peroxide values (mequiv O_2 kg^{-1} oil) measured on fresh oils and after three months of oil storage; O/L, ratio between oleic and linoleic acids; H_2O, water content (mg H_2O kg^{-1} oil); OSI, oxidative stability index (hours); AOP, antioxidant power expressed as QE0 quercetin equivalent with potential set to 0 mV (μg quercetin mL^{-1} phenolic extract).

Table 3. Quantification Express as mg Analyte kg^{-1} Olive Oil of the Different Phenols by CE (mean ± SD, $n = 7$) [a]

sample	simple phenols[b]	lignans[c]	secoiridoids[c]	total
S1	4.4 ± 0.6	12.6 ± 3.2	150.0 ± 7.2	167.0 ± 4.9
S2	1.9 ± 0.2	11.9 ± 2.7	264.5 ± 9.3	278.4 ± 9.2
S3	4.6 ± 0.2	3.7 ± 0.5	233.9 ± 11.4	242.2 ± 11.9
S4	4.7 ± 0.2	8.9 ± 0.6	193.8 ± 8.6	207.5 ± 9.3
S5	4.2 ± 0.4	13.4 ± 3.8	139.4 ± 9.9	156.9 ± 9.8
S6	5.1 ± 0.2	11.7 ± 2.3	98.3 ± 11.6	115.1 ± 11.3
S7	4.0 ± 0.3	8.8 ± 2.0	80.0 ± 10.5	92.8 ± 11.9
S8	2.4 ± 0.9	10.8 ± 2.6	74.8 ± 5.2	88.0 ± 6.5
S9	3.5 ± 0.3	10.6 ± 2.00	53.7 ± 9.2	66.1 ± 9.8
S10	4.6 ± 0.4	6.2 ± 1.4	141.0 ± 17.7	151.8 ± 19.2
S11	2.7 ± 0.1	5.8 ± 0.4	39.3 ± 2.3	48.4 ± 3.1
S12	3.4 ± 0.2	27.5 ± 1.5	63.7 ± 2.8	98.1 ± 4.5
S13	2.9 ± 0.2	19.7 ± 1.2	37.5 ± 0.2	62.1 ± 1.4
S14	2.3 ± 0.0	7.8 ± 0.2	24.4 ± 0.9	34.6 ± 1.1
S15	5.2 ± 0.2	29.5 ± 1.5	125.1 ± 2.8	165.5 ± 3.7
S16	6.9 ± 0.3	19.5 ± 1.3	116.8 ± 8.0	144.8 ± 9.8
S17	1.7 ± 0.1	3.1 ± 0.4	144.8 ± 9.7	149.6 ± 10.1
S18	3.7 ± 0.1	2.6 ± 0.4	77.6 ± 2.8	83.9 ± 3.2
S19	2.1 ± 0.1	2.3 ± 0.3	20.0 ± 0.5	24.7 ± 0.9
S20	3.4 ± 0.2	6.5 ± 0.4	102.6 ± 4.6	112.5 ± 4.8
S21	1.8 ± 0.6	7.6 ± 1.6	24.2 ± 1.1	33.6 ± 1.4
S22	1.8 ± 0.1	10.3 ± 0.7	115.1 ± 7.3	127.2 ± 7.4
S23	4.0 ± 0.3	6.7 ± 0.8	87.6 ± 8.9	98.3 ± 10.0
S24	5.4 ± 0.2	9.6 ± 1.1	158.4 ± 5.7	173.4 ± 5.9
S25	1.4 ± 0.5	3.5 ± 0.3	15.1 ± 1.0	20.0 ± 1.7
S26	1.1 ± 0.0	2.6 ± 0.2	18.0 ± 1.3	21.7 ± 1.5
S27	2.4 ± 0.8	16.7 ± 3.3	39.7 ± 3.4	58.8 ± 3.2
S28	2.6 ± 0.1	3.3 ± 0.3	40.7 ± 0.8	47.2 ± 0.7
S29	2.9 ± 0.1	8.6 ± 1.3	66.6 ± 4.4	78.1 ± 4.1
S30	1.5 ± 0.1	3.8 ± 0.4	20.0 ± 0.4	25.3 ± 0.5
S31	1.9 ± 0.1	1.2 ± 0.3	11.6 ± 0.7	14.7 ± 0.9
S32	1.0 ± 0.0	3.7 ± 0.1	14.2 ± 0.3	18.9 ± 0.3

[a] Simple phenols, sum of tyrosol and hydroxytyrosol; lignans, sum of pinoresinol and acetoxypinoresinol; secoiridoids, sum of seven peaks: OA (a) + DOA, DOA (b), Lig Agl (b), OA (b), EA (a), OA (c) + Lig Agl (c) + DOA (c) + EA (b,c), DOA (d) + EA (d). [b] Quantified with a calibration curve of 3.4-dihydroxyphenilacetic acid at $\lambda = 200$ nm. [c] Quantified with a calibration curve of oleuropein glucoside at $\lambda = 200$ nm.

from fresh olive oils (*11, 13*). Another evaluation of PV was carried out after three months of storage because this period is long enough to observe the beginning of the oxidative reactions and to see differences in the PV. As shown in **Table 2**, after three months of storage the average of PV reached a mean of 11.70. The samples attacked to a higher degree were for the most part those that presented a stronger increase in the PV.

As reported by other authors (*12, 16–25*) *Bactrocera oleae* attack has been positively correlated with both free acidity ($r = 0.77$, $p < 0.05$) and PV ($r = 0.58$, $p < 0.05$). These correlations were higher when the analyses were carried out after three months of oil storage: $r = 0.78$ for acidity (data not shown) and $r = 0.63$ for PV (in both cases for $p < 0.05$). In particular, when the percentage of infested olives was modest (<10%), the oxidative status of corresponding virgin olive oil was not affected. These results are in accordance with previous studies (*24, 25*), which emphasized that free acidity and peroxides together with sensory characteristics are the principal quality parameters and are related to fruit integrity. Fly attack can be considered an influential factor in the premature aging process of virgin olive oil. In fact, this kind of olive infestation may cause an acceleration of oxidative and hydrolytic degradation favored by the presence of exit holes that expose the olive pulp to the action of microorganisms and oxygen.

Water Content. The water content of samples varied from 800 to 3330 ppm, with an average value of 1481 ppm. It is important to highlight that no influence of fly attack was found on the water content of olive oils.

Nevertheless, water is affected by the processing system of olive fruits (continuous, pressure, or traditional). Generally, samples with a higher water content were those produced with a traditional processing system (average value 2170 ppm), whereas samples obtained by continuous processing systems had a lower content of water (average value 1250 ppm).

OSI. This parameter varied greatly from 7.5 h (S30) to 42.1 h (S4). As has been previously noted, OSI depends on several factors (*8, 14*). Aparicio et al. attributed the oxidative stability to several variables; however, fatty acid composition and the content of phenolic compounds are those with the greatest influence. The oxidation rates of linoleic and oleic acids explain why stability is higher when the content of the monounsaturated acids is high and the content of polyunsaturated acids is low. Thus, the O/L ratio has the most marked relationship with stability (*8*). An olive oil has a good stability index if this value is higher than 7 (*33*), although samples S12, S13, S15, S21, and S30 had a ratio lower than 5. The infestation did not cause significant changes in the fatty acid composition, which was affected mostly by olive ripening and olive cultivar (S12, S13, S21, and S30 were produced by *Gentile* olive cv).

In general, our results agree with those previously reported. In fact, positive correlations with OSI have been found for both the phenolic content and O/L ratio ($r = 0.81$, $p < 0.05$; $r = 0.57$, $p < 0.05$), whereas the oxidative stability was negatively correlated with the degree of olive infestation ($r = -0.54$, $p < 0.05$). The lower stability with increased infestation may be

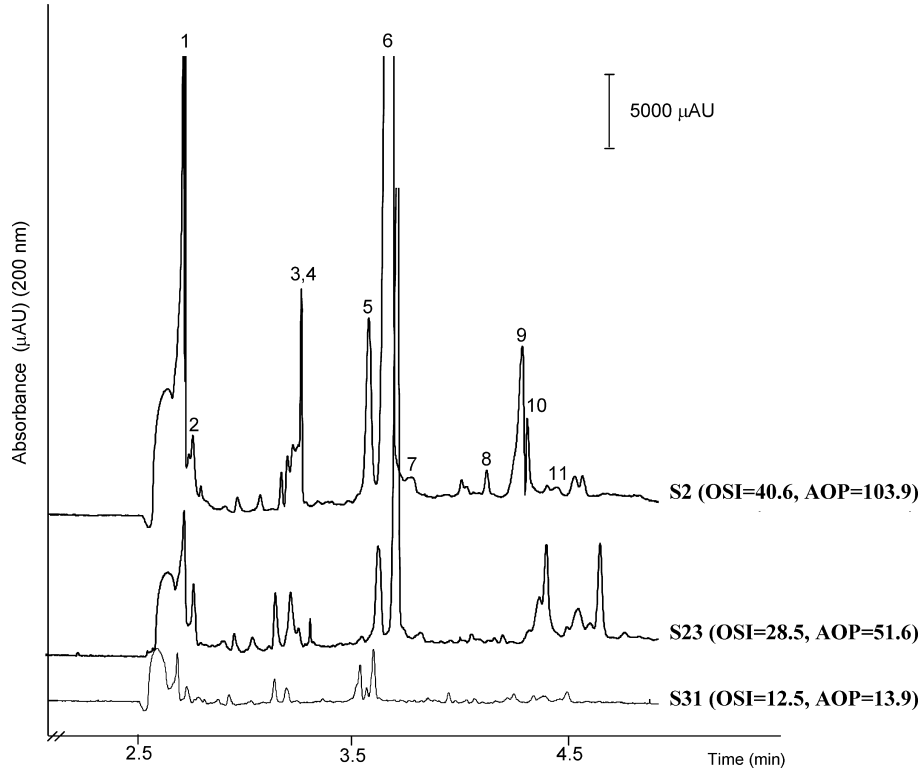

Figure 2. Overlay of electropherograms relative to phenolic extracts of three oil samples (S2, S23, and S31) differing in phenol content, values of oxidative stability (OSI, in hours) and antioxidant power (AOP, in quercetin equivalent), and the degree of fly attack (2.5%, 25%, and 60%).

explained by the decrease in phenols and *o*-diphenols in damaged olives. Additionally, we noted that a negative correlation exists between the PV and OSI ($r = -0.61$, $p < 0.05$), which was more pronounced after three months of storage of olive oil ($r = -0.71$, $p < 0.05$).

Phenolic Compounds. The content of phenolic compounds presented a high variability depending on the variety of olives, different typology of transformation and production system (**Table 3**). In general, samples strongly attacked by the fly showed a particularly low phenolic content; for example, oil obtained from olives with a percentage of attack higher than 30% always showed phenolic content values lower than 80 mg kg^{-1} of olive oil. It can be observed that the *o*-diphenol content showed a high correlation with OSI values, whereas it was negatively influenced by the degree of olive infestation ($r = 0.86$, $p < 0.05$; $r = -0.50$, $p < 0.05$). Among the *o*-diphenols, the oleuropein (OA) and decarboxy-methyl oleuropein aglycons (DOA), belonging to secoiridoids, were highly involved in both of these effects (in particular, OA(a) + DOA(a) $r = 0.74$, $p < 0.05$; $r = -0.53$ $p < 0.05$).

Therefore, fly attack resulted in the loss of phenols, *o*-diphenols, and in particular of some secoiridoid derivatives. Consequently, when phenols decrease as the percentage of infested olives increases, the stability of the resulting oils is compromised. It is likely that this effect is due to an increase of polyphenoloxidase activity caused by larval damage to tissues and by the presence of exit holes that expose the olive pulp to oxygen (*24*).

Different samples with low, medium, and high percentages of fly attack were chosen (**Figure 2**), and an evident decrease of phenolic content was observed when the percentage of fly attack increases. However, it is not possible to state that a direct correlation exists between phenolic content and the percentage of fly attack because as we said before, there are many variables that influence the phenolic profile (cultivar, growing area, climatic conditions, peaking system, technological plant, oil stored, etc.) (*31*).

If we consider different real samples, the influence of all other variables determines a large qualitative and quantitative variation on the phenolic profile. Therefore, we conclude that phenolic content itself cannot be considered as a process parameter or as an indicative marker of the percentage of fly attack. For example, samples from the same cultivar and with the same percentage of fly attack, 5, 6, 7, 8, and 9, present different behaviours regarding their phenolic content.

Antioxidant Power. AOP varied from 5.19 (S17) to 167.01 (S15) μg mL^{-1} quercetin equivalents. As previously observed (*32*), the AOP values of the phenolic fraction of virgin olive oils are closely related to their radical scavenging activity as they represent the most readily oxidizable compounds. The AOP shows a weak positive correlation with OSI ($r = 0.38$, $p < 0.05$), confirming the influence of the phenolic fraction, particularly the readily oxidizable, on the oxidative stability of olive oil. Our results showed a positive correlation between AOP and the phenol and *o*-diphenol contents ($r = 0.69$, $p < 0.05$; $r = 0.59$, $p < 0.05$) as well as simple phenols ($r = 0.55$; $p < 0.05$). Significant positive correlation also exists between AOP and individual phenols, or groups of phenols. An especially high influence was exerted by OA(a) + DOA(a), which also showed a negative correlation with percentage of fly attack ($r = -0.53$, $p < 0.05$). In addition, we observed that a negative correlation exists between the PV and AOP ($r = -0.38$; $p < 0.05$) and that this correlation is still more prominent between AOP and PV after three months of oil storage ($r = -0.49$; $p < 0.05$). The contribution of the quantity of phenolic compounds to oxidative stability and their different levels of AOP are evident in **Figure 2** in which several electropherograms (S2, S23, and S31) have been overlaid. These findings suggest that the AOP value may be used as a predictive index of antioxidant capacity that could be exerted by phenols during virgin olive oil storage.

Nowadays, the content of phenolic compounds is increasingly used in industrial mills as a useful parameter to evaluate the quality of olive oil. This correlation is right because it is obvious that phenolic content influences the stability of olive oil and the sensory characteristics. However, this parameter is not that important for evaluating the health status of the olives (attacked by *Bactrocera oleae*).

The aim of this work was to find the most appropriate method to know if an olive oil comes from olives attacked by the olive fly. There has been evidence that a simple parameter such as free acidity is stronger and more useful for judging the quality of an olive oil right after production because it is independent of all of the other technological parameters.

It is important to highlight that this is the first time that the effect of fly attack on the phenolic fraction, considering several percentages of attack, has been studied. Although it is not possible to say that a direct correlation exists between these two parameters, it has been proved that olive oils from olives with a high percentage of fly attack (S30, S31, and S32, more than 50% of fly attack) present a very low amount of phenols.

This argument is even more important if we observe that the quality of the row material is highly correlated with oil stability after a few months of storage. Peak identification numbers: **1**, Lig Agl (a); **2**, TY, **3**, Pin; **4**, Ac Pin; **5**, OA (a) + DOA (a); **6**, DOA (b); **7**, Lig Agl (b); **8**, OA (b); **9**, EA (a); **10**, OA (c) + Lig Agl (c) + DOA (c) + EA (b,c); **11**, HYTY. Detection wavelength: 200 nm.

ABBREVIATIONS USED

O/L, oleic/linoleic acid ratio; UV, ultraviolet; PV, peroxide value; RSD, relative standard deviation; GC, gas chromatography; OSI, oxidative stability index; AOP, antioxidant power; QE, quercetin equivalent; HSD, honest significant difference; TY, tyrosol; HYTY, hydroxytyrosol, Pin, pinoresinol; Ac Pin, acetoxypinoresinol; OA, oleuropein aglycon, DOA, decarboxymethyl oleuropein aglycon; Lig Agl, ligstroside aglycon; EA, elenolic acid.

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Al finalizar el trabajo experimental expuesto en este capítulo se puede concluir de forma resumida que se han alcanzado tres objetivos muy interesantes:

1.- Estudiar por primera vez el perfil fenólico del aceite de oliva con el fin de comprobar su variación debido al ataque por la mosca mediante una técnica separativa como es la electroforesis capilar. Su importancia radica en que la información obtenida es mucho más completa que con ensayos en los que sólo se determina el contenido en polifenoles totales, como es el caso del Folin Ciocalteau.

2.- Comprobar que, a pesar de que el contenido en compuestos fenólicos es cada vez más un importante parámetro de calidad a tener en cuenta a la hora de clasificar un aceite de oliva, no existe una relación directa entre porcentaje de ataque por la mosca del olivo e influencia sobre el contenido fenólico del aceite.

3.- Que un parámetro tan simple como la acidez libre es capaz de juzgar mucho mejor la calidad de un aceite ya que no depende de tantas variables como en el caso del contenido fenólico.

Bloque II: Miel

La miel es una sustancia elaborada por las abejas melíferas a partir del néctar de las flores, o de las secreciones procedentes de partes vivas de las plantas, que las abejas liban, transforman, combinan con sustancias específicas propias y almacenan y dejan madurar en los panales de la colmena.

La miel ha formado parte en muchas culturas de la medicina tradicional [219], aunque ha sido empleada en la mayoría de los casos como edulcorante. A pesar de ello no ha sido hasta los últimos años cuando han salido a la luz evidencias acerca de sus beneficios para la salud y su capacidad antioxidante [220,221]. Se ha empleado obteniendo buenos resultados en el tratamiento de quemaduras, desórdenes gastrointestinales, asma, heridas infectadas y úlceras en la piel [222]. Por otra parte y gracias a su capacidad antioxidante, se ha utilizado como conservante en alimentos [223], al prevenir reacciones de deterioro oxidativo en éstos, como por ejemplo la oxidación lipídica en la carne [224] o el pardeamiento enzimático en frutas y vegetales [225,226].

La composición de la miel es muy compleja, está constituida por una mezcla de alrededor de 200 componentes, siendo básicamente una matriz sobresaturada de azúcares, entre los cuales la fructosa y la glucosa son los más importantes [227]. Además posee una gran variedad de componentes minoritarios entre los que se encuentran los compuestos fenólicos (dentro de los cuales los flavonoides tienen una

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gran importancia), enzimas (glucosa oxidasa y catalasa), ácido ascórbico, carotenoides, ácidos orgánicos, aminoácidos, proteínas y α-tocoferol [228].

A continuación se muestra una tabla en la que se resume la composición media de la miel.

Hidratos de carbono (75-80%)	**Agua (15-20%)**	**Ácidos (0.1-0.5%)**	**Minerales (0.1-1.5%)**	**Proteínas y aminoácidos (0.2-2%)**	**Enzimas y Vitaminas**	**Constituyentes del aroma**	**Otros**
Monosacáridos (70-75%) Fructosa Glucosa **Disacáridos** Sacarosa Maltosa Isomaltosa Gentobiosa Maltulosa Trealosa Turanosa Kojibiosa **Trisacáridos y azúcares superiores** Erlosa Isomaltotriosa Maltotriosa Melecitosa Rafinosa Isomaltopentaosa		Ác. glucónico (70-80% de la acidez total) Ác. acético Ác. butírico Ác. cetoglutárico Ác. cítrico Ác. fórmico Ác. fumárico Ác. láctico Ác. maleico Ác. málico Ác. oxálico Ác. piroglutámico Ác. succínico Ác. tartárico	Potasio Cloro Sodio Calcio Fóforo Magnesio Silicio Hierro Manganeso Aluminio Antimonio Plata Bario Berilio Bismuto Boro Cobalto Cromo Galio Germanio Yodo Litio Molibdeno Níquel Oro Osmio Plomo Estaño Estroncio Titanio Vanadio Zinc	Materias nitrogenadas de la abeja y la cría **Aminoácidos** Prolina Fenilalanina Ác. aspártico Ác. glutámico Isoleucina Leucina Valina Alanina Arginina Cistina Glicina Histidina Lisina Metionina Serina Tirosina Treonina Triptófano	**Enzimas** Amilasa Sacarasa Glucosaoxidasa Catalasa Fosfatasa **Vitaminas** Ác. ascórbico (C) Riboflavina (B2) Ác. pantoténico Niacina (PP) Tiamina (B1) Piridoxina (B6) Biotina (H) Ác. fólico	**Alcoholes** Metanol, Etanol Propanol etc. **Aldehídos y cetonas** Formaldehído Acetaldehído Propianaldehído Hidroximetilfurfural **Ésteres** Acetato de metilo Acetato de etilo etc.	Glicéridos Esteroles Fosfolípidos Ác. palmítico Ác. oleico Ác. laurico Trazas de cera **Compuestos fenólicos** Polen Esporas de hongos Levaduras osmófilas

De acuerdo con investigaciones previas, es posible afirmar que la capacidad antioxidante de la miel se debe principalmente a la presencia de los compuestos

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fenólicos que contiene, aunque no hay que olvidar que esta actividad antioxidante se debe, sin lugar a dudas, a una acción sinérgica entre diferentes tipos de compuestos [229,230].

Como es posible imaginar, debido a la gran variedad de mieles procedentes de infinidad de tipos de flores diferentes, la composición y capacidad antioxidante de cada una de ellas va a depender en gran medida del tipo de flor de la que proviene el néctar, de la estación del año, de factores ambientales (tipo de terreno, clima, factores genéticos), así como del procesado al que es sometida la miel [231].

Diferentes trabajos muestran que en ocasiones hay una relación clara entre el origen floral de la miel y el perfil flavonoide de ésta. Este tipo de análisis son de gran interés ya que la presencia predominante de uno o varios compuestos puede servir para determinar el origen de una miel. Por ejemplo la hesperetina se puede usar como biomarcador de la miel de azahar, 8-metoxi-kaempferol es el principal compuesto fenólico de la miel de romero, la luteolina es característica de la miel de lavanda y la quercetina de la miel de girasol [232,233].

Otra característica a tener en cuenta es que normalmente, las mieles de tonalidad más oscura presentan una mayor capacidad antioxidante (atribuída principalmente a su capacidad reductiva de los radicales libres, y a su actividad antimicrobiana) con respecto a aquellas de color claro. Se ha demostrado que el color de la miel, además de depender de parámetros tales como la alcalinidad de la miel, su contenido en cenizas, se ve influenciado por compuestos capaces de colorear como los carotenoides y flavonoides [234,235].

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Cabe destacar que las características terapéuticas de la miel, especialmente su capacidad antimicrobiana es especialmente acentuada si la comparamos con la de otros productos naturales como las aceitunas de mesa, las setas o el zumo de uvas y el vino [235].

A pesar de todo lo expuesto anteriormente, la miel también puede provocar efectos no deseados para la salud. Diferentes autores han encontrado evidencias de efectos como la arritmia cardiaca, hipotensión, problemas respiratorios, etc [236,237].
Debido a su actividad antimicrobiana, la miel impide en muchos casos el crecimiento y la supervivencia de determinados organismos por el bajo pH y alto contenido en azúcares que presenta cuando se encuentra sin diluir [238]. Por lo tanto, se espera que la miel contenga un pequeño número y una variedad muy limitada de microorganismos. Sin embargo, ente ellos se incluyen ciertas levaduras y bacterias formadoras de esporas, y microorganismos como *Bacillus cereus, Clostridium perfringes*, o *Clostridium botulinum*, que bajo ciertas condiciones pueden causar enfermedades a la especie humana [239-241].

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Capítulo 6

Avances en el análisis de los compuestos fenólicos en productos derivados de las abejas.

Publicación incluída en este capítulo:

Advances in the analysis of phenolic compounds in products derived from bees.
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Al igual que se hizo con en el bloque dedicado al aceite de oliva, este primer capítulo del bloque destinado a la miel consiste en una revisión bibliográfica. En este caso, el tema general del review fueron los avances analíticos para la determinación de los compuestos fenólicos en los productos derivados de las abejas. Entre ellos se encuentran la miel y el propóleo, ya que en el resto de productos derivados de la colmena no está descrita la presencia de compuestos fenólicos.

El trabajo se encuentra dividido en dos partes fundamentales: una dedicada a la miel y otra al propóleo. Cada una de ellas consta de diversos apartados en los que se describen los métodos de preparación de muestra y las diferentes metodologías analíticas puestas a punto para el análisis de los compuestos fenólicos en función del tipo de técnica (métodos espectrofotométricos, cromatográficos y electroforéticos).

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Advances in the analysis of phenolic compounds in products derived from bees

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Abstract

Honey and propolis are rich in phenolic compounds, which act as natural antioxidants, and are becoming increasingly popular because of their potential role in contributing to human health. These compounds can also be used as indicators in studies into the floral and geographical origin of the honey and propolis themselves. We present here an overview of current analytical methods for measuring polyphenols in honey and propolis. The analytical procedure to determine individual phenolic compounds involves their extraction from the sample, analytical separation and quantification. The techniques reviewed are based on spectrophotometry as well as analytical separation techniques such as gas chromatography, high-pressure liquid chromatography and capillary electrophoresis.

Keywords: Phenolic compounds; Honey; Propolis; Analysis

1. Introduction about interest of phenolic analysis

Quality control, nutritional value and the monitoring of hazardous residues in foodstuffs have all become major topics of public interest [1]. The effects of growing conditions, processing, transport, storage, genetics and other factors concerning chemical and biochemical components are also important issues in food science [2]. In recent years there has been growing interest in functional foods, i.e. foods that can provide not only basic nutritional and energetic requirements but also additional physiological benefits [3]. The term "functional food" was used for the first time in Japan in the 1980s and was applied to processed food which contained ingredients that conferred the benefits of some physiological functions. Nowadays a functional food can be defined as a food that produces a beneficial effect in one or more physiological functions, increases well-being and/or decreases the risk of suffering from a particular medical condition. The functionality of a food is usually related to some of the ingredients that it contains and at present consumers prefer these ingredients to have a natural rather than synthetic origin. Thus they are commonly extracted from plants, food by-products and other natural sources [4].

Among the functional ingredients the group most widely studied is the family of antioxidants. Traditionally, this kind of compounds have played an important role in food science and technology because of their usefulness in preserving foodstuffs against oxidative degradation [5]. Interest in antioxidant compounds has increased nowadays in the light of recent evidence regarding the important role of antioxidants in human health. In fact several preventative effects against different diseases such as cancer, coronary diseases, inflammatory disorders, neurological degeneration, aging, etc., have been related to the consumption of antioxidants [6,7].

Phenolic compounds or polyphenols, are one of the most important groups of compounds occurring in plants, where they are widely distributed, comprising at least 8000 different known structures [8]. Polyphenols are also products of the secondary metabolism of plants. These compounds are reported to exhibit anticarcinogenic, anti-inflammatory, anti-atherogenic, antithrombotic, immune modulating and analgesic activities, among others and exert these functions as antioxidants [9–13]. In general, phenolic compounds can be divided into at least 10 types depending upon their basic structure: simple phe-

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nols, phenolic acids, coumarins and isocoumarins, naphthoquinones, xanthones, stilbenes, anthraquinones, flavonoids and lignins. Flavonoids constitute the most important polyphenolic class, with more than 5000 compounds already described [6].

Due to the importance of polyphenols in food this manuscript reviews their analysis in different products derived from bees. Several natural products are manufactured by bees to construct their hives and produce honey. These include beeswax, royal jelly, beebread, propolis and honey itself. There are no studies analysing the phenolic profile of beeswax, royal jelly and beebread and so this review confines itself to the analysis of polyphenols in honey and propolis.

In general, an analytical procedure for the determination of individual phenolic compounds involves three basic steps: extraction from the sample, analytical separation and quantification. Several methods have been developed to analyse polyphenols in honey and propolis: colorimetric reactions, thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC) and lately, capillary electrophoresis (CE).

2. Honey

2.1. Introduction

Honey has been used as a food since the earliest times. Only in recent years, however, has evidence emerged of its antioxidant capacity [14]. It is also used as a food preservative [15–17], preventing deteriorative oxidation reactions in foods, such as lipid oxidation in meat [18,19] and the enzymatic browning of fruits and vegetables [20–22]. Antioxidants specifically retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals. Nevertheless, the antioxidant activity of honey varies greatly depending on the floral source [23,24] and external factors such as the season and environment, and finally its processing.

Honey is reported to contain at least 181 substances [25] and is considered as part of traditional medicine. Apitherapy has recently become the focus of attention as a form of folk and preventive medicine for treating certain conditions and diseases as well as promoting overall health and well being [26]. It has been reported to be effective in gastrointestinal disorders [27,28], in the healing of wounds and burns [29,30], as an antimicrobial agent [28–32] and to provide gastric protection against acute and chronic gastric lesions [33,34].

Honey is a supersaturated solution of sugars, to which the main contributors are fructose (38%) and glucose (31%). It also has a wide range of minor constituents, many of which are known to have antioxidant properties [35,36]. These include flavonoids and phenolic acids [37,38], certain enzymes (glucose oxidase, catalase) [25], ascorbic acid [25], Maillard reaction products [25], carotenoid-like substances [42], organic acids [37] and amino acids and proteins [39]. The natural antioxidants, especially flavonoids, exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions [40].

The quality of honey is judged by its botanical or floral origin and chemical composition [37] and price of honey is based on its quality [41]. Traditionally, the floral source of a honey has been identified by the analysis of bee pollens present in the honey. Tan et al. however [42] have suggested that chemical approaches might be more accurate and easily undertaken in the characterisation of the floral source of a honey [43]. The analysis of their phenolic compounds, including flavonoids, has been suggested [44] and this technique tends to be used to study their floral and geographical origins. Before this, researchers tried to use the analysis of amino acids [38,45] to complement pollen analysis in the determination of the floral origins of honey. Even so, the analysis of phenolic compounds has been regarded as a very promising way of studying the floral and geographical origins of honeys [44–49]. In these studies, the flavanone hesperetin has been used as a marker for citrus honey [50–52], the flavonol kaempferol for rosemary honey [50,53] and quercetin for sunflower honey [54]. Some phenolic acids, such as ellagic acid in heather honey, have also been used as floral markers [37,55,56], and the hydroxycinnamates (caffeic, *p*-coumaric and ferulic acids) in chestnut honey [37]. Pinocembrin, pinobanksin and chrysin are the characteristic flavonoids of propolis and these flavonoid compounds have been found in most European honey samples [54]. In some honeys, such as those derived from lavender and acacia, no specific phenolic compounds have been found as suitable floral markers [54]. Other possible phytochemicals markers may be found, such as abscisic acid for heather honey [56]. Abscisic acid has also been detected in rapeseed, lime tree and acacia honeys [54]. A study of the phenolic contents of honey may also determine the presence of antimicrobial activity [57,58].

2.2. Sample preparation

Isolation of the phenolic compounds from the sample matrix is generally a prerequisite to any comprehensive analytic 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 components of interest and free from interfering matrix components [59]. The extraction procedure used in most of the studies published is a solid phase extraction consisting of the following steps. The honey samples are mixed with five parts of water (pH 2 with HCl) until completely fluid and then filtered through cotton to remove solid particles. The filtrate is then passed through a column of Amberlite XAD-2 [60]. The phenolic compounds remain in the column while sugars and other polar compounds elute with the aqueous solvent, resulting in a flavonoid recovery of >95% [60,61]. The column is washed with acidic water (pH 2 with HCl) and subsequently with distilled water. The whole phenolic fraction is then eluted with methanol and dried under reduced pressure at 40 °C. There is a modification to this extraction in which the filtrate is mixed with Amberlite and stirred with a magnetic stirrer for 10 min before filling the column.

It is possible to carry out the next step, the clean-up, in two different ways. In the first one, the residue obtained after the evaporation of the methanol is resuspended in distilled water and extracted with diethyl ether. The ether extracts are combined and diethyl ether is removed by flushing with nitrogen. The dried residue is then redissolved in methanol and filtered [43,53,54,56,62–71]. In the second one, the residue is dissolved in methanol and the solution passed through a Sephadex LH-20 column. The phenolic fraction is evaporated to dryness under reduced pressure, redissolved in methanol and filtered [36,50,61,72,73].

Weston et al. [65] have demonstrated that phenolic acids seem to be eluted with the sugar fractions during the fractionation of honey on a XAD-2 column, as Ferreres et al. [74] mentioned that sugars and polar compounds were washed with water. In addition, by using diethyl ether, they aimed to eliminate the non-flavonoid phenolic compounds, which contaminated the flavonoid peaks; thus the main diethyl ether extract contents were flavonoids.

Aljadi et al. [75,76] recover the phenolic compounds from honey using a C18-SPE cartridge. Honey samples are prepared, subjected to base hydrolysis and extracted with ethyl acetate (liquid–liquid extraction) as described by Wahdan [77]. The fraction extracted with ethyl acetate is evaporated under dryness, then the dry honey extract is redissolved in acidified deionised water and the phenolics are adsorbed onto preconditioned isolute C18 columns. The cartridges are preconditioned by passing methanol and acidified water. The adsorbed phenolics are then eluted from the cartridges by passing methanol–water solution 25% (v/v) at a drop wise flow rate. The recovered fractions are combined, dried under nitrogen and subjected to further analysis. Extraction using a SPE-C18 cartridge is a simple technique that employs inexpensive disposable extraction columns and provides many advantages, such as a reduction of solvent consumption and high recoveries of the analytes.

