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DOCTORAL THESIS

CHARACTERIZATION OF POLYPHENOLS IN TUNISIAN OLIVE WITH ANTICANCER CAPACITY USING LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

CARACTERIZACIÓN DE POLIFENOLES DE OLIVO TUNECINO CON CAPACIDAD ANTICANCERIGENA MEDIANTE CROMATOGRAFIA LIQUIDA ACOPLADA A ESPECTROMETRIA DE MASAS

Presented by

AMANI TAAMALLI

Submitted for the degree of Doctor in Chemistry

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CHARACTERIZATION OF POLYPHENOLS IN TUNISIAN OLIVE WITH ANTICANCER CAPACITY USING LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY

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That the work presented in this thesis doctoral entitled "**CHARACTERIZATION OF POLYPHENOLS IN TUNISIAN OLIVE WITH ANTICANCER CAPACITY USING LIQUID CHROMATOGRAPHY COUPLED TO MASS SPECTROMETRY**" has been carried out under my direction and that of both Drs. Antonio Segura-Carretero and David Arráez-Roman in the laboratories of the Department of Analytical Chemistry in the Functional Food Research and Development Centre (CIDAF) of the Health Technological park in Granada, and shows all requirements of eligibility to obtain the Doctor degree for the University of Granada.

In Granada, May 2012

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SUMMARY

The olive fruit, its oil, and the leaves of the olive tree have a rich history of nutritional, medicinal, and ceremonial uses. A large number of scientific studies have suggested that these beneficial properties are related to the high level of antioxidants, particularly phenolic compounds, in this plant species. This explains the increasing interest focused on these compounds. The present doctoral thesis deals with the analysis of phenolic compounds in the main product and by-product of Tunisian olive, namely olive oil and olive leaves. The results are presented as two main sections according to the matrix used.

The first section (SECTION I), dedicated to the olive oil, is divided into three chapters (Chapters 1-3).

The first chapter includes a review on the polyphenols occurring in Tunisian olive products (olive fruit and olive oil) and some by-products (olive leaves and olive-mill wastewater). The various methods used for the analysis as well as the bioactive properties of these compounds reported in recent years are described.

The second chapter concerns the characterization of extra-virgin olive oils from the two main Tunisian varieties and another four varieties grown in restricted geographical zones. The aim is to explore their phenolic profile and improve the potential of the secondary varieties under study. The phenolic compounds, determined using high-performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF-MS), showed significant variation among the six varieties. The parameters studied were analysed chemometrically to discriminate among the cultivars. In this sense, the principal-component analysis (PCA) showed that variables such as oleic, linoleic, quinic and vanillic acids, apigenin, luteolin, taxifolin, oleuropein aglycon, pinoresinol acetate, elenolic acid, and oxidative stability discriminate the different varieties of extra-virgin olive oil studied. The agglomerative hierarchical clustering (HAC) results agreed with

those found by PCA. Moreover, linear discriminant analysis (LDA) enabled the classification of the oil samples according to their geographical origin.

In the third chapter, the behaviour of the phenolic profile of extra-virgin olive oils from 13 accessions belonging to the variety “Chemlali” was studied according to the production zone. The phenolic-profile data gained using HPLC–ESI-TOF–MS were used for oil classification by geographical area. The production area has significantly influenced the phenolic composition of the ‘Chemlali’ olive-oil variety, oils produced from ‘Oueslatia’, ‘Ain Zena’, ‘Siliana’ and ‘Bir Ali Ben Khelifa’, among others, being the richest in polyphenols. A discriminant analysis model gave a correct classification pathway with 100% of the correct predicted membership by selecting only 13 compounds (oleuropein aglycon, 10-hydroxy-oleuropein aglycon, hydroxy-elenolic acid, elenolic acid, ligstroside aglycon, decarboxylated oleuropein aglycon, hydroxy-decarboxylated oleuropein aglycon, decarboxylated ligstroside aglycon, ferulic acid, coumaric acid, quinic acid, and luteolin).

The second section (SECTION II), dedicated to olive leaves, is divided into three chapters (Chapters 4-6).

The fourth chapter describes the optimisation of a microwave-assisted extraction (MAE) for the extraction of phenolic compounds from olive leaves and their analysis using a combination of HPLC coupled to ESI-TOF–MS and electrospray ion trap tandem mass spectrometry (ESI-IT-MS2). The experimental variables that affect the MAE process, such as the solvent type and composition, microwave temperature, and extraction time, were optimised using a univariate method. The optimised MAE method combined with HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2 enabled the identification of a large number of phenolic compounds. Thus the proposed procedure proved useful as an alternative extraction method for characterizing phenolic compounds from olive leaves, due to its efficiency, speed, and automatization.

The fifth chapter includes the comparison of advanced extraction techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE), together with the traditional solid-liquid extraction and the evaluation of the cytotoxic capacity of different olive-leaf extracts against JIMT-1 breast-cancer-cell line. The phenolic profile analysed using a HPLC coupled to ESI-TOF-MS and ESI-IT-MS2 proved to be influenced by the olive variety and the extraction method used, mainly by the solvent employed. Among the different extraction methods, the largest number of phenolic compounds was identified by MAE. The evaluation of the cytotoxic activity of the extracts, realized through the MTT assay, showed that the extract derived from 'El Hor' variety was the most potent. The cytotoxic activity seemed to depend on the presence of some specific phenolic compounds.

And the sixth chapter concerns the characterization of phenolic compounds and their metabolites in the cytoplasm of JIMT-1 human breast-cancer cells treated with olive-leaf extracts. In this chapter, the metabolomic study was focused on the olive-leaf extract that showed the highest cytotoxicity for cancer cells (according to the results described in the previous chapter). The metabolites were characterized using HPLC-TOF-MS.

RESUMEN

Resumen

El fruto del olivo, su aceite y las hojas del mismo tienen una amplia historia en cuanto a sus usos alimenticios, medicinales y ceremoniales. En este sentido, un gran número de estudios científicos han sugerido que estas propiedades beneficiosas están relacionadas con el alto contenido en antioxidantes, particularmente compuestos fenólicos, presentes en esta especie vegetal. Esto explica el creciente interés que existe hoy en día sobre el estudio de estos compuestos fenólicos. La presente memoria está centrada en el análisis de los compuestos fenólicos presentes en el aceite de oliva Tunecino, el producto principal del olivo, como en un sub-producto importante, la hoja. Así, los resultados obtenidos de estos estudios se presentan en dos secciones principales de acuerdo con la matriz utilizada.
La primera sección (Sección I), dedicada al aceite de oliva, se divide en tres capítulos (capítulos 1-3).

En el primer capítulo se lleva a cabo una revisión de los compuestos fenólicos presentes en los productos del olivo Tunecino (aceituna y aceite de oliva) y en algunos sub-productos derivados (hojas de olivo y agua residuales) así como los diferentes métodos utilizados para su análisis y las propiedades bioactivas de los mismos.

El segundo capítulo versa sobre la caracterización de aceite de oliva virgen extra de las dos variedades principales de Túnez y otras cuatro variedades cultivadas en zonas geográficas limitadas. El objetivo es estudiar su perfil fenólico y evaluar el potencial de las variedades secundarias que se están estudiando. Los compuestos fenólicos, analizados mediante cromatografía líquida de alta resolución acoplada a espectrometría de masas de tiempo de vuelo mediante ionización por electrospray (HPLC-ESI-TOF-MS), mostraron una variación significativa entre las seis variedades. Los parámetros estudiados fueron analizados quimiometricamente para discriminar entre los diferentes cultivos. En este sentido, el análisis de componentes principales (PCA) mostró que las variables tales como los ácidos grasos oleico y linoleico,

apigenina, luteolina, taxifolina, oleuropeína aglicona, acetato de pinoresinol, los ácidos elenólico, quínico y vanílico, y la estabilidad oxidativa fueron suficientes para discriminar las diferentes variedades de aceite de oliva virgen extra estudiadas. Las agrupaciones jerárquicas (HAC) de los resultados coinciden con los encontrados por PCA. Por otra parte, el análisis por discriminación lineal (LDA), permitió la clasificación de las muestras de aceite de acuerdo a su origen geográfico.

En el tercer capítulo, los perfiles fenólicos de 13 sub-variedades de oliva virgen extra, pertenecientes a la variedad "Chemlali", han sido estudiados de acuerdo a la zona de producción. Así, los datos de los perfiles fenólicos, obtenidos mediante HPLC-ESI-TOF-MS, fueron utilizados para la clasificación de los aceites de acuerdo a su origen geográfico. Los datos reflejan que la zona de producción ha influido en la composición fenólica de la variedad 'Chemlali'. Así, los aceites producidos a partir de la variedad Oueslatia, Ain Zena, Siliana y Bir Ali Ben Khelifa, entre otros, han mostrado un mayor contenido en compuestos fenólicos. Un modelo de análisis discriminante dio una correcta clasificación con 100% de los miembros predichos por la selección de sólo 13 compuestos (oleuropeína aglicona, 10-hidroxi-oleuropeína aglicona, el ácido hidroxi-elenólico, ácido elenólico, ligustrósido aglicona, oleuropeína aglicona descarboxila, hidroxi-oleuropeína aglicona descarboxila, ligustrósido aglicona descarboxilado, el ácido ferúlico, ácido cumárico, ácido quínico, y la luteolina).

La segunda sección (Sección II), dedicada a las hojas de olivo, se divide en tres capítulos (capítulos 4-6).

El cuarto capítulo describe la optimización de una técnica de extracción asistida por microondas (MAE) para la extracción de compuestos fenólicos en hojas de olivo Tunecino y su posterior análisis mediante HPLC acoplado a ESI-TOF-MS y a espectrometría de masas con analizador de trampa de iones (ESI-IT-MS^2). Las variables experimentales que afectan al proceso MAE, tales como el tipo de disolvente y su composición, la temperatura y el tiempo de extracción, se optimizaron utilizando un método univariante. Así, mediante MAE y el uso de HPLC-ESI-TOF-MS y HPLC-ESI-IT-MS2 se pudieron identificar un gran número de

compuestos fenólicos presentes el la hoja de olivo. Por todo ello, la técnica de MAE propuesta demostró su utilidad para ser utilizada como una técnica alternativa de extracción de compuestos fenólicos en hojas de olivo, principalmente debido a su eficiencia, velocidad y automatización.

El capítulo cinco incluye un estudio comparativo de diferentes técnicas de extracción avanzadas, tales como extracción asistida por microondas (MAE), extracción con fluidos supercríticos (SFE) y extracción por líquidos presurizados (PLE), junto con la extracción sólido-líquido tradicional. Posteriormente, se llevó a cabo una evaluación de la capacidad citotóxica de los diferentes extractos de hoja de olivo frente a líneas celulares JIMT- 1 de cáncer de mama. Los perfiles obtenidos, mediante HPLC-ESI-TOF-MS y HPLC-ESI-IT MS^2, fueron diferentes en función de la variedad de la hoja y el método de extracción utilizado principalment por el disolvente empleado para la extracción. Entre los diferentes extractos analizados, fue en el extracto de MAE en el que se identificaron un mayor número de compuestos fenólicos. La evaluación de la actividad citotóxica de los extractos, realizados a través de ensayo MTT, demostraron que el extracto derivado de la variedad “El Hor” fue el más potente.

El sexto capítulo versa sobre la caracterización de compuestos fenólicos y sus metabolitos en citoplasmas de células de la línea JIMT-1 de cáncer de mama las tratadas con extractos de hoja de olivo. En este capítulo, el estudio metabolómica se centró en el extracto de hoja de olivo que mostró la mayor citotoxicidad en las células cancerosas (de acuerdo con los resultados descritos en el capítulo anterior). Las muestras fueron analizadas mediante HPLC-ESI-TOF-MS y los datos obtenidos fueron tratados estadísticamente, llevándose a cabo una comparación entre los perfiles metabólicos del citoplasma control y tratado, encontrando de esta manera posibles candidatos. Así se pudieron identificar 3 compuestos de la familia de los flavonoides como luteolina, apigenina y diosmetina.

RESUME

L’olivier est connu dans l’histoire pour les utilisations nutritionnelles, médicinales et rituelles de ses fruits, l’huile extraite et ses feuilles. Un grand nombre de recherches scientifiques ont suggéré que ces propriétés bénéfiques sont dues à leur contenu élevé en antioxydants, les composés phénoliques en particulier. C'est ce qui explique l'intérêt croissant porté sur ces composés. La présente thèse doctorale porte sur l'analyse des composés phénoliques présents dans le principal produit de l’olivier: l’huile d’olive ainsi que dans l’un des principaux sous-produits : les feuilles d’olivier. Les résultats obtenus durant la réalisation de ce travail sont présentés sous forme de deux sections principales en fonction de la matrice utilisée.

La première section (section I) est dédiée à l'huile d'olive et est divisée en trois chapitres (chapitres 1-3).

Le premier chapitre comprend une revue sur les phénols caractérisés dans les produits de l'olivier, à savoir le fruit de l'olivier et l'huile d'olive, et quelques sous-produits tels que les feuilles d'olivier et les margines ainsi que les différentes méthodes utilisées pour leur analyse et leurs propriétés bioactives décrites durant les dernières années.