Another type of solid-phase extraction for phenolic compounds in honey, used by Inoue et al. [26], is a GL-Pak PLS-2 cartridge. Honey samples are dissolved in distilled water. The sample solution is transferred into an SPE cartridge preconditioned with methanol and distilled water. This is then washed with water and eluted with methanol. The solutions are evaporated to dryness under a stream of nitrogen. The samples are redissolved by adding methanol.

For the extraction of homogentisic acid from honey an aliquot of homogenized honey is dissolved in water in screw-capped tubes. Ethyl acetate is added to each tube and the mixture is agitated in a rotary shaker. The phases are allowed to separate and the organic extracts are centrifuged. After centrifugation, anhydrous sodium sulphate is added to the combined extracts and evaporated to dryness by a rotary evaporator. The residue is taken up with acetone and the acid isolated by preparative TLC using H_2SO_4 10^{-2}N ($R_f = 0.7$) [78].

To extract a similar quantity of honey an Amberlite XAD-2 column requires more solid phase than that used in the other different types of SPE but more phenolic compounds are identified than with the other types of SPE.

2.3. Spectrophotometric determination of phenolic compounds

The colorimetric assay based on the reaction of Folin-Ciocalteu reagent is a method widely used for the determination of total phenols in honey [17,75,79,80]. 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 main disadvantage of the colorimetric assay is its low specificity, as the color reaction can occur with any oxidizable phenolic hydroxy group. An interesting approach to the content of total extractable phenolic compounds in different food samples involving the comparison of chromatographic and spectrophotometric methods has recently been reported, accounting for the possible influence of other substances as interfering compounds [81].

A typical protocol using the Folin-Ciocalteu method could be as follows. Each honey sample is diluted with distilled water and filtered. This solution is then mixed with Folin-Ciocalteu reagent for 5 min and sodium carbonate is added. After incubation at room temperature the absorbance of the reaction mixture is measured at 760 nm against a methanol blank. Gallic acid is used as standard to produce the calibration curve. The mean of three readings is used and the total phenolic content is expressed in mg of gallic acid equivalents/100 g of honey [82]. A modification of the Folin-Ciocalteu method has been carried out by Vinson et al. [83].

2.4. Chromatographic determination of the phenolic profile of honey

The need for knowing the profiles and identifying individual honey compounds requires the replacement of traditional methods by separative techniques. High-performance liquid chromatography (HPLC) is without doubt the most useful analytical technique for characterizing polyphenolic compounds, though gas chromatography and capillary electrophoresis are used in some instances.

GC was employed in its beginnings in an attempt to facilitate the determination of polyphenolic compounds. It has been used to determine polyphenols in honey in some published studies [47,52,76]. GC–MS has also been employed for the analysis of flavonoids in honey and, in this case, the derivatization step was unnecessary [84].

Studies with HPLC are described in Table 1, giving mobile phases, type of elution employed, stationary phase, extraction system, detection system used, compounds identified and several pertinent observations. The HPLC mode most widely used has been reversed-phase HPLC. In this case the stationary phase consists of a non-polar octadecylsilane (C_{18}) bonded phase and the mobile phase is a polar solvent.

The majority of published chromatography studies describe the use of an elution mobile gradient phase in recognition of the complexity of the phenolic profile. Several mobile phases have been used but the most common are binary systems comprising an aqueous component and a less polar organic solvent.

Table 1
Separation of phenolic compounds of honey using HPLC methods

Column	Mobile phases	Elution	Detection	Extraction system	Identified compounds	Observations	Reference
XTerra RP18 (15 cm × 0.39 cm, 5 μm)	A: water:formic acid (99.5:0.5); B: methanol	Gradient	DAD λ = 285 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	*p*-Hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, *cis–trans*-abscisic acid, cinnamic acid, pinobanksin, quercetin, pinocembrin, kaempferol, chrysin, galangin	Antioxidants of honeys from various floral sources	[23]
Lichrocart RP-18 (18.1 cm × 0.4 cm, 5 μm)	A: water:formic acid (95:5); B: methanol	Gradient	DAD λ = 280 and 340 nm	SPE (Amberlite XAD-2) clean-up: Sephadex LH-20	Ellagic acid, myricetin, chalcone, glycoside, quercetin, luteolin, 8-methoxikaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, chrysin, genkwanin, tectochrysin	Flavonoids in *Apis mellifera* and *Melipona* spp. honeys	[36]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (95:5); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, kaempferol 8-methyl ether, pinocembrin, quercetin 3,3′-dimethyl ether, isorhamnetin, chrysin, pinobanksin, tectochrysin	Flavonoids, phenolic acids and abscisic acid in Leptospermum honeys	[43]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Grandient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Pinocembrin, pinobanksin, chrysin, galangin, tecthochrysin, quercetin, kaempferol, 8-methoxykaempferol, caffeic acid, *p*-coumaric acid, *cis–trans*-abscisic acid, ferulic acid, apigenin, quercetin 3,7-dimethyl ether, quercetin 3,3′-dimethyl ether, hesperetin	Flavonoid profile of European unifloral honeys	[54]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	*Trans–trans*-abscisic acid, *cis*, *trans*-abscisic acid, pinobanksin, pinocembrin, chrysin, galangin	Analysis of abscisic acid and flavonoids in heather honey	[56]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Pinobanksin, quercetin, luteolin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, chrysin, galangin, tectochrysin	Analysis of 15 flavonoids in rosemary honey	[62]
Lichrocart RP-18 (10 cm × 0.4 cm, 5 μm)	A: water:formic acid (95:5); B: methanol	Gradient	DAD λ = 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Quercetin glycoside, luteolin glycoside, 8-methoxykaempferol glycoside, kaempferol glycoside, quercetin, luteolin, methylated luteolin, 8-methoxykaempferol, isorhamnetin, genkwanin	Flavonoids in stinglessbee honey	[64]
LiChrospher 100 RP-18 (12 cm × 0.4 cm, 5 μm)	A: water:formic acid (95:5); B: methanol	Gradient	UV λ = 270 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Caffeic acid, phenyllactic acid, methyl syringate, cinnamic acid, pinobanksin, pinocembrin, chrysin, galangin	Antibacterial phenolic components of manuka honey	[65]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Myricetin, tricetin, quercetin, luteolin, kaempferol	Flavonoids markers of Eucalyptus honey	[66]

Table 1 (*Continued*).

Column	Mobile phases	Elution	Detection	Extraction system	Identified compounds	Observations	Reference
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl eher, kaempferol, pinobanksin, pinocembrin, chrysin	Flavonoids in Eucalyptus Australian honeys	[67]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Gallic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, *o*-coumaric, ferulic acid, ellagic acid, abscisic acid	Analysis of seven phenolic acids and two abscisic acid isomers in Eucalyptus honey	[68]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, 8-methoxy kaempferol, pinocembrin, quercetin 3,3′-dimethyl ether, isorhamnetin, chrysin, pinobanksin, genkwanin	Flavonoids in Melaleuca, Guioa, Lophostemon, Bansia and Helianthus honeys	[69]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Myricetin, tricetin, quercetin, luteolin, quercetin 3-methyl ether, kaempferol, kaempferol 8-methyl ether, pinocembrin, quercetin 3,3,′-dimethyl ether, isorhamnetin, chrysin, pinobanksin	Quantitative analysis of Flavonoids in Australian Eucalyptus honeys	[70]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Gallic acid, chloroenic acid, coumaric acid, ferulic acid, ellagic acid, syringic acid	Phenolics acids in Melaleuca, Guioa, Lophostemon, Bansia and Helianthus honeys	[71]
C_{18} (15 cm × 0.46 cm, 5 μm)	A: water:acetic acid (99:1); B: methanol:acetic acid (99:1)	Gradient	DAD λ = 280 nm	Extraction with ethyl acetate and SPE (C_{18})	Gallic acid, caffeic acid, ferulic acid, benzoic acid, cinnamic acid	Isolation and identification of phenolic acids in Malaysian honey	[76]
Discovery RP Amide C_{16} (15 cm × 0.46 cm, 5 μm)	A: water:acetic acid (95.5:0.5); B: methanol: acetic acid (95.5:0.5)	Gradient and isocratic	Multichannel Electrochemical detector and mass spectrometry	SPE: GL-Pak PLS-2 cartridge	Methyl syringate	Identification of phenolic compounds in manuka honey	[26]
Lichrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290 and 340 nm, NMR	SPE (Amberlite XAD-2) clean-up: extraction with diethyl ether	Ellagic acid, pinobanksin, hesperetin, quercetin, luteolin, 3-methylquercetin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, pinocembrin, phenylethyl caffeate, pinobanksin 3-acetate, dimethylallyl caffeate, quercetin 3,7-dimethyl ether, chrysin, galangin, galangin 3-methyl ether, myricetin 3,7,4′,5′-methyl ether, pinocembrin 7-methylether, tecthochrysin	Flavonoids in Tunisian honeys	[63]
Spherisorb ODS2 (25 cm × 0.46 cm, 5 μm)	Methanol/H_2SO_4 10^{-2}N (10:90, v/v)	Isocratic	DAD λ = 292 nm NMR, MS	Extraction with ethyl acetate and TLC	Homogentisic acid	Determination of homogentisic acid in strawberry-tree honey	[78]

For example, a method available for the analysis of the phenolic fraction of honey is a reversed-phase HPLC using gradient elution with an aqueous solution of formic acid and methanol as solvents.

With regard to the detection system employed in HPLC, it should be emphasised that UV–vis detection with a diode array detector is undoubtedly the most common, although electrochemical detection systems [26,47,85–87] and mass detectors [26,78] have been used to a lesser extent.

The most frequent wavelengths used have been 290 and 340 nm. Because some phenolic compounds show several absorption maxima, the use of simultaneous multiple UV (photodiode array) is recommended for identification purposes, and also because this detector offers chromatograms at any wavelength accompanied by the absorption spectrum of each eluted band. In this way the absorption spectrum can be combined with retention parameters for the possible identification of an unknown compound as well as to measure the purity of the elution band in question. At 290 nm it is known that all polyphenolic compounds absorb, but nonetheless, some studies recommend using different wavelengths to achieve maximum sensitivity, and also, if possible, a suitable selectivity depending upon the polyphenolic compounds contained in the honey in question.

Polyphenols are usually identified by comparing retention times, UV spectra and chromatograms. NMR spectrometry is often also used as a complementary technique for structural assignment [56,63,66,78].

2.5. Electrophoretic determination of the phenolic profile

Capillary electrophoresis has also been used as an alternative technique to HPLC for the analysis of phenolic compounds in honey. CE combines short analysis times and high separation efficiency of polyphenols in honey. The use of this rapid analytical technique, allowing a faster screening of phenolic compounds, is highly recommended. The union of speed, resolution, simplicity and low operating costs make the technique an attractive option for the development of improved methods for determining phenolic compounds in honey.

Despite of the advantages that CE seems to have, there are few publications dealing with the determination of polyphenols in honey by this technique.

The operative modes used are borate-based CZE and borate-based micellar electrokinetic chromatography (MECK) with sodium dodecylsulfate (SDS) as micellar agent. The MECK methods study honey flavonoids [50,88] and the CZE method analyzes the whole polyphenolic fraction of honey [73].

A summary of optimized conditions of capillary electrophoresis methods (effective length of capillary, internal diameter of capillary, wavelength of detection, voltage, temperature, injection time, buffer concentration) where honey samples are analysed as well as the phenolic compounds studied are set out in Table 2.

3. Propolis

3.1. Introduction

Propolis, or bee glue, is a dark-coloured resinous substance collected by honeybees from leaf buds and cracks in the bark of various tree species [89]. Bees may also use material actively secreted by plants, or exuded from wounds in plants (lipophylic material on leaves, mucilages, gums, resins, lattices, etc.). Once collected, this material is enriched with salivary and enzymatic secretions. The resulting substance is used by bees to seal holes in their hives, strengthen the thin borders of the comb, exclude

Table 2
Summary of optimized conditions of capillary electrophoresis methods where honey samples are analized

Instrumental variables						Experimental variables			Identified compounds	References
L_{ef} (cm)	i.d. (μm)	λ_d (nm)	V (kV)	T (°C)	t_{inj} (s)	Type of buffer	[Buffer] (mM)	pH		
63	70	280	20	30	2	Sodium borate/SDS + 10% methanol	200/50	8	Pinobanksin, naringenin, hesperetin, 8-methoxykaempferol, myricetin, quercetin, luteolin, eriodictyol, pinocembrin, kaempferol, apigenin, chrysin, galangin	[50]
63	50	340	21	25	2	Boric acid/SDS	200/50	8.5	Eriodictyol, naringenin, hesperetin, pinobankin, pinocembrin, myricetin, quercetin, kaempferol, luteolin, apigenin, chrysin, galangin, genkwanin, tectochrysin	[88]
50	50	280	20	30	–	Sodium borate + 20% methanol	100	9.5	Hydroxymethylfurfural, phenylethylcaffeate, dimethylallylcaffeate, pinobanksin, naringenin, hesperetin, cinnamic acid, chlorogenic acid, *m*-coumaric acid, quercetin, luteolin, syringic acid, ferulic acid, pinocembrin, *o*-coumaric acid, kaempferol, *p*-coumaric acid, apigenin, vanillic acid, chrysin, galangin, ellagic acid, rosmarinic acid, *p*-hydroxybenzoic acid, caffeic acid, gallic acid, 2,4-dihydroxybenzoic acid	[73]

L_{ef}, effective length of capillary; i.d., internal diameter of capillary; λ_d, wavelength of detection; V, voltage; T, temperature; t_{inj}, injection time; [Buffer] buffer concentration.

draught and make the entrance of the hive weathertight or easier to defend. Propolis is also used as an "embalming" substance to cover hive invaders which the bees have killed but cannot transport out of the hive [90].

Propolis has been used extensively in folk medicine since it possesses various biological activities such as antiseptic, antifungal, antibacterial, antiviral, anti-inflammatory, anaesthetic and antioxidant properties [89,91,92] among others. It can increase the body's natural resistance to infections and lower blood pressure and cholesterol levels. Applied externally, propolis relieves various types of dermatitis. In addition, it is used in mouthwashes and toothpastes to prevent caries and treat gingivitis and stomatitis [93] and it is claimed to be useful in cosmetics and as a constituent of health foods [94].

The plant origin of propolis determines its chemical diversity. Bee glue's chemical composition depends on the species of local flora present at the site of collection and thus in the geographic and climatic characteristics at the site [95]. In the world's temperate zones the dominant propolis source is the bud exudate of poplar (*Populus*) [90,96] whereas in the tropical regions there are no poplars and bees have to find different plant sources for bee glue. In spite of possible differences in composition due to the different plant sources, most propolis samples share considerable similarity in their overall chemical nature. It is made up of 50% resin (composed of flavonoids and related phenolic acids), 30% wax, 10% essential oils, 5% pollen and 5% other organic compounds [97]. Polyphenols (including flavonoids, phenolic acids and their esters), due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters and to scavenge free radicals, are considered to be the main pharmacologically active molecules in propolis [98].

More than 180 compounds, mainly polyphenols, have been identified as constituents of propolis [94]. As mentioned already, the concentration of phenolic compounds may vary substantially according to the origin of the samples and such differences are likely to affect its biological activities and consequently its clinical properties [99]. Therefore the assay of these components is of great importance.

3.2. Sample preparation

Propolis cannot be used as a raw material; it must be purified by extraction with solvents. This process should remove the inert material and preserve the polyphenolic fractions. Extraction with ethanol is particularly suitable to obtain dewaxed propolis extracts rich in polyphenolic components [100] and this is the most commonly used solvent, especially at concentrations of 70% [101–108] and 80% [109–114], although other concentrations have also been used, such as 95% [115] and absolute ethanol [116,117]. Compared with absolute ethanol, extraction with aqueous ethanol results in wax-free tinctures, containing higher amounts of phenolic substances [118]. Park and Ikegaki [119] used various concentrations of ethanol as solvent and measured the absorption spectra of the different extracts. The 80% ethanolic extract showed highest absorption at 290 nm, which means that the highest concentration of flavonoids (especially of kaempferide, acacetin and isorhamnetin) was liberated from the propolis when using this solvent. With other ethanol concentrations, however, it is possible to extract higher quantities of other flavonoids, for example, with 60% ethanol the most extracted compounds were isosakuranetin, quercetin and kaempferol; and with 70% ethanol, pinocembrin and sakuranetin.

Extraction with pure water [101,119,120] (these extracts are likely to contain phenolic acids which are very soluble in water), methanol [62,63,100,121], hexane and acetone [122,123] and chloroform [124] has also been used.

The analysis of raw propolis is more frequent than the analysis of commercial propolis preparations [100,114,121,125]. The preparation of crude propolis begins by dehydrating the sample so that the dried propolis (cooled) can be ground into a fine powder. Then, in one procedure, a weighted sample is dissolved in the solvent (the most frequently used proportion is 1:10, w/v) and left for 24 h at room temperature [63,105,107,108,111,114,117,126]. It is then filtered and the procedure repeated several times [103,104,106,121] as successive extractions ensure the complete recovery of the phenolics. Alternatively, the sample is dissolved by shaking at 70 °C for 30 min [112,113,119]. After dissolution the insoluble portion is separated by filtration and the solvent is then evaporated to dryness under reduced pressure [63,103,104,107,108] and redissolved. Instead of this, the mixture can be centrifuged to obtain the supernatants [111,112,114,115,119,121,126], which can then be directly used for analysis.

It must be stressed that, as in any analytical study, sampling is extremely important, because this procedure determines the final result.

3.3. Spectrophotometric determination of phenolic compounds

The increasing use of propolis preparations in medicine requires the development of suitable approaches for the quantitative determination of their active components. Rapid spectrophotometric methods are assumed to be especially useful for the routine control of propolis [111,118,127,128]. These methods are aimed at the determination of either total flavonoids and total phenolics [118,127] or total flavanones/dihydroflavonols and total flavones/flavonols [128].

Popova et al. [106] pointed out that the quantification of the active compounds within groups with the same or close chemical structures correlates better with their biological activity and is more informative than the quantification of individual components. They assume, therefore, that this is a correct approach to characterising and standardising propolis preparations. Other advantages are its simplicity, good repeatability and acceptable accuracy.

Quantitative determinations of flavonoids in propolis are conducted by two colorimetric methods [129]. The aluminium chloride method is used to determine the flavone and flavonol content; it is based on the formation of a complex between the aluminium ion Al(III) and the carbonyl and hydroxyl groups of the flavonoid. The test solution, methanol and aluminium chloride in methanol (w/v) are mixed and left for 30 min. The

absorbance is then measured at 425 nm [106,127]. To quantify flavanones and dihydroflavonols the 2,4-dinitrophenylhydrazine (DNP) method is used. This is based upon the interaction of these compounds with DNP in acidic media to form coloured phenylhydrazones. The test solution and DNP solution diluted with methanol are heated at 50 °C for 50 min in a water bath. After cooling to room temperature, the mixture is diluted with potassium hydroxide in methanol (w/v). The resulting solution is diluted with methanol. Absorbance is measured at 486 nm [128,129]. The sum of flavonoid contents determined by the above two methods closely represents the real content of total flavonoids [129].

It is possible to determine the total flavonoid content with the Folin-Ciocalteu method, which is the most widely used for the spectrophotometric quantification of total phenolics [130]. Briefly, the test solution, distilled water, Folin-Ciocalteu reagent and sodium carbonate solution are mixed. The sample is left for incubation and the absorbance is measured at 760 nm [106,117].

3.4. Chromatographic determination of the phenolic profile of propolis

The complete characterization of propolis activity involves both qualitative and quantitative chemical analysis. Chromatographic techniques such as fine chromatography, gas and, in particular, HPLC provide the profile and identification of the individual phenolic compounds. They are discussed in this section. Special attention is given to detection systems, due to their importance in the characterization of polyphenolic compounds. Detection is routinely achieved by ultraviolet absorption, often involving a photodiode array detector. Coupled techniques, particularly mass spectroscopy, are being used increasingly for routine work.

3.4.1. Thin-layer chromatography

In TLC, the choice of stationary phase as well as a suitable solvent depends upon the polyphenolic structures being studied.

A classical stationary phase of silica gel (precoated plates) is widely used [108–113,131,132] to separate more apolar flavonoids such as flavonols and isoflavonoids [81]. Samples are eluted with different mobile phases: ethanol/water (55:45, v/v) [110,112], petroleum ether/ethyl acetate (70:30) [108], petroleum ether/acetone/formic acid (35:10:5) [132], chloroform/ethyl acetate (60:40) [113], toluene/chloroform/acetone (40:25:35) [111], *n*-hexane/ethyl acetate/acetic acid (31:14:5) [111,132,133] or (60:40:3) [111] and chloroform/methanol/formic acid (44.1:3:2.35) [131].

Medic-Saric et al. [133] used two-dimensional TLC with densitometric evaluation with *n*-hexane/ethyl acetate/glacial acetic acid (31:14:5, nu/nu) (System A) and chloroform/methanol/formic acid (44:3.5:2.5) (System B) as mobile phases.

Visualization is performed in short- and long-wavelength UV light and in some cases spraying with different reagents. A common wavelength is 366 nm [108,110,112,132].

3.4.2. Gas chromatography

GC determines phenolic compounds both qualitatively and quantitatively. It is usually necessary, however, to derivatize the compounds to make them suitable for GC analysis. During the last 10 years GC has been extensively employed by several researchers [103–105,108,124,134–147]. An alternative is high-temperature, high-resolution gas chromatography (HT-HRGC) [107,112,122,123,148], which is an established technique for separating complex mixtures and identifying high-molecular-weight compounds that do not elute when analysed on ordinary GC columns.

GC coupled with mass spectrometer (MS) is the method most widely used, since MS allows the acquisition of molecular mass data and structural information together with the identification of compounds. Propolis, however, contains components that are not volatile enough for direct GC–MS analysis even upon derivatization or HT–GC–MS [116]. Table 3 shows some GC temperature ranges used, characteristics of the column employed, analysis time, detection system, type of derivatization, compounds identified and several observations about a few notable published works.

3.4.3. Liquid chromatography and high-performance liquid chromatography

High-performance liquid chromatography (HPLC) currently represents the most popular and reliable analytical technique for the characterization of polyphenolic compounds, as witnessed by the number of papers published on the subject. HPLC coupled to MS, and even to nuclear magnetic resonance spectroscopy (RMN), has improved the analysis of non-volatile species and allows us to establish definitive structures [149].

Electrospray ionisation (ESI) [150] permits the direct ionisation and transference of molecules to mass spectrometers and has extended the applicability of MS for a variety of new classes of molecules with thermal instability, high polarity and high mass.

Mirodikawa et al. [120] have established a suitable LC–MS method for the determination of the chemical constituents and therefore the quality of propolis.

Several authors have analysed polyphenolic compounds in propolis of diverse origins using different HPLC methods with different extraction systems and coupling diverse detector systems [62,63,100,106,109,110,112,113,115,117,119,126,134,146,151–157]. Table 4 summarises the information provided by some representative papers and gives the characteristics of the column employed, the mobile phases, the type of elution employed, the detection and extraction systems used, compounds identified and several pertinent observations.

3.5. Capillary electrophoresis analysis of phenolic compound in propolis

Because of the previously mentioned characteristics of capillary electrophoresis, this technique could well prove to be an interesting choice for the analysis of phenolic compounds. Nevertheless, to our knowledge, there are few reports about its use

Table 3
GC conditions for determination of phenolic compounds in propolis

Temperature range (°C)	Column	Analysis time (min)	Detection	Derivatization	Identified compounds	Observations	Reference
85–310	DB1 column (30 m × 0.32 mm i.d.)	85	MS	Pyridine + BSTFA	Pinostrobin chalcone, hexamethoxy flavone, pinostrobin, pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin, naringenin, dihydrocinnamic acid, cinnamic acid, *p*-coumaric acid, isoferulic acid, ferulic acid, caffeic acid	Composition and activities of Egyptian propolis	[103,104]
100–310	HP5-MS capillary column (23 m × 0.25 mm i.d.), 0.5 mm film thickness	42	MS	Pyridine + BSTFA	Pinocembrin, pinobanksin, pinobanksin *O*-acetate, chrysin, galangin, pentenyl caffeates, benzyl caffeates, phenethyl caffeate	Composition of European propolis	[105]
100–310	HP5-MS capillary column (23 m × 0.25 mm i.d.), 0.5 μm film thickness	42	MS	Pyridine + BSTFA	Cinnamic acid, benzyl cinnamate, cinnamyl cinnamate, pinocembrin, pinobanksin, pinobanksin 3-acetate, chrysin, galangin, phenylethyl caffeate, cinnamyl caffeate, vanillin, *p*-coumaric acid, ferulic acid, caffeic acid, dehydroabietic acid	Composition and antibacterial activity of Turkish propolis TLC analysis too	[108]
50–285	HP1 methyl silicone capillary column (25 m × 0.25 mm i.d.)	55	MS	Methylation	Cinnamic acid, vanillin, ethyl cinnamate, vanillic acid, *p*-coumaric acid, ferulic acid, ethyl ferulate, 3-methylbut-2-enyl ferulate, 3-methylbut-3-enyl ferulate	Major organic constituents in New Zealand propolis. HPLC analysis too	[134]
40–390	Borosilicate capillary column (20 mm × 0.3 mm i.d.) coated with PS-086[a] (d_f = 0.1 μm) connected to a 2 m piece of 0.25 mm i.d., high-temperature fused silica (which served as an interface)	54	MS	BSTFA	Ethyl hydrocinnamate, hydrocinnamic acid, inositol, cinnamic acid, ferulic acid, caffeic acid, pinostrobin	Composition and microbicidal activity of Brazilian and Bulgarian propolis	[107]
50–285	CBP5 column (30 m × 0.25 mm i.d.)	55	MS	Methylation	Coumaric acid, ferulic acid, pinobanksin, kaempferol, apigenin, isosakuranetin, pinocembrin, dimethylallyl caffeic acid, pinobanksin 3-acetate, chrysin, galangin, kaempferide, tectochrysin	Propolis and plant resins HPLC and TLC analysis too	[112]
40–390	Glass column (22 m × 0.2 mm i.d.) coated with PS-086[a]	54	FID MS	Trimethylsilylation: bis (trimethylsilyl)-trifluoro acetamide (BSTFA)	Inositol, *p*-cinnamic acid, ferulic acid, isoferulic acid, caffeic acid	Flavonoids in acetone no derivatizated, derivatization of methanol extract	[122]
40–380[b]	Fused silica capillary (10 m × 0.3 mm i.d.) coated with 0.1 μm film of Silaren-30[c]	55	FID MS	No	Hydrocinnamic acid, vanillin, cinnamic acid, benzyl cinnamate, naringenin 3′,4′-dimethoxy, betuleol, kaempferid	Hexane and acetone crude extracts	[123]
40–370[d]		50					

[a] 15% phenyl, 85% methyl polysiloxane.
[b] Program A.
[c] 30% diphenylpolysiloxane, 40% sildiphenylene ether, 30% dimethyl polysiloxane.
[d] Program B.

Table 4
HPLC conditions for determination of phenolic compounds in propolis

Column	Mobile phase	Elution	Detection	Extraction system	Identified compounds	Observation	References
LiChrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290, 340 nm	In MeOH for 2 h at room temperature	Pinobanksin, quercetin, 8-methoxykaempferol, kaempferol, apigenin, isorhamnetin, quercetin 3,3′-dimethyl ether, pinocembrin, quercetin 7,3′-dimethyl ether, chrysin, galangin, techtochrysin	Flavonoids in rosemary nectar, honey and propolis	[62]
LiChrocart RP-18 (12.5 cm × 0.4 cm, 5 μm)	A: water:formic acid (19:1, v/v); B: methanol	Gradient	DAD λ = 290, 340 nm	In MeOH for 24h at room temperature, evaporated and redissolved in MeOH	Pinobanksin, pinocembrin, phenylethyl caffeate, pinobanksin 3-acetate, dimethylallyl caffeate, chrysin, galangin, myricetin 3,7,4′,5′-methyl ether, pinocembrin 7-methyl ether	Phenolics in Tunisian honey and propolis	[63]
Spherisorb ODS-2 (25 cm × 0.7 cm, 5 μm)	Methanol:water (58:42, v/v)	Isocratic	UV, EIMS, NMR	In a Soxhlet apparatus with MeOH, centrifuged, evaporated, clean-up	Myricetin 3,7,4′,5′-methyl ether, pachypodol	One fraction of the chromatographed sample is purified by semipreparative HPLC	[63]
Symmetry C_{18} column (22 cm × 0.46 cm, 5 μm)	A: 30 mM NaH_2PO_4 (pH 3); B: acetonitrile	Gradient	PAD λ = 265, 290, 360 nm	Commercial preparations diluted in MeOH	3,4-Dihydroxy-cinnamic acid, 4-hydroxy-cinnamic acid, 3-hydroxy-cinnamic acid, quercetin, kaempferol, galangin, naringenin, pinocembrin, chrysin	Quality control of commercial propolis APCI mass spectra obtained	[100]
Intersil 5 ODS-2 column (25 cm × 0.46 cm i.d.) with a Chromosphere ODS guard column (1 cm × 0.3 cm i.d.)	A: water: acetic acid (95:5, v/v); B: methanol	Gradient	UV λ = 290 nm	In 70% EtOH for 24 h at room temperature (×2)	Flavones and flavonols, flavanones and dihydroflavonols and total phenolics (caffeic acid, *p*-coumaric acid, ferulic acid, kaempferol, pinocembrin, phenethyl caffeate, isopentyl caffeate, chrysin, galangin, pinostrobin, benzyl caffeate)	Active constituents of poplar-type propolis; verification of the spectrophotometric quantification results.	[106]
YMC Pack ODS-A (RP)	Acetic acid:methanol:water (5:75:60, v/v/v)	Isocratic	DAD λ = 254 nm	In 80% EtOH for 30 min at 70 °C, centrifuged	Quercetin, kaempferol, apigenin, isorhamnetin, rhamnetin, pinocembrin, sakuranetin, isosakuranetin, chrysin, acacetin, galangin, kaempferide, tectochrysin	Antimicrobial activity of Brazilian propolis; TLC analysis too	[110]
YMC Pack ODS-A RP-18 (25 cm × 0.46 cm, 5 μm)	A: water; B: methanol	Gradient	DAD λ = 268 nm	In 80% EtOH for 30 min at 70 °C, centrifuged	Coumaric acid, ferulic acid, pinobanksin, kaempferol, apigenin, isosakuranetin, pinocembrin, dimethylallyl caffeic acid, pinobanksin 3-acetate, chrysin, galangin, kaempferide, techtochrysin	Botanical origin and composition of Brazilian propolis; TLC, GC analysis too	[112]
ODS column (25 cm × 0.4 cm i.d., 5 μm)	A: H_2O:0.1% H_3PO_4; B: CH_3CN:0.1% H_3PO_4	Gradient	UV λ = 254 nm	Aqueous-ethanolic extract and partition between inmiscible solvents	Evidenced the presence of phenolic compounds by the intense fluorescence	Antibacterial activity of Brazilian propolis. TLC analysis too	[113]
Superspher 100 RP-18 (12.5 cm × 0.4 cm, 4 μm)	A: Methanol:acetic acid, 1 M (50:50); B: Methanol:acetic acid, 1 M (40:60); C: acetonitrile	Gradient	UV λ = 254 nm	In 95% EtOH for 7 days at room temperature, centrifuged, evaporated and redissolved	Pinocembrin, galangin	Activity against *Streptococcus pyogenes* of Italian propolis	[115]

Table 4 (*Continued*).