Le deuxième chapitre s'intéresse à la caractérisation des huiles d'olive extra vierge des deux principales variétés tunisiennes et d'autres variétés secondaires -cultivées dans des zones géographiques restreintes- dans le but d’explorer leurs profils phénoliques et évaluer les potentialités des variétés secondaires. Les composés phénoliques, déterminés en utilisant la chromatographie liquide à haute performance couplée à la spectrométrie de masse à temps de vol (HPLC-ESI-TOF-MS), ont montré une variation significative entre les six variétés. Des analyses statistiques ont été appliquées aux traits étudiés pour la discrimination entre les cultivars étudiés. L’analyse en composantes principales (ACP) a montré que des variables tels que les les acides gras oleique et linoleique, apigenin, luteolin, taxifolin, oleuropein aglycon, pinoresinol acetate, les acides quinique et vanillique, l’acide elenolique anisi que la stabilité oxydative permettent la discrimination entre les huiles d’olive extra vierges

des différentes variétés. L'agglomération hiérarchique (CAH) vient appuyer les résultats trouvés par la ACP. En plus, l'analyse discriminante linéaire (ADL) a permis la classification des huiles selon leur origine géographique.

Dans le troisième chapitre, la composition phénolique des huiles d'olive extra vierges de 13 accessions appartenant à la variété 'Chemlali' a été étudiée en fonction de la zone de production. Les données des profils phénoliques obtenus par HPLC-ESI-TOF-MS ont été utilisées pour la classification des huiles en fonction de l'aire géographique. La zone de production a fortement influencé la composition phénolique de la variété 'Chemlali'. Les huiles produites à 'Oueslatia', 'Ain Zena', 'Siliana' et 'Bir Ali Ben Khelifa' étaient les plus riches en polyphénols. Il convient de noter que les profils obtenus par la technique HPLC-ESI-TOF-MS étaient utiles pour classer les huiles en fonction de l'aire géographique. Une classification correcte à 100% des membres prédits a été obtenue en sélectionnant seulement treize composés phénoliques, à savoir oleuropein aglycon, 10-hydroxy-oleuropein aglycon, hydroxy- acid elenolique, acid elenolique, ligstroside aglycon, oleuropein aglycon decarboxylée, hydroxy- oleuropein aglycon decarboxylaée, ligstroside aglycon decarboxylé, les acides ferulique, coumarique et quinique, et luteolin).

La deuxième section (section II) est consacrée pour les feuilles d'olivier et est divisée en trois chapitres (chapitres 4-6).

Le quatrième chapitre décrit l'optimisation d'une méthode d'extraction assistée aux micro-ondes (MAE) pour l'extraction des composés phénoliques de feuilles d'olivier et de leur analyse en utilisant une combinaison de HPLC couplée à ESI-TOF-MS et à la spectrométrie de masse à trappe d'ions (ESI-IT/MS2). Les variables expérimentales qui affectent le processus MAE, tels que le type de solvant et sa composition, la température, et le temps d'extraction, ont été optimisés en utilisant une méthode univariée. La méthode de MAE optimisée combinée avec HPLC-ESI-TOF-MS et HPLC-ESI-IT-MS2 a montré être utile comme une méthode d'extraction alternative pour la caractérisation des composés phénoliques de feuilles d'olivier en raison de son efficacité, rapidité et automatisation.

Le cinquième chapitre comprend la comparaison des techniques d'extraction telles que l'extraction assistée aux micro-ondes (MAE), l'extraction au fluide supercritique (SFE) et l'extraction liquide sous pression (PLE), ainsi que la méthode conventionnelle et un test *in vitro* de la capacité cytotoxique des extraits contre une lignée cellulaire de cancer de sein JIMT-1. Le profil phénolique analysé par HPLC combinée à ESI-TOF–MS and ESI-IT-MS^2 a montré une variation en fonction de la variété d'olivier ainsi que de la méthode d'extraction utilisée, principalement par le solvant. Parmi les différentes méthodes d'extraction, celles assistée aux micro-ondes a permis l'identification du plus grand nombre de composés phénoliques. L'évaluation de la capacité cytotoxique des extraits, réalisée par le test MTT, a montré que l'extrait de la variété 'El Hor' obtenu par la SFE était le plus puissant. La capacité cytotoxique parait dépendre de certains composés phénoliques spécifiques.

Et le sixième chapitre s'intéresse à la caractérisation des composés phénoliques et de leurs métabolites dans le cytoplasme des cellules humaines de cancer du sein JIMT-1 traitées. Dans ce chapitre, l'étude métabolomique a été portée sur l'extrait de feuilles d'olivier qui a montré la plus grande capacité cytotoxique sur les cellules cancéreuses (selon les résultats décrits dans le chapitre précédent). La caractérisation des métabolites a été réalisée en utilisant la HPLC-ESI-TOF-MS.

OBJECTIVES

In the Mediterranean countries the olive (*Olea europaea)* is a traditional crop of social, economic, religious, and cultural importance. In Tunisia, the olive occupies 1/3 of the national agricultural area and in economic terms represents 44% of the agricultural export of the country. Thus, Tunisia occupies the second position in the world after the European Union in the export of olive oil. Since ancient times, olive oil and the olive leaf were recommended as remedies for various diseases. Recent researches have shown that the polyphenols that they present posses important biological activities which appear to be responsible for their health beneficial effects. Consequently, there is a growing interest in the study of phenolic compounds because of their biological activities.

In this sense, the main objectives of this thesis are:

- The characterization of six varieties of Tunisian extra-virgin olive oil (Chemlali, Chetoui, El Hor, Chemchali, Jarboui, and Oueslati), determination of their phenolic profile using high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) with time-of-flight (TOF) analysis with the aim of finding any variable able to discriminate among these olive oils.
- The study of the influence of geographical area on the phenolic profile, determined using HPLC-TOF MS, of the main Tunisian olive-oil variety "Chemlali".
- The characterization of olive-leaf phenolic extract from the same Tunisian varieties cited above through the use of new and optimised non-conventional extraction techniques such as microwave extraction (MAE), pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) combined with the separative technique HPLC coupled with TOF and ion trap (IT) mass-spectrometry detectors, as well as the *in vitro* test of the cytotoxic capacity of the extracts against JIMT-1 human breast-cancer-cell lines.

- The study of the phenolic compounds and their possible metabolites in the cytoplasm of the JIMT-1 human breast-cancer cells, treated with olive-leaf extracts, using HPLC coupled to TOF-MS.

Objectivos

En los países mediterráneos, el olivo (*Olea Europaea*) es un cultivo tradicional de gran importancia social, económica, religiosa y cultural. En Túnez, el olivo ocupa un tercio de la superficie agrícola nacional y en términos económicos representa el 44% de la exportación agrícola del país. En este sentido, Túnez ocupa la segunda posición en el mundo después de la Unión Europea en la exportación de aceite de oliva. Desde la antigüedad, el aceite de oliva y la hoja de olivo han sido utilizados como remedio para la prevención de un buen número de enfermedades. Investigaciones recientes han demonstrado que los polifenoles que contienen poseen una amplia actividad biológica y parecen ser los responsables de sus efectos beneficiosos para la salud. Por todo ello, hoy día existe un interés especial en cuanto al estudio de los compuestos fenólicos debido a sus propiedades bioactivas.

En este sentido, los principales objetivos de esta tesis son los siguientes:

- La caracterización de seis variedades de aceite de oliva virgen extra Tunecino, 2 principales (Chemlali y Chetoui) y 4 secundarias (El Hor, Chemchali, Jarboui y Oueslati), determinación de su contenido fenólico mediante el uso de Cromatografía Líquida de Alta Resolución (HPLC) acoplada a espectrometría de masas (MS) con analizador de Tiempo de Vuelo (TOF) con la finalidad de descriminar entre dichos aceites.

- Estudio de la influencia geográfica de diferentes aceites, de la principal variedad tunecina "Chemlali", mediante el estudio de sus perfiles fenólicos obtenidos mediante HPLC-TOF-MS.

- La caracterización, comparación mediante HPLC-MS (TOF/IT) de la composición fenólica de extractos de hojas de olivo, de las variedades anteriormente citadas en el primer objetivo, obtenidos mediante la

optimización de diferentes técnicas de extracción no convencionales como son la extracción por microondas (MAE), la extracción por líquidos presurizados (PLE) y extracción con fluidos supercrítico (SFE), así como la evaluación de la capacidad citotóxica *in vitro* de los extractos sobre la línea celular JIMT-1 de cáncer de mama.

- Estudios de metabolómica de compuestos fenólicos y sus metabolitos en muestras de citoplasma de la línea celular JIMT-1 de cáncer de mama tratadas con extractos bioactivos de hoja de olivo mediante HPLC acoplado a TOF-MS.

Objectifs

Dans les pays Meditérranéens, l'olivier (*Olea europaea)* constitue une culture traditionnelle ayant un rôle social, économique, religieux, et culturel de grande importance. En Tunisie, l'olivier occupe le tiers des superficies cultivées du pays et représente 44% des recettes totales des exportations agricoles tunisiennes. Ainsi, la Tunisie occupe la deuxième position, à l'échelle mondiale, après l'Union Européenne dans l'exportation de l'huile d'olive. Depuis l'antiquité, l'huile d'olive ainsi que les feuilles d'olivier ont été recommandés comme remède contre certaines maladies. Des travaux récents ont montré que les polyphénols qu'ils contiennent possèdent une grande activité biologique et paraissent être reponsables de leurs bienfaits sur la santé. Ce qui explique l'intérêt croissant des recherches scientifiques pour l'étude de ces composés.

Ainsi, les objectifs de cette présente thèse doctorale sont les suivants :

- La charactérisation de six variétés Tunisiennes d'huiles d'olive extra-vierges : les deux varietiés principales (Chemlali et Chetoui) et quatre variétés secondaires (El Hor, Chemchali, Jarboui et Oueslati) et la détermination de leur composition phénolique utilisant la chromatographie liquide à haute performance (HPLC) couplée à la spectrometry de masse à temps de vol (TOF) dans le but de pouvoir discriminer entre ces huiles.

- L'étude de l'effet du site géographique sur la composition phénolique des huiles de la principale variété d'olivier Tunisienne "Chemlali", utilisant HPLC-TOF MS.

- La charactérisation des composés phénoliques des extraits des feuilles d'olivier des six variétés qu'on a cité précédemment (Chemlali, Chetoui, El Hor, Chemchali, Jarboui et Oueslati); l'étude de l'effet de la technique d'extraction

sur eux utilisant des nouvelles méthodes d'extraction telles que l'extraction assistée aux microondes (MAE), l'extraction aux fluide supercritique (SFE) et l'extraction au liquide sous pression (PLE) ainsi que la méthode d'extraction conventionnelle combinées à la technique séparative HPLC couplée à la spectroscopie de masse à temps de vol (TOF) et à trappe d'ions (IT); ainsi que le test *in vitro* de la capacité cytotoxique des différents extraits sur des cellules de cancer de sein hummain JIMT-1.

- Etude des composés phénoliques et leurs possibles métabolites dans le cytoplasme des cellules de cancer de sein JIMT-1 traitées par les extraits bioactifs des feuilles d'olivier, utilisant HPLC-TOF-MS.

Introduction

1. THE OLIVE TREE IN THE WORLD: HISTORY AND IMPORTANCE

The olive tree (*Olea Europaea*, Oleaceae) is among the oldest and most widespread fruit-bearing tree species[1]. The plant with its products has been in the forefront of the history of mankind –its civilizations, religion, politics, colonial expansions, arts and economic survival– to the point where it has become an integral part of traditions, cultures and myths[2].

The historical documentation of the first cultivation of the olive trees coincides, in general, with the evidence of the origin of the first prominent civilization that arose within or around the Mediterranean basin. The first evidence of olive cultivation comes from the Minoan age, when olives were cultivated on the island of Crete (3000-1500 BC). They were then cultivated by the Egyptians (1700 BC) and grown in an almost specialized form. Around 1000 BC, olives started to be cultivated in present-day Palestine, and between the ninth and eighth century BC, olive groves were grown in Greece and in North African coasts surrounding the Mediterranean Sea, where the olive was introduced by the Phoenicians. Through Phoenician and Greek shipping routes, olive trees reached the coast of Sicily and Spain, where they were widely spread in the fifth century BC. Between the sixth and fourth century BC their cultivation was established in many regions of Italy and Spain by the Romans. By the first century AD, olives were a economic crop for the Romans, who imported oil from the most remote colonies of the empire, mainly Spain and North Africa[3].

1. Liphschitz N., Gophna R., Hartman M., Biger G. (1991). The beginning of olive (Olea europaea) cultivation in the old world: a reassessment. *J. Arch. Sci. 18*: 441–453.
2. Bartolini G., Pertucelli R. (2002). Geographical diffusion. In: Classification, origin and history of the olive. Ed. H. D. Tindall. Food and Agriculture Organization of the United Nations. pp: 27-34.
3 Mastrangelo, N. 1982. L'olivo. Officine Grafiche Calderini, Bologna, Italy.

The development of olive cultivation in North Africa is of particular interest and was due to the land-reclamation program in this area by the Romans[4]. The Phoenicians reportedly introduced the olive into North and West Africa and possibly into Libya and Tunisia when they founded Carthage (9th century BC.)[5-7.] It may also have been introduced by the Greeks (8th-7th century BC.) who brought it into Cyrenaica. In Carthage, a Phoenician colony and maritime power of the western Mediterranean had a flourishing olive-growing industry and its accompanying trade was developed in this region[2]. After the fall of Rome, sedentary agriculture in North Africa declined and suffered from several nomadic invasions[8,9], but increased again after the 15th century, up to the present extent of cultivated area in the Mediterranean **(Figure 1)**.

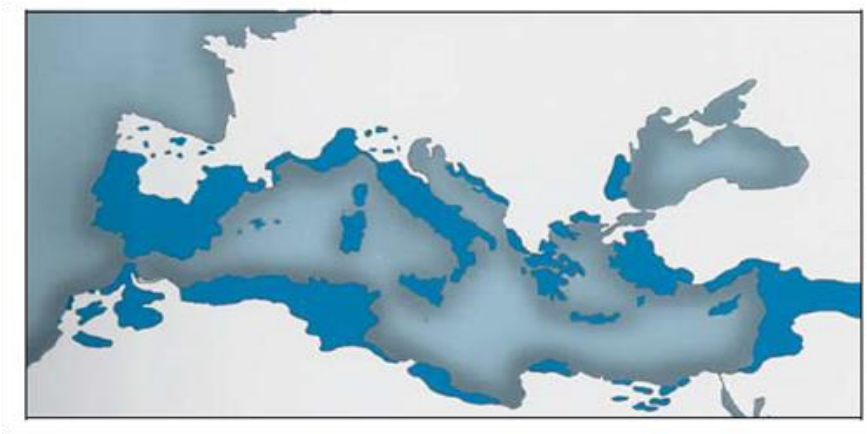


Figure 1. Geographical distribution of olive cultivation[10]

4. Camps-Fabrer H. (1984). L'olivier et son importance économique dans l'Afrique du Nord Antique. *Olivae, 2*: 9-22.
5 De Candolle A. (1883). Origine des plantes cultivées. Germer Bailliere, Paris.
6. Acerbo G. (1937). La marcia storica dell'olivo nel Mediterraneo. Atti della Società per il Progresso delle Scienze, Riun. XXV, Vol. I, Fasc. 2: 1-22.
7. Camps-fabrer H. (1953). L'olivier et l'huilerie dans l'Afrique romaine. Publ. Service des antiquités. Alger pp. 11-12, Conseil Oléicole International, Madrid (Espagne), 30 - 33.
8. Goodchild R. (1950). Roman Tripolitania: reconnaissance in the desert frontier zone. *Geogr. J, 115*: 161-178.
9. Goodchild, R., 1952: The decline of Libyan agriculture. *Geogr. Mag. 25*: 142–156.
10. Ghedira K. (2008). L'olivier. *Phytothérapie, 6*: 83-89

In ancient times, olive oil had many documented uses. It was used as a main dietary component, for cosmetic and basic medicinal purposes, and for lamp fuel. Many religious rituals involved the use of olive oil. The Greeks ceremoniously rubbed olive oil onto athlete's skin then scraped it off with the sweat and dust after competition. It was also used to make soap and to anoint the dead[11]. The leafy branches of the olive tree, a symbol of abundance, glory, and peace, were used to crown the victors of games and wars. As symbols of benediction and purification, olive branch was ritually offered to deities and powerful figures, as found in Tutankhamen's tomb[12].

Today, although to a lesser extent, the olive tree is also grown in countries such as Argentina, Chile, Australia, and South Africa.

The olive (*Olea Europaea*, L) is a species of major economic importance to many countries. Currently, 98.7% of the olive tree is located in the countries bordering the Mediterranean, especially Spain, Italy, Greece, Portugal, Turkey, Syria, Tunisia, Morocco and Algeria.

The world production of olive oil has increased from 1,453 thousand tonnes in 1995/1996 to an estimated 3,098 thousand tonnes in 2011/2012[13]. The share of the different countries in the average production of olive oil during this period reflects the predominance the European Union, followed by Tunisia, Syria, Turkey and Morocco **(Figure 2)Erreur ! Source du renvoi introuvable.**.

11. Vossen P. (2007). Olive oil: history, production, and characteristics of the world's classic oils. *Hortscience, 42*: 1093-1100.
12. El Bassam N. (2010). Energy crops guide. In: Handbook of bioenergy crops: a complete reference to species, development and applications. MPG books, UK. 93-398.
13. International olive oil council. Market newsletter N 56 December 2011. Available on the web site: http://www.internationaloliveoil.org

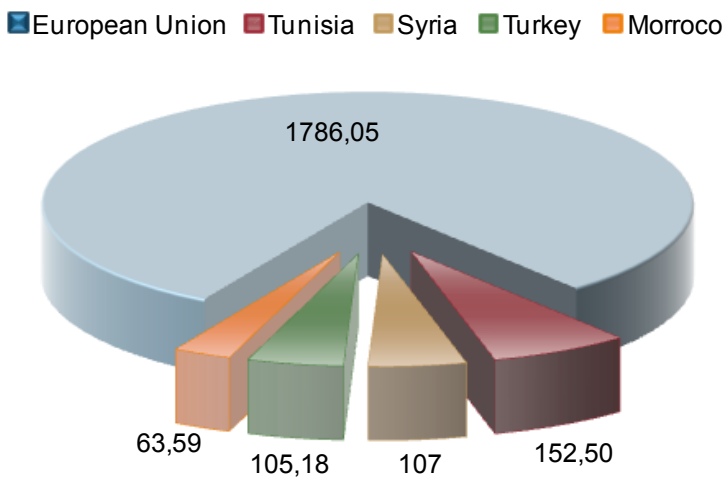


Figure 2. World production of olive oil

Similarly, olive-oil consumption has increased from 1,852 thousand tonnes in 1995/1996 to reach 3,078.5 thousand tonnes as estimated for the current year which is a level similar to that of production.

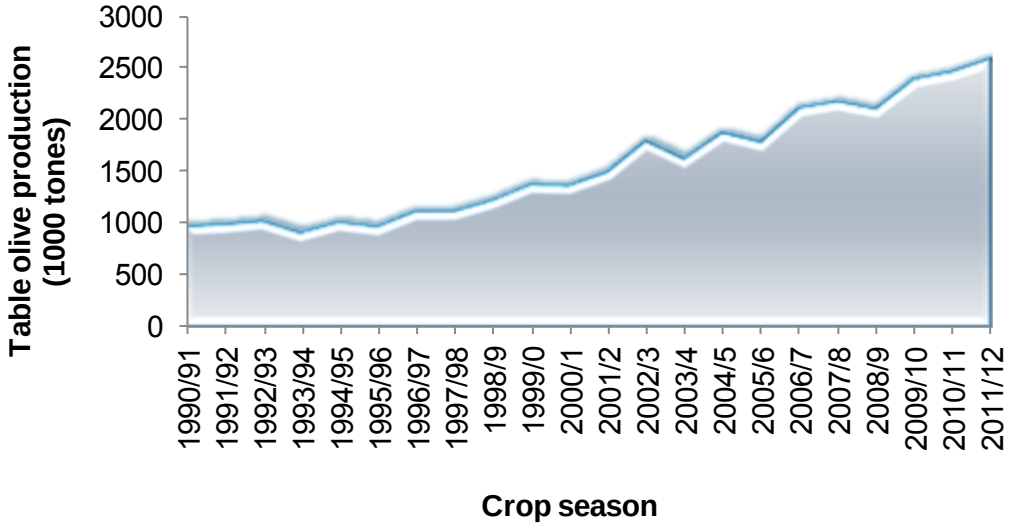


Figure 3.World production of table olives

World production in table olives has also increased remarkably during the last few decades from 950 thousand tonnes in the 1990/1991 harvest to 2,440 thousand

tonnes in 2010/1011 season **(Figure 3)** The production for 2011/2012 is estimated at 2,565 thousand tonnes, for an increase of 5% over that registered in 2010-2011, while consumption is anticipated to jump by 8% over the level of 2010/2011.

Around 61% of world consumption of table olives is concentrated in four countries or customs unions (EU 26%; Egypt 13%; Turkey 12%; USA 10%). Over the last 10 crop years, consumption has risen by 4% per crop year on average. Russia and Brazil stand out amongst the countries that are primarily table-olive importers[13].

2. THE OLIVE SECTOR IN TUNISIA

2.1. Situation of the olive sector

Tunisia, the most important olive-growing country of the southern Mediterranean region, dedicates over 30% of its cultivated land to olive growing (1.68 million ha). It is making great efforts to restructure and modernize its sector as well as to improve olive-oil quality and expand acreage. Olives are found in all the regions of Tunisia, from north to south, They are cultivated with other crops such as cereals in the north, citrus fruits and vine in the Cap Bon peninsula, and strictly as a monoculture in the Sahel and Sfax.

The geographical distribution of the olive groves, numbering roughly 64 million trees, shows that olive used for oil extraction is concentrated mainly in the central regions (Sousse, Monastir, Mahdia, Sfax, Kairouan, Kasserine, and Sidi Bouzid), and covering some 1,144,400 hectares (34.4 million trees). In the north (Tunis, Ariana, Ben Arous, Nabeul, Bizerte, Beja, Jendouba, Le Kef, Siliana and Zaghouan) and southern (Gafsa, Gabes, Medenine, Tozeur, and Tataouine Kebeli) olive groves occupy 214.6 and 307.500 hectares (21.3 and 8.4 million trees), respectively. The regions of Sousse, Monastir, Mahdia, Sfax, Medenine, Kairouan, Sidi Bouzid, Nabeul, Siliana, Zaghouan,

Kasserine, Kef, and Gafsa are the main areas of olive-oil production, accounting for 87% of the national plantation[14].

Planting densities, which vary according to edapho-climatic conditions, are some 27 trees/ha in the south, 30 trees/ha in the centre, and 99 trees/ha in the north. The national average is 38 trees/ha. The olive groves present several varieties (**Figure 4**), each marking one the edapho-climatic characteristics of its production region. The main varieties are: Chemlali, Chetoui, Oueslati, Jarboui, Zalmati, Zarrazi, Barouni and Chemchali.

According to their use, the Tunisian varieties are categorized into two classes:

Dual-purpose varieties, e.g. Chetoui, Oueslati, Zalmati, Chemlali, Gerboui, Chemchali, Rkhami, Zarrazi. Table-olive varieties, e.g. Meski, Sayali, Tounsi, Besbessi, Marsaline, Beldi, Fouji, Bidh Hamam, Limli, and Limouni.

Chemlali and Chetoui, which are the most cultivated varieties in the territory, are considered the main varieties in Tunisia.

- Chemlali, found in 60% of the olive-crop area, primarily in the north-eastern, central, southern regions. This cultivar is extremely vigorous and hardy, bears late, and has high alternate productivity; the fruit is small and forms compact clusters. The oil yield is medium to high, reaching as much as 25%. The tree is drought resistant, moderately tolerant of salinity, but sensitive to olive knot.

- Chetoui, grown in 35% of the country's olive acreage, is found mostly along the northern coastal strip of Tunisia. Of medium hardiness, it has a hardy rooting ability; its starts bearing at an intermediate age, and its productivity is low but constant. The fruit ripens in December and is picked in January and February. It is used primarily for oil production, giving a medium oil yield, although it can also be used for black

14. Ministère de l'Agriculture (2006). Direction Générale des Etudes et du Développement Agricole, Enquêtes sur les structures agricoles 2004-2005, Tunisie 2006.

pickling. It is tolerant for cold and salinity but requires ample water. Also it resists the most common olive diseases although it is sensitive to olive leaf spot.