Column	Mobile phase	Elution	Detection	Extraction system	Identified compounds	Observation	References
YMC Pack ODS-A (RP)	Acetic acid:methanol:water (5:75:60, v/v/v)	Isocratic	DAD λ = 254 nm	In 10–95% EtOH for 30 min at 70 °C, centrifuged; in water too	Isosakuranetin, sakuranetin, quercetin, kaempferol, pinocembrinm, kaempferide, acacetin, isorhamnetin	Evaluation of the preparations to see which have maximum absorption	[119]
Capcell Pak ACR 120 C_{18} column (25 cm × 0.2 cm i.d., 5 μm)	A: 0.1% formic acid:water; B: 0.08% formic acid:acetonitrile	Gradient	PAD λ = 195–650 nm MS (ESI)	In EtOH for 24 h at room temperature, centrifuged	Caffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, quercetin, pinobansin 5-methyl ether, apigenin, kaempferol, pinobanksin, cinnamylideneacetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, phenethyl caffeate, tectochrysin, artepillin C	Antioxidant activity of propolis of various geographic origins	[126]
Capcell Pak ACR 120 C_{18} column (25 cm × 0.2 cm i.d., 5 μm)	A: 0.1% formic acid:water; B: 0.1% formic acid:acetonitrile	Gradient	PDA λ = 195–650 nm MS (ESI)	In EtOH for 24 h at room temperature, centrifuged	Caffeic acid, *p*-coumaric acid, 3,4-dimethoxycinnamic acid, pinobansin 5-methyl ether, apigenin, kaempferol, pinobanksin, cinnamylideneacetic acid, chrysin, pinocembrin, galangin, pinobanksin 3-acetate, phenethyl caffeate, cinnamyl caffeate, tectochrysin	Antioxidant activity of propolis from Korea; colorimetric measurements too	[117]
LiChrospher 100 RP-18 (11.9 cm × 0.4 cm, 5 μm)	(1) A: formic acid; B: methanol; (2) A′: H_3PO_4 (pH 2.0); B′: MeCN	Gradient	PAD λ = 268 nm	Samples supplied as ethanolic tinctures	Cinnamic acid, pinobanksin, pinocembrin, pinobanksin 3-acetate, 1,1′-dimethylallylcaffeic acid, chrysin, galangin, pinocembrin 7-methyl ether, chrysin 7-methyl ether, galangin 7-methyl ether	Major organic constituents in New Zealand propolis; GC–MS analysis too	[135]
YMC PACK ODS column (25 cm × 2 cm)	0.1% trifluoroacetic acid in CH_3CN:H_2O (6:4)	Isocratic	UV, MS, 2D NMR	In EtOH for 12 h at room temperature, concentrated	Isonymphaeol-B, nymphaeol-A, nymphaeol-B, nymphaeol-C	New prenylflavonoid isolated from propolis from Okinawa; structure determined; extract previously chromatographed	[149]
Chromsep RP-18 (25 cm × 0.46 cm i.d., 5 μm)	A: methanol; B: water:acetonitrile (97.5:2.5, v/v)	Gradient	UV λ = 310 nm	–	Chrysin and others	Establishing ideal conditions for analysis	[155]

Table 5
CE conditions for determination of phenolic compounds in propolis

Instrumental variables						Experimental variables			Identified compounds	References
L_{ef} (cm)	i.d. (μm)	λ_d (nm)	V (kV)	T (°C)	t_{inj} (s)	Type of buffer	[Buffer] (mM)	pH		
56	50	200 (DAD)	30	25	2	(a) Sodium phosphate	25	(a) 7	(a) 3,4-Dimethoxycinnamic acid, *p*-coumaric acid, cinnamic acid, benzoic acid	[102]
						(b) Sodium borate		(b) 9.3	(b) Methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, *p*-coumaric acid, cinnamic acid, benzoic acid	
50	75	214	18	25	4	Borate + 0.5%MeOH	100	9.5	Rutin, chrysin, myricetin, kaempferol, hesperetin, daidzein, genistein, apigenin, quercitrin, luteolin, galangin	[114]
50	75	262	23	25	12	H_3BO_3–$Na_2B_4O_7$	40–60	9.2	Rutin, ferulic acid, apigenin, luteolin, quercetin, caffeic acid	[121]
50	50	254	15	25	–	Sodium tetraborate	30	9	Pinocembrin, acacetin, chrysin, catechin, naringenin, galangin, luteolin, kaempferol, apigenin, myricetin, quercetin, cinnamic acid, caffeic acid, resveratrol	[125]
56	50	200 (DAD)	30	25	2	Borate/SDS + 10% (v/v) acetonitrile	25/50	9.3	Pinocembrin, chrysin, galangin	[102]
50	75	214	−15	25	4	Sodium borate/SDS	30/50	8.5	Unsatisfactory conditions in the separation of some flavonoids	[114]
55	50	214	14	–	8	Borax/SDS + 5% (v/v) EtOH	30/12	9	Hesperetin, cinnamic acid, nicotinic acid	[158]

L_{ef}, effective length of capillary; i.d., internal diameter of capillary; λ_d, wavelength of detection; V, voltage; T, temperature; t_{inj}, injection time; [Buffer] buffer concentration.

with propolis and so far its applications are basically on the determination of flavonoids.

Different modes of operation are applicable with CE. Capillary zone electrophoresis (CZE) [102,114,121,125] is based on differences in the electrophoretic mobility of compounds caused by their charge and size. Micellar electrokinetic chromatography (MEKC) [102,114,141,158], in which surfactants such as sodium dodecyl sulphate (SDS) are added to the separation buffer, is also capable of separating neutral compounds.

The effects of some of the variables, such as buffer pH, buffer concentration, separation voltage and injection time, are studied in order to optimize the analytical conditions [114,121,158]. A summary of the optimized conditions of CE methods is provided in Table 5.

As flavonoids are weakly acidic their separation requires a buffer of pH > 10 to be successful. Chi et al. [99] determined flavonoids and phenolic acids in propolis by CZE using a buffer with pH 10.1. Nonetheless, important flavonoids such as myricetin and quercetin, for instance, may decompose in such an alkaline medium [50]. Therefore, if possible MEKC is used for the determination of flavonoids in natural samples [102].

4. Conclusions

The quality of honey and propolis depends on its chemical composition and floral origin. Their polyphenolic content is strongly affected by the floral, geographical origin and climatic characteristic of the site. For this reasons, the identification and quantification of the polyphenols of honey and propolis are of great interest.

Furthermore, they have a very important antioxidant capacity that is provided by polyphenols such as flavonoids and phenolic acids. These antioxidants report beneficial effects in human health. It has been commented that consumption of these bee products contributes to the treatment of stomach ulcer, sore throat and wounds and burns. Numerous studies have proven their versatile pharmacological activities: antibacterial, antifungal, antiviral, anti-inflammatory, hepatoprotective, antioxidant, antitumor, etc.

As a result, many analytical procedures have been carried out directed towards the determination of the complete phenolic profile of honey and propolis. The techniques employed in the last years have been GC, HPLC and CE, mainly combined with diode array detection and mass spectrometry

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Capítulo 7

Problemas para la estimación cualitativa y cuantitativa de los compuestos fenólicos de la miel mediante electroforesis capilar acoplada a detección por UV-Vis.

Publicación incluída en este capítulo:

Problems of quantitative and qualitative estimation of polyphenols in honey by capillary electrophoresis with UV-vis detection.
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La importancia del estudio de la fracción fenólica de la miel radica, como ya se ha explicado en la introducción, en los efectos biológicos beneficiosos que los compuestos pertenecientes a esta fracción son capaces de aportar. Además el perfil fenólico de la miel despierta gran interés debido a que podrían encontrarse marcadores varietales, ya que el carácter antioxidante varía en gran medida dependiendo del tipo de miel. Por tanto conociendo en profundidad este perfil y las diferencias entre variedades sería posible caracterizar el origen floral y geográfico de una miel.

Como se destaca en el review del capítulo anterior, esta fracción de la miel ha sido estudiada por técnicas tales como la cromatografía de gases, HPLC e incluso EC. De todas ellas la electroforesis capilar había sido poco utilizada hasta la fecha, por ello decidimos poner a punto varios métodos electroforéticos, comenzando por una metodología acoplada a un detector UV, que pudiese mejorar resultados anteriores reduciendo tiempos de análisis y disminuyendo los costes de éstos (menor consumo de reactivos con respecto a HPLC).

A la hora de realizar este trabajo se eligieron 6 variedades diferentes de miel: romero, tomillo, castaño, de la sierra, azahar y mil flores. A continuación se escogió el método de extracción a emplear en la preparación de nuestra muestra y por último se llevó a cabo la puesta a punto del método electroforético optimizando las siguientes variables:

- pH y tipo de la disolución reguladora.
- tipo y tiempo de inyección.
- voltaje de la separación.
- diámetro y longitud del capilar.
- longitud de onda de detección.
- temperatura.

Llegando a la conclusión de que los parámetros con los que se obtenía una óptima separación eran los siguientes: disolución reguladora de 100 mM de acetato amónico a pH 9.5, inyección hidrodinámica de 6 s, voltaje de 20 kV, capilar de sílice fundida de 50 μm de diámetro interno y 57 cm de longitud total (50 hasta la ventana de detección) y longitud de onda de detección de 280 y 340 nm

Problems of quantitative and qualitative estimation of
polyphenols in honey by capillary electrophoresis with UV-vis detection

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ABSTRACT

A capillary electrophoresis method to determine and quantify some compounds of the polyphenolic fraction of honey has been carried out. A detailed method optimization was performed to separate the phenolic compounds present in honey using a methanol-water extract of Rosemary honey. This manuscript reveals the difficulties presented to carry out the peak identification using UV-vis coupled to capillary electrophoresis as detection system in honey. Honey is a complex mixture of compounds that requires very effective separation techniques to allow the identification of the constituents of the polyphenolic fraction. In order to study this fraction of honey, a capillary electrophoresis method was proposed. The study of the polyphenolic fraction was firstly accomplished in rosemary honey. This honey was spiked individually with 22 commercial standards that have been found previously in honey. Only chrysin, pinocembrin, kaempferol, ferulic acid and p-coumaric acid could be identified, though chrysin and pinocembrin were overlapped. Because of this, only kaempferol and ferulic and p-coumaric acids were quantified. Furthermore, the method was applied in another five extracts of honey from different floral sources and the results obtained were similar. Therefore, other detectors such as the mass spectrometer should be employed to assign without any doubts the identity of the peaks present in the electropherogram of an extract of the polyphenolic fraction of honey.

INTRODUCTION

Honey has been an ingredient of traditional medicine on account of its dietary and curative properties since ancient times (1). It is considered as a functional food because it can provide not only basic nutritional and energetic requirements but also an additional physiological benefit (2). The group most widely studied among the functional ingredients is the family of antioxidants. These antioxidants act as preservatives in food (3-5), specifically they retard deterioration, rancidity or discoloration due to oxidation caused by light, heat and some metals. Furthermore, it has been reported to be effective in gastrointestinal disorders (6, 7), in the healing of wounds and burns (8, 9), as antimicrobial agent (7-11), and to provide gastric protection against acute and chronic gastric lesions (12, 13). However, the composition of honey varies greatly depending on the honey floral source (14, 15). Honey is a remarkably complex natural liquid that is reported to contain at least 181 substances (16), and there are an important number of polyphenolic compounds that can be presents in it. Because of this, the analysis of the polyphenolic fraction of honey is not an easy work. The need for knowing the profiles and identifying individual honey compounds requires the use of separate techniques. High-performance liquid chromatography (HPLC), has been the most useful analytical technique for characterizing polyphenolic compounds, though gas chromatography (GC) and capillary electrophoresis (CE) have also been used in some instances (17). As detection system, the UV-vis detection has been the most common. This detection system is very easy to operate and permits the determination of a wide range of compounds and functional groups, but at the same time, the UV-vis detector has a very low sensibility. Due to the importance of polyphenols, in this manuscript we present a capillary electrophoresis method coupled with UV-vis detector for determining the polyphenolic fraction of honey and we comment on the problems of identification that appear using UV-vis detection in complex samples as honey.

EXPERIMENTAL SECTION

Samples and reagents

Commercial honeys from different origins and different floral sources were analized. Rosemary honey, citrus honey and multifloral honey were acquired from Apisol, S.A. Montroy (Valencia, Spain). Chestnut-tree honey and mountain range honey were purchased from Al-Andalus Delicatessen, S. Coop. And. (Lanjarón, Granada, Spain); and thyme honey was obtained from Apilore, S.L. (Écija, Sevilla, Spain). The standards of the polyphenolic compounds kaempferol, quercetin, hesperetin, chrysin, naringenin, p-coumaric acid, caffeic acid, myricetin, ferulic acid, 3-hidroxycinnamic acid, chlorogenic acid, galangin, ellagic acid and abscisic acid were acquired from Sigma-Aldrich (St. Louis, MO); luteolin, apigenin, pinocembrin, tectochrysin, rosmarinic acid, isorhamnetin, rhamnetin and genkwanin were purchased from Extrasynthèse (Genay, France). All chemicals were of analytical reagent grade and used as received. Ammonium acetate was from Panreac (Barcelona, Spain) and ammonia from Merck (Darmstadt, Germany); which were used as running buffers at different concentrations and pHs. Methanol was acquired from Panreac. Distilled water with a conductivity of 18.2 MΩ was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). Amberlite XAD-2 employed for the sample preparation was purchased from Supelco (Bellefonte, PA, USA).

Apparatus

The CE instrument used was a Beckman 5500 capillary electrophoresis connected to a UV-vis detector. A GOLD software (Beckman Coulter, Fullerton, CA, USA) installed in a PC was used for system control and data handling. All capillaries (fused silica) used were obtained from Beckman Instruments and had an inner diameter (i.d.) of 50 μm, a total length of 57 cm and an effective separation length of 50 cm. The temperature was controlled using a fluorocarbon based cooling fluid.

Sample preparation

Extraction was performed following published methods (18, 19, 20), modified accordingly 30 g of rosemary honey samples were

thoroughly mixed with five parts (150 mL) of distilled water, adjusted to pH 2 with concentrated HCl, until completely fluid by stirring with a magnetic stirrer at room temperature. The fluid samples were then filtered through cotton wool to remove solid particles. The filtrate was mixed with 40 g Amberlite XAD-2 (pore size 9 nm, particles size 0.3-1.2 mm) and stirred in a magnetic stirrer for 10 min, which was considered enough to adsorb honey phenolics with a recovery rate more than 80 percent (18, 21). The Amberlite particles were then packed in a glass column (42 3.2 cm) and the column was washed with acidified water (pH 2 with HCL, 100 mL) and subsequently rinsed with distilled water (300 mL) to remove all sugars and other polar constituents of honey. The phenolic compounds remained adsorbed on the column (22) and were eluted with methanol (300 mL). The methanolic extract was concentrated to dryness under reduced pressure in a rotary evaporator at 50ºC. The residue was resuspended in distilled water (5 mL) and extracted with diethyl ether (5 mL 3). The diethyl ether extracts were combined and the ether was removed to dryness under reduced pressure in a rotary evaporator at 30ºC. The dried residue was then redissolved in 0.5 mL of methanol, filtered through a 0.45 m membrane filter. Finally distilled water was added to the extract to obtain a 50:50 methanol:water solution and analysed by CE-UV-vis.

RESULTS AND DISCUSSION

Effects of experimental and instrumental variables in the CE method

The effect of pH was the first experimental variable tested by adjusting the buffer (ammonium acetate) pH between 8.5 and 10 by adding a proper amount of NH3. Three different buffers were tested: ammonium acetate, sodium tetraborate and ammonium chloride. All of them were prepared in a 9-10 pH range, and ammonium acetate was the buffer that gave the best resolution for phenolic compounds in a satisfactory time. The buffer concentration was investigated in a range between 20-120mM. We found that 100 mM of ammonium acetate at pH 9.5 represented the best compromise for the resolution of the compounds studied an a reasonable analysis time. The applied voltage was varied from 15 to 30 kV. The voltage used to obtain the sorter analysis time and maintain a good resolution was 20 kV.

Capillary electrophoresis method

The experimental and instrumental conditions chosen to carry out the analyses were the followings: 100 mM ammonium acetate was used as a buffer at a pH 9.5, samples were injected hydrodynamically for 6s and the electrophoretic separations were performed at 20 kV of voltage and 25ºC of temperature. UV detection was achieved at 280 and 340 nm. In order to maintain the reproducibility of the migration times between analyses, the capillary was conditioned by rinsing with 1 M NaOH for 2 min, distilled water for 2 min and running buffer for 5 min to equilibrate the capillary. Every time a new capillary was used, it was preconditioned by rinsing with 1 M NaOH for 10 min, distilled water for 5 min and running buffer for 20 min. The different phenolic compounds were identified by comparison of migration times obtained from real samples and standards, and spiked extracts of honey with standards at several concentration levels.

Identification and quantitation of polyphenols in several Spanish honeys

Firstly, the study of identification and quantitation of polyphenols was carried out in rosemary honey. In order to perform the peak identification, we spiked the extract of rosemary honey with standards of polyphenolic compounds that have been found previously in honey (23-28). All the standards that have been used for peak identification are shown in Table 1, together with their corresponding structures.

A standard solution of each analyte was prepared and the extracts of rosemary honey were spiked individually with each one at different concentration levels in order to ensure the identity of the peaks.
All the analyses were done at two wavelengths, 280 and 340 nm.
In order to choose the wavelengths to carry out the analyses, the UV spectra of each analyte was done. The 280 and 340 nm values were selected because these wavelengths were the maximum of absorbance of the standards of the polyphenolic compounds studied.
Despite spiking the extract with each standard individually, and achieving good electrophoretic separation, peak identification was not easy using UV-vis as detection system.
This fact is due to the complexity of the honey samples and the similarity among the structures of the polyphenols that causes the migration times of the analytes to be too similar.
UV-vis detector cannot differentiate among compounds with the same or very similar retention times and, because of this, peak identification has been very difficult. In Figure 1 it can be seen

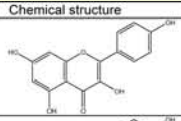
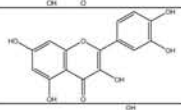
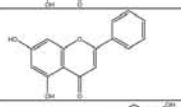
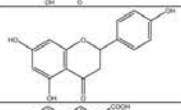
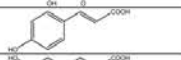
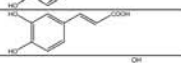
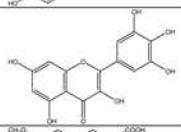
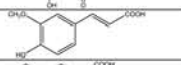
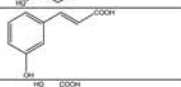
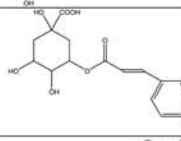
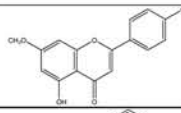
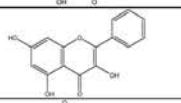
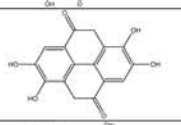
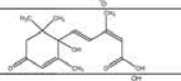
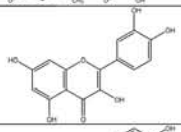
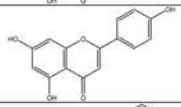
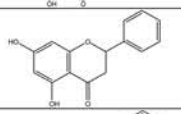
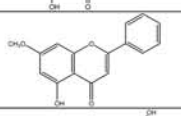
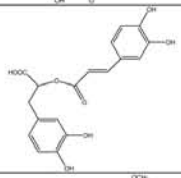
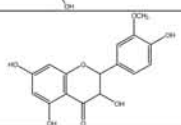
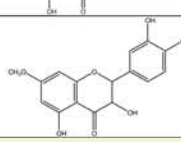
Analites	Chemical structure
Kaempferol	
Quercetin	
Hesperetin	
Chrysin	
Naringenin	
p-coumaric acid	
Caffeic acid	
Myricetin	
Ferulic acid	
3-hydroxycinnamic acid	
Chlorogenic acid	
Genkwanin	
Galangin	
Ellagic acid	
Abscisic acid	
Luteolin	
Apigenin	
Pinocembrin	
Tectochrysin	
Rosmarinic acid	
Isorhamnetin	
Rhamnetin	

Table 1. Chemical structures of the phenolic compounds studied

three electropherograms, one of them shows an electropherogram of an extract of honey, the second one presents the electropherogram of an extract of honey spiked with a standard where the identification is not clear The third electropherogram shows the profile of an extract of honey spiked with another standard where there are no doubts in the

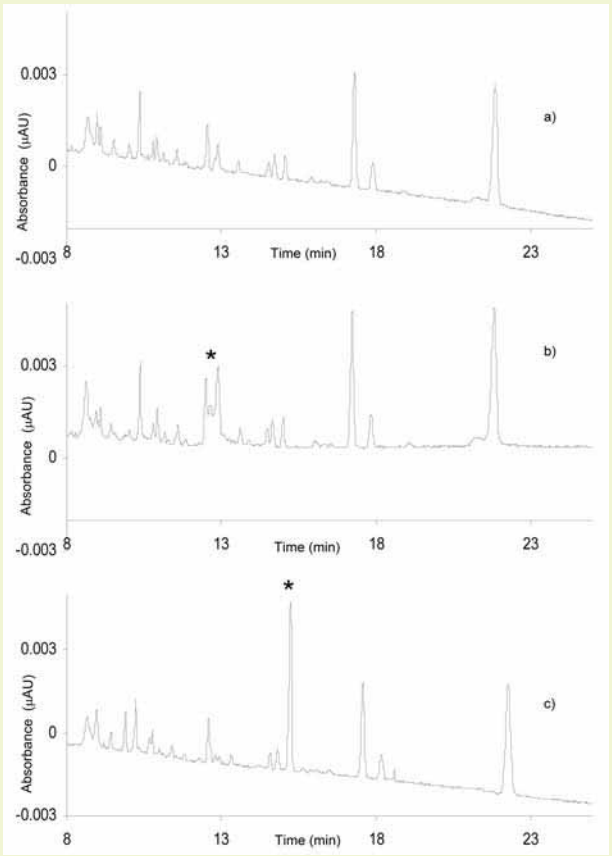


Figure 1. a) Electropherogram of a Rosemary honey extract, b) electropherogram of a Rosemary honey extract spiked with galangin and c) electropherogram of a Rosemary honey extract spiked with ferulic acid

identification.
Therefore, in spite of 22 standards being used, and achieving good resolution by CE, only five compounds were successfully identified (kaempferol, ferulic acid, p-coumaric acid, pinocembrin and chrysin) and two of them have overlapping peaks (pinocembrin and chrysin). Because of this, the quantitation was carried out for three analytes. The calibration curve of ferulic acid and p-coumaric acid were done at 280 nm and the calibration curve of kaempferol was carried out at 340 nm because the analytes have an absorbance maximum at these wavelengths. In Figure 2 it is showed an electropherogram obtained for an extract of rosemary honey under optimized conditions where it can be seen the peaks corresponding to the analytes identified.
Standard calibration graphs were prepared for kaempferol, ferulic acid and p-coumaric acid. The detection limit (LOD), quantitation limit (LOQ), 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 (29). Three replicates of each analyte at different concentrations were done in order to set up the calibration. All calibration curves showed good linearity between different concentrations depending on the analytes studied. The calibration plots indicate good correlation between peak areas and analyte concentrations; regression coefficients were higher than 0.99 for kaempferol, ferulic acid and p-coumaric acid. All the analytical

Analyte	RSD (%) (intermediate value)	Detection limit (DL) (µg/mL)	Quantification limit (QL) (µg/mL)	Calibration range(µg/mL)	Calibration equations	r^2
Kaempferol λ=340 nm	2.66	0.129	0.430	0.430-10	y=5.472E-5x-1.812E-5	0.9991
Ferulic acid λ=280 nm	3.98	0.367	1.225	1.225-25	y=5.424E-5-1.703E-5	0.9990
p-coumaric acid λ=280 nm	3.72	0.130	0.434	0.434-50	y=1.922E-5+1.78E-5	0.9995

RSD: Relative Standard Deviation

Table 2. Analytical parameters of proposed method

parameters of the proposed method are summarized in Table 2.
The samples of rosemary honey extracts were injected in the

Analyte	Rosemary honey
Kaempferol	5.64 ± 0.27
Ferulic acid	13.47 ± 0.66
p-coumaric acid	41.07 ± 2.03

Table 3. Quantitative results obtained for the analysis of Rosemary honey. (n=7) (Value= X ± SD)µg/100 g of honey

CE instrument seven times (n=7).
The quantitative results are presented in Table 3. Using the described CE method, another five varieties of honey were analyzed. The electropherograms obtained with these extracts of honey can be observed in Figure 3. It can be seen that these honeys have a polyphenolic fraction as complex as rosemary honey, so the problems for identification are the

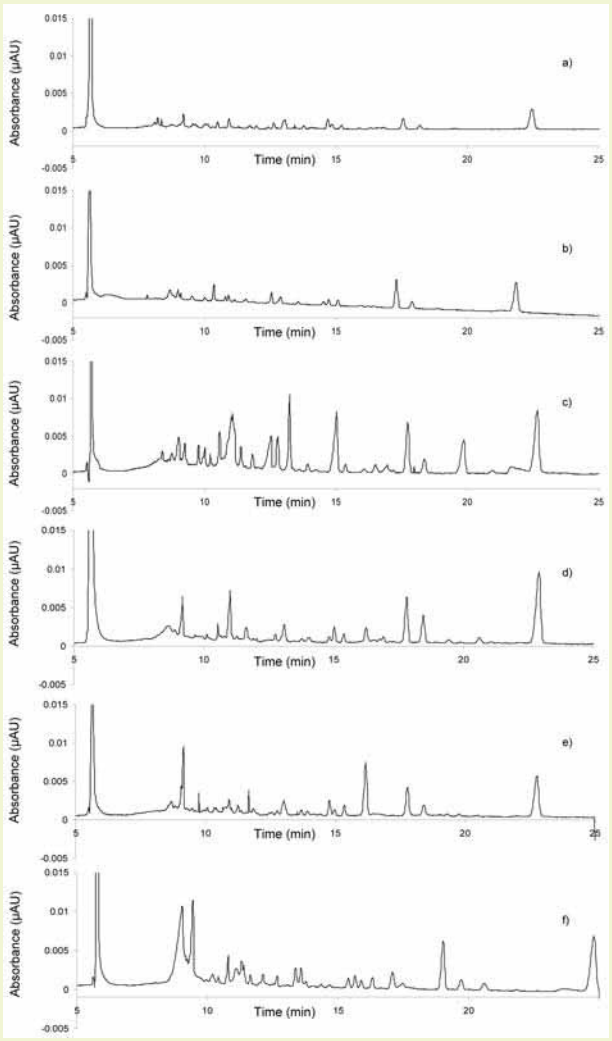


Figure 3. Electropherogram of phenolic fraction extracted from rosemary honey samples from different floral sources. (a) Citrus honey, (b) rosemary honey, (c) chestnut-tree honey, (d) thyme honey, (e) multifloral honey and (f) mountain range honey. Detection was performed at 280 nm

same.

CONCLUSION

In conclusion the present work reports a qualitative and quantitative capillary electrophoresis method of honey phenolic compounds. Six different kinds of honey has been analyzed to compare their polyphenolic profiles. This study has provided a view about the problems of carrying out the identification of polyphenols employing UV-vis detection in complex polyphenolic samples as honey. This fact makes

difficult the qualitative and quantitative analysis of these compounds in honey and, in order to achieve a good identification a more reliable detection system such as a mass spectrometer should be employed.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the contract C019 of the Consejería de Agricultura y Pesca of the Junta de Andalucía. They also gratefully acknowledge the financial support from the Ministerio de Educación y Ciencia for the Project CTQ2005-01914/BQU and for the grant AP-2004-5488.

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BUSINESS

LANGMEAD & SENSITECH
Langmead specializes in the production of conventional and organic greens for the retail, catering and food processing industry. Being in control of their cold chain forms a critical part of Langmead's overall supply chain, and for this reason, the company has chosen to work with **Sensitech**.
The company report that with today's customers demanding superior quality produce throughout the year, it is a critical advantage for the retailer if they can meet such demand. On the supplier side, it is equally important to deliver consistent product quality throughout the year, and thus cold chain monitoring provides an additional benefit - brand protection. For these reasons, Langmead closely monitors its salad products with Sensitech's TempTale® temperature recorders, to ensure that their products reach the retailer in optimum condition.
www.sensitech.nl
www.langmeadfarms.co.uk

COGNIS & BRINGWELL
Global nutritional ingredients and speciality chemicals company, **Cognis**, and **Bringwell International AB**, announced that they have agreed to sign an exclusive Trademark Sub-licence Agreement for Tonalin® CLA, when sold as dietary supplements through health food stores in Norway and Sweden.
Bringwell International AB is a leading company with patented own-developed and in-licensed brands and ingredients. In Norway, Bringwell is represented by Medtech Pharma Norge, the leading company in health food supplements in that country.
www.cognis.com

NATUREX'S BUSINESS
Over 2005 as a whole, **Naturex** generated sales of EUR 50.15 million, an increase of 44.1 percent, including the 6-month consolidation of Pure World. At constant USD exchange rates, growth comes out at 45.7 percent. In proforma terms, sales increased by 82.2 percent and reached EUR 63.40 million of which 71 percent was generated in the USA and Canada. Nutraceuticals now account for 61 percent of activity and food ingredients for 33 percent. In 2005, Naturex achieved further high returns despite making a major acquisition.
www.naturex.com

FRUTAROM…IN KOREA
Frutarom is investing in clinical trials in South Korea.
Othmar Schmidiger, manager of the Israeli firm's Korean operations, reported that the company wanted to be able to offer extracts that are supported by science and can be promoted using health claims.
The firm's green mate was tested in trials last year and is currently going through the regulator's approval process. It has been shown to have a positive effect on the body's fat metabolism.
Schmidiger reports: "With this we will be able to introduce finished product concepts, like slimming foods and beverages that mention weight loss on the package […] Frutarom's olive leaf extract, already tested in trials in Germany, is also likely to be studied in Korea with the aim of being added to the KFDA's list of approved functional foods".
www.frutarom.com

NOVOZYMES IS GROWING
Steen Riisgaard, president and CEO of **Novozymes**, said: "Novozymes has made a good start to 2006" and continued, "We achieved pleasing top-line growth of 11 percent, positively affected by exchange rates. And underlying sales are also showing a higher rate of growth than in 2005 […] In addition, the first quarter has once again demonstrated the company's ability to continuously optimise production and therefore increase profitability, despite rising energy and raw material prices. Sales are developing as expected, and the outlook for growth in both sales and in earnings remains unchanged".
For 2006 growth in sales is expected at 7 to 9 percent.
www.novozymes.com

AWARD

INTERNET ADVERTISING COMPETITION
Fortitech, Inc.'s advertising campaign, "The Ultimate Fortification Destination", has won an Internet Advertising Competition (IAC) award from the Web Marketing Association based in Boston, Mass.
The global campaign was designed by the Albany, N.Y. based marketing communications agency, Media Logic and programmed by Arcadam.
The award is listed as "The Best Food Industry Online Campaign". The promotion launched in early 2005 and was compared with virtually all other corporate Web sites within the food industry and medium.
Maria Battista, director of marketing at Fortitech, explained: "The key to the campaign was to offer something of value to users to incent them to click through and register on our Web site […] By offering this added value, we successfully managed to drive a significant number of decision-makers and potential customers to register".
The "Ultimate Fortification Destination" campaign was designed to be a comprehensive effort to promote interest and understanding of the vast nutritional resources available on the newly redesigned Web site.
The award-winning campaign featured traditional full-page advertisements, email blasts, hot-linked web banners, buttons and skyscrapers on various Web sites and E-newsletters worldwide serving the food and nutrition industry.
www.fortitech.com

A pesar de la capacidad de resolución de la electroforesis capilar, en este trabajo se pone de manifiesto que debido a la complejidad de la matriz estudiada junto con la universalidad y baja selectividad y sensibilidad de la detección UV-Vis, la identificación de los compuestos polifenólicos se convierte en una tarea difícil. En muchos casos es imposible discernir entre los analitos ya que su similitud de estructuras hace que migren a tiempos muy parecidos.

El estudio de la fracción polifenólica se efectuó en miel de romero sobre la que se doparon individualmente 22 patrones comerciales que previamente habían sido descritos como presentes en miel. De todos ellos, sólo se pudieron identificar con cierta seguridad crisina, pinocembrina, kaempferol, ácido ferúlico y ácido p-cumárico; aunque crisina y pinocembrina aparecían como un pico solapado, por lo que la cuantificación sólo se hizo para el kaempferol y los ácidos ferúlico y p-cumárico. Estos resultados se aplicaron sobre otras cinco variedades de miel: de tomillo, de castaño, de la sierra, de azahar y de mil flores, obteniéndose perfiles muy similares y donde la identificación presentaba la misma problemática que en la miel de romero.

Este trabajo permitió concluir que, para el análisis de compuestos fenólicos en muestras tan complejas como la miel, no es suficiente con el empleo de un detector UV-Vis sino que es necesario el uso de sistemas de detección mucho más potentes como es la espectrometría de masas.

Capítulo 8

Identificación de los compuestos fenólicos en miel de romero mediante CE-ESI-MS (IT).

Publicación incluída en este capítulo:

Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis–electrospray ionization-mass spectrometry.
(*Journal of Pharmaceutical and Biomedical Analysis* **2006**, 41, 1648–1656)

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Departamento de Química Analítica de la Universidad de Granada.

A causa de la problemática para caracterizar el perfil fenólico de la miel mediante UV-Vis expuesta en el capítulo anterior, se decidió desarrollar una nueva metodología de análisis por electroforesis capilar pero esta vez acoplada a un detector de masas (ESI-MS (IT)). Así, de este modo, se combinaban la rapidez y la eficiencia de la electroforesis capilar con una herramienta selectiva y capaz de dar información estructural de los compuestos como es la espectrometría de masas. Con este detector es posible comparar tanto los tiempos de migración de patrones y analitos de la muestra, como las masas de éstos confirmando de manera más fiable la identidad de los compuestos en cuestión.