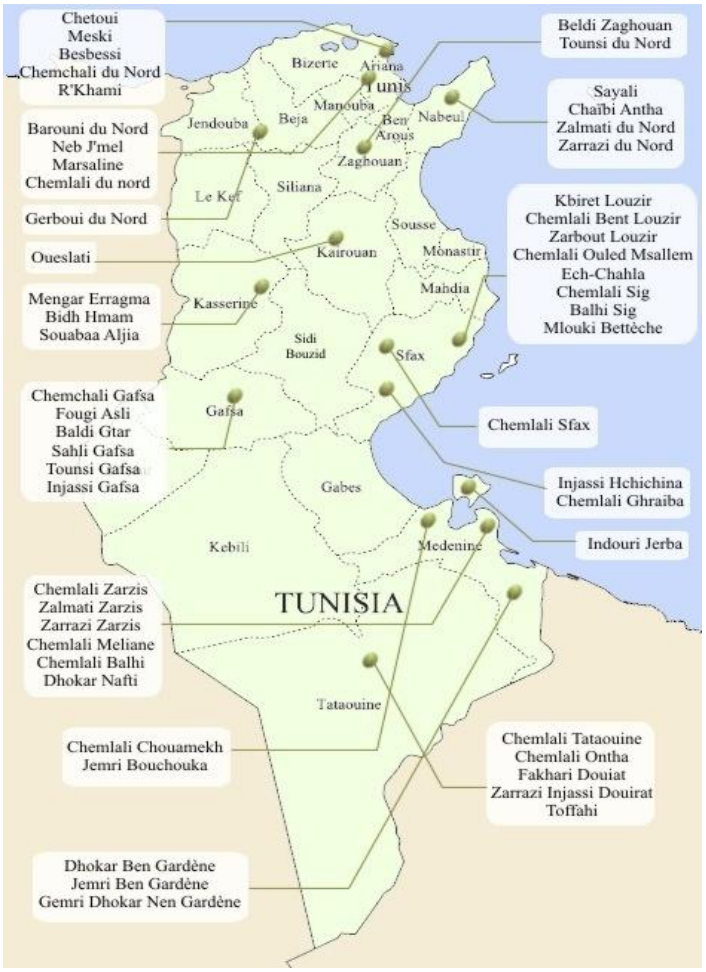


Figure 4. Geographical distribution of Tunisian olive varieties (Source: ONH Tunisia[15])

15. Office Nationale de l'Huile (ONH). Available on the website: www.onh.com.tn. http://www.internationaloliveoil.org/estaticos/view/136-country-profiles. Accessed January 5, 2012.

2.2. Importance of the olive sector

Olive cultivation plays a vital role in the social and economic life of Tunisia and accounts for approximately 15% of the total value of final agricultural production. Olive oil represents 14% of the total production value of agro-food industry and 47% of the agricultural and food exports as well as 4.5% of the exports of goods and services[16]. In least affluent areas of the country, olive growing helps strike a regional balance because it is often the only feasible crop. This helps to keep people in rural areas which otherwise might undergo depopulation.

Olive cultivation constitutes the main activity for about 269,000 farmers of a total of 471,000 farmers in the country i.e. 57% of the total. This sector provides between 25 and 30 million agricultural workdays per year, representing 20% of the country's agricultural employment, and also includes industrial oil extraction involving 1,702 mills (719 classic, 450 super-press, 515 continuous chains and 18 mixed) with a crushing capacity of approximately 38,463 tonnes of olives per day. The geographical distribution of these mills shows a high concentration of units and ability to extract oil in the regions of Sfax (410), Mahdia (229), Sousse (195), Monastir (187), Medenine (137), Kairouan (115), Sidi Bouzid (85), Nabeul (59), Gafsa (50), Gabes (35), Zaghouan (30), Beja (28), and Mannouba (17), which represent 92.7% of the mills.

In volume terms, Tunisia is the world's largest producer, behind the European Union. The olive oil produced in Tunisia, is destined mainly to export, ranking Tunisia second after the European Union in olive-oil exportation. Over the past decade, Tunisia produced an average of about 150,000 tonnes of olive oil, of which 75% were exported, representing 19.11% of worldwide olive-oil exportation.

16. International olive oil council (IOOC). Olive growing in Tunisia. E.108/Doc. no. 4, 26:4. Available on the website: http://www.internationaloliveoil.org/estaticos/view/136-country-profiles. Accessed January 5, 2012.

3. CHEMICAL COMPOSITION OF OLIVE OIL AND OLIVE LEAF

Olive oil is the lipophilic product of olive fruit. When olive fruits are harvested at maturity and processed, olive oil, which has a delicate and unique flavour, is produced. One of the main by-products of the olive-oil industry is olive leaves. In fact, leaves represent 10% of the weight of olives in oil extraction[17]. Leaves also constitute the major by-product resulting from pruning; it has been estimated that pruning alone produces 25 kg of by-products (twigs and leaves) per tree annually.

In terms of chemical composition, olive-oil components can be divided into two categories: saponifiable and unsaponifiable fractions[18]. These categories of components are also found in olive leaves.

In olive oil, the saponifiable fraction represents more than 98% of the total oil weight and is composed mainly of 98 to 99 % of triacylglycerols and 1 to 2% of minor glyceridic components such as waxes, mono- and di-acylglycerol free fatty acids and phospholipids[19]. In olive leaves, the lipid fraction represents 1.0 to 1.3% of the total weight of fresh leaves.

The triacylglycerols of olive oil are composed of three fatty acids attached to a glycerol backbone. Biosynthesis of triacylglycerols in olives generally follows the 1,3 random distribution which means that fatty acids on the triacylglycerols are randomly distributed in the 1 and 3 positions; in the 2 position, there is always an unsaturated fatty acid. Trisaturated triacylglycerols such as PPP, EEE, PEP, EPE, etc., and

17. Espínola F. (1997). Cambios tecnológicos en la extracción del aceite de oliva virgen. *Alimentación, Equipos y Tecnología, 21*: 314-389.

18. Ollivier D., Souillol S., Guérère M., Pinatel C.., Arlaud J. (2000). Données récentes sur la composition en acides gras et en triglycérides d'huile d'olive vierges françaises. *Le Nouvel olivier*, *13*: 13-18.

19. Ryan D., Robards K., Lavee S. (1998). Evolution de la quantité de l'huile d'olive. *Olivae 72*: 23-41.

triunsaturated triacylglycerols that include a linolenic acid such as PoPoLn are absent in olive oil[20]. The major triacylglycerols present in olive oils are OOO (40-60%), POO (10-20%), OOL (10-20%), POL (5-7%) and SOO (3-7%)[21,22] where O=oleic acid, L= linoleic acid, P= palmitic acid and S= stearic acid. The tiacylglycerols constitute an authenticity parameter for the differentiation of olive oil from other oils. In olive leaves, trioleine and palmitodioleine are reportedly the major triglycerols[23].

As mentioned above, most of the fatty acids in olive oil are present as triacylglycerols. The fatty-acid profile of a typical virgin olive oil consists of six main fatty acids: oleic and palmitoleic acids, which are monounsaturated; palmitic and stearic acids, which are saturated; and linoleic and linolenic, which are polyunsaturated fatty acids. Historically, the healthful properties of virgin olive oil were attributed to a high proportion of monounsaturated fatty acids, namely oleic acid. Recent findings have shown that high intake of monounsaturated fat may protect against Alzheimer's disease, whereas intake of saturated or trans-unsaturated fats may be detrimental[24]. Furthermore, the dietary replacement of saturated fat by monounsaturated fat derived from olive oil improved postprandial lipid metabolism and thrombosis and significantly reduced plasma-cholesterol concentrations.

Oleic acid is represented with much higher concentrations (55-83%) than the other fatty acids; linoleic (3.5-21%), palmitic (7.5-20%), stearic (0.5-5%), palmitoleic (0.3-3.5%) and linolenic acids (≤1%). Other fatty acids found in olive oil at low concentrations are myristic, margaric, heptadecanoic, arachidic, behenic, and

20. Tiscorina E., Fiorino N. & Evangelisti F. (1982). Chemical composition of olive oil and variations induced by refining. *Riv. Ital. Sostanze Grasse 59*: 519-556.

21 Kiritsakis A.K. (1990). Flavor components of olive oil- A review. *J. Am. Oil Chem. Soc. 75*: 675-681.

22. Boskou D. (1996). Olive Oil ; Chemistry and Technology. American Oil Chemist's Society. Press: champaign, IL, USA, pp 52-83.

23. Guinda A., Lanzón A., Ríos J.J., Albi T. (2002). Aislamiento y cuantificación de los componentes hoja del olivo: Extracto de hexano. *Grasas y Aceites 53*:240-245.

24. Panza F., Solfrizzi V., Colacicco A.M., D'Introno A., Capurso C., Torres F., Del Parigi A., Capurso S., Capurso A. (2004). Mediterranean diet and cognitive decline. *Public Health Nutr. 7*: 959-63.

lignoceric acids[25,26]. The six fatty acids were also reported to be present in olive leaves by Zarrouk et al. (1981) and among them palmitic, oleic, and linolenic acids were the major ones[27], which were also found later by other authors[28].

Only when the fatty acids are bound in triacylglycerol units is the oil considered to be of good quality. A triacylglycerol unit may lose one fatty acid to become a diacylglycerol, or if it loses two fatty acids it is a monoacylglycerol. The fatty acid lost from the triacylglycerol is then called a 'free fatty acid'. The free fatty acid content or acidity index is an indicator of the lipase-activity as well as the quality and freshness of olive fruits, and the storage potential and the stability of the oil. Oil is considered to be of a good quality when the acidity index is low[29].

The unsaponifiable fraction of olive oil is present at very low amounts (about 2% of oil weight) and constitutes a complex matrix composed of several chemical compounds such as hydrocarbons, aliphatic and triterpenic alcohols, sterols, volatile compounds, and antioxidants such as carotenoids and phenolic compounds[30].

Linear or polycyclic, the hydrocarbons are the major constituents of the unsaponifiable fraction of olive oil. Squalene ($C_{30}H_{50}$), a biochemical linear triperpene precursor of sterols, is the main hydrocarbon of olive oil and represents about 40% of the total weight of olive oil unsaponifiables. In fact, olive oil is richer in squalene than

25. Quiles Joules L. (2006). Olive Oil and Health. Wallingford, Oxfordshire, UK. CABI Publishing. http://site.ebrary.com/lib/iyte.
26. Garcia-Gonzalez D.L., Aparicio-Ruiz R., Aparicio R. (2008). Virgin olive oil-chemical implications on quality and health. *Eur. J.Lipid Sci. Technol. 110*: 1-6.
27. Zarrouk M., Cherif A. (1981). Action du chlorure de sodium sur la teneur en lipides de plants d'Olivier (Olea europaea L.). Z. Pjlanzenphysial. Bd. 105:85-92.
28. Seyyed Nejad M., Niroomand A. (2008). Study on lipid changes of leaves and fruits olive adapted to high temperature condition Inkhuzestan. *Pak. J. Biol. Sci. 10:* 4535-4538.
29. Ryan D., Robards K., Lavee S. (1998). Evolution de la qualité de l'huile d'olive. *Olivae 72*: 23-41.
30. Servili M., Selvaggini R., Esposto S., Taticchi A., Montedoro G., Morozzi G. (2004). Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A 1054*: 113–127.

most vegetable oils[31]. Carotenoids are also hydrocarbons present as a minor fraction in olive oil. The most important carotenoids present are β-caretene and lycopene[32]. They are also responsible for the olive-oil colour. It has been reported that β-caretene concentrations in virgin olive oil range from 0.5 to 4 mg/kg. Other hydrocarbons such as polycyclic aromatic (e.g. phenanthrene, pyrene, fluorenthrene, 1,2-benzanthracene, chrysene) are also found in olive oil[33]. In fresh olive leaves, the hydrocarbons constitute 37 to 42 %[34]. Guinda et al. (2002) have studied five Spanish varieties of olive leaves and found that among the identified saturated hydrocarbons, C29, C32, and C33 were the major ones whereas among tocopherols and carotenoids, alpha-tocopherol and beta-carotene presented the major compounds, respectively[23]. Hydrocarbon amounts ranged from 2530 to 3400 ppm in Spanish olive-leaf varieties and Squalene concentrations varied also between 38 and 152 ppm.

Aliphatic and triterpenic alcohols are important constituents in olive oil. Saturated straight chain of aliphatic alcohols with even-numbered carbon atoms (C_{18} to C_{28}) are found in olive oils at concentration of about 350 mg/kg of oil. The main aliphatic alcohols present in olive oil are hexacosanol (C_{26}), octacosanol (C_{28}), and tetracosanol (C_{24}). Tricosanol (C_{23}), pentacosanol (C_{25}) and heptacosanol (C_{27}) may be present in traces[35]. Triterpenic alcohols, also called 4,4-dimethylsterols constitute 1000 to 1500

31. Lozano-Sanchez J. (2009). Composicion del aceite de oliva. In El aceite de oliva virgin: Tesoro de Andalucia. Eds. Fernandez-Gutierrez A. and Segura-Carretero, A. Fundacion Unicaja. Impreso en España. 197-224.
32. Ramirez-Tortoza M.C. (2006). Chemical composition, types and characteristics of olive oil. In: Olive oil and health. Eds. Quiles J.L; Ramirez-Tortoza M.C. and Yakoob, *P. CAB International*. UK, USA. 45-61.
33. Fedeli E. (1977). Lipids of olives. Prog. Chem. *Fats and Other Lipids*, *15*: 57-74.
34. Boudhrioua N., Bahloul N., Ben Slimen I., Kechaou N. (2009). Comparison on the total phenol contents and the color of fresh and infrared dried olive leaves. *Ind. Crops. Prod. 29*: 412-9.