El protocolo seguido en el desarrollo de este trabajo puede resumirse en los siguientes pasos:

1. Preparación de la muestra. Se llevó a cabo siguiendo el mismo proceso de extracción que el capítulo anterior. De esta forma los resultados de esta metodología podían ser comparados con los anteriores.

2. Optimización del método electroforético de modo que éste fuese compatible con el espectrómetro de masas. Los parámetros óptimos fueron: 100 mM de acetato amónico a pH 10 y 10% de 2-propanol, inyección hidrodinámica de 20 s a 0.5 psi, voltaje de 25 kV, capilar de sílice fundida de 50 μm de diámetro interno y 100 cm de longitud total.

3. Optimización de los parámetros de la fuente de ionización (ESI): líquido adicional compuesto por 2-propanol/agua 60:40 (v/v) y 0.1% (v/v) de trietilamina, velocidad de flujo de 3 μL/min, velocidad de flujo del gas de secado 7 L/min, temperatura de 350º C, presión del gas de nebulización de 6 psi y estabilidad de compuesto de 25%.

4. Identificación de los compuestos pertenecientes a la fracción fenólica de la miel empleando la información proporcionada por el espectrómetro de masas.

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Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis–electrospray ionization-mass spectrometry

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Abstract

Complex extracts of rosemary honey constituents often require very effective separation techniques to allow the identification of different compounds. Capillary electrophoresis (CE) coupled to mass spectrometry (MS) detection can provide structure-selective information about the analytes in such matrices and has turned out to be an attractive alternative to HPLC methods. A simple and cost-effective analytical method involving solid-phase extraction (SPE) and capillary zone electrophoresis coupled to electrospray ionization-ion trap mass spectrometry (CZE-ESI-MS) to identify and characterize phenolic compounds in rosemary honey is described. The SPE, CE and ESI-MS parameters were optimized in order to maximize the number of phenolic compounds detected and the sensitivity of their determination. All CE–ESI-MS experiments were performed with uncoated fused-silica capillaries and an alkaline volatile buffer system consisting of 100 mM NH_4Oac with 10% of 2-propanol at pH 10. Since sheath liquids can made significant effects on the sensitivity in typical CE–ESI-MS application, the effect of type and flow rate of the sheath liquid on the sensitivity of phenolic compounds were investigated. As result, the best sensitivity was obtained with a sheath liquid containing 2-propanol/water 60:40 (v/v) and 0.1% (v/v) of triethylamine at 3 μL/min in the negative ion mode. We describe the first method for the analysis of phenolic compounds in rosemary honey at mg/L levels by using a simple SPE before CE–ESI-MS analysis.

Keywords: Capillary electrophoresis; Electrospray ionisation; Mass spectrometry; Phenolic compounds; Rosemary honey

1. Introduction

In the past several years, there has been increasing evidence of the antioxidant capacity of honey. Honey can prevent deteriorative oxidation reactions in foods, such as lipids oxidation in meat [1,2] and enzymatic browning of fruits and vegetables [3,4]. Honey has therefore great potential to serve as a natural food antioxidant. In a previous study, it was demonstrated that honey is similar in antioxidant capacity to many fruits and vegetables on a fresh weight basis, as measured by the oxygen radical absorbance capacity assay [5]. The antioxidant activity of honey, however, varies greatly depending on the honey floral source [5,6]. There is a lack of knowledge about the profiles of antioxidant substances in honey from various floral sources. The variation in these profiles might be responsible for the widely varying abilities of honeys to protect against oxidative reactions [7].

Honey is a remarkably complex natural liquid that is reported to contain at least 181 substances [8]. The composition of honey is rather variable and primarily depends on the floral source; however, certain external factors also play a role, such as seasonal and environmental factors and processing. Honey is a supersaturated solution of sugars and a wide range of minor constituents is also present in honey, many of which are known to have antioxidant properties [7]. The antioxidant activity of phenolic compounds might significantly contribute to the human health benefits of plant foods [9,10] and beverages such as red wine and tea [10–12]. Honey contains a great number of phenolic

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compounds, which are generally acknowledged to be of considerable importance because of its chemoprotective effect in human beings.

Rosemary honey is produced from *Rosmarinus officinalis* (Lamiaceae). This honey has a very good consumer acceptance and commercial value in European countries because of its mild flavor and light color [13].

Even though the characterization of phenolic compounds from honey has been succesfully carried out using GC [14,15] and HPLC [16–19], CE [20–22] 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, so the technique has emerged as a good alternative in pharmaceutical, forensic and food research laboratories.

The hyphenation of CE to MS combines the high speed and efficiency of CE with the selectivity and sensitivity inherent to MS. 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 [23]. In general, if a separation technique is coupled with MS the interpretation of the analytical results can be more straightforward [24–26]. In this sense, ESI has emerged as a highly useful technique which allows direct coupling with electrophoretic separation techniques [27]. Furthermore, collisionally induced dissociation can be used to obtain fragment ions of structural relevance for identifying target compounds in a highly complex matrix.

The aim of the present work has been to develop the first simple SPE-CE–ESI-MS method for the identification and characterization of phenolic compounds in rosemary honey samples.

2. Experimental

2.1. Chemical and samples

Rosemary honey samples were collected from a commercial centre market (Apisol, S.A. Montroy (Valencia)).

All chemicals were of analytical reagent grade and used as received. Ammonium acetate was from Panreac (Barcelona, Spain) and ammonia from Merck (Darmstadt, Germany) were used to prepare CE running buffers at different concentrations and pH values. Buffers were prepared 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 from Aldrich (Steinheim, Germany), sodium hydroxide, 2-propanol used in the sheath flow and on the buffer, methanol used on the buffer and in the extraction procedure and diethyl ether all HPLC grade were obtained from Panreac (Barcelona, Spain). All solutions were filtered through a 0.45 μm Millipore (Bedford, MA, USA) membrane filters before injection into the capillary.

2.2. CE–ESI-MS

The analyses were made in 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. Bare fused-silica capillary of 50 μm i.d. came from Beckman Coulter Inc. (Fullerton, CA, USA). A detection window was created at 10 cm for the UV detector and 100 cm was the total length (corresponding to the MS detection length).

Before first use, the bare capillaries were conditioned with 0.1 M sodium hydroxide during 20 min followed by a water rinse for other 10 min. At the end of the day the capillary was flushed with water for 10 min and air for 5 min.

Capillary conditioning of the columns was done by flushing for 2 min sodium hydroxide, 4 min with water, and then for 10 min with the separation buffer (during all the capillary conditioning was used a pressure of 20 psi).

The instrument was controlled by a PC running the 32 Karat System software from Beckman.

MS experiments were performed on a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker Daltonik GmgH, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Electrical contact at the electrospray needle tip was established via a sheath liquid by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA). For the connection between the CE system and the electrospray ion source of the mass spectrometer, the outlet of the separation capillary was fitted into the electrospray needle of the ion source and a flow of conductive sheath liquid established the electrical contact between capillary effluent and water for electrospray needle. The mass spectrometer was run in the negative ion mode and the capillary voltage was set at 4000 V. The ion trap scanned at 50–650 *m/z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap 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 Results and Discussion. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonics.

2.3. Solid-phase extraction procedure

Extraction was performed according to methods described previously [28–30] with some modifications. About 30 g of rosemary honey samples were thoroughly mixed with five parts (150 mL) of distilled water, adjusted to pH 2 with concentrated HCL, until completely fluid by stirring with a magnetic stirrer at room temperature. The fluid samples were then filtered through cotton wool to remove solid particles. The filtrate was mixed with 40 g Amberlite XAD-2 (pore size 9 nm, particles size 0.3–1.2 mm) and stirred in a magnetic stirrer for 10 min, which was considered enough to adsorb honey phenolics with a recovery rate more than 80% [28,31]. The Amberlite particles were then packed in a glass column (42 cm × 3.2 cm) and the column

was washed with acidified water (pH 2 with HCl, 100 mL) and subsequently rinsed with distilled water (300 mL) to remove all sugars and other polar constituents of honey. The phenolic compounds remained adsorbed on the column [32] and were eluted with methanol (300 mL). The methanolic extract was concentrated to dryness under reduced pressure in a rotary evaporator at 50 °C. The residue was resuspended in distilled water (5 mL) and extracted with diethyl ether (5 mL × 3). The diethyl ether extracts were combined and the ether was removed to dryness under reduced pressure in a rotavapory evaporator at 30 °C. The dried residue was then redisolved in 0.5 mL of methanol, filtered through a 0.45 μm membrane filter. Finally distilled water was adding to the extract until obtaining a 50:50 methanol:water solution and analysed by CE–ESI-MS.

3. Results and discussion

3.1. Development of CE–ESI-MS method

The methanol–water extracts of rosemary honey were obtained as described in the Section 2.3. Extracts were used to optimize the electrophoretic and MS conditions.

Initially, the electrophoretic conditions were optimized according to the following criteria: migration behaviour, sensitivity, analysis time and peak shape. First, buffers containing different concentrations of ammonium acetate at basic 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 in ESI. Amonium acetate concentration was varied from 20 to 120 mM (in steps of 20) in an attempt to improve the resolution and minimize the analysis time; pH values from 8.5 to 10.5 (in steps of 0.5) and concentrations of methanol or 2-propanol from 0 to 10% (in steps of 5%) were assayed to obtain the best peak shape, resolution and efficiency among the phenolic compounds. Finally, the addition of 2-propanol as organic modifier raised the best resolution (Fig. 1). The best parameters turned out to be 100 mM ammonium acetate at pH 10 and 10% 2-propanol. The voltage 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 a N_2 pressure of 0.5 psi for 20 s (1 psi = 6894.76 Pa). These conditions were chosen for the subsequent optimization of the ESI parameters. During buffer optimization we used the best 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 triethylamine at a flow rate of 3 μL/min, a drying gas flow rate of 7 L/min at 350 °C, nebulizer gas pressure of 6 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

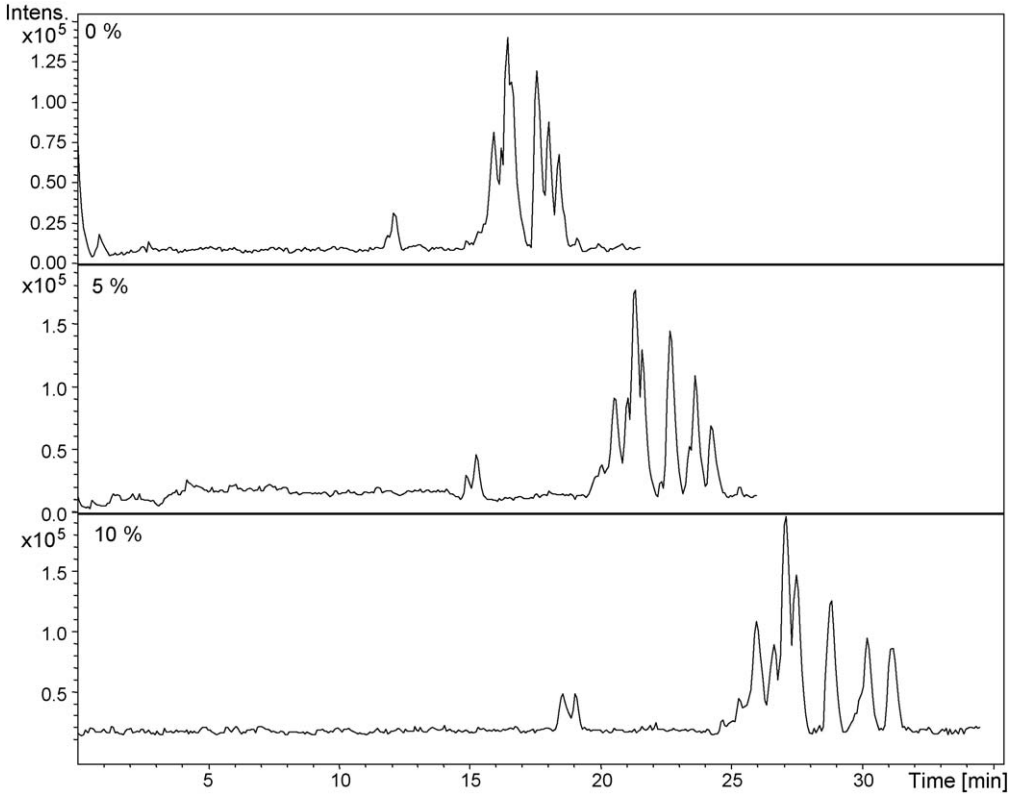

Fig. 1. Optimization of percentages of 2-propanol added to the running buffer. Initial conditions: 50 μm i.d. fused silica capillary, 100 cm total length; buffer: 100 mM ammonium acetate pH 10; voltage: 20 kV; injection time: 20 s at 0.5 psi; sheath liquid: 2-propanol/water 50:50 (v/v) at flow rate 0.20 mL/h; drying gas: 5 L/min; temperature: 300 °C; nebulizing gas pressure: 4 psi. MS analyses were carried out using negative polarity. Compound stability: 100%. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

compounds extracted from rosemary honey 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 [33,34]. Generally, small amounts of volatile triethylamine (TEA) or ammonium hydroxide can be used for ESI-negative detection [35].

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. For the optimization of the ESI parameters was used the signals corresponding to the high peak, because this family has the same behaviour in this optimization.

Initially we tested 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 without 0.1 and 0.2% (v/v) TEA. Using 80:20 sheath liquid with and without TEA, the current broke down after 10 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. However, the use of a sheath liquid of 60:40 (v/v) 2-propanol/water plus 0.1% (v/v) TEA provided higher current stability and MS signal. Therefore, 60:40 (v/v) 2-propanol/water with 0.1% (v/v) TEA was selected as sheath liquid.

We then optimized the other ESI-MS parameters, drying gas temperature and flow, nebulizing gas pressure, compound stability and sheath liquid flow (Fig. 2A–E) using the height of the MS signal. Initially the value for each parameter was the best found in the preliminary studies; after re-optimizing each parameter we then used the new value to complete the optimization of the other parameters.

As can be seen, a temperature of 350 °C (Fig. 2A), drying gas flow at 7 L/min (Fig. 2B), and nebulizer gas pressure at 6 psi (Fig. 2C) provided the best signals.

It can also be seen in Fig. 2D that compound stability plays an important role in detecting rosemary honey 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

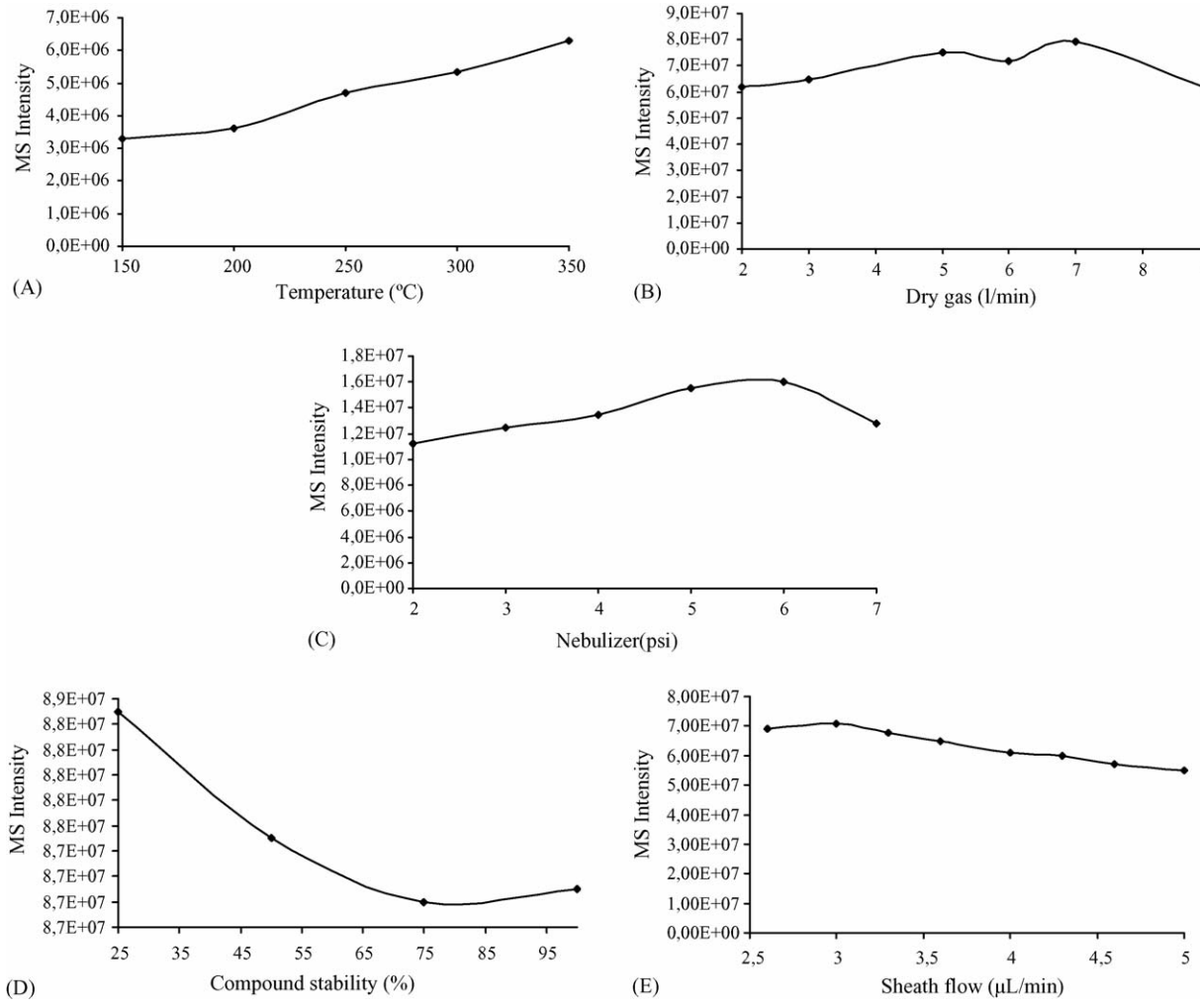

Fig. 2. Optimization of ESI-MS parameters. Conditions: buffer: 100 mM ammonium acetate and 10% 2-propanol at pH 10; voltage: 25 kV; injection time: 20 s at 0.5 psi. MS analyses were carried out using negative polarity. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

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 the voltage applied by the MS instrument and, therefore, the higher the solute fragmentation that can take place at that point. We chosen 25% as the best value.

The best sheath-liquid flow was one of 3 μL/min (Fig. 2E). This effect has also been mentioned in the literature [36]; 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. 3 were obtained for methanol–water extracts of rosemary honey. The prolonged analysis time is a consequence of the long capillary lengths that are needed to couple a CE instrument to the MS. This is not a problem as such, but counteracts one of the main advantages of CE, namely its speed.

The repeatability of the CE–ESI-MS analysis, expressed by the RSD of five consecutive injections was 0.89% for the analysis time and 2.8% for the high peak area, adequate for the goal of the present work.

3.2. Characterization of rosemary honey phenolic compounds by CE–ESI-MS

The potential of the CE–ESI-MS method was checked by characterizing the SPE extracts obtained from a rosemary honey. In the first time a qualitative analysis is demonstrated in order to identify phenolic compounds in rosemary honeys. The extracts of rosemary honey were analyzed according to the procedure described in the Section 3.1. When honey extracts were analyzed, some coexistent substances tended to affect on the inner surface of capillary, which would decrease the electro-osmotic flow (EOF) and the peak height gradually. In order to improve the reproducibility of this method, when the capillary was used for analysis of honey samples, it was flushed sequentially with 0.2 M sodium hydroxide for 1 min, water for 3 min, and finally equilibrated with background electrolyte solution for 8 min before each injection.

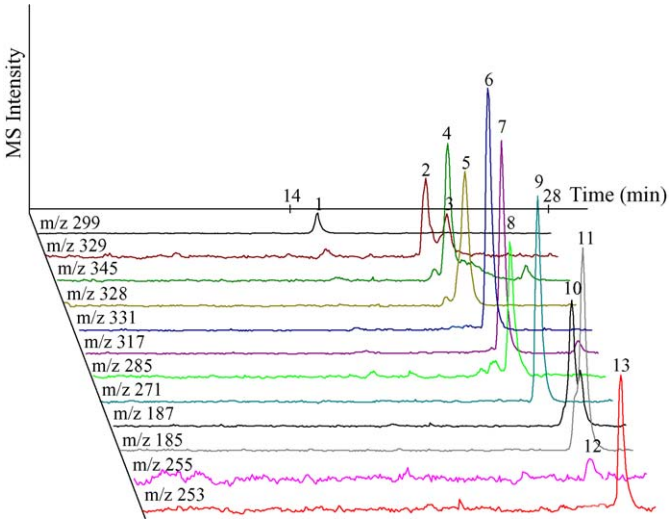

Fig. 4. Extracted ion electropherogram of the compound detected. (1) Kaempferid, (2) Quercetin 3′,3′-dimethyl eter, (3) Quercetin 7,3′-dimethyl eter, (4) N.I., (5) N.I., (6) Monogalloyl, (7) Myricetin, (8) Kaempferol, (9) Pinobanksin, (10) N.I., (11) N.I., (12) Pinocembrin, (13) Chrysin. All conditions as in Fig. 3. N.I: not identified.

The peaks of the main phenolic compounds of rosemary honey were identified by comparing both migration time and MS data obtained from rosemary honey samples with standards.

Fig. 4 shows the extracted ion electropherograms of several compounds detected in the methanol-water extract of a rosemary honey and the Fig. 5A and B the mass spectra of the identified compounds; the following compounds were identified: (1) Kaempferid ($[M-H]^-$ 299 *m/z*), (2) Quercetin 3′,3′-dimethyl eter ($[M-H]^-$ 329 *m/z*), (3) Quercetin 7,3′-dimethyl eter ($[M-H]^-$ 329 *m/z*), (6) Monogalloyl-glucose ($[M-H]^-$ 331 *m/z*), (7) Myricetin ($[M-H]^-$ 317 *m/z*), (8) Kaempferol ($[M-H]^-$ 285 *m/z*), (9) Pinobanksin ($[M-H]^-$ 271 *m/z*), (12) Pinocembrin ($[M-H]^-$ 255 *m/z*), (13) Chrysin

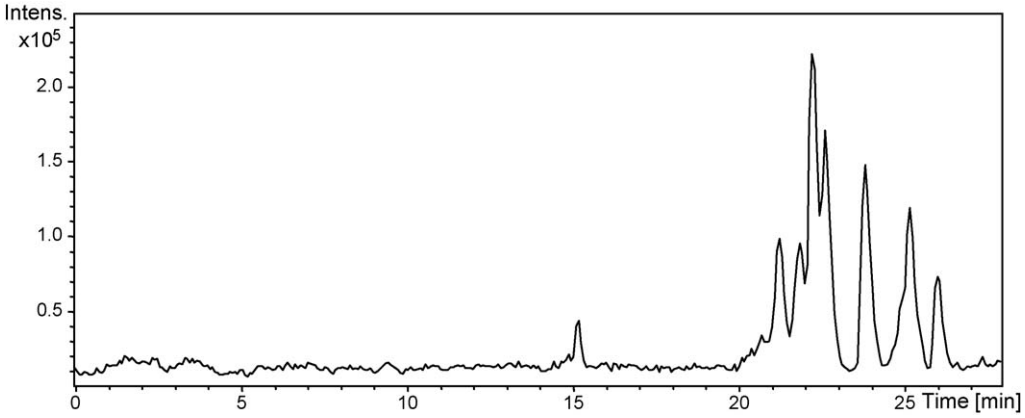

Fig. 3. Base peak electropherogram of rosemary honey sample using the optimal conditions. CE-MS conditions: buffer: 100 mM ammonium acetate 10% 2-propanol at pH 10; voltage: 25 kV; injection time: 20 s at 0.5 psi; sheath liquid: 2-propanol/water 60:40 (v/v) containing 0.1% (v/v) triethylamine; flow rate: 0.18 mL/h; drying gas: 7 L/min, 350 °C; nebulizing gas pressure: 6 psi. MS analyses were carried out using negative polarity. Compound stability: 25%. MS scan 50–650 *m/z* (target mass 350 *m/z*). Sample: rosemary honey extract.

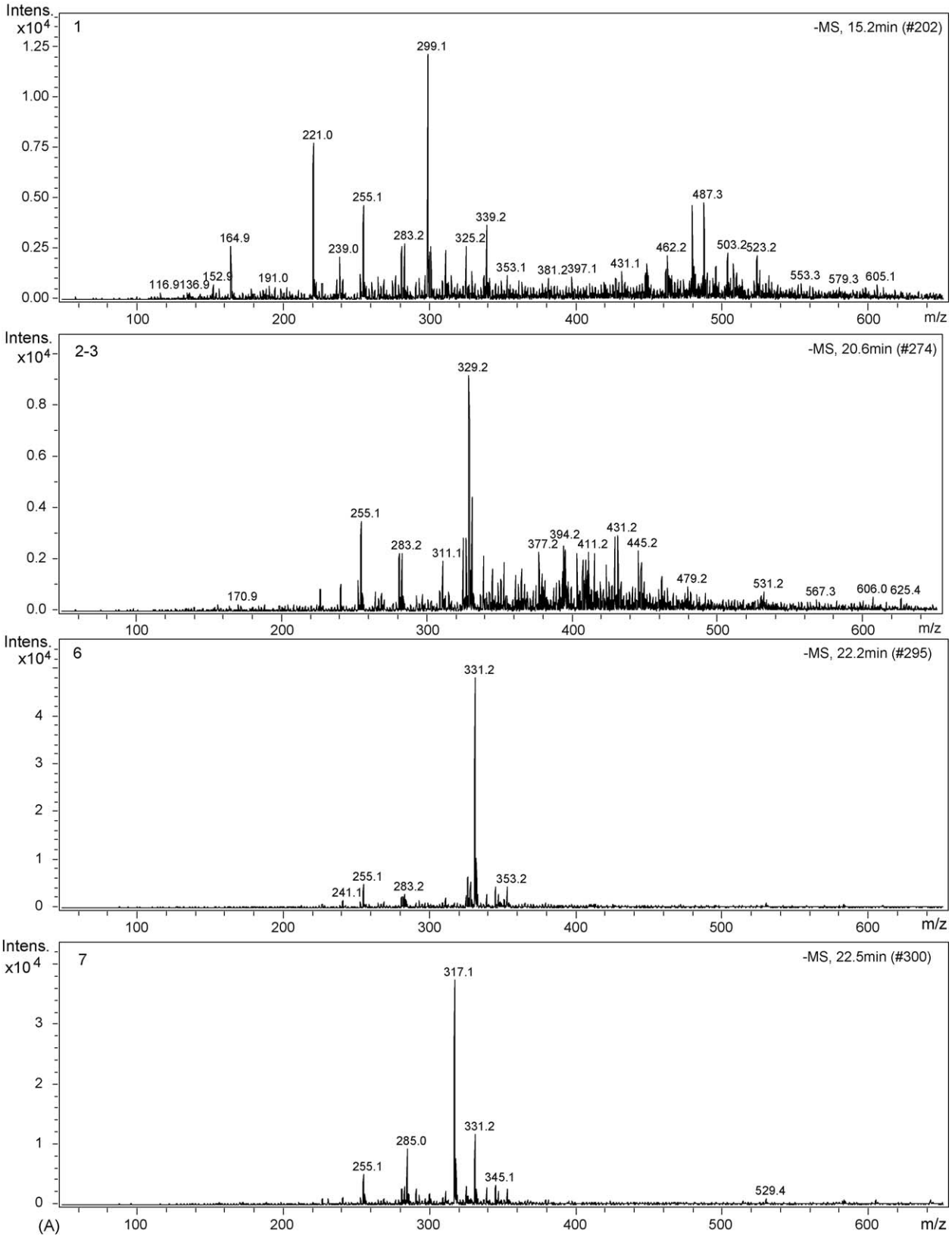

Fig. 5. (A and B) MS spectra of the identified peaks in a methanol–water rosemary honey extract. All conditions as in Fig. 3.

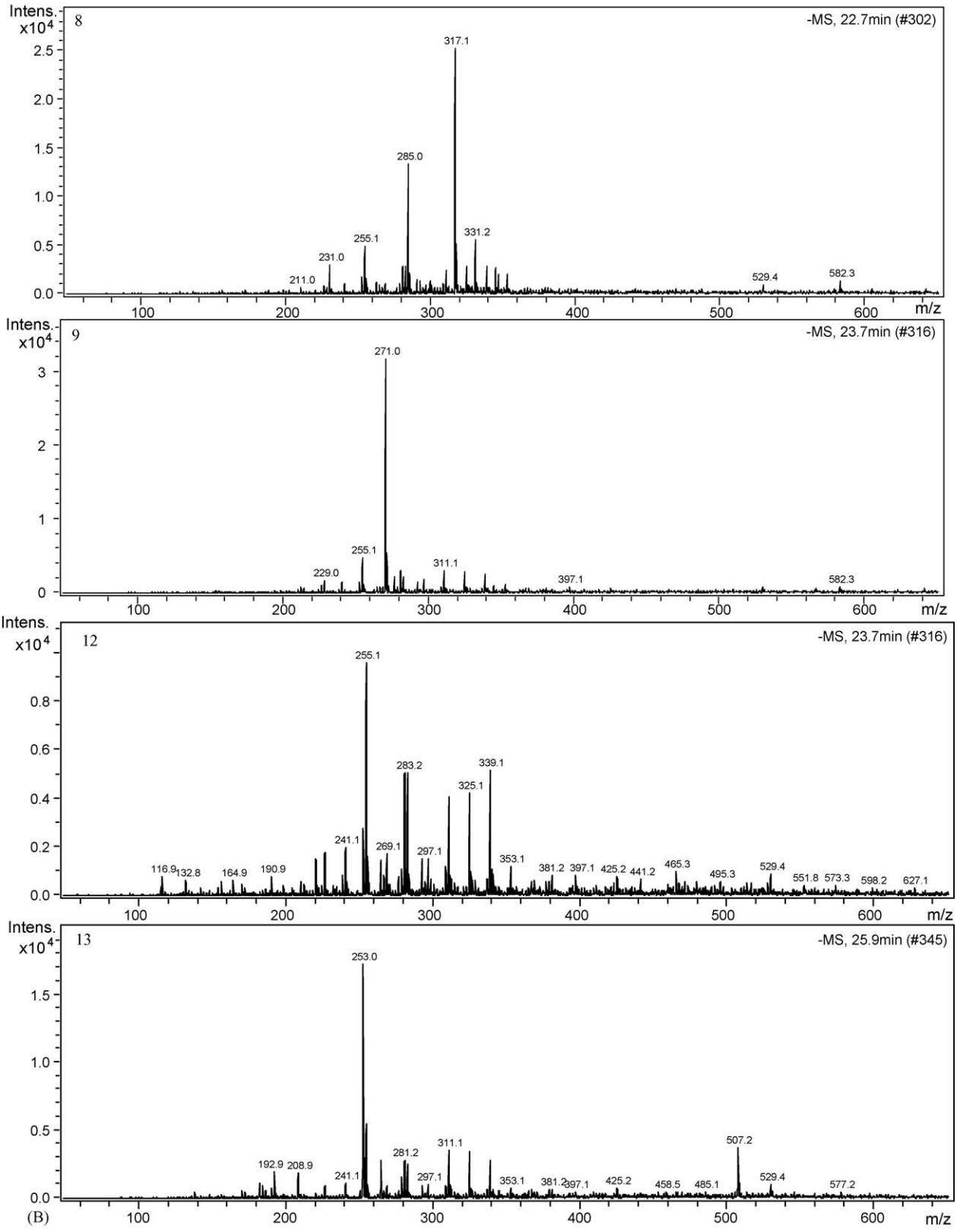

Fig. 5. (*Continued*).

($[M-H]^-$ 253 *m/z*). The compounds (1), (2), (3), (7), (8) are flavonols, (6) is a tannin compound, (9), (12) are flavanones and (13) is a flavone compound.

This 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 firstly, for the concentration of several of the compounds under study in the sample of rosemary honey and secondly, because of carrying out the detection in negative mode.

In any case, detection limits were calculated for several polyphenols and the values were around 1–25 mg/L with a RSD between 3 and 6% in all cases at these concentrations.

As has been reported in previous papers, the use of phenolic compound analysis generally using HPLC was used in the identification of honeys [37] and has been used as a tool for studying the floral and geographical origins of honeys. In these studies the authenticity of the floral origin of honey has been demonstrated [29,30]. The flavonol, Kaempferol ($[M-H]^-$ 285 *m/z*) has been used as marker for rosemary honey [38]. In addition Pinocembrin, Pinobanksin (flavanones) and Chrysin (flavone) ($[M-H]^-$ 255, 271, 253 *m/z*, respectively) are the characteristic flavonoids of propolis, and these flavonoid compounds have been found in most European honey samples [39].