35. Kiritsakis P., William W.C. (2000). Analysis of edible oils. In: Handbook of olive oil: analysis and properties. Eds. John L. Harwood,Ramón Aparicio. Aspen Publishers, Inc, USA. 129-250.

mg/kg of olive oil and are represented mainly by β-amirin, butyrispermol, cycloartenol and 24-methylencycloartenol[31]. Numerous alcohols, namely, docosanol, tetracosanol, hexacosanol, octacosanol, and triacontanol, germanicol, alpha-amyrin, beta-amyrin and an isomer of alpha-amyrin have been identified in olive leaves[23]. Nevertheless, alpha-amyrin and beta-amyrin are the major alcohols.

Sterols are tetracyclic compounds biosynthesised from squalene. In olive oil, sterols are present in the range of 1800 to 4939 mg/kg[36]. These can be present in a free form or esterified with fatty acids. The sterols differ by the number and position of the double bonds and their lateral chain nature. The main sterols found in olive oil are β-sistosterol (70 to 90%), Δ-5-avenasterol (5 to 20%), campesterol (1 to 5%), and stigmasterol (0.5 to 2%). The major sterol in olive leaves was β-sistosterol, according to Guinda et al. 2002[23], who recorded concentrations varying between 614 and 250 ppm.

Other sterols are found at low amounts in olive oil, including cholesterol, campestenol, Δ-7-campesterol, clerosterol, Δ-5-24 stigmastadienol, Δ-7- stigmastenol and Δ-7- avenasterol[37,38]. Intermediates of sterol biosynthesis such as 4-α-methylsterols are also found in olive oil but in very low amounts and they are mainly represented by obtusifoliol, gramisterol, cycloeucalenol, and citrostadienol[22]. These are Δ-7and Δ-8 sterols except the cycloeucalenol.

Flavours and aromas of the oils are generated by a number of volatile constituents which are present at very low concentrations. The aroma compounds of olive oil are

36. Mulinacci N., Giaccherini C., Innocenti M., Romani A., vincieri F.F., mattei, A. (2005). Analysis of extravirgin olive oils from stoned olives. *J. Sci. Food Agric.* 85: 662-670.
37. Caselli S., Modi G., Nizzi Griffi F., Fiorino P. (1993). Variabilité de la composition en acides gras, en stérols et en alcools de l'huile d'olive de cultivars de la Toscane. *Olivae 47*: 46-50.
38 De Blas O.J., Valle Gonzalez A. (1996). Determination of sterols by capillary gas chromatography. Differentiation among different types of olive oil: virgin refined and solvent-extracted. *J. Am. Oil Chem. Soc. 73*: 1685-1689.

classified as aromatic and aliphatic hydrocarbons, alcohols, aldehydes, ketones, esters, phenols and phenol derivatives, oxygenated terpenes and furan derivatives. Aldehydes are always found in greater quantities in olive oil compared to other flavour compounds. Different sensory descriptions of virgin olive-oil flavour, include green, sweet, fruity, with bitter, and pungent being the most prominent sensory perceptions of consumers. The concentrations of this complex mixture are reported to be between 250 and 300 ppm[39,40]. Hexanal, trans-2-hexenal, 1- hexanol and 3-methylbutanol represents the major compounds[41,40].

Tocopherols, important compounds in olive oil, are classified by some authors as lipophilic phenolics (Ramirez-Tortoza). . Their concentrations are reported to be in range of 5-300 mg/kg or 12-400 mg/kg in good-quality oils. Tocopherols belong to the family of vitamin E. In fact, they constitute a group of molecules composed of a 6-OH-chromane ring and a lateral chain of 16 carbon atoms, of isoprenic structure[42]. The carbon chain exists in two forms: a form including three insaturations which characterize the tocotrienols and a saturated form which characterizes the tocopherols. The 6-OH-chromane ring exists in 4 forms distinguished by the position of the substituent methyl groups which gives rise to four forms of tocopherols and tocotrienols, α, β, γ, and δ.

The α-tocopherol is the main homologue of vitamin E present in olive oil. It represents about 90-95% of total tocopherols, the forms β and γ amounts do not exceed 10% and the form δ in lesser amounts. The determination of tocopherol content of olive oil can be used for detecting adulteration with seed oils[35]. The major

39. Fedeli E. (1997). Technologie de production et de conservation de l'huile. In «Encyclopédie Mondiale de l'Olivier», Conseil Oléicole International, Madrid (Espagne), 251-291.
40. Kiritsakis A.K. (1998). Olive oil from the tree to the table, Kiritsakis A.K., Lenart E.B., Willet W. C. and Hernandez, eds., Food and Nutrition Press, Connecticut, USA, 348.
41. Kiritsakis A.K. (1993). La chimie de l'arôme de l'huile d'olive. *Olivae 45*: 28-33.
42. Léger C.L. (2000). La vitamine E: état actuel des connaissances, rôle dans la prévention cardiovasculaire, biodisponibilité. *Ocl-Ol Corps Gras li. 7:* 258-265.

tocopherol identified in olive leaves, α-tocopherol varies in concentration from 41 to 125 μg/g[23].

Colour is an important attribute for the determination of olive-oil characteristics and is related to the quality for the majority of consumers. There are two types of pigments present in olive oil: chlorophylls and carotenoids and are responsible for the colour of the oil and are also involved in the mechanisms of auto-oxidation and photo-oxidation. The quantity of chlorophylls in olive oil depends on a number of factors such as the variety, the degree of maturity of the olives, methods of oil extraction, and some other biological and technical factors[33].

Chlorophyll is present as chlorophylls a and b. The loss of the magnesium cation transforms them into pheophytins *a* and *b*. The form *a* differs from the form *b* by the substituent group in the carbon at the position number 3, which is a methyl group in the form *a* and a formyl group in the form *b*. In virgin olive oil from mature olives the concentrations of chlorophyll are about 1 to 10 mg/kg and those of pheophytins are about 0.2 to 24.0 mg/kg. Pheophytin is the dominant pigment in the chlorophyll fraction with concentrations of about 70 and 80%. If the oil is extracted from black olives, pheophytin is the only chlorophyll pigment present[43]. In olive leaves, chlorophylls (*a* and *b*) were identified with amounts of 11.6 and 16 mg/g of dry weight varying according to the variety[44].

The carotenoids are the pigments responsible for the yellow colour of the olive oil. Chemically, they are classified as terpenoids composed of eight isoprene units. Their concentrations vary between 1 and 20 mg/kg. They are present in olive oil as lutein,

43. Rahmani M., Saarani Csallany A. (1991). Chlorophyll and β-carotene pigments in Morrrocain virgin olive oils measured by high-performance liquid chromatography. *J. Am. Oil Chem. Soc. 68*: 672-674.

44. Guerfel M., Baccouri O., Boujnah D., Chaibi W., Zarrouk M. (2009). Impacts of water stress on gas exchange, water relations, chlorophyll content and leaf structure in the two main Tunisian olive (Olea europaea L.) cultivars. *Sci. Hort. 119*: 257-263.

β-carotene, violaxanthine and neoxanthine. Among them, lutein is the major component.

Because of the characteristics of minor components, some molecules are present almost exclusively in virgin olive oil since the processes (mainly refining) involved in the production of these oils remove them in the case of phenolic compounds.

3.1. Phenolic compounds

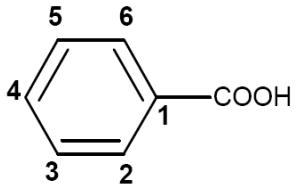
Phenolic compounds make important contributions to the nutritional properties, sensory characteristics, and the shelf life of olive oil in addition to their biological activities. The presence of phenolic fraction is a special characteristic of virgin olive oil among vegetable. Olive leaves constitute one of the richest parts of the olive tree in phenols where they are present mainly in the glycosylated form. This fraction is a complex matrix composed of numerous compounds from different chemical classes: phenolic acids, phenolic alcohols, secoiridoids, flavonoids, and lignans.

3.1.1. Simple phenols

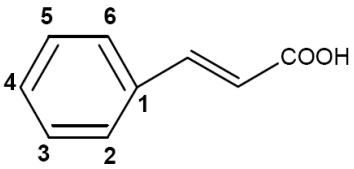
The phenolic acids are the first class of phenolic compounds observed in virgin olive oil[45]. This class can be divided into two main groups: benzoic acids (with the basic chemical structures of C_6-C_1) such as gallic, benzoic, vanillic, protocatecuic, syringic, hydroxybenzoic acids; and cinnamic acids (with the basic chemical structures of C_6-C_3) such as caffeic, coumaric, ferulic, cinnamic, and sinapinic acids.

Salicylic acid, chlorogenic acid and were reported to be present in addition in olive leaves (**Figure 5**).

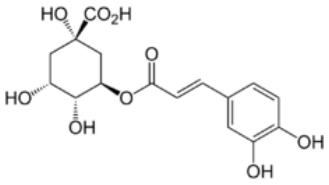
45. Vàsquez R.A. (1978). Les polyphénols de l'huile d'olive et leur influence sur les caractéristiques de l'huile. *Rev. Fr. Corps Gras 39:* 25- 32.



3-Hydroxybenzoic acid : 3-OH
p- Hydroxybenzoic acid : 4-OH
3,4-Dihydroxybenzoic acid : 3,4-OH
Gentisic acid : 2,5-OH
Vanillic acid : 3-OCH3, 4-OH
Gallic acid: 3,4,5-OH
Syringic acid : 3,5-OCH3, 4-OH
Salicylic acid: 2-OH



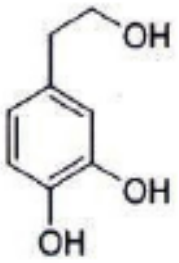
o-Coumaric acid : 2-OH
p-Coumaric acid : 4-OH
Caffeic acid : 3,4-OH
Ferulic acid : 3-OCH3, 4-OH
Sinapinic acid : 3,5-OCH3, 4-OH



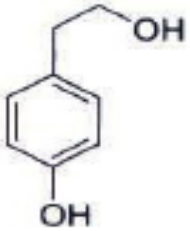
Chlorogenic acid

Figure 5. Structures of phenolic acids identified in olive oil and olive leaves

The main phenolic alcohols identified in virgin olive oil are hydroxytyrosol and tyrosol **(Figure 6)**. Hydroxytyrosol, tyrosol, caffeic acid, coumaric acids, and *p*-hydroxybenzoic acid influence the sensory characteristics of VOO[41]. The glucosylated forms of hydroxytyrosol and tyrosol were identified in addition in olive leaves.



Hydroxytyrosol



Tyrosol

Figure 6. Structures of main phenolic alcohols identified in virgin olive oil and olive leaves

3.1.2. Secoiridoids

The secoiridoids are the main components in virgin olive oil together with the lignans. In olive leaves the secoiridoids and flavonoids are dominant groups sharing the total phenol content[46]. Secoiridoids are produced from the secondary metabolism of terpenes. These compounds are characterized by the presence of elenolic acid or its derivates 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 and the elenolic acid glucoside (oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*). Secoiridoids of virgin olive oil in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous β-glucosidases during crushing and malaxation[47]. Those derived from the hydrolysis of oleuropein contribute to the intensity of the bitterness of VOO. The most abundant secoiridoids of virgin olive oil identified are the dialdehydic form of elenolic acid linked to hydroxytyrosol or tyrosol.

In olive leaves, secoiridoids such as oleuropein, oleuropein diglucoside, oleuroside, demetyloleuropein, 10-hydroxyoleuropein, oleuropein aglycon, deacetoxy-oleuropein aglycon, ligstroside, ligstroside aglycon, verbascoside, nuzhenide, oleoside, and elenolic acid glucoside were also identified[48-51]. Oleuropein has been

46. Makris D.P., Boskou G., Andrikopoulos N.K. (2007). Polyphenolic content and in vitro antioxidant characteristics of wine industry and other agri-food solid waste extracts. *J. Food Comp. Anal. 20*: 125–132.
47. Bendini A., Cerretani L., Carrasco-Pancorbo A., Gómez-Caravaca A.M., Segura-Carretero A., Fernández-Gutiérrez A., Lercker. G. (2007). 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 12*: 1679-1719
48. Fu S., Arráez-Roman D., Segura-Carretero Menéndez A., J. A., Menéndez-Gutiérrez M.P., Micol V., Fernández-Gutiérrez A. (2010). Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem. 397*: 643–654.

frequently reported to be the most prominent phenolic compounds. Its content is very high, reaching 60-90 mg/g of dry leaf weight[52]. Nevertheless, in certain cases the content of oleuropein has been found to be lower than that of major flavonoids[53].

The following figure shows the structures of the main identified secoiridoids in VOO and olive leaves **(Figure 7)**.