In our research the extract ion electropherogram shows two peaks which correspond with $[M-H]^-$ 329 *m/z*. These peaks correspond at two compounds described by Tomás-Barberán and co-workers [40]. They claimed that the first peak $[M-H]^-$ 329 *m/z*, corresponds to Quercetin 3′,3′-dimethyl eter and the second one corresponds to Quercetin 7,3′-dimethyl eter. In addition, a third form (Quercetin 3,7-dimethyl eter) exits but is barely detected by UV detection. We could confirm that the third form was non-detected using CE–ESI-MS because the sheath liquid has a significant effect on the sensitivity (diluted of sample) of the compounds. These results confirm the developed study.

The other four compounds $[M-H]^-$ 345, 328, 185 and 187 *m/z* could not be identified, although some possible structures come out after carrying out the MS–MS experiments. A clearer assignment of these compounds is now being carried out in our lab.

4. Conclusions

In conclusion the present work describes the first qualitative SPE-CE–ESI-MS method to study phenolic compounds, in rosemary honey samples after their extraction by SPE. Different parameters were optimized and a successful CE–ESI-MS separation was obtained by using a running buffer consisting of 100 mM ammonium acetate and 10% of 2-propanol at pH 10. The sheath liquid used in the ESI-MS interface was a mixture of isopropanol/water 60:40 (v/v) in presence of 0.1% TEA obtaining a good repeatability of the method studied and a relative standard deviations (RSDs) of peak areas/migration time were 2.8 and 0.89%. Under these conditions, several phenolic compounds were identified in rosemary honey at mg/L levels. The compounds were identified using the electrophoretic results, the molecular weight and the structural information of MS obtained using CE.

In comparison to the chromatographic methods, the proposed method is a good alternative for simultaneous analysis of phenolic components in rosemary honey due to this technique provides fast and efficient separations in this type of analysis and used reduced sample and solvents consumption. Also, the hyphenation of CE to MS combines the advantages of CE with the selectivity and sensitivity inherent to MS.

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La principal novedad de este capítulo 8 se encuentra en que era la primera vez que se empleaba la espectrometría de masas acoplada a electroforesis capilar para la determinación de la fracción fenólica de la miel.

Como puede observarse, los tiempos de análisis conseguidos eran del orden de los 25 min, muy similares a los obtenidos en el capítulo anterior, aunque considerablemente menores que los obtenidos mediante HPLC (alrededor de los 60 min), por lo que desde este punto de vista la electroforesis capilar supone una ventaja frente a HPLC. Por otro lado, esta nueva metodología EC-MS permitió detectar con claridad 13 compuestos, de los cuales 9 fueron identificados, frente a los 5 analitos identificados mediante CE-UV-Vis.

Bloque III: Nuez

El nogal se considera como uno de los árboles de frutos comestibles más antiguo conocidos por el hombre. Las referencias históricas lo remontan al año 7000 a.C. en Persia [242], aunque no fueron introducidos en Europa y América hasta el siglo XVI y XVII respectivamente.

La nuez es la semilla del nogal, pertenecen a la familia Juglandaceae, al género *Juglans*, siendo *Juglans regia* L. la conocida como nuez común.

Su importancia en la dieta radica en que posee un alto valor nutritivo, y además influye a nivel médico, ya que su composición (basada sobre todo en ácidos grasos poliinsaturados o PUFAs) actúa a nivel bioquímico y fisiológico en el organismo [243].

Las nueces son únicas dentro de los frutos secos debido a su alto contenido en PUFAs, sobre todo ácido linoleico y α-linolénico. La relación entre estos ácidos grasos también es única, linoleico/α-linolénico de 4/1, ya que ha demostrado disminuir el riesgo de enfermedades cardiovasculares debido a la reducción de la concentración de colesterol en sangre [244].

La energía y composición que contienen 100 g de la porción comestible de la nuez es la siguiente: energía (654 kcal); grasas (65.2 g); proteínas (15.2 g); fibra (6.7 g); fósforo (346 mg); potasio (441 mg); folatos (98 μg); y vitamina E (2.9 mg). El contenido lipídico consiste en SFAs (6.1 g), predominantemente ácidos palmítico (4.4 g) y esteárico (1.7 g); ácidos grasos monoinsaturados o MUFAs (8.9 g) de los cuales el ácido oleico integra casi el total; y PUFAs (47.2 g), predominantemente ácidos linoleico (38.1 g) y α-linolénico (9.1 g). Además contiene 72 mg de fitosteroles. La composición en aminoácidos es más alta en ácido glutámico (2.8 g), arginina (2.3 g), ácido aspártico (1.8 g), leucina (1.2 g), serina (0.93 g), glicina (0.82 g), valina (0.75 g), fenilalanina (0.71 g), alanina (0.7 g), prolina (0.7 g), isoleucina (0.62 g), y treonina (0.6 g) [245]. Otros compuestos minoritarios como los compuestos fenólicos tienen también una importancia destacada en las nueces, siendo su concentración de >1500 mg de

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equivalentes de ácido gálico/100 g [246] . Este valor representa más del doble de la concentración de compuestos fenólicos encontrada en otro tipo de frutos secos, y es superior al de otros alimentos considerados como ricos en polifenoles. Se puede afirmar que un puñado de nueces contiene más compuestos fenólicos que un vaso de zumo de manzana (117 mg en 240 mL) , una barra de chocolate (205 mg en 43 g de chocolate) , e incluso que un vaso de vino (372 mg en 150 mL) [247].

Los compuestos fenólicos de la nuez gozan de gran interés ya que han demostrado poseer diversas propiedades beneficiosas. Uno de los compuestos más importantes de la nuez, la juglona, se conoce por su actividad antimicrobiana y por su capacidad para disminuir la incidencia de tumores en el intestino de las ratas [248]. Por otra parte, Kris-Etherton *y col.* [249], argumentaron que el ácido elágico y los flavonoides presentes en nuez, tienen el efecto potencial de modular el colesterol del suero, confiriéndosele a un determinado grupo de flavonoides efectos cardioprotectores. Otros autores [250] también han demostrado que la catequina inhibe la oxidación del colesterol LDL y protege a las células linfáticas contra los efectos citotóxicos provocados por el LDL oxidado.

No sólo la parte comestible del fruto de la nuez es rica en compuestos fenólicos, sino que otras zonas como la piel, la cáscara, e incluso la corteza y hojas del nogal son ricas en fenoles [251].

Las nueces con cáscara contienen 16 g/kg de fenoles totales expresados como equivalentes de ácido gálico.

La mayoría de los antioxidantes, entre los que se encuentran los compuestos fenólicos, se disponen en la piel que recubre la parte comestible del fruto, quedando menos de un 10% de éstos cuando se elimina esta piel. La cáscara de la nuez es un medio de

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protección contra la oxidación, por lo que las nueces conservadas con su cáscara tendrán una mayor vida media y su contenido en antioxidantes será mayor que en aquellos casos en los que la cáscara se elimina [252].

La piel de la nuez ha demostrado ser rica en ácido elágico, metilgalato, ácido gálico y elagitaninos [253]. Análisis del fruto han llevado a la identificación de numerosos ácidos fenólicos en el fruto [254]: p-hidroxibenzoico, vanílico, gentísico, protocatecuico, siríngico, p-cumárico, gálico, ferúlico, cafeico y sinápico. Sin embargo, las formas libres de los ácidos fenólicos son minoritarias en la nuez, predominan las formas esterificadas y glicosiladas comprendiendo el 76% del total de los ácidos fenólicos presentes en nuez.

Se han desarrollado numerosos trabajos con el fin de caracterizar los compuestos fenólicos en nuez, por ejemplo Colaric *y col.* [255] identificaron ácido clorogénico, cafeico, p-cumárico, ferúlico, sinápico, ácido elágico, siríngico, siringaldehido, y juglona en el fruto y piel de la nuez. Otros estudios más recientes [256,257] han detectado en la semilla de la nuez taninos hidrolizables, glasreginina A-C, junto con adenosina y arginina.

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Capítulo 9

Desarrollo de un método rápido para la determinación de compuestos fenólicos y otros compuestos polares en nuez mediante CE-ESI-MS (TOF).

Publicación incluída en este capítulo:

Development of a rapid method to determine phenolic and other polar compounds in walnut by capillary electrophoresis–electrospray ionization time-of-flight mass spectrometry.

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La presencia de compuestos fenólicos desempeña un papel importante en otras matrices alimentarias como son los frutos secos, contribuyendo positivamente a la capacidad antioxidante de estos alimentos. De entre todos los frutos que componen este grupo, la nuez ha demostrado ser la más rica como fuente de antioxidantes [258], compuestos que como es sabido proporcionan numerosos beneficios para la salud. Por eso la caracterización de ellos en la nuez ha despertado gran interés en la comunidad científica y por esta razón ha sido el objetivo del capítulo 9 de la memoria de tesis.

Como se había ido haciendo a lo largo de la investigación llevada a cabo durante la tesis doctoral, en este caso también se propuso emplear la técnica de electroforesis capilar (capaz de disminuir tiempos de análisis, mantener una buena resolución y reducir los gastos de reactivos) acoplada a espectrometría de masas en lugar de HPLC. Así se puso a punto y se demostró la potencialidad de una nueva metodología alternativa a las ya estudiadas por HPLC, al mismo tiempo que se usaba por primera vez para este fin el espectrómetro de masas con tiempo de vuelo (MS (TOF)), capaz de determinar masas exactas que posteriormente son procesadas obteniéndose información acerca de las posibles fórmulas moleculares.

Al igual que en el desarrollo de otras metodologías de análisis de compuestos fenólicos, en este caso se comenzó por la elección de la muestra y su preparación. A continuación se optimizaron los parámetros de trabajo tanto a nivel de separación (electroforesis capilar) como a nivel de detección (MS (TOF)), y una vez optimizado el método se pasó a la identificación de los compuestos pertenecientes a la fracción en estudio. Para llevar a cabo esta identificación, se compararon los tiempos de migración de los analitos con los de los patrones, así como los espectros de masas de cada uno de ellos (teniendo en cuenta que el MS (TOF) es capaz de proporcionar masas exactas). Además se empleó también un detector de masas con trampa de iones con el que se obtuvieron los espectros de MS/MS de los analitos detectados. De este modo se corroboró la

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identificación realizada mediante MS (TOF) y se completó información acerca de los compuestos "desconocidos" o no identificados.

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Development of a rapid method to determine phenolic and other polar compounds in walnut by capillary electrophoresis–electrospray ionization time-of-flight mass spectrometry

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ABSTRACT

The aim of this work was to develop a capillary electrophoresis–mass spectrometry (CE–MS) method to identify and quantify phenolic and other related polar compounds in walnut samples. The extraction capacity of several solvent mixtures of phenolic compounds from walnut by conventional solid–liquid extractions was tested, and CE and electrospray ionization MS parameters were optimized. The finalized procedure is able to determine many well-known phenolic compounds present in walnuts and provide relevant information about the presence of minor polar compounds. A new compound in walnut ((2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6-*O*-β-D-glucopiranosyl ester, $[M-H]^-$ 403.161 *m*/*z*) with a structure similar to glansreginins was also identified. Phenolic compounds correspond to 14–28% of total polar compounds quantified. Aglycone and glycosylated ellagic acid represent the principal components and account for 64–75% of total phenols in walnuts. However, the sum of glansreginins A, B and (2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester was in the range of 72–86% of total quantified compounds. In addition, this is the first time that separation by CE with detection by electrospray ionization time-of-flight MS has been applied to the analysis of phenolic and other polar compounds in walnut samples, providing results in less than 15 min.

1. Introduction

Nuts, including walnuts, are a traditional food in the Mediterranean, South America and Asia, and are ingredients in sauces, stuffing, entrees, snacks, appetizers and desserts. Walnuts are one of the oldest tree foods known to humans, with historical references dating back to Persia in 7000 B.C. [1].

Walnuts are very rich in fats (52–70%) (oleic, linoleic and linolenic acid are predominant), proteins (14–24%) [2] and contain free amino acids [3].

Walnuts are also good sources of antioxidants including vitamin E and other compounds with biological activity such as flavonoids, other polyphenols and sterols [4].

Walnuts are stable, especially in shells. When walnuts are kept in a cool, dry environment they have a shelf life of 12 months [5]. Walnuts are also a rich source of phenolic compounds [6] which contribute to their antioxidant capacity; they inhibit the oxidation of human plasma and low-density lipoproteins (LDLs) in vitro [7] and may influence the low mortality rates from coronary heart disease and cancer in Mediterranean populations as walnuts are important constituents of the daily Mediterranean diet [8] as already mentioned. In addition to the favorable lipid profile of nuts, their phenolic content must be considered as a potential contributor to their apparent antiatherogenic effect. Additionally, phenolic compounds have anti-inflammatory and anti-mutagenic activities [7].

The phenolic content of walnut may affect the storage stability, and could be used as important criteria for evaluation of walnut quality [9]. The presence of phenolic compounds has also been related to the slightly astringent flavor of walnut fruits [10]. Analysis of phenols, polyphenols, and pigments in foods is fundamental to evaluate both the food and raw material due to their contribution to the color, taste, flavor characteristics and health benefits.

Most phenolic compounds commonly identified in walnut are phenolic acids and condensed tannins, and ellagic acid and flavonoids potentially have serum cholesterol-modulating effects [11]. Different phenolic acids and flavonoids have been identified in walnut fruit extracts by high-performance liquid chromatography (HPLC) [12–14], and in recent studies of walnuts some authors

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reported 16 additional polyphenols, including 3 new hydrolyzable tannins, namely glansrins A–C [15–17]. However, edible walnut fruits have not been widely investigated. CE can play a crucial role as a simple, rapid, economical and accurate method for the determination of these compounds in foods [18].

The aim of the present work was to develop a fast CE–electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) method to determine phenolic and other related polar compounds in walnut (<15 min). The different steps to be developed were as follows: (a) optimization of the most significant variables influencing the individual separation–detection of walnut phenolic compounds using CE–ESI-TOF-MS; (b) selection of the most adequate method to extract phenolic compounds from walnut; (c) identification and quantification of phenolic compounds; and (d) comparison among different varieties of walnut fruits.

To our knowledge, this is the first time that CE coupled to MS has been used to study the polar fraction of walnuts. This methodology provides short analysis times and allowed for the identification of a new compound in walnut.

2. Materials and methods

2.1. Samples

Walnuts samples used in this study were from three different varieties of walnut called Chandler, Howard and Hartley. All were purchased in local markets in 2007.

2.2. Chemicals and standards

All chemicals were analytical reagent grade and used as received. Ammonium hydrogen carbonate was from Fluka (Buchs, Switzerland) and ammonium acetate from Merck (Darmstadt, Germany), which were used as running buffers at different concentrations and pHs. All the organic solvents, methanol, ethanol, hexane, acetone and 2-propanol were from Panreac (Barcelona, Spain). They were used for the different extraction systems and sheath liquids. Double-deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium hydroxide was from Panreac. All the standard compounds were purchased from Sigma–Aldrich (St. Louis, MO, USA). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v).

2.3. Sample preparation

The samples (pellicle and kernel) were cut into thin slices and then ground in a mortar to a fine texture. Once crushed, walnuts were defatted with hexane for 30 min under agitation at room temperature. Phenolic compounds were extracted from the raw material by sonication using 4/1 (v/v) ethanol/H_2O at a solid sample to solvent ratio of 1/10 (w/v) at 40 °C for 30 min. The extraction was repeated twice, after centrifugation at 1000 rpm for 10 min (Allegra 21R, Beckman, Palo Alto, CA, USA) supernatants were combined and the solvent was evaporated under vacuum at 40 °C using a rotary evaporator (Büchi, Switzerland), and then reconstituted with 4 mL of 50/50 (v/v) water/methanol. The extracts were filtered with a nylon filters 0.2 μm (Millipore, Bedford, MA, USA) and stored at −20 °C until use.

2.4. CE–ESI-MS

2.4.1. Instrumentation

To identify phenolics, electrophoretic analyses were performed using a P/ACE System MDQ (Beckman Instruments, Fullerton, CA, USA) coupled to an MS detector. MS experiments were performed on a Bruker Daltonics microTOF spectrometer (Bruker Daltonik, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). A CE–ESI interfacing sheath liquid flow system by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA) was used. Due to the complexity of the sample and the necessity to confirm data to identify some compounds, MS/MS analysis with P/ACE System MDQ coupled with Esquire 2000 ion trap (IT)-mass spectrometer (Bruker Daltonik) was also carried out.

2.4.2. CE experimental conditions

Uncoated fused silica tubing (50 μm i.d. × 375 μm o.d.; Beckman, Fullerton, CA, USA) was used. The effective capillary length was 90 cm. The optimum separation condition of CE was obtained using 40 mM ammonium acetate (pH 9.5). A voltage of 30 kV with normal polarity was applied for 15 min. Before the first use the capillaries were conditioned with 1 M NaOH for 20 min followed by a water rinse for another 10 min. Between runs capillary conditioning was done by flushing 1 M NaOH, water and separation buffer for 3, 2 and 5 min, respectively. At the end of the day, the capillary was flushed with water for 10 min and air for 5 min. Standards and samples were injected hydrodynamically for 20.0 s at the anodic end in low-pressure mode (0.5 psi (1 psi = 6894.76 Pa)). After each electrophoretic cycle, the capillary was rinsed with HPCE-grade water (2 min). All washing steps were performed at the same temperature of the run. The running buffer was changed after two runs. The capillary electrophoretic analysis was repeated three times for each extract ($n = 12$) and three times for each calibration point ($n = 3$). Data were collected with the Karat Software Version 5.0 data system (Beckman Coulter, Fullerton, CA, USA).

2.4.3. MS experimental conditions

TOF-MS transfer parameters were optimized by direct infusion experiments with Tunning mix (Agilent Technologies) and walnut extracts. Good sensitivity at a reasonable resolution was obtained (5000–10,000 at 250 m/z). The trigger time was set to 50 μs (45 μs for setting transfer time and 5 μs pre-pulse storage time), corresponding to a mass range of 50–1100 m/z. Spectra were acquired by summarizing 30,000 single spectra, defining the time resolution to 1.5 s.

The analyses carried out in the IT-MS were run in the negative ion mode and the capillary voltage was set at 3600 V. The IT scanned at 100–1100 m/z range at 13.000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 110.00 ms, the target count at 20,000 and the trap drive level at 100%. The instrument was controlled by a personal computer running the Esquire NT software from Bruker Daltonics.

The accurate mass data of the molecular ions were processed by DataAnalysis 3.3 software (Bruker Daltonik). It provides a list of possible elemental formulae by means of the Generate Molecular Formula (GMF) Editor, which uses a CHNO algorithm [19]. This provides information about elemental composition, sigma and m/z values. Calibration of the MS was performed using sodium formate.

2.5. Statistical analysis

One-way analysis of variance (ANOVA, Tukey's honest significant difference multiple comparison) was evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

Table 1
Analytical parameters of the method proposed

Analyte	RSD	LOD (μg/mL)	LOQ (μg/mL)	Calibration range (μg/mL)	Calibration equations	r^2
Catechin	2.57	0.185	0.618	10–100	$y = 44{,}640x - 320{,}445$	0.9962
Gallic acid	2.71	0.276	0.922	1–10	$y = 29{,}950x - 3841$	0.9916
Ferulic acid	2.04	0.337	1.124	1.15–20	$y = 24{,}556x - 7388$	0.9902
Vanillic acid	3.11	0.981	3.270	1–50	$y = 8442x + 75{,}009$	0.9983
p-Coumaric acid	1.89	0.708	2.359	5–80	$y = 11{,}705x + 98{,}716$	0.9955
Chlorogenic acid	2.37	0.990	3.302	10–100	$y = 8362x + 9045$	0.9902
Ellagic acid	4.68	2.085	6.950	10–250	$y = 10{,}563x + 8471$	0.9906

3. Results and discussion

3.1. CE–ESI-TOF-MS optimization

Optimization of the method was done using the Chandler walnut extracts. Initially, the electrophoretic conditions were optimized according to the following criteria: efficiency, sensitivity, run time and peak shape.

The optimization began by testing voltage and the type, concentration and pH of the buffer. Separation was evaluated using two different background electrolytes (BGEs) such as ammonium acetate and ammonium carbonate. Ammonium acetate/NH_3 and ammonium carbonate/NH_3 were used as running buffers at a concentration of 60 mM and pH 9.5. The best separation, in terms of efficiency, baseline stability and analyte migration time was obtained with the ammonium acetate/NH_3 running buffer. Besides carbonate, BGE showed a current higher than 50 μA, and it has been reported that currents lower than 50 μA usually reduce the risk of peak broadening due to Joule heating even when capillaries are not properly thermostated close to or inside the sprayer [20].

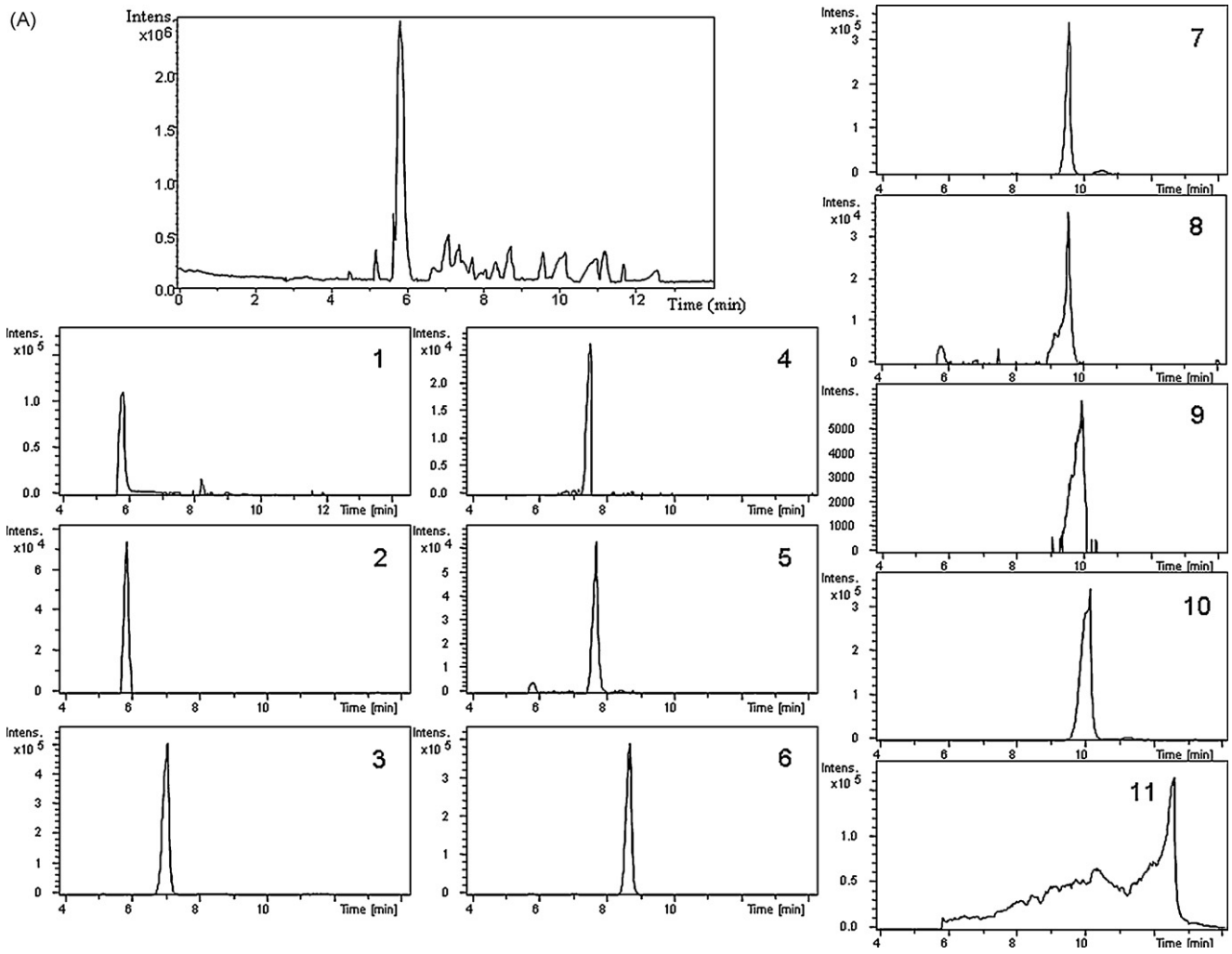

Fig. 1. (A) BPE obtained by CE–ESI-TOF in walnut (Chandler variety) under optimized conditions, and EIEs of identified compounds: (1) catechin, (2) galloylglucose, (3) glansreginin B, (4) ferulic acid glycoside, (5) vanillic acid glycoside, (6) glansreginin A, (7) (2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester, (8) cumaroylquinic acid, (9) chlorogenic acid, (10) ellagic acid pentoside dimer and (11) ellagic acid. (B) BPE obtained by CE–ESI-TOF of walnut (Chandler variety) under optimized conditions, and EIEs of unidentified compounds: (a) $[M-H]^-$ 1025.3391 *m/z*, (b) $[M-H]^-$ 429.2125 *m/z*, (c) $[M-H]^-$ 413.2179 *m/z*, (d) $[M-H]^-$ 333.0591 *m/z*, (e) $[M-H]^-$ 267.0705 *m/z*, (f) $[M-H]^-$ 265.1459 *m/z*, (g) $[M-H]^-$ 146.0462 *m/z*, (h) $[M-H]^-$ 261.1336 *m/z* and (i) $[M-H]^-$ 289.0820 *m/z*. Optimized conditions: running buffer 40 mM ammonium acetate/NH_3, pH 9.5, voltage 30 kV, 20 s injection time, sheath liquid 2-propanol/water 50/50 (v/v) at a flow rate of 0.22 mL/h, a drying flow rate of 5 L/min, at 220 °C using a nebulizing gas pressure of 0.6 bar.

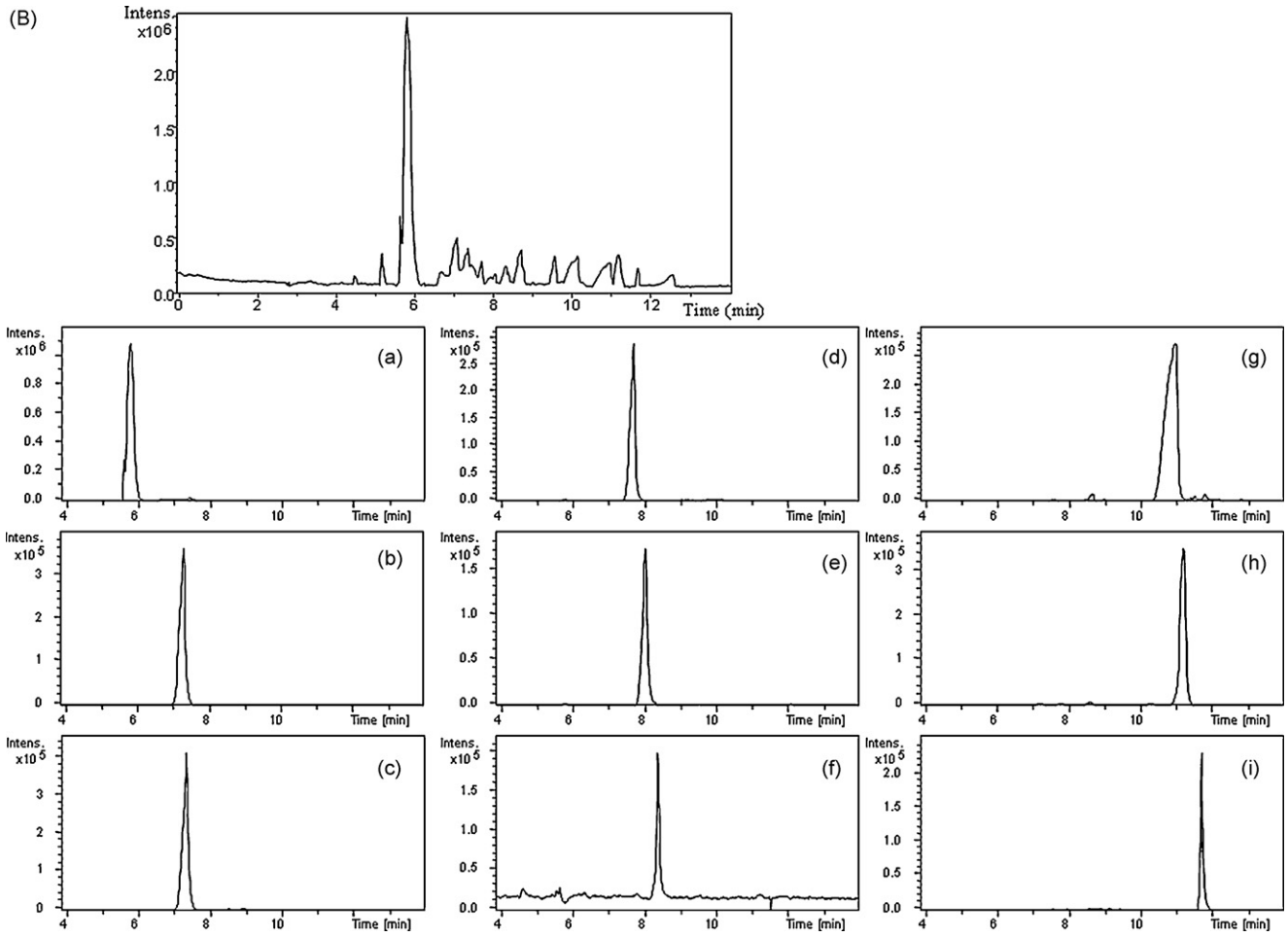

Fig. 1. (*Continued*).

The concentration of selected BGE buffer (ammonium acetate/NH_3) was varied between 20 and 80 mM in 20 mM intervals. The pH was then studied between 8.5 and 10.5 in pH intervals of 0.2. The best compromise in terms of efficiency, selectivity and migration time was found for 40 mM ammonium acetate/NH_3 pH 9.5. Under such basic conditions, the polyphenols and other polar compounds in walnut were negatively charged. In the studied range, almost all of the compounds presented similar charges and profiles. For this reason, the optimum pH value selected was 9.5 since it gave the best peak shape.

The addition of 2-propanol as an organic modifier in the range of 0–10% was then tested, but no improvements were observed.

Next the applied voltage was varied between 10 and 30 kV, and a voltage of 30 kV was chosen to provide the shortest analysis time, good efficiency and acceptable electrical current values (around 31 μA).

The ESI-TOF-MS operating conditions were optimized by adjusting the sheath liquid flow rate, nebulizer pressure, dry gas flow rate and ESI chamber temperature, selecting the MS intensity of several peaks as optimization criteria while a sample solution was injected and separated in the CE–ESI-MS system. The choice of these variables was a compromise between maintaining efficient and well-resolved electrophoretic separation and improving ionization performance.

The composition of the sheath liquid (methanol/water and 2-propanol/water) and the percentage of organic solvent between 40 and 80% were also studied. With 2-propanol/water 50/50 (v/v) and a flow rate of 3.6 μL/min, the best separation was obtained in terms of signal response and signal stability. A value of 3.6 μL/min was selected as the optimum sheath liquid flow because lower flows reduced the ionization yield due to the instability of the spray, while at higher flow rates dilution of the electrophoretic bands was too high and the intensity of the MS signal for these compounds was reduced. The other ESI parameters were chosen according to the sheath liquid flow of 3.6 μL/min: the most suitable ones were a nebulizer pressure of 0.6 bar, dry gas flow equal to 5 L/min and dry gas temperature 220 °C.

Finally, the optimum conditions were running buffer 40 mM ammonium acetate/NH_3, pH 9.5, voltage 30 kV, 20 s injection time, sheath liquid 2-propanol/water 50/50 (v/v) at a flow rate of 0.22 mL/h, a drying flow rate of 5 L/min, at 220 °C and using a nebulizing gas pressure of 0.6 bar. ESI-IT-MS optimum conditions were sheath liquid 2-propanol/water 50/50 (v/v) at a flow rate of 3.6 μL/min, a drying flow rate of 5 L/min, at 250 °C using a nebulizing gas pressure of 5 psi.

3.2. Sensitivity and repeatability

The sensitivity of the method was studied by defining the limits of detection (LODs) and limits of quantification (LOQs) for individual compounds in standard solutions (Table 1). LOD and LOQ were, respectively, set at S/N = 3 and S/N = 10 where S/N is the signal-to-noise ratio.

Table 2
Compounds identified in an extract walnut by CE–ESI-TOF

Peak	Molecular formula	Selected ion	*m/z* experimental	*m/z* calculated	MS/MS fragments	Error (ppm)	Sigma value	Tolerance (ppm) in generated molecular formula	Migration time (min)	Classification order (number of possibilities)	Possible compound
	$C_{10}H_{12}N_2O$	$[M-H]^-$	175.0885	175.0877		−4.8	0.0080	5	4.48	1st (1)	Serotonin
	$C_6H_{13}N_4O_2$	$[M-H]^-$	173.1044	173.1058		−7.9	0.0027	10	5.16	1st (1)	Arginine
a	$C_{36}H_{66}O_{33}$	$[M-H]^-$	1025.3391	1025.3414	845/683/341/179/162	2.2	0.0097	5	5.80	1st (6)	Polysaccharide
1	$C_{15}H_{14}O_6$	$[M-H]^-$	289.0700	289.0718	245/205/179	1.8	0.0084	5	5.85	1st (1)	Catechin
2	$C_{20}H_{20}O_{14}$	$[M-H]^-$	483.0779	483.0780	313/169	0.2	0.0045	5	5.87	1st (3)	Digalloylglucose
3	$C_{24}H_{38}O_{15}$	$[M-H]^-$	565.2152	565.2138	403/343/241/197	−2.5	0.0807	5	7.07	1st (4)	Glansreginin B
b	$C_{21}H_{34}O_9$	$[M-H]^-$	429.2125	429.2130	369/387	1.1	0.0234	5	7.30	1st (2)	Unidentified
c	$C_{21}H_{34}O_8$	$[M-H]^-$	413.2179	413.2181	371/353	0.4	0.0097	5	7.34	1st (2)	Unidentified
4	$C_{16}H_{20}O_9$	$[M-H]^-$	355.1032	355.1035	193	0.8	0.2289	5	7.54	1st (1)	Ferulic acid glucoside
d	$C_{16}H_{14}O_8$	$[M-H]^-$	333.0591	333.0616	241/153	4.9	0.0819	5	7.69	1st (1)	Unidentified
5	$C_{14}H_{18}O_9$	$[M-H]^-$	329.0868	329.0878	247/167	3.1	0.0551	5	7.71	1st (1)	Vanillic acid glucoside
e	$C_9H_{16}O_9$	$[M-H]^-$	267.0705	267.0722	249/231/175/113	6.3	0.0041	10	8.22	1st (1)	Unidentified
f	$C_{15}H_{21}O_4$	$[M-H]^-$	265.1459	265.1445	249/113	5.0	0.0274	5	8.36	1st (1)	Unidentified
6	$C_{28}H_{35}NO_{13}$	$[M-H]^-$	592.2039	592.2036	403/343/241/197	−0.5	0.0441	5	8.69	3rd (47)	Glansreginin A
7	$C_{18}H_{27}O_{10}$	$[M-H]^-$	403.1610	403.1610	343/241/197	0.0	0.0478	5	9.54	1st (2)	(2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester
8	$C_{16}H_{18}O_8$	$[M-H]^-$	337.0916	337.0929	255/163	3.8	0.0189	5	9.54	1st (1)	Cumaroylquinic acid
9	$C_{16}H_{18}O_9$	$[M-H]^-$	353.0878	353.0861	–	4.7	0.0264	5	9.91	1st (1)	Chlorogenic acid
10	$C_{38}H_{28}O_{24}$	$[M-H]^-$	867.0866	867.0898	433/301/169	−1.4	0.0221	5	10.11	1st (4)	Ellagic acid pentoside dimer
g	$C_5H_9NO_4$	$[M-H]^-$	146.0462	146.0459	128	−2.1	0.0032	5	10.93	1st (2)	Unidentified
h	$C_{12}H_{21}O_6$	$[M-H]^-$	261.1336	261.1344	243/199/159/125	3.0	0.0015	5	11.15	1st (1)	Unidentified
i	$C_{14}H_{14}N_2O_5$	$[M-H]^-$	289.0820	289.0830	174/132	3.3	0.0061	5	11.63	1st (6)	Unidentified
11	$C_{14}H_6O_8$	$[M-H]^-$	300.9975	300.9990	–	4.8	0.0111	5	12.53	1st (1)	Ellagic acid

Intraday and interday precisions were developed to evaluate the repeatability of CE–ESI-TOF-MS method. An ethanol–water extract was injected ($n = 6$) on the same day (intraday precision) for 3 consecutive days (interday precision, $n = 18$). The relative standard deviations (RSDs) of analysis time and peak area were determined.

The repeatability of the analysis time, expressed by the RSD, was 0.24%, while the interday repeatability was 0.81%. The intraday repeatability of peak area, expressed by the RSD, was 3.9%, whereas the interday repeatability was 5.8%.

3.3. Identification of phenolic compounds in walnuts

The identification of phenolic compounds was carried out comparing their migration times and mass spectra provided by TOF-MS with those of authentic standards when available. Remaining compounds were identified by the interpretation of their mass spectra obtained by the TOF-MS and the MS/MS spectra acquired with the IT-MS.

Fig. 1A and B shows, respectively, the base peak electropherogram (BPE) of an extract of Chandler walnut and the extracted ion electropherograms (EIEs) for identified (signed by numbers) and unidentified (signed by letters) main compounds in walnut extracts.

All the compounds studied are summarized in Table 2. This table includes molecular formula, selected ion, calculated and experimental *m/z*, MS/MS fragments, error, sigma value, tolerance (ppm) in generated molecular formula, migration time, classification order (number of possibilities) and possible compounds. Fig. 2 shows the structure of the identified compounds.

The identification by MS-TOF was carried out using the Generate Molecular Formula Editor. First of all, a low tolerance was chosen (in most cases the tolerance was 5 ppm). After that, options with a low sigma value (<0.05) and a low error (<5 ppm) were taken into account in most cases. The last step was to consider the position of the molecular formula in the table of possible compounds. In fact, most of the identified compounds are in position number 1 in Table 2.

Table 2 also includes some unidentified compounds with a relatively high abundance in walnut. Even though identification of these compounds was not possible with the generated data by TOF analysis, this data together with the MS/MS fragments obtained by the ion trap mass spectrometer were included as it is felt they are important compounds.

Most of the compounds found in this work have been previously described in walnuts. Phenolic acids and their derivatives, catechin and ellagic acid have also been identified before in walnut by other authors [13,21], as well as glansreginins A and B [17].

Concerning the identified compounds, catechin, chlorogenic acid and ellagic acid were confirmed by comparing their migration times and mass spectra with those of standard compounds.

To identify compounds for which no commercial standards were available (ferulic acid glycoside, vanillic acid glycoside, cumaroylquinic acid, ellagic acid pentoside dimer, and glansreginins A and B), we checked the generated molecular formula (Table 2) obtained by TOF analysis and also studied the MS/MS fragments of each. $[M-H]^-$ 355.1032 *m/z* gave 193.05 as a fragment (ferulic acid) and loss 162 corresponding to a fragment of glucose. $[M-H]^-$ 329.0868 *m/z* gave 197.04 as a fragment (vanillic acid), and the difference between them is 162 corresponding to a fragment of glucose. $[M-H]^-$ 337.0916 *m/z* gave 163 as a fragment (coumaric acid), $[M-H]^-$ 867.0866 *m/z* fragments in 433/301/169 in the same way as ellagic acid pentoside dimer [22]. $[M-H]^-$ 592.2039 *m/z* and $[M-H]^-$ 565.2152 *m/z* give both 403/343/241/197 as fragments corresponding to the fragments previously described by Ito et al. to be glansreginins A and B, respectively [17].

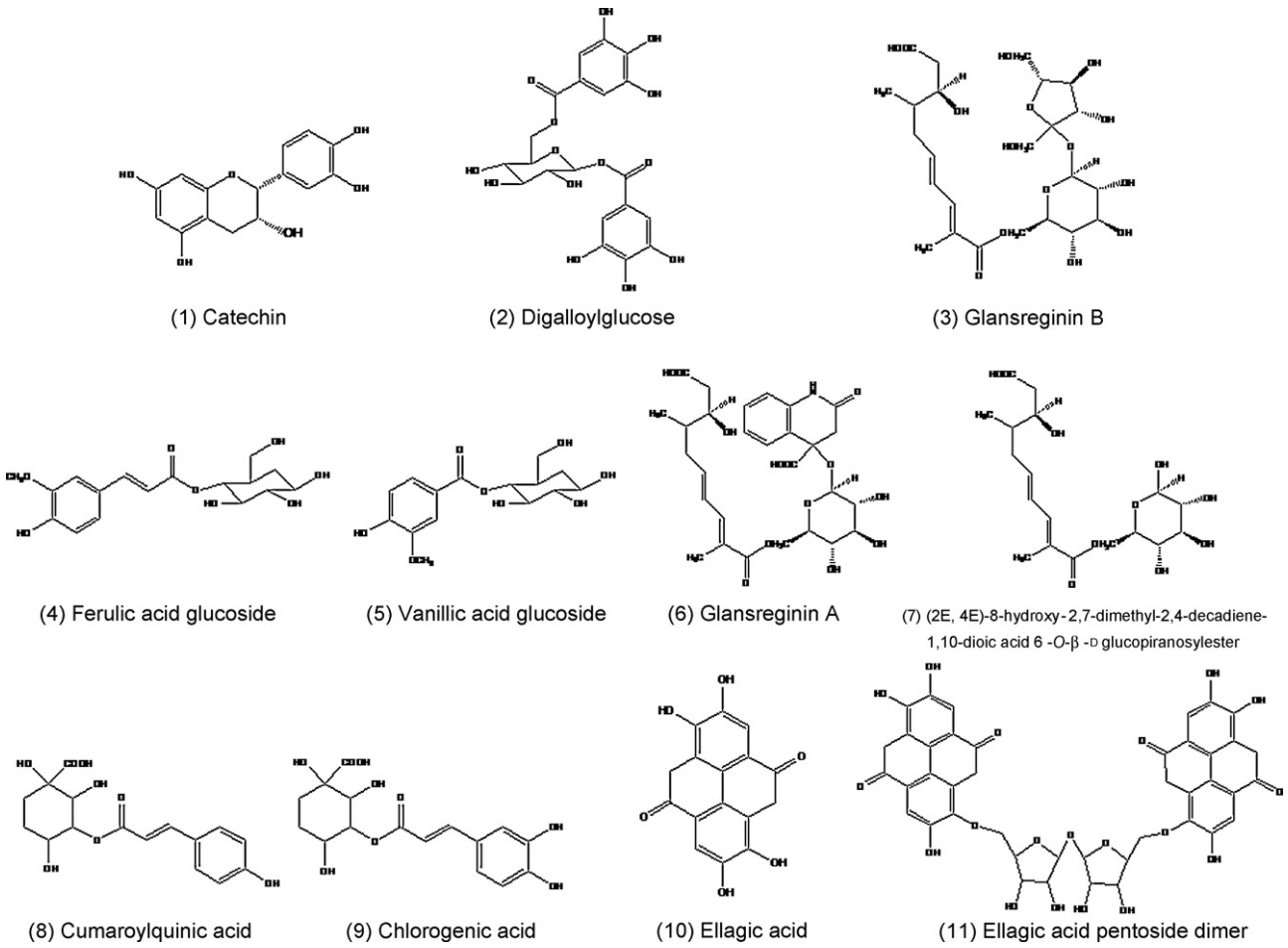

Fig. 2. Structures of identified compounds in the phenolic fraction of walnut.

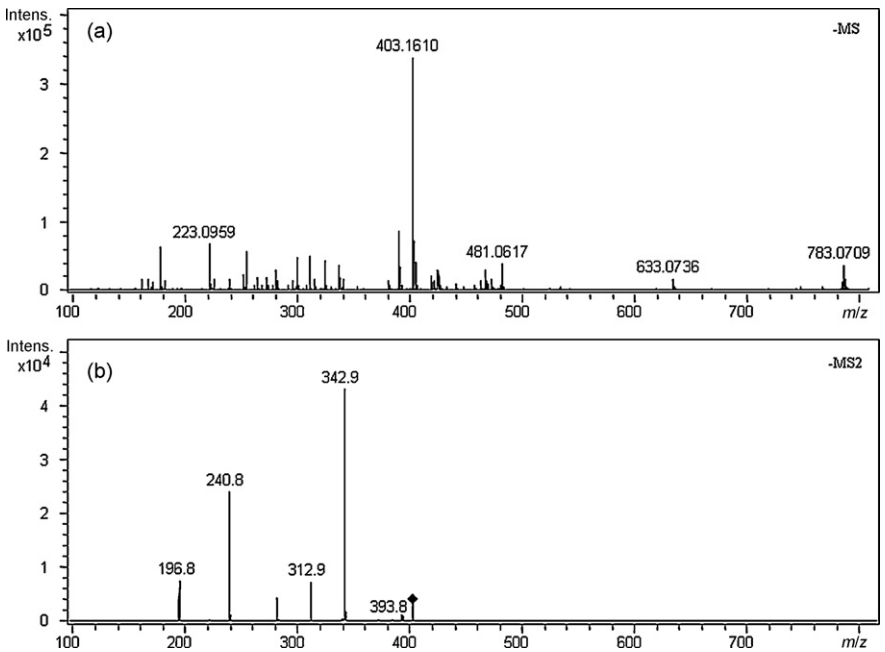

Fig. 3. (a) TOF-MS spectrum (sheath liquid 2-propanol/water 50/50 (v/v) at a flow rate of 0.22 mL/h, a drying flow rate of 5 L/min, at 220 °C and using a nebulizing gas pressure of 0.6 bar) and (b) MS/MS spectrum (sheath liquid 2-propanol/water 50/50 (v/v) at a flow rate of 0.22 mL/h, a drying flow rate of 5 L/min, at 250 °C using a nebulizing gas pressure of 5 psi).

Table 3
Results expressed in mg analyte/kg of dry weight walnut of real samples ($n = 7$; value = $X \pm$ SD)

Analyte	Chandler	Howard	Hartley
Catechin	4.4 ± 0.2 (b)	6.6 ± 0.3 (a)	4.0 ± 0.4 (b)
Digalloylglucose	1.6 ± 0.3 (b)	1.1 ± 0.2 (b)	2.3 ± 0.5 (a)
Glansreginin B	92.1 ± 30.6 (a)	35.5 ± 5.5 (b)	99.7 ± 0.1 (a)
Ferulic acid glucoside	1.6 ± 0.3 (a)	0.9 ± 0.0 (b)	1.5 ± 0.2 (a, b)
Vanillic acid glucoside	4.8 ± 0.5 (a)	6.4 ± 0.7 (a)	7.5 ± 0.9 (a)
Glansreginin A	76.3 ± 15.6 (b)	335.6 ± 22.9 (a)	76.3 ± 14.3 (b)
Cumaroylquinic acid	4.7 ± 0.1 (b)	8.3 ± 1.0 (a)	4.1 ± 0.8 (b)
Chlorogenic acid	2.4 ± 0.5 (a)	2.0 ± 0.2 (a)	1.5 ± 0.2 (a)
Ellagic acid pentoside dimer	36.1 ± 3.6 (a)	33.0 ± 3.9 (a)	37.2 ± 3.1 (a)
Ellagic acid	24.7 ± 2.1 (a)	12.4 ± 0.3 (b)	6.9 ± 1.3 (b)
(2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester	40.8 ± 6.21 (b)	48.3 ± 4.3 (a)	32.3 ± 3.9 (b)

Different letters (a and b) in the same row indicate significantly different values ($p < 0.05$, HSD Tukey test).

Special attention was paid to compound number 7 with $[M-H]^-$ 403.161 *m/z*. It was observed that all the MS/MS fragments obtained for this compound are the same as the MS/MS fragments obtained for glansreginins A and B (343/241/197), and that this compound appears at a migration time totally different (Table 2). Based on this, it likely corresponds to a compound that maybe involved in the synthesis of glansreginin A or B without the 2-hydroxycinchoninic acid or sucrose, respectively. Its structure corresponds with the IUPAC name (2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester. Fig. 3 shows the MS spectra obtained by TOF and the MS/MS spectra acquired by IT for this compound. It is important to highlight that to our knowledge, this compound has not been described before in walnuts.

Additional unidentified compounds have been included in Table 2 as they are an important part of the polar fraction of walnut. The MS/MS analyses were also carried out for these compounds (Table 2).

Compound (a) $[M-H]^-$ 1025.3391 *m/z* and its fragments appear as the most abundant peak in the electropherogram. Due to this, the MS/MS analysis of compound and the fragments were performed, and the masses were 845/683/341/179/162 *m/z*. It appears to be a compound that fragments into sugar moieties ($C_6H_{12}O_6$), which is consistent with a polysaccharide.

A monoamine neurotransmitter and amino acid, serotonin ($[M-H]^-$ 175.0885 *m/z*) and arginine ($[M-H]^-$ 173.1044 *m/z*), respectively, were identified by mass spectra and Generate Molecular Formula Editor. Both compounds are presumed to be positively charged at working pH since their migration time is lower and they appear at the beginning of the electropherogram.

3.4. Quantification of phenolic compounds in walnut

Seven standard calibration graphs for the quantification of the principal compounds found in the electrophoretic profile were prepared using seven commercial standards (catechin, ferulic acid, gallic acid, vanillic acid, *p*-coumaric acid, chlorogenic acid and ellagic acid). All calibration curves showed good linearity between different concentrations depending on the analytes studied. The calibration plots indicate good correlation between peak areas and analyte concentrations, and regression coefficients were higher than 0.990 in all cases (Table 1).

Catechin, chlorogenic acid and ellagic acid were quantified by the calibration curves obtained from their respective commercial standards. The other compounds, which had no commercially standards, were tentatively quantified on basis of other compounds that have similar structures. Digalloylglucose was quantified using a gallic acid calibration curve, ferulic acid glycoside using a ferulic acid calibration curve, vanillic acid glycoside using a vanillic acid calibration curve, cumaroylquinic acid with *p*-coumaric acid, and ellagic acid pentoside dimer with ellagic acid; glansreginins A–B and (2*E*,4*E*)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6′-*O*-β-D-glucopiranosyl ester were quantified with a chlorogenic acid calibration curve. It has to be taken into account that the response of the standards can be different from the response of the analytes present in the walnut sample, and consequently the quantification of these compounds is only an estimation of their actual concentrations.

Using the described extraction system and CE–MS method, the three walnut varieties were analyzed, and the quantitative results are presented in Table 3.

The most abundant compounds were the two dicarboxylic acid derivatives, glansreginins A and B (the sum of glansreginins A and B was in the range of 168.4–371.1 mg of chlorogenic acid/kg dry weight). Howard walnuts were different from the other varieties as they had the highest quantity of glansreginin A and the lowest amount of glansreginin B. Chandler walnut had the highest amounts of ellagic acid: twice that found in Howard and four times more than in Hartley. Ellagic acid pentoside dimer was found in the range of 33.0–37.2 mg analyte/kg dry weight, and all three varieties had similar amounts.

As far as the amount of quinic acids derivatives is concerned, chlorogenic acid was more abundant in Chandler, while cumaroylquinic acid was more abundant in Howard (twice the amount as in the other two varieties).

Regarding phenolic acid glycosides, vanillic acid glycoside was more abundant than ferulic acid glycoside for all three varieties. Howard walnut had the lowest amount of ferulic acid glycoside. Hartley walnut had the highest concentration of digalloylglucose and the lowest amount of catechin. Howard was richest in catechin.

4. Concluding remarks

CE coupled to a TOF-MS was used to study the phenolic fraction of walnuts for the first time. The method utilized successfully identified and quantified the major compounds of this fraction in walnut in less than 15 min. It is also important to highlight that, to our knowledge, the compound identified as number 7 was described for the first time in walnut, and that this methodology permits comparison of the phenolic content in different varieties of walnut.

This CE methodology represents an advantage over HPLC methodologies since it can study the polar fraction of walnuts in a short time. Moreover, consumption of solvents and sample in CE is very low compared to HPLC.

Acknowledgments

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La metodología descrita en este capítulo ha demostrado presentar una serie de ventajas que la hacen ser muy interesante.

- En primer lugar es uno de los pocos trabajos en los que se ha empleado la electroforesis capilar para el estudio de la fracción fenólica de la nuez, y la primera vez que esta técnica se ha acoplado a espectrometría de masas para este fin.
- También es de resaltar la corta duración de los análisis, este método es capaz de arrojar resultados en menos de 15 minutos, lo que resulta muy útil si se quisiera utilizar para análisis rutinarios.
- Es posible identificar y cuantificar 11 compuestos del perfil.
- Es posible la aplicación de este método sobre diferentes variedades de nuez, permitiendo la comparación del contenido fenólico entre ellas.
- Además, hay que prestarle especial interés a la identificación de un nuevo compuesto nunca antes determinado en nuez (ácido 8-hidroxi-2,7-dimetil-2,4-decadien-1,10-dioico 6-O-β-D-glucopiranosilester).

De todas formas, a pesar de todas estas ventajas y de la potencialidad que ha demostrado esta metodología, se pensó que podía ser interesante continuar el estudio de esta matriz empleando para ello otro tipo de detector como la resonancia magnética nuclear (RMN). Así, por un lado, se podrían identificar sin lugar a dudas los compuestos ya "conocidos", y por otro intentar descifrar la estructura de aquellos que no ha sido posible identificar hasta ahora.

Bloque IV: Teucrium polium

Teucrium es un género de plantas perennes de la familia Lamiaceae, de porte arbustivo o herbáceo, común en hábitats insolados tipo tomillar o formando parte del sotobosque e incluso empleado en jardinería. El género consta de más de 300 especies, muchas con gran diversificación subespecífica. Es oriundo de las regiones del Medio Oriente y del Mediterráneo, donde ha sido empleado como hierba medicinal durante más de 2000 años [259]. *Teucrium polium* es una especie del género *Teucrium* (conocida comúnmente con el nombre de zamarrilla),de flores blanquecinas o rosas, sus hojas se han usado tradicionalmente en la cocina y con fines medicinales, en particular para el tratamiento de dolencias de estómago. La infusión preparada a partir de esta planta se usa contra el cólico, dolor de cabeza, como agente depurativo, antiespasmódico, contra las piedras en el riñón,…[260].

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Las plantas medicinales como *Teucrium polium* se consideran una fuente importante de compuestos antioxidantes y sus beneficios terapéuticos en muchas ocasiones se les atribuyen a sus propiedades antioxidantes [261,262]. Se ha demostrado que tiene efectos diuréticos, antipiréticos, diaforéticos, antiinflamatorios, antihipertensivos, anoréxicos, analgésicos [263,264], bactericidas [265] y antidiabéticos [266].

Las plantas pertenecientes al género *Teucrium* están constituídas por multitud de compuestos entre los que destacan ésteres de ácidos grasos [267], diterpenos [268], monoterpenos [269], sesquiterpenos [270], y compuestos fenólicos [271,272]. Éstos últimos han demostrado poseer una fuerte actividad antioxidante [273], existiendo estudios *in vitro* e *in vivo* que comparan la actividad antioxidante de *Teucrium polium* con la del α-tocoferol [274,275].

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Entre los compuestos característicos del género *Teucrium* se encuentran los fenilpropanoides glucósido, de entre los cuales el verbascósido y el poliumoside son característicos de *Teucrium polium* [276]. Es de destacar que el grupo de los flavonoides contenidos en *Teucrium polium* se encuentran en su gran mayoría en forma de metoxi derivados [277,278].

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Capítulo 10

Identificación de antioxidantes en *Teucrium polium* mediante LC-SPE-NMR y DPPH *on-line*.

Trabajo incluído en este capítulo:

Combining LC-SPE-NMR and *on-line* DPPH for the identification of Teucrium polium phytochemical radical scavengers.
(Enviado a *Food Chemistry*)

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El trabajo descrito en este capítulo se llevó a cabo durante el periodo de estancia realizado en el centro de NMR de la Universidad de Ioannina (Grecia). En él se demuestra la fuerte potencialidad para el análisis de compuestos fenólicos de la cromatografía líquida acoplada a resonancia magnética nuclear.

La matriz empleada en este caso fue una planta medicinal autóctona de la región mediterránea denominada *Teucrium polium* (zamarrilla) y ampliamente usada para combatir sobre todo dolencias de estómago. Esta planta ha demostrado tener una importante capacidad antioxidante, debida en parte a su contenido fenólico, por lo que se decidió caracterizar su perfil fenólico al mismo tiempo que se estudiaba la capacidad antioxidante de la fracción completa y de cada uno de los compuestos detectados realizando medidas de DPPH *on-line*.

El primer paso en este estudio fue la extracción de los compuestos de interés para su posterior análisis. Se llevaron a cabo tres tipos de extracciones diferentes (agua, metanol, y una tercera en tres pasos: hexano, acetato de etilo y metanol). A continuación se puso a punto la separación por HPLC, y una vez optimizada se planteó el análisis por NMR. Para ello se disponía de un módulo intermedio entre HPLC y NMR de extracción en fase sólida constituído por diferentes cartuchos en los que se fueron atrapando múltiples veces los analitos de interés con el fin de obtener una concentración suficiente capaz de ser detectada por NMR. Acto seguido se transferían uno a uno los compuestos y se llevaban a cabo los experimentos de NMR necesarios (1D y 2D) para poder determinar sus estructuras.

Además se realizaron las medidas de la actividad antioxidante mediante DPPH off-line y *on-line*. Estas medidas *on-line* permitían medir la actividad antioxidante de cada analito por separado, y atribuir cuáles de ellos contribuían más a la actividad total.

Combining LC-SPE-NMR and on-line DPPH for the identification of *Teucrium polium* phytochemical radical scavengers

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KEYWORDS

Teucrium Polium, phytochemicals, radical scavengers, phenylpropanoid glycosides, flavonoids, LC-SPE-NMR, on-line DPPH

Abstract

Teucrium polium (*Lamiaceae*) is a popular medicinal plant that is used in the daily diet. The antioxidant activity of *Teucrium polium* extracts has been widely investigated, demonstrating their *in vitro* and *in vivo* antioxidant effect. Recent biological studies indicate their possible therapeutic efficacy due to its cytotoxic and hypoglycemic properties. In this work we attempt to analyze *Teucrium polium* extracts in terms of its composition and radical scavenging activity, using LC-SPE-NMR and the on-line DPPH method. Off-line DPPH measurements were also carried out and the correlation of results was attempted. NMR data revealed the presence of the phenylpropanoid glycosides verbascoside and poliumoside, the flavones of apigenin and its derivatives and two methoxyflavonols. On-line DPPH experiments showed that the scavenging activity is mostly attributed to the main component of the extracts the phenyl propanoid poliumoside. A high correlation between off-line and on-line DPPH experiments was found. Since the antioxidant potency is a well-established biomarker**,** the above strategy of combing the LC-SPE-NMR and the on-line DPPH method, which can pinpoint the active phytochemicals without the tedious isolation step is highly recommended.

1. Introduction

Lamiaceae family is considered to be the most studied group of flora comprising a great list of different genera and species of aromatic plants. *Teucrium* is a genus of the *Lamiaceae* family that includes 300 species [1]. One of the most known species in Greece is *Teucrium polium*, commonly named as gold germander. *Teucrium polium* aerial plants are frequently used in the daily diet as a spice, appetizer, and refreshing beverage as well as for medicinal purposes [2]. The thorough use of *Teucrium polium* plant material prompted scientists to study both the activity and composition of its extracts.

The antioxidant activity of *Teucrium polium* extracts has been mostly investigated, providing a plethora of data. In detail, the *in vitro* antioxidant activity of *Teucrium polium* extracts has been evaluated using inhibition of DPPH [2-6] and ABTS [4] assays, inhibition of hydroxyl radicals [6, 7], protection of a *β*-carotene/linoleic acid model system [2, 6, 7], the ammonium thiocyanate method [2], the phossphomolybdenum and the Trolox equivalent antioxidant capacity tests [5]. Metal catalysed protein oxidation (B2002) and iron induced lipid peroxidation in rat liver microsomes [7, 8] methods have also been reported. The above results demonstrate that *Teucrium polium* extracts possess antioxidant activity *in vitro.* The antioxidant potential *in vivo* was also explored and in all experiments the activity was comparable to that of *β*-tocopherol [9]. Recently, the effect of *Teucrium polium* extracts on oxidative stress and apoptosis was evalutated and the corresponding prevention was attributed mostly to the high antioxidative potential [10, 11]. In addition, the cytotoxic effect on tumor cells [12-

[15], and their hypoglycemic effect [16, 17] suggest that these extracts have the potential to act as therapeutic agents.

Studies on the composition of *Teucrium polium* extracts reported the presence of phenylpropanoid glycosides, which are characteristic for *Teucrius* genus [1, 18]. The subgroup of flavonoids is also usually found in the form of methoxy-derivatives [2, 19, 20]. The aim of this work was to analyze the phytochemical content of crude *Teucrium polium* extracts using the hyphenated technique of LC-SPE-NMR and to combine structural data with antioxidant activity information obtained from off-line and on-line DPPH experiments, without the use of any separation or isolation step. This is a part of an on-going effort on the search of bioactive phytochemicals in plant extracts.

2. Materials and Methods

2.1. Chemicals

Methanol, hexane and ethyl acetate for soxhlet extraction were obtained from Riedel de Haen (Seelze, Germany). Acetonitrile-d_3 (NMR quality) was purchased from Deutero (Kastellaun, Germany). 2,2-Diphenyl-1-picrylhydrazyladical (DPPH$^{\bullet}$) and Folin & Ciocalteu's phenol reagent were purchased from Sigma (St. Louis, MO) and Merck (Darmstat, Germany) respectively. Acetonitrile and water were of HPLC grade and were obtained from Scharlau (Barcelona, Spain). Acetic acid was analytical grade provided by Merck (Darmstadt, Germany).

83 *2.2. Sample preparation*

84 Dried aerial parts of the flowering plant of *Teucrium polium* were collected
85 from Prosilio of Tzoumerka (Epirus, Greece) on summer 2007. The plant material
86 was air-dried at room temperature in the dark and kept in glass dispensers until study.
87 The plant was characterized in the Laboratory of Botany, Department of Biological
88 Applications and Technologies (Dr A. Kyparissis). Four different extracts were
89 prepared. The aqueous extract (aqueous *Teucrium polium* extract, ATP) was obtained
90 from 20 g of plant that were boiled with 100 mL distilled water for 1 hour and
91 filtered. The water was totally removed using a freeze-dryer (CHRIST Alpha 1-2,
92 Germany) to obtain the dried extract. The methanol extract (methanol *Teucrium*
93 *polium* extract, MTP) was prepared form 10 g of plant that were extracted with 200
94 mL of methanol in a Soxhlet apparatus for 6 hours. The extract was concentrated in a
95 rotary evaporator and kept in sealed dark flasks after minutes of nitrogen flushing.
96 The same procedure was repeated and subsequent extraction with solvents of
97 increasing polarity in a Soxhlet apparatus was carried out. The sequence of the
98 solvents was hexane, ethyl acetate and methanol and, thus, the ethyl acetate (EATP)
99 and a second methanol extract (MTPb) were obtained.

100

101 *2.3. 1,1-Diphenyl-2-picrylhydrazyl assay (DPPH assay) and total phenolic content*
102 *assessment*

103 In order to evaluate the scavenging activity using the $DPPH^{\bullet}$ assay [21], 2mL of
104 each sample were mixed with 1 mL of 0.3 mM solution of $DPPH^{\bullet}$ in methanol, and

105 the absorbance of the mixture was measured after 30 min incubation time in the dark
106 at 517 nm . Several concentrations of each sample were tested and the % of free
107 radical scavenging activity was determined by the following equation:
108 % Scavenging activity = 100-[(Ab of sample –Ab of blank) x 100/Ab of control]
109 EC_{50} values are referred to the lower concentration of the compounds under study
110 required for the 50% of the antioxidant reactivity.
111 For the total phenol content assessment 0.2mL of each sample were mixed
112 with 4.8 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent and the mixture
113 was left for 3 minutes. 1 ml of aqueous saturated Na_2CO_3 solution was added under
114 stirring and after dilution with distilled water to a total volume of 10 mL the mixture
115 was left to incubate for 1h at room temperature in the dark. The absorbance of the
116 solution at 725 nm was then measured. A calibration curve of gallic acid at various
117 concentrations 50 to 400 mg/kg was obtained and the content of total phenols was
118 referred as gallic acid equivalent (GAE) (mg/kg of dry plant extract). $DPPH^{\bullet}$ and total
119 phenol content measurements were carried out on an UV/Vis spectrophotometer
120 Jenway 6505 (Essex, England).
121
122 *2.4. Instrumentation and chemical analysis using 1D and 2D NMR, LC-SPE-NMR*
123 *and MS*
124 NMR data acquisition was carried out on a Bruker AV-500 spectrometer
125 (Bruker BioSpin, Rheinstetten, Germany). ^1H NMR spectra of the extracts were
126 obtained using 20 mg of material in 0.6 mL of solution in CD_3CN. The NMR system

was controlled by software TopSpin 1.3. Chemical shifts are expressed in δ (parts per million) referenced to the solvent peak (1.94 ppm for CD_3CN). ^1H NMR and ^1H-^1H correlated spectroscopy (^1H-^1H COSY) were used to assign signals. The parameters for the ^1H-^1H COSY spectrum were as follows: spectral width of 5000 Hz, acquisition time was 0.41 s, relaxation delay of 1 s, and 32 transients were acquired (12 dummy scans) for each of 512 increments.