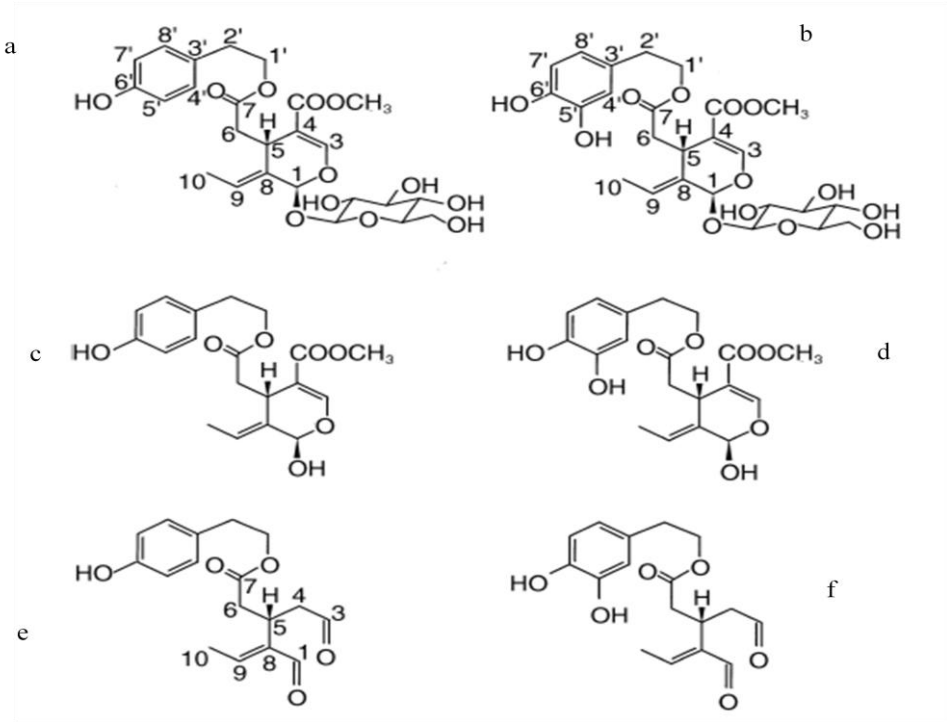


Figure 7. Structures of main secoiridoids identified in virgin olive oil and olive leaves: a. Ligstroside; b. Oleuropein; c. Ligstroside aglycon; d. Oleuropein aglycon; e carboxymethyl

49. Laguerre M., López Giraldo L.J., Georges Piombo M.C., Figueroa-Espinoza M.P., Benaissa M., Combe A., Rossignol Castera A., Lecomte J., Villeneuve P. (2009). Characterization of Olive-Leaf Phenolics by ESI-MS and Evaluation of their Antioxidant Capacities by the CAT Assay. *J. Am. Oil Chem. Soc. 86*: 1215-1225.

50. Fleuriet A., Macheix J.J., Andary C., Villemur P. (1984). Mise en évidence et dosage par chromatographie liquide à haute performance du verbascoside dans le fruit de six cultivars d'*Olea europaea* L. C.R. Acad. Sci. Paris, Ser. III. 7:253-256.

51. Soler-Rivas C., Espín J.C., Wichers H.J. (2000). Oleuropein and related compounds. *J. Sci. Food Agric. 8*: 1013-1023.

52. Le Tutour B, Guedon D. (1992). Antioxidant activities of *Olea europaea* leaves and related phenolic compounds. Phytochem. 31: 1173-1178.

53. Japón-Luján R., Ruiz-Jiménez J., Luque de Castro M.D. (2006). Discrimination and classification of olive tree varieties and cultivation zones by biophenol contents. *J. Agric. Food Chem. 54*: 9706-9712.

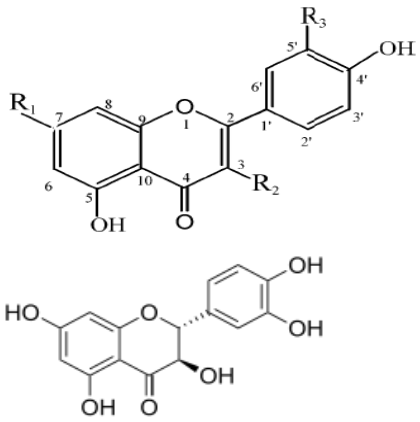
dialdehydic form of ligstroside aglycon; f. Decarboxymethyl dialdehydic form of oleuropein aglycon

3.1.3. Flavonoids

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 on 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 **(Figure 8).** Flavonoids such as luteolin and apigenin are reported to be flavonoids present in virgin olive oil[54]. Luteolin may originate from rutin or luteolin-7- glucoside, and apigenin from apigenin glucosides.

Regarding olive leaves, a significant amount of flavonoids was found. The literature reports several compounds of this class as occurring in olive leaves: luteolin glucoside, luteolin-7-o-rutinoside, apigenin-7-o-glucoside, apigenin 6,8-di-C-glucoside, rutin, quercetin-3-o-glucoside, chrysoeriol-7-o-glucoside, taxifolin, quercetin, chryseriol, luteolin, diosmetin, and apigenin[52-55]. Two luteolin glucoside isomers, luteolin 7-o-glucoside and luteolin 4'-o-glucoside, are found to be the most abundant forms ranging in level from about 0.4-1.6 to 0.1-0.8 mg/g of fresh weight, respectively[56].

55. Bouaziz M., Sayadi S. (2005). Isolation and evaluation of antioxidants from leaves of a Tunisian cultivar olive tree. *Eur. J. Lipid Sci. Technol. 107*:497–504.
56. Liakopoulos G., Stavrianakou S., Karabourniotis G. (2006). Trichome layers versus dehaired lamina of *Olea europaea* leaves: differences in flavonoid distribution, UV-absorbing capacity, and wax yield. *Environ. Exp. Bot*. 55: 294–304.



rutunoside R1=o-rutinose, R2=H, R3=H; luteolin o-glucoside Apigenin glucoside: R1=o-Glucose, R2=H, R3=H
R1=o-glucose; R2=H, R3=OH; rutin R1=OH, R2=o-rutinose, Apigenin rutunoside: R1=o-Rutinose, R2=H, R3=H
R3=OH; luteolin R1=OH, R2=H, R3=OH; quercetin R1=OH, Luteolin o-glucoside: R1=o-Glucose, R2=H, R3=OH
R2=OH, R3=OH; apigenin R1=OH, R2=H, R3=H
Rutin: R1=OH, R2=o-Rutinose, R3=OH
Luteolin: R1=OH, R2=H, R3=OH
Quercetin: R1=OH, R2=OH, R3=OH
Apigenin: R1=OH, R2=H, R3=H

Taxifolin

Taxifolin

Figure 8. Structures of main flavonoids identified in virgin olive oil and olive leaves

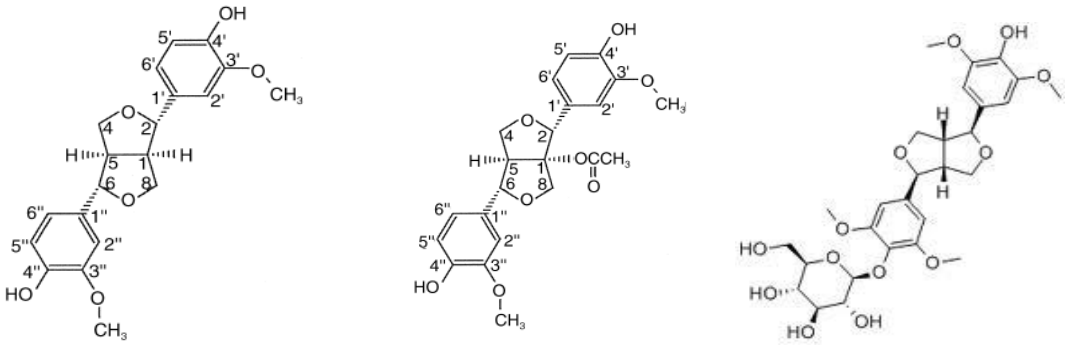
3.1.4. Lignans

The last group of phenolic compounds to be identified in virgin olive oil is the lignans such as acetoxypinoresinol, pinoresinol and hydroxypinoresinol **(Figure 9)**[57]. Chemically, these compounds are related to lignin. Their concentration in virgin olive oil is reported to reach 100 mg/kg[58]. Another lignin compound, syringaresinol **(Figure 9)**, was found in olive leaves but was not quantified[48].

3.2. Factors affecting the phenolic profile in olive oil and leaves

The phenolic composition of olive oil and olive leaves depends on several factors. The statement that the quality of extra-virgin olive oil (EVOO) is genetically dependent is a clear and well-known concept that now appears self-evident[59]. Several studies

57. Brenes M., Hidalgo F. J., Garcìa A., Rios J. J., Garcìa P., Zamora R., Garrido A. (2000). Pinoresinol, and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J. Agric. Food Chem. 77*: 715-720.
58. Owen R. W., Mier W., Giacosa A., Hull W. E., Spiegelhalder B., Bartsch H. (2000). Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* 46: 976-988.
59. Inglese P., Famiani F., Galvano F., Servili M., EspostoS., Urbani S. (2011). Factors affecting virgin olive oil composition. In : Horticultural Reviews Volumen 38, Jules Janick. John Wiley and Sons Inc. Hoboken, New Jersey. Printed in USA.



Acetoxypinoresinol Pinoresinol Syringaresinol

Figure 9. Structures of main lignans identified in virgin olive oil and olive leaves

concerning the effect of the cultivar on the phenolic compounds of virgin olive oil (VOO) have shown the importance of this parameter[60-62]. However, many factors are involved, including the cultivation site[63,64], climate[65], degree of maturation[66], crop season[67], irrigation[68] and production process[69].

60. Tura D., Gioletti C., Pedò S., Failla O., Bassi D., Serraiocco A. (2007). Influence of cultivar and site of cultivation on levels of lipophilic and hydrophilic antioxidants in virgin olive oils (Olea Europea L.) and correlations with oxidative stability. *Sci. Hortic. 112*: 108-119.
61. Solinas, M., Angerosa, F. and Marsilio, V., 1988. Indagine su alcuni componenti dell'aroma degli oli vergini di oliva in relazione alia varietà delle olive. *Rivista Riv. Ital. Sostanze Grasse 45*: 361-368.
62. Aparicio R., Morales M.T., Alonso, V. (1997). Authentication of European extra-virgin olive oils by their chemical compounds, sensory attributes and consumers attitudes. *J. Agric. Food Chem. 45*, 1076-1083.
63 Vinha A.F., Ferreres F., Silva B.M., Valentao P., Gonçalves A., Pereria J.A., Oliveira M.B., Seabra R.M., Andrade P.B. (2005). Phenolic profiles of Portuquese olive fruits (Olea europaea L.): influences of cultivar and geographical origin. *Food Chem. 89*: 561-568.
64. Criado M.N., Morello J.R., Motilva M.J., Romero M.P. (2004). Effect of growing area on pigment and phenolic fractions of virgin olive oils of the Arbequina variety in Spain. *J. Am. Oil Chem. Soc. 81*: 633-640.
65. Servili M., Esposto S., Fabiani R., Urbani S., Taticchi A., Mariucci F., Selvaggini R., Montedoro G. F. (2009). Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacol*. *17*:76-84.
66. Kalua C.M., Allen M.S., Bedgood D.R., Bishop A.G., Prenzler P.D. (2005). Discrimination of olive oils and fruits into cultivars and maturity stages based on phenolic and volatile compounds. *J. Agri. Food Chem. 53*: 8054-8062.
67. Gomez-Alonso S., Salvador M.D, Fregapane G. (2002). Phenolic compounds profile of Cornicabra virgin olive oil. *J. Agric. Food Chem. 50*: 6812-6817.

During the early stages of fruit maturation the total content of phenolic compounds increases and then decreases more or less rapidly with the intensification of the pigmentation of the epicarp and the mesocarp. Oils from very mature olives present lower phenolic contents[30,70]. There are also variations between the ratio of the individual phenols[71,72]. The concentrations of oleuropein aglycon derivatives decrease with the beginning of pigmentation with an increase in the phenolic alcohols such as hydroxytyrosol and tyrosol[73]. The different equilibrium between phenolic compounds toward the simplest forms appears to be related to greater activity of glucosidases and of the esterase during the early stages of fruit ripening[72-65].

Studying the influence of different irrigation-system strategies, Gómez-Rico et al. (2007) found a significant decrease in the polyphenol content in Cornicabra olive-oil variety as the amount of water supplied increased[74]. Water stress during a given period of the olive cycle (pit hardening and fruit growth) influenced not only the total amount of phenolic compounds in the oil but also their profile. Romero et al. (2002)

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68. Tovar M.J., Motilva M.J., Luna M., Girona J., Romero, M.P. (2001). Analytical characteristics of virgin olive oil from young trees (Arbequina cultivar) growing under linear irrigation strategies. *J. Am. Oil. Chem. Soc. 78*: 1-7.

69. Ranalli A., Contento S., Schiavone C., Simone N. (2001). Malaxing temperature affects volatile and phenol composition as well as other analytical features of virgin olive oils. *Eur. J. Lipid Sci. Tech. 103*: 228-238.

70. Baccouri O., Guerfel M., Baccouri B., Cerretani L., Bendini A., Lercker G., Zarrouk M., Ben Miled Daoud D. (2008). Chemical composition and oxidative stability of Tunisian monovarietal virgin olive oils with regard to fruit ripening. *Food Chem. 109*: 743-754.

71. Salvador M.D., Aranda F., Gomez-Aonso S., Fregapane G. (2001). Cornicabra virgin olive oil: A study of five crop seasons. Composition, quality and oxidative stability. *Food Chem. 74*: 267-274.

72. Briante R., Patumi M., Limongelli S., Febbraio F., Vaccaro C., Di Salle A., La Cara F., Nucci R. (2002): Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of olea europaea L. Plant Sci. 162:791-798.

73. Morelló J.R, Romero M.P., Ramo T., Motilva M.J. (2005). Evaluation of l-phenylalanine ammonia-lyase activity and phenolic profile in olive drupe (Olea europaea L.) from fruit setting period to harvesting time. . *Plant Sci. 168*: 65-72.

74. Gómez-Rico A., Salvador M.D., Moriana A., David Pérez c, Olmedilla N., Ribas F., Fregapane G. (2007). Influence of different irrigation strategies in a traditional Cornicabra cv. olive orchard on virgin olive oil composition and quality. *Food Chem. 100*: 568-578.

found that the concentration of lignans, vanillic acid, and vanillin increased in the oils from the most irrigated treatments while the secoiridoid derivatives increased in the oils from the most stressed irrigation treatments[75].

Other studies reported the impact of temperature on the phenolic content of VOO. Ripa et al. (2008) found a negative correlation between the temperature and the phenolic content in Italian olive oils[76], in contrast to the observations of Tura et al. (2008) for Italian cultivar Casaliva [77]. Some authors have reported that polyphenol contents decline with altitude[78] whereas in other reports cite the converse behaviour[79].

Olive-storage conditions also strongly affect the phenolic composition in the fruit before mechanical oil extraction[19,30]. Consequently, EVOO after olive storage registers a lower amount of phenols in comparison to that pressed from fresh fruits[80-65]. Olive-oil storage has also showed an effect on its phenolic composition. Considerable

75. Romero M.P., Tovar M.J., Girona J., Motilva M.J. (2002). Changes in the HPLC phenolic profile of virgin olive oil from young trees (Olea europaea L. Cv. Arbequina) grown under different deficit irrigation strategies. *J. Agric. Food Chem. 50*: 5349-5354.

76. Ripa V., De Rose F., Caravita M.A., Parise M.R., Perri E., Rosati A., Pandolfi S., Paoletti A., Pannelli G., Padula G., Giordani E., Bellini E., Buccoliero A., Mennone C. (2008). Qualitative evaluation of olive oils from new olive selections and effects of genotype and environment on oil quality. *Adv. Hort. Sci. 22*: 95-103.

77. Tura D., Failla O., Pedò S. Gigliotti, C., Bassi D., Serraiocco A. (2008). Effects of seasonal weather variability on olive oil composition in northern Italy. *Acta Hort. 791*: 769-776.

78. Osman M., Metzidakis I., Gerasopoulas D., Kiritsakis A. (1994). Qualitative changes in olive oil of fruits collected from trees grown at two altitudes. *Riv. Ital. Sostanze Grasse 71*: 187-190.

79. Issaoui M., Flamini G., Brahmi F., Dabbou S., Ben Hassine K., Taamali A., Chehab H., Ellouz M., Zarrouk M., Hammami M. (2010). Effect of the growing area conditions on differentiation between Chemlali and Chétoui olive oils. *Food Chem. 119*: 220-5.

80. Angerosa F., Servili M., Selvaggini R., Taticchi A., Esposto S., Montedoro G.F. (2004). Volatile compounds in virgin olive oil: Occurrence and their relationship with the quality. *J. Chrom. A 1054*: 17–31.

decreases in secoiridoid derivatives and 3, 4-DHPEA-AC resulted after the storage period whereas lignans were the more stable phenolic compounds[81].

The extraction process also affects the phenolic composition of EVOO. Olive-oil extraction involves technological procedures that affect the phenolic content of the olive oil and hence oil stability and quality through some chemical and enzymatic reactions of various endogenous enzymes of olive fruit. Crushing and malaxation are the most significant steps of the mechanical oil-extraction process that affect phenolic composition of EVOO. In fact the main hydrophilic phenols such as oleuropein aglycons originate during this phase by the hydrolysis of oleuropein, demethyl oleuropein and ligstroside and catalysed by the endogenous β-glucosidases. During malaxation, the concentration of secoiridoid aglycons and phenolic alcohols diminish in olive pastes and in the related oils, with increasing temperature and processing time[59].

The influence of crop season on the phenolic fraction of VOO was studied by Morello et al. (2006). Their study indicated that the main differences between crop seasons were observed in secoiridoid derivatives, vanillin, tyrosol, apigenin, luteolin, and lignans[82].

There has been growing interest in the study of olive leaves mainly for their biological activity. Nevertheless, there is lack of information concerning the factors that may affect its phenolic fraction. It was reported that the concentration of phenolic compounds in olive leaf varies depending on the quality, origin, and variety of the

81. Morello J.R., Motilva M.J., Tovar M.J., Romero M.P. (2004). Changes in commercial virgin olive oil (cv Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chem. 85*: 357-364.

82. Morello J.R., Romero M.P., Motilva M.J. (2006). Influence of seasonal conditions on the composition and quality parameters of monovarietal virgin olive oils. *J. Am. Oil Chem. Soc. 83*: 683-690.

olive tree[83]. Some authors have found that the amounts of oleuropein in the olive leaves were markedly altered by colour/age and genetic factors, whereas negligible variations were ascribable to factors such as the quantification method and the collecting period[84].

Many studies have been reported on the classification of olive oils according to their cultivars or geographical origins or PDO by means of various chemometric methods applied to fatty acids and triacylglycerols[85], sterol compositions[86,87], sensory attributes[47], volatile compositions[88], and phenolic composition[89]. Phenolic acids, hydroxytyrosol, and tyrosol have been found more suitable variables than other phenolics for classifying virgin olive-oil varieties by means of PCA and stepwise discriminant analyses[90,91]. Considering the oleuropein concentration, statistical analyses were also applied to olive-leaf samples with the aim of evaluating the

83. Campeol E., Flamini G., Cioni P.L. Morelli, I.; Cremonini, R.; Ceccarini, L. (2003). Volatile fractions from three Cultivars of *Olea europea* L. collected in two different seasons. *J. Agric. Food Chem. 51*: 1994-1999.

84. Ranalli A., Contento S., Lucera L., Di Febo M., Marchegiant D. Di Fonzo V. (2006). Factor affecting the content of iridoid oleuropein in olive leaves (*Olea europaea L.*). *J. Agri. Food Chem. 54*: 438-448.

85. Stefanoudaki E., Kotsifaski F., Koutsaftakis A. (1999). Classification of olive oils of the two major Cretan cultivars based on their fatty acid composition. *J. Am. Oil Chem. Soc. 76*: 623-626.

86. Rui Alves M., Cunha S.C., Amaral J.S., Pereira J.A, Oliveira M.B. (2005). Classification of PDO olive oils on the basis of their sterol composition by multivariate analysis. *Anal. Chim. Acta, 549*: 166-178.

87. Lerma-García M.J., Simó-Alfonso E.F., Méndez A., Lliberia J.L., Herrero-Martínez J.M. (2011). Classification of extra virgin olive oils according to their genetic variety using linear discriminant analysis of sterol profiles established by ultra-performance liquid chromatography with mass spectrometry detection. *Talanta 75*: 937-943.

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effectiveness of some influencing factors, including genetic profile, harvest period, and colour/age in the discrimination among the samples studied [84].

4. PHENOLIC COMPOUNDS IN OLIVE AND BIOACTIVITY

The evergreen olive tree (*Olea europaea* L.) treasured over the centuries for its fruits and the oil, is becoming a source of natural antioxidants and other bio-active ingredients. The pharmacological properties of olive oil, fruit, and leaves have been recognized as important components of medicine and a healthy diet because of their phenolic content[92].

High levels of total cholesterol (TC), low-density lipoprotein (LDL-C) and LDL-C oxidation have been known as factors for atherosclerosis risk, which is a main cause of cardiovascular disease. Consumption of phenol-rich VOOs demonstrated a beneficial effect on plasma lipoproteins. Studies involving animals have demonstrated that ingestion of phenol rich VOO led to a decrease in circulating TC, LDL-C[93] and an increase in HDL-C[94] upon phenol-rich VOO consumption. Furthermore, *in vivo* human studies showed that the ratio of total cholesterol (TC) to high-density lipoprotein-cholesterol (HDL-C) decreases with increasing phenolic concentration of VOO consumed. HDL-C level also rose linearly with phenolic content of the oil[95].

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In the same way, the administration of polyphenol-rich olive-leaf extracts significantly lowered the serum levels of total cholesterol (TC), triglycerides and LDL-C while at the same time elevating the serum level of HDL-C in rats fed a cholesterol-rich diet. In addition, these extracts lowered the content of the lipid peroxidation indicators in the liver, heart, kidneys, and aorta compared with those of rats fed on a cholesterol-rich diet and were capable of boosting the serum antioxidant potential and hepatic superoxide dismutase and catalase activities[96].

In vivo human and animal studies have demonstrated diminished LDL oxidation with greater ingestion of olive-oil phenolic compounds[97]. Phenolic compound hydroxytyrosol were found to be capable of preventing LDL oxidation[98].

VOO phenolic compounds have been also shown to inhibit human platelet activity *in vitro*[99], and compounds such as hydroxytyrosol[100], oleuropein aglycone, and luteolin[101] were potent inhibitors of platelet aggregation.

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101 Dell' Agli, M., Maschi, O., Galli, G. V., Fagnani, R., Dal Cero, E., Caruso, D., Bosisio, E. (2008). Inhibition of platelet aggregation by olive oil phenols via cAMP-phosphodiesterase. *Br. J. Nutr. 99*: 945-951.

In non-smoking male humans, olive-leaf polyphenols also inhibited *in vitro* platelet activation in healthy, possibly via the H_2O_2-scavenging properties of these compounds[102].

Oxidative damage to DNA is a precursor for human carcinogenesis[103]. Research concerning DNA damage (as measured by the comet assay and 8-oxo-deoxyguanosine) showed that the intake of phenol-rich EVOO (up to 592 mg/kg) lessens oxidative DNA damage *in vivo* in humans by up to 30%[104,105]. In agreement with these findings, a recent animal study has also reported the protective effect of olive-oil phenolic compounds against DNA damage[106].

Animal experimentation has demonstrated the beneficial effect of using oleuropein derived from olive-leaf extract as an effective hypoglycaemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing both enzymatic and non-enzymatic defences against diabetes[107].

It is well established that the pathophysiology of common disease states such as cancer, cardiovascular disease, arthritis and neurodegenerative disease are

102. Singh I., Mok M., Christensen A.M., Turner A.H., Hawley J.A. (2008). The effects of polyphenols in olive leaves on platelet function. *Nutr. Metab. Cardiovasc. Dis. 18*: 127-132.
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107. Al-Azzawie H.F., Saeed Alhamdani M.S. (2006). Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits. *Life Sci. 78*: 1371-1377.

associated with chronic inflammation[108],[109]. *In vivo* studies have demonstrated the anti-inflammatory property of VOO phenolic compounds. These studies have reported a significant decrease in inflammatory markers, namely plasma thromboxane B_2 (TXB_2) and leukotrine B_4 (LTB_4)[110], Interleukin-6 (IL-6) and C-reactive protein (CRP)[111]. Hydroxytyrosol reported had significant anti-inflammatory properties in an animal model of inflammation and attenuated tumour necrosis factor alpha (TNFa) and interleukin 1 beta (IL- 1b) expression, which are pro-inflammatory cytokines often observed in inflammatory disease[112]. Oleuropein aglycon inhibits TNFa-induced matrix metalloproteinase 9 (MMP-9) in a monocyte cell line, and this has implications for health as monocytes together with the molecules they express play a significant role in inflammation-based disease-development capacity[113]. Furthermore, the phenolic compound, oleocanthal has been found to share the same mechanistic anti-inflammatory pathway as the non-steroidal anti-inflammatory drug, ibuprofen. *In vitro*, oleocanthal has been shown to inhibit both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inflammatory

108. Mc Geer P.L., Mc Geer E.G., Schwab C. (2009). Inflammatory processes exacerbate degenerative neurological disorders. Curr. Hypoth. Res. Mile Alzheim. Dis. *4*: 117-124.
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enzymes in a dose-dependent manner, and is more potent than ibuprofen in inhibiting these enzymes at equimolar concentrations[114].

Moreover, *In vitro* research has reported antimicrobial properties of VOO phenolic compounds. The dialdehydic form of decarboxymethyl ligstroside aglycon, in particular, showed potent antimicrobial activity against different bacterial strains [115].

Research on phenolic compounds from olive leaves reported that verbacoside can be used to repair brain's oxidative damage caused by heroin consumption[116], apigenin-7-glucoside to fight against Alzheimer's[117] or liver diseases[116] and luteolin-7-glucoside was found to avoid the abnormal proliferation of aortic vascular smooth-muscle cells that is a common cause of pathogenesis such as atherosclerosis and restenosis[118].