LC-UV-SPE-NMR measurements were carried out on a chromatographic separation system consisting of an Agilent G13311A solvent delivery pump and a Bruker DAD UV detector (Bruker BioSpin, Rheinstetten, Germany). The samples were injected using an Agilent G1311A autosampler with a 100 µL loop. The Bruker/Spark Prospect 2 solid phase extraction unit (Bruker BioSpin and Spark, Emmen, The Netherlands) was used to automatically trap the chromatographic peaks on Hyspere GP cartridges (2 mm i.d, 10-12 µm) after postcolumn addition of water using a Knauer K 120 HPLC pump (Berlin, Germany). The trapped peaks were dried with dry nitrogen gas and eluted with deuterated acetonitrile into a Bruker AV-500 NMR spectrometer equipped with 3 mm LC SEI ^{13}C-^1H probe head with an active volume of 60 µL from Bruker BioSpin. The NMR system was controlled by software TopSpin 1.3.

The chromatographic separation was carried out on a 250 mm x 4.6mm i.d., 5-μm, Discovery C18 column from Supelco (Pennsylvania, USA). The flow rate was 0.6 mL/min, and the injection volume was 20 μL. Gradient elution was performed using solvent A (0.1% acetic acid in water) and solvent B (acetonitrile) with the

following linear gradient: at 0 min 15% B, at 20 min 15% B, at 30 min 40% B, at 40 min 80% B, at 45 min 80% B and at 55 min 5% B. During the chromatographic separation, water with 0.1% acetic acid was added to the eluent leaving the column with a second pump (make-up pump), to provide proper retention of the peaks under study on the SPE cartridges (Spark, Holland). When the chromatographic separation and trapping was completed, the cartridges were dried with nitrogen gas for 30 min to remove the residual solvents, and subsequently, the analytes were transferred to the NMR probe head with CD_3CN. The total amount of CD_3CN that was used for the elution and transfer of each sample was 380 μL. All cartridges were automatically reconditioned with 1 mL of acetonitrile at a flow rate of 1 mL/min after use and equilibrated with 1 mL of water at a flow rate of 1 mL/min before each use. Chemical shifts are expressed in δ (parts per million) referenced to the solvent peak. ^{1}H NMR spectra were acquired for all compounds trapped; 2D ^{1}H-^{1}H COSY, ^{1}H-^{1}H total correlated spectroscopy (^{1}H-^{1}H TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (^{1}H-^{13}C HSQC) and ^{1}H -^{13}C heteronuclear multiple bond correlation (^{1}H-^{13}C HMBC) were obtained when necessary. After their NMR data acquisition the samples were collected and were further analyzed by mass spectrometry.

ESI-MS in the negative mode was performed using an Agilent G2455A ion trap mass spectrometer equipped with Agilent software (Agilent Technologies, Waldbronn, Germany). The collected samples were dissolved in acetonitrile and infused in the ESI source with flow rate 100 μL/min. Operating conditions were: accumulation time, 300 ms; dry temperature, 350 ^{o}C; capillary voltage, 3500V;

nebulizer, 30 psi; dry gas, helium at 8 L/min. Ion trap full scan analysis was conducted from *m*/*z* 50 to 1300 with an upper fill time of 200 ms.

2.5. On-line DPPH• radical-scavenging analysis

The on-line DPPH• radical-scavenging analysis was performed by a hyphenated LC-DPPH• method introduced by van Beek' group [22]. The instrumentation used was based on the LC–UV-SPE-NMR system in which several modifications were applied. In particular, the make-up pump that is introduced to the LC-UV-SPE-NMR system for postcolumn addition of water, was also used in the LC-DPPH set up for DPPH• solution delivery (Fig.1).

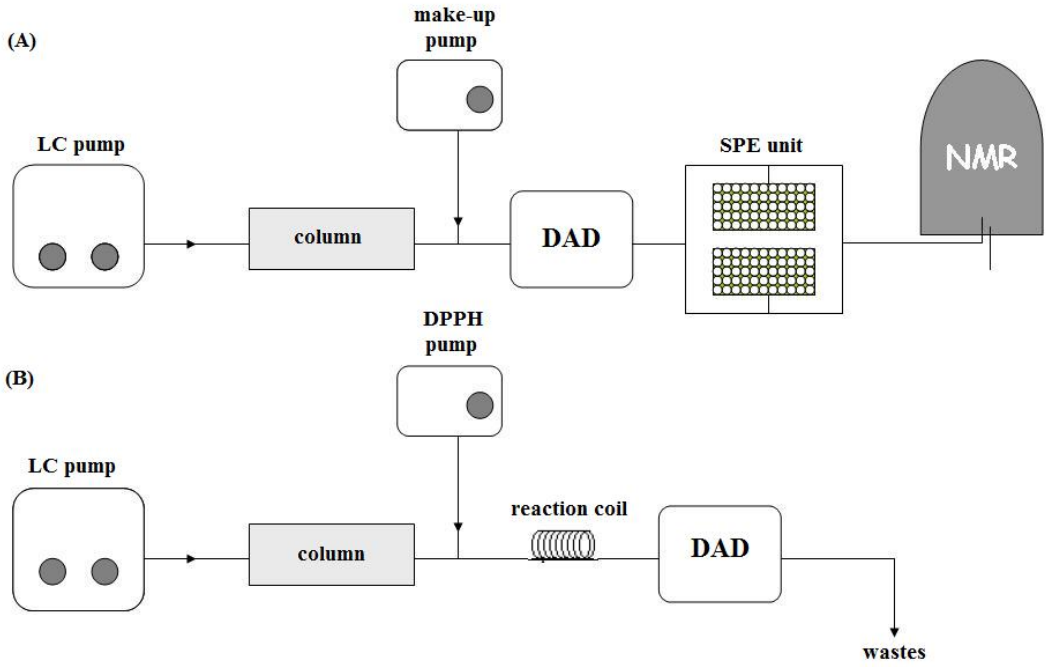


Figure 1.: LC-SPE-NMR and LC-DPPH set-up.

A reaction coil of Teflon was then placed (15mX0.3mm i.d., tubing of a larger inner diameter is unsuitable due to undesirable peak broadening) to ensure adequate reaction of the eluents and $DPPH^{\bullet}$. The $DPPH^{\bullet}$ solution ($5x10^{-5}$ mM) was inserted to the eluents at a flow rate of 0.2 mL/min. The separated analytes reacted post-column with the $DPPH^{\bullet}$ and the bleaching of the $DPPH^{\bullet}$ solution was recorded at 517 nm using the Bruker DAD UV detector of the LC-UV-SPE-NMR system.

3. Results and Discussion

3.1. $DPPH^{\bullet}$ radical-scavenging activity and total phenol content of Teucrium polium extracts

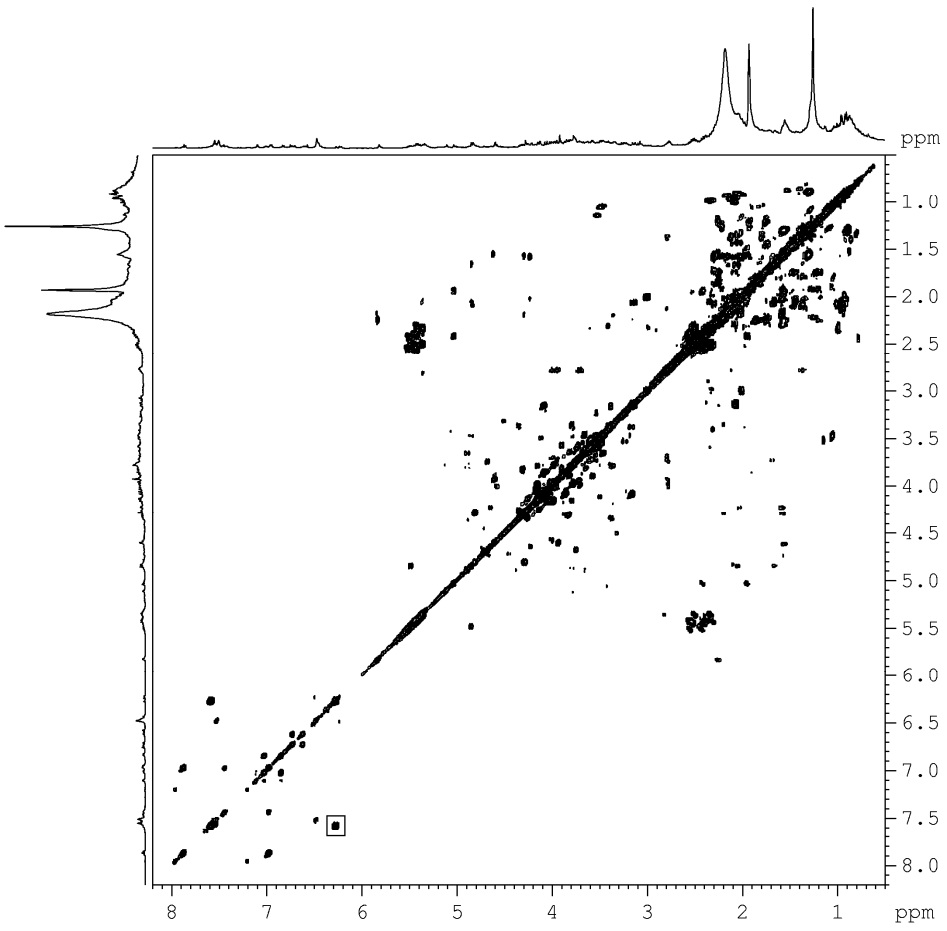
In order to primarily characterize *Teucrium polium* extracts, the $DPPH^{\bullet}$ radical scavenging test and total phenol content measurements were carried out. It is widely accepted that higher concentration levels in phenolics corresponds to lower EC_{50} values, since phenolics are usually responsible for radical scavenging activity. The results showed that the methanol extracts provided higher scavenging activity with EC_{50} of 52 µg/mL for MTP and 58 µg/mL for MTPb. The scavenging activity of aqueous and ethyl acetate extracts showed EC_{50} values of 74 and 212 µg/mL, respectively. The phenolic content study revealed that the aqueous extract was the most rich in phenolics, providing a phenolic content equivalent to 164 mg of gallic acid per kg of extract. Methanol extracts had a similar phenolic content corresponding to 144 and 149 mg/kg of extract, while ethyl acetate reached 92 mg/kg of extract. To elucidate the correlation between the phenolic content of *Teucrium extracts* and their

radical scavenging activity linear regression analysis was performed, quoting the correlation coefficient (*r*). It was found that the phenolic content of the extracts showed strong correlation with their $DPPH^{\bullet}$ scavenging activity (r=0.92), which indicates that the scavenging activity is attributed to the phenolic group of phytochemicals.

3.2 1D and 2D NMR studies of teucrium extracts

The next step was to identify the phytochemicals present in *Teucrium polium* extracts. The analysis of a complex plant extract usually includes fractionation of the mixture, separation of the individual components using liquid chromatography, and structure elucidation using various spectroscopic methods (UV, IR, NMR, MS). In an initial attempt to characterize the phytochemical content of *Teucrium polium* extracts, 1D and 2D NMR spectroscopy was used, which, according to previous studies of our group, reveal the presence of phenolic subgroups within a short analysis time, avoiding the time consuming separation step [23-25]. Comparison of the ^{1}H-NMR spectra of *Teucrium* extracts showed that the aqueous and methanol extracts share a similar ^{1}H-NMR profile, while that of ethyl acetate is significantly different. In particular, the aromatic spectral region of the aqueous and methanol extracts exposed the spin system of a cinnamic acid moiety group that can be easily recognized due to the characteristic set of doublets at 7.6 and 6.3 ppm, and their J= 15.8 Hz. 2D ^{1}H-^{1}H DQF-COSY spectra of the extracts clearly confirmed the presence of a cinnamic acid moiety as suggestively shown for MTP in Fig. 2. The cinnamic acid moiety

227 compound seems to be the main component of all samples; the respective cross peak
228 on the 2D map of the EATP is weak. Taking into consideration the crowed aliphatic
229 region of the 2D spectra, the presence of glycosides was also pointed out.



230

231 Figure 2.: 500 MHz ^{1}H-^{1}H DQF-COSY spectrum of the MTP extract. The cinnamic
232 (caffeic) acid moiety is revealed.

233

234

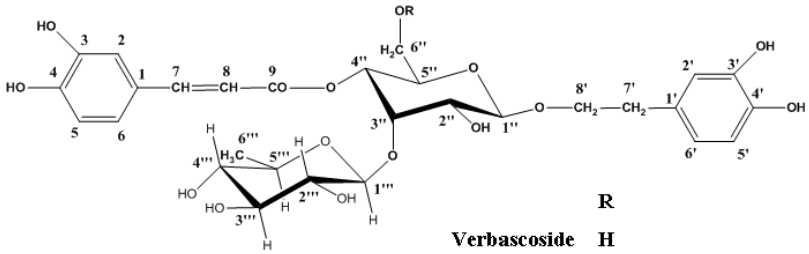
Previous work has shown that the resonances at the region of 11-14 ppm of the ^{1}H-NMR spectrum are attributed to the strong hydrogen bond between OH(5) and CO(4) of the flavonoid structure. The corresponding region of the ^{1}H NMR spectra of *Teucrium* extracts revealed two main signals indicating two differerent flavonoid species that resonate at ~13 and 12.8 ppm respectively. It is notable that the OH(5) resonance is more deshielded in the flavones compared to that of the flavonols. This can be ascribed to the presence of the OH(3) group in flavonols that attenuates the electron density of the CO(4) and, thus, decreases the strength of the intramolecular hydrogen bond.

Thus, 1D and 2D NMR data acquisition disclosed the differentiation of aqueous, methanol and ethyl acetate *Teucrium* extracts, the presence of a main component possessing a cinnamic acid moiety, the presence of glycosides and the presence of two flavonoids species within a short analysis time.

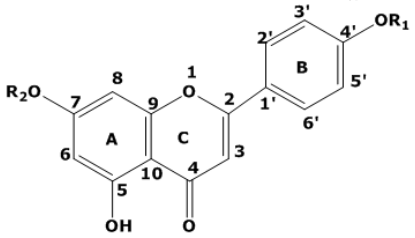
3.3. Separation and Identification of the Teucrium phytochemicals

The chromatographic separation of the *Teucrium* extracts confirmed the differentiation of aqueous, methanol and ethyl acetate samples indicated by NMR. In particular, ATP, MTP, and MTPb share a similar -8 compound- chromatographic profile, while EATP seems to contain mainly the compounds denoted as peaks 6, 7 and 8, in higher concentration levels compared with those of aqueous and methanol extracts. Thus, compounds 1-5 were analyzed with LC-SPE-NMR instrumentation using the MTP sample and for the compounds 6-8 the EATP extract was preferred.

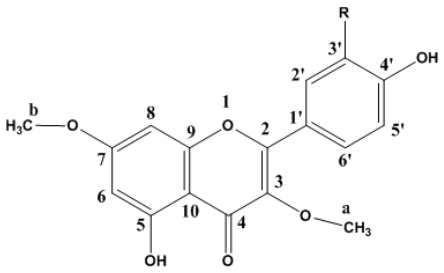
^{1}H-NMR spectrum of the predominant peak 2 pointed the spin system of caffeic acid moiety as well as the characteristic resonance of sugar anomeric protons. Homonuclear and heteronuclear 2D NMR experiments revealed the structure of poliumoside as the main component of *Teucrium* extracts. This phenyl propanoid glycoside has been reported as the typical substance of the *polium* section of the genus *Teucrium* [26]. Its structure consisting of two caffeic acid units conjugated with two rhamnose and one glucose molecules was identified mainly due to 2D ^{1}H-^{1}H COSY, TOCSY, ^{1}H-^{13}C HSQC and HMBC experiments that revealed the spin system of each unit and their in between connection. The denoted peak 1 was then attributed to verbascoside since the signals of the second rhamnose unit are missing on the ^{1}H-NMR spectrum. MS data confirmed the assignment of both phenyl propanoid glycosides. Peaks 3-6 were attributed to apigenin and apigenin derivatives. Comparison of their NMR spectra demonstrates the position of substitution of each compound and leads to structure elucidation of all apigenin derivatives. Peaks 7 and 8 were assigned as dimethoxy derivatives of quercetin and kaempferol, respectively. In Fig. 3 the structures of the identified molecules are shown.



	R
Verbascoside	H
Poliumoside	Rhamnose



	R_1	R_2
Apigenin	H	H
Apigenin-7-O-glucoside	H	Glu
Apigenin-7-O-rutinoside	H	Rut
Apigenin-4'-O-glucoside	Glu	H



	R
3,7-dimethoxykaemferol	H
3,7-dimethoxyquercetin	OH

Figure 3.: Structures of the compounds present in *Teucrium polium* extracts.

All spectroscopic data are quoted below.

Verbascoside (1): Negative ESI-MS: *m/z* 623 [M-H]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 7.61 (1H, d, *J*=15.8Hz, H-7), 7.13 (1H, d, *J*=1.8Hz, H-2), 7.06 (1H, dd, J_1=8.0Hz, J_2=1.9Hz, H-6), 6.87 (1H, d, *J*=8.3Hz, H-5), 6.78 (1H, d, *J*=1.9 Hz, H-2'), 6.76 (1H, d, *J*=8.0 Hz, H-5'), 6.66 (1H, dd, J_1=8.0Hz, J_2=1.9Hz, H-6'), 6.29 ((1H, d, *J*=15.8Hz, H-8), 5.15 (1H, d, *J*=0.9 Hz, anomeric proton of rhamnose H-1'''), 4.38 (1H, d, *J*=7.8 Hz, anomeric proton of glucose H-1''), 4.02 (1H, dd, J_1=9.6 Hz, J_2=2.5 Hz, H-8'b),

3.73 (1H, dd, J_1=9.6 Hz, J_2=2.5 Hz, H-8'a), 4.88-3.30 (sugar protons), 2.81 (1H, t, *J*=7.1 Hz, H-7'), 1.06 (1H, d, *J*=6.2 Hz, H-6''').

Poliumoside (2): Negative ESI-MS: *m/z* 769 [M-H], 623 [M-Rhamnose]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 7.61 (1H, d, *J*=15.8Hz, H-7), 7.13 (1H, d, *J*=1.8Hz, H-2), 7.06 (1H, dd, J_1=8.0Hz, J_2=1.9Hz, H-6), 6.87 (1H, d, *J*=8.3Hz, H-5), 6.77 (1H, d, *J*=1.9 Hz, H-2'), 6.75 (1H, d, *J*=8.0 Hz, H-5'), 6.66 (1H, dd, J_1=8.0Hz, J_2=1.9Hz, H-6'), 6.29 ((1H, d, *J*=15.8Hz, H-8), 5.14 (1H, d, *J*=0.9 Hz, anomeric proton of rhamnose H-1'''), 4.64 (1H, d, *J*=1.2 Hz, anomeric proton of rhamnose H-1''''),4.37 (1H, d, *J*=7.8 Hz, anomeric proton of glucose H-1''), 3.97 (1H, dd, J_1=9.6 Hz, J_2=2.5 Hz, H-8'b), 3.68 (1H, dd, J_1=9.6 Hz, J_2=2.5 Hz, H-8'a), 4.88-3.30 (sugar protons), 2.81 (1H, t, *J*=7.1 Hz, H-7'), 1.16 (1H, d, *J*=6.2 Hz, H-6''''), 1.06 (1H, d, *J*=6.2 Hz, H-6''').

Apigenin-7-*O*-rutinoside (3): Negative ESI-MS: *m/z* 577 [M-H]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.94 (1H, s, OH-5), 7.89 (2H, d, *J*=8.9 Hz, H-2', H-6'), 6.98 (2H, d, *J*=8.9 Hz, H-3', H-5'), 6.72 (1H, d, *J*=2.4 Hz, H-8), 6.64 (1H, s, H-3), 6.46 (1H, d, *J*=2.0 Hz, H-6), 5.01 (1H, d, *J*=7.5 Hz, anomeric proton of glucose H-1''), 4.65 (1H, d, *J*=1.1 Hz, anomeric proton of rhamnose H-1'''), 4.4-3.1 (sugar protons), 1.12 (1H, d, *J*=6.3 Hz, H-6''').

Apigenin-7-*O*-glucoside (4): Negative ESI-MS: *m/z* 431 [M-H], 269 [M-glucose]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.93 (1H, s, OH-5), 7.90 (2H, d, *J*=8.9 Hz, H-2', H-6'), 6.98 (2H, d, *J*=8.9 Hz, H-3', H-5'), 6.75 (1H, d, *J*=2.4 Hz, H-8), 6.65 (1H, s, H-3), 6.45 (1H, d, *J*=2.1 Hz, H-6), 5.04 (1H, d, *J*=7.6 Hz, anomeric proton of glucose H-1''), 4.0-3.3 (sugar protons).

Apigenin-4'-*O*-glucoside (5): Negative ESI-MS: *m/z* 431 [M-H], 269 [M-glucose]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.89 (1H, s, OH-5), 7.97 (2H, d, *J*=9.1 Hz, H-2', H-6'), 7.20 (2H, d, *J*=9.1 Hz, H-3', H-5'), 6.50 (1H, d, *J*=2.2 Hz, H-8), 6.65 (1H, s, H-3), 6.23 (1H, d, *J*=2.2 Hz, H-6), 5.03 (1H, d, *J*=7.6 Hz, anomeric proton of glucose H-1''), 4.0-3.3 (sugar protons).

Apigenin (6): Negative ESI-MS: *m/z* 269 [M-H]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.93 (1H, s, OH-5), 7.87 (2H, d, *J*=8.8 Hz, H-2', H-6'), 6.96 (2H, d, *J*=8.8 Hz, H-3', H-5'), 6.58 (1H, s, H-3), 6.48 (1H, d, *J*=1.9 Hz, H-8), 6.22 (1H, d, *J*=2.0 Hz, H-6).

5,3',4'-trihydroxy-3,7-dimethoxyflavone (7): Negative ESI-MS: *m/z* 329 [M-H]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.89 (1H, s, OH-5), 7.45 (1H, s, H-2'), 7.43 (1H, s, H-6'), 6.96 (1H, d, *J*=8.6 Hz, H-5'), 6.76 (1H, s, H-8), 6.60 (1H, s, H-6), 3.93 (3H, s, 7-OCH_3), 3.77 (3H, s, 3-OCH_3).

5,4'-trihydroxy-3,7-dimethoxyflavone (kumatakenin) (8): Negative ESI-MS: *m/z* 313 [M-H]; ^{1}H-NMR (500 MHz, CD_3CN) δ: 12.89 (1H, s, OH-5), 7.88 (2H, d, *J*=8.7 Hz, H-2', H-6'), 6.97 (2H, d, *J*=8.7 Hz, H-3', H-5'), 6.76 (1H, s, H-8), 6.62 (1H, s, H-6), 3.93 (3H, s, 7-OCH_3), 3.77 (3H, s, 3-OCH_3).

3.4. On-line DPPH$^{\bullet}$ for antioxidant pinpointing

In an attempt to attribute the antioxidant activity of *Teucrium* extracts to the identified phytochemicals the on-line DPPH$^{\bullet}$ set-up described in Fig. 1 was used. This method combines a separation technique with fast post-column chemical detection that can rapidly pinpoint the active compounds in complex mixtures [27]. In Fig. 4 the

chromatographic analysis of MTPb together with the respective DPPH• chromatograms depicted as negative peaks, is presented.

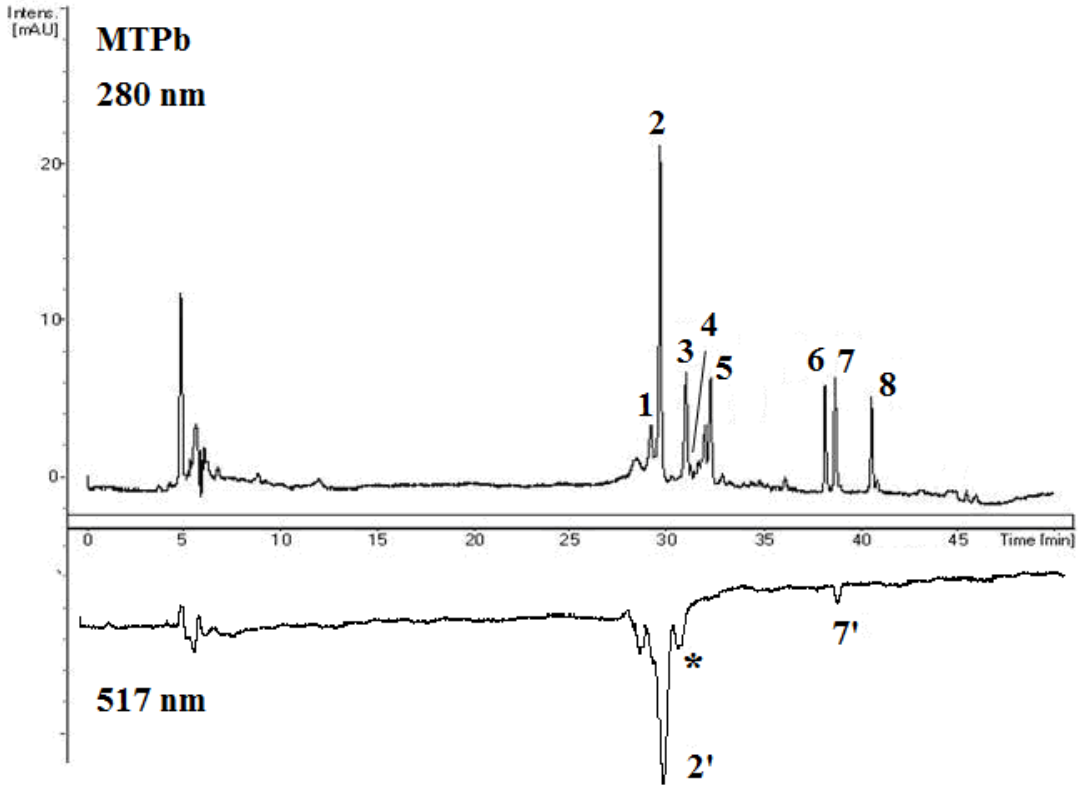


Figure 4.: Chromatographic analysis of MTPb extract together with the respective DPPH• chromatograms depicted as negative peaks.

The targeted antioxidant components are easily indicated without the need of laborious isolation procedures. Fig. 4 clearly indicates that poliumoside is the dominant active compound of the extracts. Concerning the flavonoids present in *Teucrium* extracts apigenin and its derivatives are not expected to evoke significant bleaching due to its slow reaction with DPPH• [28]. Indeed, the peaks no 3-6 do not seem to react with the radical solution under the experimental conditions used. The

345 dimethoxy quercetin derivative is pinpointed as radical scavenger while kumatakenin
346 is not active. This is due the single hydroxyl substitution on the B-ring, which in
347 combination with the methoxy substitution in position 3 is considered to yield lower
348 scavenging activity [28].

349 To elucidate the contribution of each one of the components to the overall
350 scavenging activity of *Teucrium* extracts each induced bleaching was expressed as the
351 % total negative peak area (Table 1).

352

353 Table 1. Off-line and on-line scavenging activity of *Teucrium extracts.* The on-line
354 measurements are expressed as the % total negative peak area.

355

Sample	EC_{50} µg/mL (off-line DPPH)	Total negative peak area of extracts	% of total negative peak area of antioxidants				
			verbascoside	poliumoside	3,7-dimethoxy quercetin	Unknown compound 1[a]	Unknown compound 2[b]
ATP	74	4733	13	65	ND	22	ND
MTP	52	5484	11	67	5	17	ND
MTPb	58	4826	12	67	3	18	ND
EATP	212	3258	-	57	23	10	10

356 *ND: not detected*

357 [a]*: the uknown compound 1 coelute at 280 and 254 nm with apigenin-7-O-rutinoside*

358 [b] *the unknown compound 2 is present only in EATP extract*

359

360 As indicated, poliumoside is the most active component in all extracts holding
361 up from 57-67% of extracts total activity. All extracts have a negative signal at the
362 point where apigenin-7-*O*-rutinoside is eluted. Taking into cosideration the slow

363 reaction of apigenin with DPPH, in combination with the relative test with stadard
364 apigenin-7-*O*-rutinoside, it is suggested that an unknown compoud is co-eluted
365 together with peak 3 that has scavenging activity. A second unknown peak which is
366 present only in the EATP sample also seems to act as radical scavenger possessing the
367 10% of EATP total activity.

368 In addition, the correlation between off-line and in on-line DPPH
369 measurements was investigated. The EC_{50} values obtained from the off-line
370 experiments were compared with the total negative peak areas of the *Teucrium*
371 extracts and a high correlation was found (r = 0.96). The above results clearly suggest
372 this method as a simple and easy tool to pinpoint the radical scavengers in a complex
373 mixture such as those of plant extracts. Considering that the antioxidant potency is a
374 well established biomarker**,** which indicates beneficial biological effects of plant
375 derived substances it can be widely utilized to reveal the possible bioactive
376 components.

377

378 **4. Conclusions**

379 The results of the *Teucrium polium* extracts study on both composition and
380 scavenging activity showed that poliumoside is the most abundant and active
381 component of the extracts. The daily and often unconscionable use of various herbal
382 decoctions necessitates the development of rapid and accurate analytical techniques.
383 Since the antioxidant potency is a well-established biomarker**,** the above strategy of

384 combing the LC-SPE-NMR and the on-line DPPH method, which can pinpoint the
385 active phytochemicals without the tedious isolation step is highly recommended.
386
387 **Acknowlegments**
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LC-SPE-NMR es una herramienta de enorme potencia para la separación de muestras complejas y posterior identificación de sus componentes. Se combinan la capacidad de separación de HPLC (se dan lugar a métodos robustos y fiables), con NMR que proporciona datos definitivos acerca de la estructura de los compuestos a los que nos enfrentamos. El único problema que surge es la sensibilidad de NMR, por ello necesitamos concentrar las fracciones de los analitos que entran en NMR, empleando para ello el módulo de extracción en fase sólida.

En este trabajo se han logrado separar e identificar 8 compuestos pertenecientes a la fracción fenólica de *T. polium* y se ha medido la actividad antioxidante tanto de la fracción como de cada uno de los compuestos. De este modo se ha podido demostrar que el poliumoside es el compuesto más abundante en esta fracción y a su vez el que presenta mayor actividad antioxidante.

Este tipo de análisis resulta de gran interés sobretodo en casos en los que la matriz analizada sea de uso común en la dieta diaria, como resulta ser en el caso de *T. polium* amplamente usada en la zona mediterránea como hierba medicinal y consumida en la mayoría de los casos en forma de infusión.

Anexo

Caracterización de la fracción fenólica del aceite de oliva mediante LC-SPE-NMR

Como ya se ha resaltado anteriormente, los compuestos fenólicos, y en particular los compuestos fenólicos del aceite de oliva, poseen un gran interés debido a todas las propiedades beneficiosas para la salud que se le han atribuido.

En los últimos años se han hallado incluso grandes indicios de que actúan sobre ciertos tipos de cáncer, entre los que encuentra el cáncer de mama [279,280].

Ya que el grupo donde se ha desarrollado mi tesis está vinculado a estos estudios y que ésta ha estado enfocada al estudio de estos compuestos (teniendo un gran peso el bloque dedicado al aceite de oliva), se planteó realizar un estudio más profundo de la fracción fenólica del aceite. De este modo podríamos ahondar aún más en el conocimiento de las estructuras de los compuestos que constituyen esta fracción (muchos de los cuáles son todavía desconocidos).

En la actualidad existen trabajos enfocados directamente en esta línea [281,282] en el que se han caracterizado la mayoría de los compuestos pertenecientes a la fracción fenólica de aceite, pero no se ha llegado a la identificación de aquellos compuestos todavía desconocidos.

Este trabajo se comenzó durante el periodo de estancia desarrollado en el Servicio de Resonancia Magnética Nuclear de la Universidad Autónoma de Barcelona, y está aún sin concluir. A pesar de esto y por haber sido llevado a cabo durante el disfrute del periodo predoctoral, se ha querido incluir un esbozo del trabajo realizado y de las líneas futuras a seguir.

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1. Materiales y métodos

1.1 Muestra

La muestra empleada fue un aceite de oliva virgen extra de la variedad Cornezuelo que previamente había sido analizado y mostraba un alto contenido fenólico.

1.2. Extracción de la fracción fenólica

Se llevó a cabo siguiendo el protocolo puesto a punto durante esta tesis y que se expone en el capítulo 2 [283].

1.3. Sistema LC-SPE-RMN

El equipo de HPLC empleado fue un Agilent 1200 con cuatro bombar dotado de un desgasador a vacío y un inyector automático (Agilent, Waldbronn, Germany), un detector de batería de diodos de Bruker-Biospin (Rheinstetten, Germany) y un espectrómetro de masas Esquire 6000 ion trap (IT)-mass spectrometer (Bruker Daltonics, Bremen, Germany). Las fracciones obtenidas de la separación mediante HPLC se recolectaron en una unidad Prospekt II peak-trapping (Bruker-Biospin/Spark Holland) equipada con cartuchos 10 × 2 mm Hysphere (Spark Holland). Los cartuchos fueron acondionados previamente con agua, después de atrapar las fracciones de interés fueron secados con nitrógeno y la transferencia de su contenido hacia el equipo de RMN se realizó con acetonitrilo deuterado. El LC-SPE se acopló a un espectrómetro Bruker AVANCE 500 con un CryoFit con celda de 60 µl de volumen activo (Bruker-Biospin). El espectrómetro Bruker AVANCE 500 estaba equipado con una cryosonda triple 5 mm TCI de detección inversa $^{1}H/^{13}C/^{15}N$. El software empleado para controlar el proceso de manera automatizada fue HyStar 2.3 (Bruker Daltonics, Bremen, Germany).

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1.4. Separación cromatográfica de los compuestos fenólicos

La separación cromatográfica se realizó empleando el siguiente método cromatográfico: columna Gemini C18 5 μm, 25 x 3 cm, usando como Fase A agua con el 0.1% de ácido acético y como Fase B acetonitrilo con el 0.1% de ácido acético, y aplicando el siguiente gradiente: de 0 a 20 min, 95% (A):5% (B) a 80% (A):20% (B); de 20 a 30 min, 80% (A):20% (B) a 70% (A):30% (B); de 30 a 40 min, 70% (A):30% (B) a 70% (A):30% (B); de 40 a 50 min, 70% (A):30% (B) a 65% (A):35% (B); de 50 a 60 min, 65% (A):35% (B) a 50% (A):50% (B); de 60 a 70 min, 50% (A):50% (B) a 5% (A):95% (B); de 70 a 75 min, 5% (A):95% (B) a 95% (A):5% (B). El flujo se fijó a 0.5 ml/min y las inyecciones fueron de 20 μl. Los cromatogramas se registraron las longitudes de onda de 240 y 280 nm mediante un detector DAD, a la vez que registró el espectro de masas (MS (IT)).

1.5. Atrapado de compuestos

Una vez elegidas las condiciones óptimas de trabajo para la separación cromatográfica se comenzó a atrapar en los diferentes cartuchos del módulo de extracción en fase sólida (Prospekt II peak-trapping). En primer lugar se escogieron las fracciones de tiempo en las que se quería atrapar (Figura apartado 2), intentando que éstas fuesen lo más simples posibles y lo más interesantes posibles, teniendo en cuenta los datos de las masas obtenidas por espectrometría de masas.

Antes de proceder al atrapado de compuestos los cartuchos se sometieron a un proceso de acondicionado y equilibrado:

- Acondicionado: 500 μl de CH_3CN.
- Equilibrado: 500 μl de H_2O.

Cada fracción se atrapó entre 5 y 10 veces dependiendo de la concentración de compuesto de cada una de ellas.

1.6. Experimentos NMR

Espectros en una y dos dimensiones fueron registrados mediante el espectrómetro Bruker AVANCE 500 operando a 500.13 y 125.76 MHz para ^{1}H y ^{13}C respectivamente. La temperatura de la celda ha sido de 26 $\pm$ 1 ºC.

Se adquirieron experimentos de resonancia magnética nuclear del crudo de extracto de aceite de oliva, así como de las diferentes fracciones de éste una vez separadas por HPLC y atrapadas en los cartuchos de SPE.

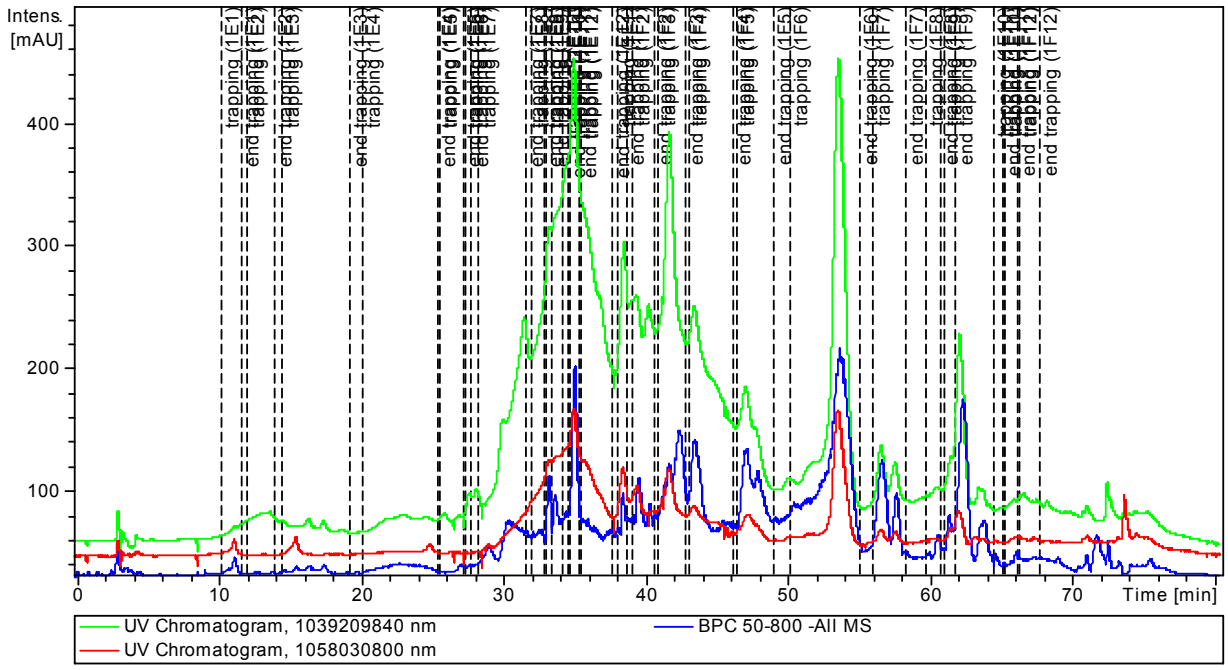
Se registraron experimentos de NMR de ^{1}H con número de barridos que oscilaba entre 64 y 3072 barridos, dependiendo de la intensidad de los picos en el cromatograma, es decir de la concentración de analito en la celda.

En los casos en que se consideró necesario las señales residuales del disolvente (CD_2HCN y trazas de H_2O) fueron suprimidas mediante una secuencia de pulsos 1D NOESY para la doble supresión de disolvente (secuencia "LC1PNF2" de la librería Bruker). La señal fid se adquirió con 16384 puntos, 12019.23 Hz de ancho espectral, 2 s de tiempo de recuperación y 80 ms de tiempo de mezcla. Para aquellas fracciones de interés en las que la concentración de analito era mayor también se registró la correlación TOCSY (Total Correlation Spectroscopy) sensible a la fase y con supresión multiple de disolvente [284] (secuencia de pulsos "MLEVDCPHWT" de la librería Bruker). Los parámetros de adquisición típicos utilizados fueron 16 barridos, 256 incrementos, 7183.91 Hz de ancho espectral en ambas dimensiones, 2048 puntos para la adquisición de la señal fid, 60 ms de tiempo de mezcla y 2.4 s de tiempo de recuperación.

2. Resultados y discusión

En la siguiente figura se pueden observar los cromatogramas obtenidos mediante UV a las longitudes de onda de 240 y 280 nm y mediante MS.

[284] Bax, A.; Davis, D. G., MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *Journal of Magnetic Resonance* **1985**, 65 (2), 355-360.



Una vez puesto a punto el método cromatográfico el siguiente paso fue aislar los compuestos para su posterior análisis mediante NMR. Aunque la resolución obtenida no fue del todo óptima, se llevó acabo el aislamiento de distintas fracciones del extracto con el objetivo de tener fracciones más simples y así facilitar la caracterización por NMR. Para ello se empleó un sistema de extracción en fase sólida acoplado en línea a la LC-SPE-RMN. De esta forma pudimos conseguir tener algunos de los analitos a una concentración suficiente para posteriormente poder ser analizados mediante RMN. En la figura anterior se pueden observar las fracciones que se aislaron en cada uno de los cartuchos de fase sólida.

A continuación se realizó la transferencia de los compuestos a la celda del espectrómetro de RMN, empleando para ello 390 µl de CD_3CN

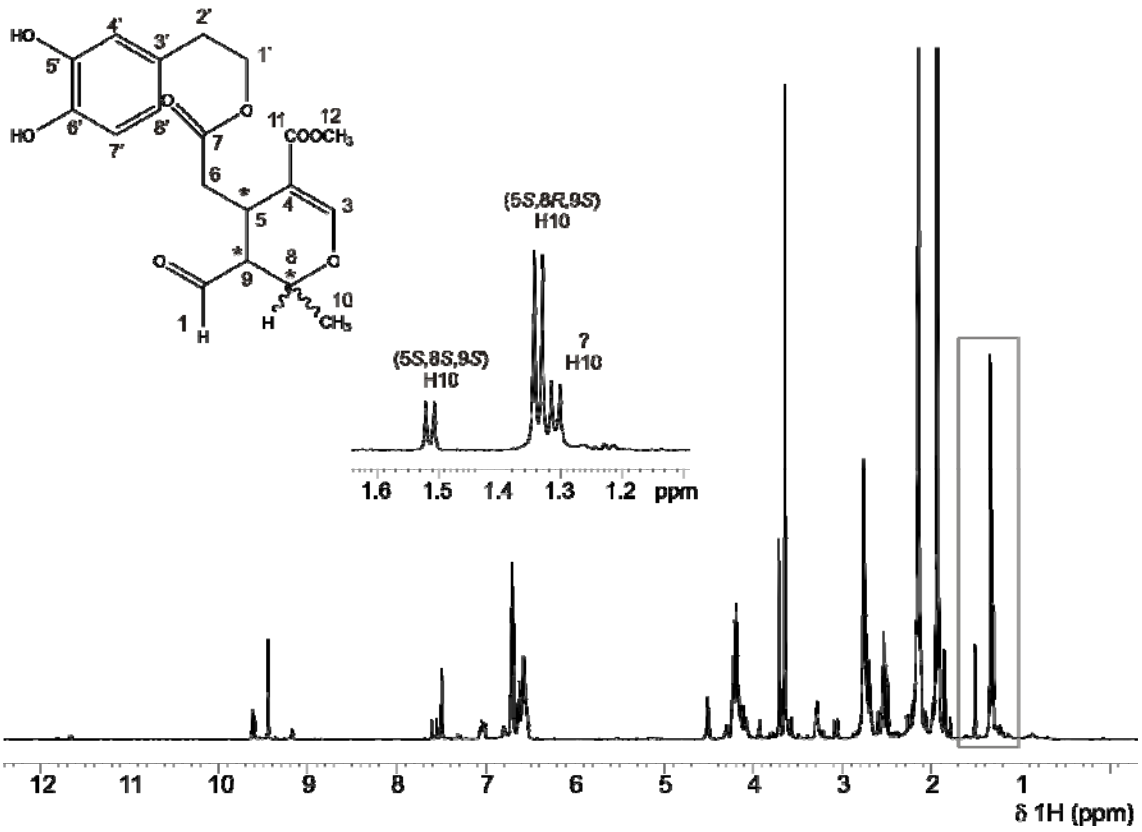
Para cada uno de los compuestos se optimizaron las secuencias de pulsos necesarias para conseguir espectros óptimos. Dependiendo de cada analito, de su concentración y de la complejidad de su estructura se adquirieron diferentes tipos de espectros de RMN: ^{1}H-RMN, COSY, TOCSY, NOESY, HMBC, HSQC, ^{13}C-RMN.

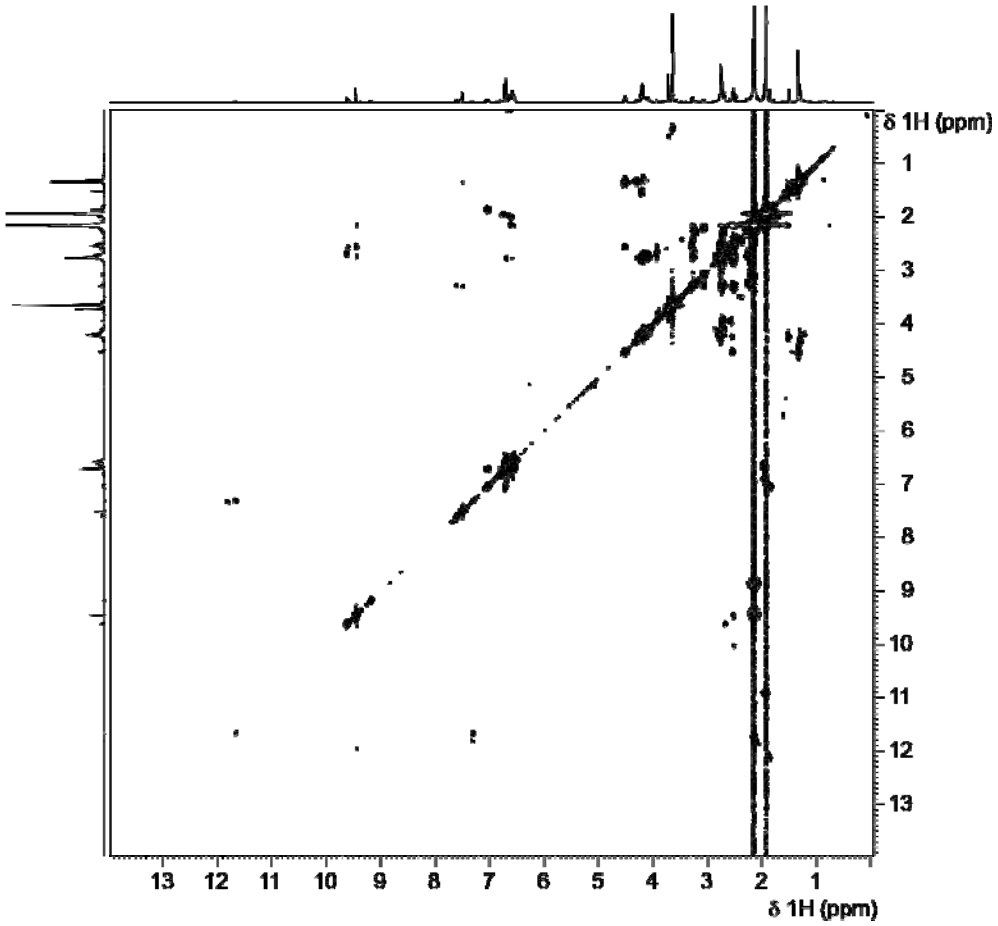
A modo de ejemplo se presentan los resultados obtenidos de la fracción F6 del cromatograma anterior. Mediante MS, a partir del BPC, se detectó en esta fracción uno

de los compuestos más importantes de la fracción fenólica del aceite de oliva como es la forma dialdehídica de la oleuropeína aglicona. Su análisis por NMR ha permitido encontrar una nueva forma diasteroisomérica de ésta no caracterizada anteriormente.

En las siguientes figuras se muestran los espectros más representativos de la fracción F6.

La primera figura consiste en un espectro de RMN de protón, donde se ha ampliado la zona en que aparecen los dobletes de los protones H-10 de los diferentes diasteroisómeros de la forma dialdehídica de la oleuropeína aglicona. Los dobletes que resuenan a δ 1.51 ppm y 1.337 ppm con constante de acoplamiento *J* 6.7 Hz pertenecen a los diastereoisómeros 5*S*,8*S*,9*S* y 5*S*,8*R*,9*S* respectivamente y han sido descritos previamente [281]. No obstante, se observa un nuevo doblete a δ 1.31 ppm con *J* 6.7 Hz. A partir de la información obtenida en el espectro COSY (siguiente figura) y TOCSY se ha podido asignar completamente el espectro de protón de la nueva molécula y concluir que se trata de un diastereoisómero de la oleuropeína aglicona no descrito hasta el momento. El análisis de los resultados de RMN obtenidos de esta fracción así como de otras continúa en fase de desarrollo.





Nuestro objetivo principal era el de caracterizar los compuestos desconocidos hasta el momento, por lo que nos centramos en las fracciones de HPLC donde se encontraban las masas desconocidas. La separación por HPLC no resultó muy satisfactoria, debido en parte a la gran cantidad de muestra inyectada con motivo de poder llegar a recolectar la mayor cantidad de cada analito posible. Esto provocó que el análisis por RMN de estas fracciones no resultase nada fácil, ya que a pesar del proceso de preconcentración llevado a cabo en la estación de extracción en fase sólida, las fracciones recogidas no contenían un único compuesto y las concentraciones conseguidas no eran muy altas por lo que en la mayoría de los casos no resultaba suficiente para su detección por el espectrómetro de NMR. Por ello habría que continuar trabajando en esta dirección intentando buscar otro tipo de columnas, quizás más apropiadas para el caso en cuestión y que permitiesen una mejor separación, simplificando las fracciones recolectadas y los análisis de NMR.

Esta investigación se encuentra aún en fase de desarrollo. Aunque ya se han obtenido muchos datos de NMR y MS que tienen que ser tratados cuidadosamente, y en los que ya nos encontramos trabajando. Como primeros resultados positivos hemos encontrado que en la fracción F6 (mostrada anteriormente) se ha identificado un nuevo isómero de la forma dialdehídica de la oleuropeína aglicona no descrito hasta la fecha. La caracterización completa de este isómero y la del resto de compuestos detectados hasta el momento por NMR se está concluyendo en la actualidad.

Conclusiones

1.- Se ha desarrollado un método para la identificación y cuantificación de algunos de los componentes mayoritarios de la fracción fenólica del aceite de oliva mediante electroforesis capilar en zona acoplada a detección UV-Vis, llegándose a determinar ocho compuestos, de los cuales 4 de ellos (ácido elenólico, ligustrósido aglicona, oleuropeína aglicona y pinoresinol) no habían sido detectados nunca con anterioridad mediante electroforesis capilar. Para la identificación, y debido a la no existencia de estándares comerciales, se han empleado estándares obtenidos mediante HPLC semipreparativa. Esta metodología ha sido aplicada a siete variedades diferentes de aceite de oliva virgen extra monovarietal (Picual, Arbequina, Lechín de Sevilla, Hojiblanca, Lechín de Granada, Picudo y Cornicabra) y se ha tratado de encontrar una relación entre la variedad y su perfil fenólico, estableciendo la posibilidad de seleccionar alguno de los compuestos como marcadores varietales.

2.- Ha sido llevada a cabo la puesta a punto de una metodología de electroforesis capilar en medio no acuoso (NACE) para la identificación de los compuestos fenólicos del aceite de oliva. La gran ventaja de esta metodología es la utilización de disolventes orgánicos, compatibles con la matriz apolar del aceite de oliva, que permiten la inyección directa de aceite en el capilar. Se han identificado 16 compuestos en aceites de oliva virgen extra procedentes de cinco variedades diferentes de aceituna: Arbequina, Picual, Hojiblanca, Lechín de Sevilla y Cornicabra; demostrándose así su potencial en cualquier variedad de aceite de oliva. Los resultados obtenidos con el método NACE desarrollado se compararon con los proporcionados con otros métodos CZE (en medio acuoso) previamente puestos a punto en nuestro grupo.

3.- Aplicando las técnicas separativas a aspectos tecnológicos se ha evaluado el efecto de diferentes sistemas de filtración muy usuales en pequeñas almazaras en el contenido fenólico de aceite de oliva virgen mediante HPLC-DAD-MS. Los aceites utilizados han sido aceites provenientes de diferentes regiones de Italia, producidos en años diferentes, obtenidos mediante tecnologías diferentes y almacenados de forma distinta, permitiéndose una visión amplia y extrapolable a

una gran variedad de aceites. El contenido fenólico se ha correlacionado con el contenido en agua de los aceites, así como con su estabilidad oxidativa. Se ha demostrado que la filtración conlleva un aumento en la concentración de los compuestos fenólicos, pero sin embargo la estabilidad oxidativa disminuye después de ésta. La explicación, aparentemente contradictoria, se encuentra en que la disminución del agua hace que los compuestos fenólicos que permanecen en el aceite sean más susceptibles de ser extraídos mediante la mezcla metanol-agua con la que se realiza la extracción, y que los compuestos fenólicos (de naturaleza polar) tienen mayor actividad en emulsiones de agua en aceite.

4.- El estudio de la influencia del ataque de la mosca del olivo sobre el perfil fenólico del aceite y su comparación con el efecto producido en otros parámetros químicos de éste (acidez libre, peróxidos, ácidos grasos, contenido en agua, poder antioxidante mediante una técnica electroquímica y estabilidad oxidativa) ha llevado a la conclusión de que aunque el perfil fenólico es un parámetro que indica la calidad de un aceite de oliva, no existe una correlación clara entre el porcentaje de compuestos fenólicos y el estado de salud de las aceitunas. De todos los parámetros estudiados, la acidez libre es el parámetro más adecuado para juzgar la calidad de un aceite justo después de su producción ya que no depende de tantos factores externos como el contenido fenólico.

5.- Se ha puesto a punto una metodología mediante electroforesis capilar en zona acoplada a UV-Vis para el estudio de la fracción fenólica de la miel. La miel es una mezcla muy compleja que requiere una exhaustiva preparación de la muestra y una buena separación para poder identificar sus compuestos fenólicos, por ello el uso de un detector universal como el UV-Vis presenta gran cantidad de dificultades y después de la optimización del método sólo pudieron identificarse cinco compuestos. El método se aplicó a mieles de cinco orígenes florales diferentes obteniendo los mismos resultados.

6.- A causa de la problemática para caracterizar el perfil fenólico de la miel mediante UV-Vis, se ha desarrollado una metodología para la determinación de los compuestos fenólicos de la miel mediante electroforesis capilar pero esta vez

acoplada a un detector de masas (ESI-MS (IT)). Con este detector es posible comparar tanto los tiempos de migración de patrones y analitos de la muestra, como las masas de éstos confirmando de manera más fiable la identidad de los compuestos en cuestión y resultando una mejora considerable en comparación con el método por UV-Vis, ya que en este caso se han llegado a detectar hasta 13 analitos, de los cuales 9 se han podido identificar.

7.- Ha sido posible detectar y cuantificar 11 compuestos fenólicos en menos de 15 minutos empleando un sencillo pero potente método electroforético con detección por espectrometría de masas con tiempo de vuelo. Además se ha identificado un nuevo compuesto nunca antes determinado en nuez (ácido 8-hidroxi-2,7-dimetil-2,4-decadien-1,10-dioico 6-O-β-D-glucopiranosilester). Este método es aplicable a diferentes variedades de nuez y permite la comparación del contenido fenólico entre ellas. Los estudios de repetibilidad y reproducibilidad demuestran la robustez de este método.

8.- Se ha puesto a punto un método de HPLC acoplado en línea con un detector NMR para la caracterización de la fracción fenólica de una planta medicinal (*Teucrium polium* o zamarrilla). A esta instrumentación se le ha acoplado en línea un sistema capaz de llevar a cabo medidas de actividad antioxidante (ensayo DPPH). Esta metodología ha permitido la identificación de 8 compuestos fenólicos pertenecientes a la fracción y ha demostrado que el poliumoside es el analito presente en mayor cantidad y con mayor actividad antioxidante. NMR acoplada a HPLC ha resultado tener un gran potencial para la identificación estructural de las fracciones aisladas permitiendo la simplificación mediante la automatización de este tipo de análisis.

Conclusioni

1.- È stato sviluppato un metodo per la identificazione e quantificazione di alcuno dei componenti maggioritari della frazione fenolica dell'olio di oliva mediante elettroforesi capillare con detector UV-Vis, determinando otto composti dei quali 4 (acido elenolico, ligstroside aglicone, oleuropeina aglicone e pinoresinolo) non erano stati ancora rivelati mediante questa tecnica separativa. Per l'identificazione e a causa della mancata disponibilità di standard commerciali, tali composti sono stati ottenuti mediante collezionamento HPLC con colonna separativa. Il metodo elettroforetico è stato applicato per la determinazione della componente fenolica di sette differenti varietà di olio di oliva (Picual, Arbequina, Lechín de Sevilla, Hojiblanca, Lechín de Granada, Picudo e Cornicabra) e i dati ottenuti sono stati elaborati per l'ottenimento di alcuni composti fenolici come marker varietali.

2.- In questo capitolo è stata effettuata la messa a punto di un metodo per l'identificazione dei composti fenolici in olio di oliva mediante elettroforesi capillare in mezzo non acquoso (NACE). Il vantaggio dell'utilizzo di solventi organici, compatibili con la matrice olio, è dato dalla possibilità di iniettare direttamente l'olio in capillare. Sono stati identificati 16 composti in oli extravergini di oliva provenienti da cinque varietà di olive: Arbequina, Picual, Hojiblanca, Lechín de Sevilla e Cornicabra; dimostrando la potenzialità del metodo a discriminare differenti campioni. I risultati ottenuti con il metodo NACE sono comparabili con quelli precedentemente ottenuti in metodologia CZE (mezzo acquoso) sviluppata nel nostro gruppo.

3.- Alcune tecniche separative analitiche, come l'HPLC-DAD-MS, sono state applicate per la valutazione di alcuni aspetti tecnologici come ad esempio l'effetto di differenti sistemi di filtrazione, di comune uso in piccoli frantoi, sul contenuto fenolico di oli di oliva vergini. Gli oli, utilizzati a tale scopo, erano provenienti da differenti regioni italiane, prodotti in anni differenti e ottenuti mediani differenti tecnologie. Il contenuto in composti fenolici è stato relazionato con il contenuto in acqua e con la stabilità ossidativa. È stato dimostrato che con la filtrazione si verifica una concentrazione dei composti fenolici però porta ad una diminuzione della stabilità ossidativa. La spiegazione

dei risultati, apparentemente contraddittori, è data dal fatto che la diminuzione di acqua porta ad una maggiore propensione all'estraibilità dei composti fenolici mediante una soluzione metanolica (utilizzata per l'estrazione) e, ma, i composti fenolici (composti polari) presentano una maggior attività quando presenti in una emulsione acqua-olio.

4.- Lo studio dell'influenza che ha l'attacco della mosca dell'olivo sul profilo fenolico dell'olio e la correlazione con la variazione di altri parametri (acidità libera, numero di perossido, acidi grassi, contenuto in acqua, valutazione elettrochimica del potere antiossidante e stabilità ossidativa mediante ossidazione forzata), ha evidenziato che il profilo fenolico è un parametro di qualità dell'olio di oliva, ma non relazionabile con lo stato di integrità dell'oliva. Tra tutti i parametri studiati, l'acidità libera è risultato essere il parametro più adeguato alla valutazione della qualità di un olio durante la sua conservazione in quanto non dipende da altri fattori esterni come avviene per il contenuto fenolico.

5.- È stata messa a punto una nuova metodologia di separazione elettroforetica con detector UV-Vis per lo studio della frazione fenolica del miele. Il miele si presenta come una matrice complessa che richiede un'accurata preparazione del campione e una buona separazione strumentale per poter identificare i composti fenolici. Per questo motivo, l'impiego di un detector universale come quello UV-Vis ha presentato una serie di difficoltà a causa delle quali è stato possibile identificare solo cinque composti fenolici. Tale metodo è stato applicato a cinque mieli di diversa origine florale ottenendo gli stessi risultati.

6.- Considerata la difficoltà di identificare i composti fenolici del miele mediante detector UV-Vis, è stata effettuata la messa a punto di una nuova metodologia di separazione elettroforetica accoppiata ad un detector di massa (ESI-IT-MS) per la determinazione della frazione fenolica del miele. Con questo detector è stato possibile determinare sia il tempo di migrazione di alcuni standard e analiti del campione, sia la massa di questi ultimi confermando con una metodologia più attendibile la presenza dei composti fenolici e apportando, in definitiva, un

notevole miglioramento dell'analisi rispetto al metodo precedentemente sviluppato. Infatti, con questa nuova metodologia è stato possibile determinare 13 analiti e identificarne 9.

7.- È stato possibile identificare 11 composti fenolici in meno di 15 minuti impiegando un sensibile metodo elettroforetico accoppiato a un detector TOF. Inoltre, è stato identificato per la prima volta un nuovo composto (acido 8-idrossi-2,7-dimetil-2,4-decadien-1,10-dioico 6-O-β-D-glucopiranosilestere). È stata dimostrata l'applicabilità del metodo a differenti campioni di noci e studi di ripetibilità e riproducibilità hanno confermato la robustezza del metodo.

8.- È stato messo a punto un metodo HPLC accoppiato in linea con un detector NMR per la caratterizzazione fenolica di una pianta medicinale (*Teucrium polium*). Inoltre, al sistema prima menzionato è stato messo in linea un altro sistema in grado di effettuare la misura dell'attività antiossidante (test del DPPH). Questa metodologia ha permesso l'identificazione di 8 composti fenolici e ha evidenziato come il composto maggiormente presente e dotato di maggiore attività antiossidante è il poliumoside. La NMR accoppiata al sistema HPLC è risultata essere una tecnica con alto potenziale in grado di fornire informazioni strutturali della frazione fenolica e che si presta a semplificare le determinazioni classiche mediante sistemi automatizzati.

Final conclusions

1.- It has been developed a method for the identification and quantification of the principal components of phenolic fraction of olive oil by CE-UV-Vis. Eight compounds have been determined, and four of them (elenolic acid, ligstroside aglycon, oleuropein aglycon and pinoresinol) have never been detected before by CE. To carry out the identification some standards obtained by semipreparative HPLC were used, because commercial standards were not available. This methodology was applied to seven varieties of monovarietal extra-virgin olive oil (Picual, Arbequina, Lechín de Sevilla, Hojiblanca, Lechín de Granada, Picudo and Cornicabra). The data obtained was elaborated to obtain some compounds as varietal markers.

2.- A non aqueous CE method has been developed to determine the phenolic fraction of olive oil. The biggest advantage of this methodology is the use of organic solvents; they are compatible with an apolar matrix as olive oil and allow the direct injection of oil into the capillary. Sixteen compounds from five different varieties of olive oil (Arbequina, Picual, Hojiblanca, Lechín de Sevilla y Cornicabra) have been identified. These results were compared with the ones got with aqueous CZE obtaining good results.

3.- HPLC-DAD-MS has been used to evaluate the effect of different filtration systems, very common in small mills, on the phenolic content of virgin olive oil. Olive oils used differed in the production year, production plant, and storage conditions, parameters which provide wide information that can be extrapolated to other olive oils. Phenolic content has been correlated with water content of olive oils and also with their oxidative stability. It has been demonstrated that filtration causes an increase of the concentration of phenolic compounds, however oxidative stability decrease after it. This contradiction can be explain in the following way: water decrease makes phenolic compounds in olive oil more susceptible to be extracted by the extraction solvents (methanol/water 50/50), but at the same time these polar phenolic compounds present higher activity in water-in-oil emulsions.

4.- After the study of the influence of the fly attack on the phenolic content of olive oil and its comparation with the effect produced on othe chemical parameters (free acidity, peroxide value, fatty acid composition, water content, oxidative stability and antioxidant power of phenolic fraction by electrochemical analysis), we have concluded that besides the phenolic profile indicates the quality of an olive oil, there is no good correlation between the percentage of phenolic compounds and the health status of olives. Free acidity is stronger and more useful for judging the quality of an olive oil right after production because it is independent of all of the other technological parameters.

5.- A CE-UV-Vis method has been optimized for the study of the phenolic fraction in honey. Honey is a very complex matrix that requires an exhaustive sample preparation and a good separation in order to identify its compounds. That is why an universal detector such as UV-Vis presents some difficulties in the identification. Because of that, afer the optimization, only five phenolic compounds could be identified. This methos was used with five different kind of honey and the results obtained were the same.

6.- Because of the problems found out to characterize the phenolic profile of honey by CE-UV-Vis, we performed another methodology to determine these compounds in honey using CE-ESI-MS (IT). Mass spectrometry allows comparing standards migration times with those of the compounds under study and also the masses of them, in that way it is easier to confirm the identity of the analytes under study. This method is able to detect 13 analytes, from which 9 could be identified.

7.- Elevan phenolic compounds have been detcted and quantified thanks to a simple and powerful electrophoretic method coupled to mass spectrometry (TOF) in less than 15 min. Furthermore a new compound that had never been determined before in walnut ((2E,4E)-8-hydroxy-2,7-dimethyl-2,4-decadiene-1,10-dioic acid 6-O-d-glucopiranosyl ester) has been identified. This method can be applied to different varieties of walnut and the results can be compared. Repetibility and reproducibility studies show the robutness of the method.

8.- The characterization of the phenolic fraction of a medicinal plant known as *Teucrium polium* has been performed by HPLC-SPE-NMR. We have also coupled a system to measure antioxidant activity *on-line* (DPPH assay). Eight compounds from the phenolic fraction have been identified by HPLC-SPE-NMR. Poliumoside has proved to be the compound present in higher concentration and it has also the highest antioxidant activity. NMR coupled to HPLC is a very powerful instrumentation because provides structural information of the fractions separated by HPLC and the automatization allows to simplify this kind of analysis.