Cancer diseases came to prominence in the 20th century; with many still eluding treatment. Some scientists say that this disease is a result of a degeneration of patient's own body when exposed to potential tumour-causing substances (environmental factors) defined broadly to include tobacco, pollution, alcohol or diet. Among these factors, an unhealthy diet is the factor most likely to affect personal cancer risk. Research shows that about one-third of all cancer deaths are related to dietary factors and the lack of physical activity in adulthood[119]. Della Ragione et al.

114. Beauchamp G.K., Keast R.S.J, Morel D., Lin J., Pika J., Han Q., Lee C-H, Smith A.B., Breslin P.A.S. (2005). Phytochemistry: ibuprofen-like activity in extra-virgin olive oil. *Nature, 437*: 45-46.

115. Medina E., de Castro, A., Romero C., Brenes M. (2006). Comparison of the concentrations of phenolic compounds in olive oils and other plant oils: correlation with antimicrobial activity. *J. Agric. Food* Chem**.** *54*: 4954-961.

116. Zheng Q., Zhang Y., Zheng R., Guo D., Li, C. (2005). Effects of Verbascoside and Luteolin on Oxidative Damage in Brain of Heroin Treated Mice. *Pharmacie*, *60*:539-543.

117. Patil S.C., Singh V.P., Satyanarayan P.S.V. Jain N.K., Singh A., Kulkarni S.K. (2003). Protective Effect of Flavonoids against Aging- and Lipopolysaccharide-Induced Cognitive Impairment in Mice. *Pharmacology*, *69*: 59-67.

118. Kim T.J., Kim J.H., Jin Y.R., Yun Y.P. (2006). The Inhibitory Effect and Mechanism of Luteolin 7-Glucoside on Rat Aortic Vascular Smooth Muscle Cell Proliferation. *Archives of Pharmacal Research*, 29: 67-72.

119. López S., Pacheco Y.M., Bermúdez B., Abia R., Muriana F.J.G. (2004). Olive oil and cancer. *Grasas y Aceites, 55*: 33-41.

showed that hydroxytyrosol induced cell death in quiescent and differentiated HL60 cells, a cell line established from promyelocytic acute leukaemia but not in two colorectal cell lines (HT29 and Caco-2)[120]. Since inflammatory processes are involved in all steps of cancer transformation, these findings suggest the capability of hydroxytyrosol to reduce the lymphocytic response by inhibiting proliferation and inducing apoptosis. Hydroxytyrosol, at a concentration of 50-100 μM (comparable with its content in EVOO) caused a complete arrest of HL60 and induced apoptosis. The human malignant gingival cell line HSG1 was sensitive to phenolics, and the sequence of increasing cytotoxicity was oleuropein aglycon> oleuropein glycoside, caffeic acid> *o*-coumaric acid> cinnamic acid>> tyrosol, syringic acid, protocatechuic acid, vanillic acid[121]. Moreover, the flavonoid apigenin-7-glucoside was mainly responsible for the differentiation of HL-60 human leukaemia cells[122]. Recent investigation has shown the cell-growth-inhibition effect of EVOO phenolic extract on SW480 and HT29 human-colon adenocarcinoma cell lines. Inhibition of proliferation was accompanied by apoptosis in both colon-cancer-cell lines and a limited G2M cell-cycle arrest in the case of SW480 cells[123].

Breast cancer is the most prevalent cancer among females worldwide. Usually breast cancer either begins in the cells of the lobules **(Figure 10)**, which are the milk-producing glands, or the ducts, the passages that drain milk from the lobules to the

120. Ragione F.D., Cucciolla V., Borriello A., Pietra V.D., Pontoni G., Racioppi L., Manna C., Galleta P., Zappia V. (2000). Hydroxytyrosol, a natural molecule occurring in olive oil, induces cytochrome c-dependent apoptosis. *Biochem. Biophys. Res. Commun. 278*: 733-739.

121. Babich H., Visioli F. (2003). In vitro cytotoxicity to human cells in culture of some phenolics from olive oil. *Farmaco*. *58***:** 403-407.

122. Abaza L., Talorete T.P.N, Yamada P., Kurita Y., Zarrouk M., Isoda H. (2007). Tunisian Gerboui olive leaf extract induces growth inhibition and differentiation of human leukemia HL-60 cells. *J. Biosci. Biotech. Biochem. 71*: 1306-1312.

123. Fernandez-Arroyo S., Gomez-Martinez A., Rocamora-Reverte L., Quirantes-Pine R., Segura-Carretero A., Fernandez-Gutierrez A., Ferragut J. A. (2012). Application of nanoLC-ESI-TOF-MS for the metabolomic analysis of phenolic compounds from extra-virgin olive oil in treated colon-cancer cells. *J. Pharm. Biomed. Anal. 63*:128-134.

nipple. Less commonly, breast cancer can begin in the stromal tissues, which include the fatty and fibrous connective tissues of the breast[124].

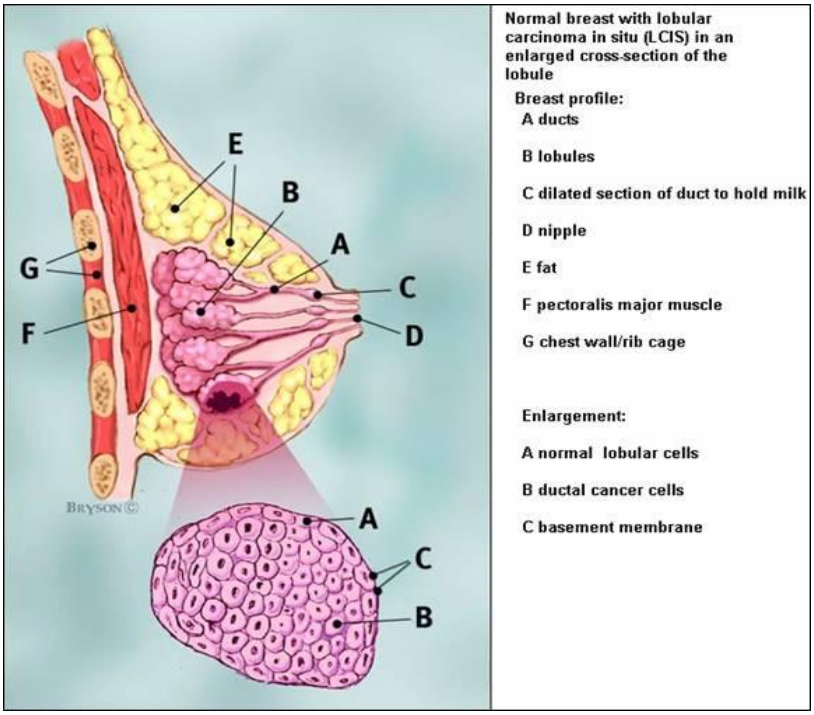

Figure 10. Lobular carcinoma *in situ* (LCIS) is a condition in which abnormal cells in the lobules of the breast[125]

Research studies investigating the association of polyphenols with breast-cancer risk have recently been increased. It was reported that secoiridoids seem to significantly combat human breast cancer by directly modulating the activities of various types of receptor tyrosine kinases, including the human epidermal growth-factor receptor (HER2) a *proto*-oncogene that plays a pivotal role in malignant transformation, tumour genesis, metastasis, and treatment failure in breast-cancer disease[126-91]. In

124. Breast cancer is an uncontrolled growth of breast cells**.** (2012). Available on the web http://www.breastcancer.org. Accessed April 24, 2012.

125. Lobular carcinoma *in situ* (LCIS) : Available on http://www.world-drugs.net/generic_nolvadex_tamoxifen_citrate.php. Accessed April 24, 2012.

126. Menendez J.A., Vazquez-Martin A., Colomer R., Brunet J., Carrasco-Pancorbo A., Garcia-Villalba R., Fernandez-Gutierrez A., Segura-Carretero A. (2007). Olive oil's bitter principle reverses acquired

addition, it was found that hydroxytyrosol-rich extract from olive leaves modulates cell-cycle progression in MCF-7 human breast-cancer cells[129].

Despite the evidence for the anticarcinogenic activity of the olive phenolic compounds, data from the literature on the molecular mechanism of the uptake and metabolism of these compounds in human are limited.

It should be noted that the biological and pharmacological activities of the phenolic extract result also from factors such as the extraction method, variations of which may directly influence the phenolic composition of the extract.

Given the importance of phenolic compounds from olive products and by-products, the extraction process constitutes a basic step in isolating and purifying compounds of interest.

5. Extraction techniques for phenolic compounds

The extraction process consists of separating one or more species from a solid or liquid matrix based on the different relative solubility that such substance or substances present in a certain solvent with respect to the rest of the components of the matrix. In other words, extraction works according to the principle that soluble components can be separated from insoluble or less soluble components by

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autoresistance to trastuzumab (Herceptin) in HER2-overexpressing breast cancer cells. *BMC Cancer 7*: 80.

127. Menendez J.A., Vazquez-Martin A., Oliveras-Ferraros C., Garcia-Villalba R., Carrasco-Pancorbo A., Fernandez-Gutierrez A., Segura-Carretero A. (2009). Extra-virgin olive oil polyphenols inhibit HER2 (erbb-2)-induced malignant transformation in human breast epithelial cells: relationship between the chemical structures of extra-virgin olive oil secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2. *Int. J. Oncol. 34*: 43-51.

128 García-Villalba R., Carrasco-Pancorbo A., Oliveras-Ferraros C., Vázquez-Martín A., Menéndez J.A., Segura-Carretero A., Fernández-Gutiérrez A. (2010). Characterization and quantifcation of phenolic compounds of extra-virgin olive oils with anticancer properties by a rapid and resolutive LC-ESI-TOF MS method. *J. Pharm. Biomed. Anal. 51*: 416-429.

129. Bouallagui Z., Han J., Isoda H., Sayadi S. (2011). Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food Chem. Toxicol. 49*: 179-184.

dissolving them in a suitable solvent. The type of extraction procedure also plays a decisive role in determining the qualitative and quantitative composition of the extract. Thus, depending on the extraction techniques selected, diverse extraction parameters should be tested in order to study the influence of solvents, temperatures, pressures, and other important parameters that might significantly influence the outcome of the extraction process employed.

At present, new promising extraction methods introduce some form of additional energy to the process in order to facilitate the transfer of solutes from sample to solvent. These methods include ultrasonic extraction, microwave-assisted extraction, supercritical fluid extraction and pressurized liquid extraction.

5.1. Conventional liquid/solid-liquid extraction

The principle of liquid-liquid (LLE) and solid-liquid (SLE) extractions is that when a sample matrix comes into contact with a solvent, the soluble components in the sample matrix move to the solvent. Thus, the solvent extraction results in the mass transfer of soluble components to the solvent in a gradient concentration. The selection of the solvent will be determined by the chemical and physical properties of the target substances. The solvent temperature must be chosen accurately depending on the raw material and on the thermal resistance of the solutes to be recovered.

5.2. Ultrasound-assisted extraction (UAE)

The ultrasound-assisted extraction does not require complex instruments and is relatively low-cost. It can be used both on a small and large scale in the phytopharmaceutical extraction industry[130]. In ultrasound-assisted extraction, only a

130. Vinatoru M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason. Sonochem. 8*: 303-313.

small portion of ultrasound spectrum is used, namely power ultrasound. Power ultrasound having frequencies between 20 kHz and 100 MHz involves passing ultrasonic energy in the form of waves through a liquid solvent containing the solid particles. As the waves hit the surface of the material, a force that is either perpendicular or parallel to the surface is generated. If the force is perpendicular to the surface, a compressive wave is formed whereas if the force is parallel to the surface, a shear wave results, giving rise to a phenomenon known as cavitation[131]. Sound waves can improve the extraction yield of plant material because they involve alternative expansions and compressions of matter inducing the creation of bubbles in liquids. The most relevant parameter to control in UAE is frequency, because small changes of this parameter can dramatically affect the yield of the extraction. Ultrasounds cause a greater penetration of solvent into cellular matrices improving mass transfer. It allows process conditions to be milder compared to traditional solvent extraction, so it is recommended for thermolabile substances.

5.3. Microwave-assisted extraction (MAE)

The application of microwave irradiation to the extraction of compounds from biological samples has recently been developed. MAE was first described in 1986 using a domestic microwave as a sample preparation technique prior to chromatography[132]. There are two types of commercially available MAE systems **(Figure 11)**: closed extraction vessels (in which every zone in the cavity and the sample it contains is evenly irradiated with randomly dispersed microwaves in the

131. Ibanez E., Herrero M., Mendiola J.A., Castro-Puyana M. (2009). Extraction and characterization of bioactive compounds with health benefits from marine resources: macro and micro Algae, cyanobacteria and invertebrates. In: Marine bioactive compounds, sources, characterization and applications. Ed. Maria Hayes. Springer New York Dordrecht Heidelberg London. 55-98.
132. Ganzler K., Salgó A., Valkó K. (1986). Microwave extraction: a novel sample preparation method for chromatography. *J. Chromato. A 371*: 299-306.

cavity) which is the most commonly used one, and open system (in which only a part of the extraction vessel containing the sample is irradiated with microwave)[133].

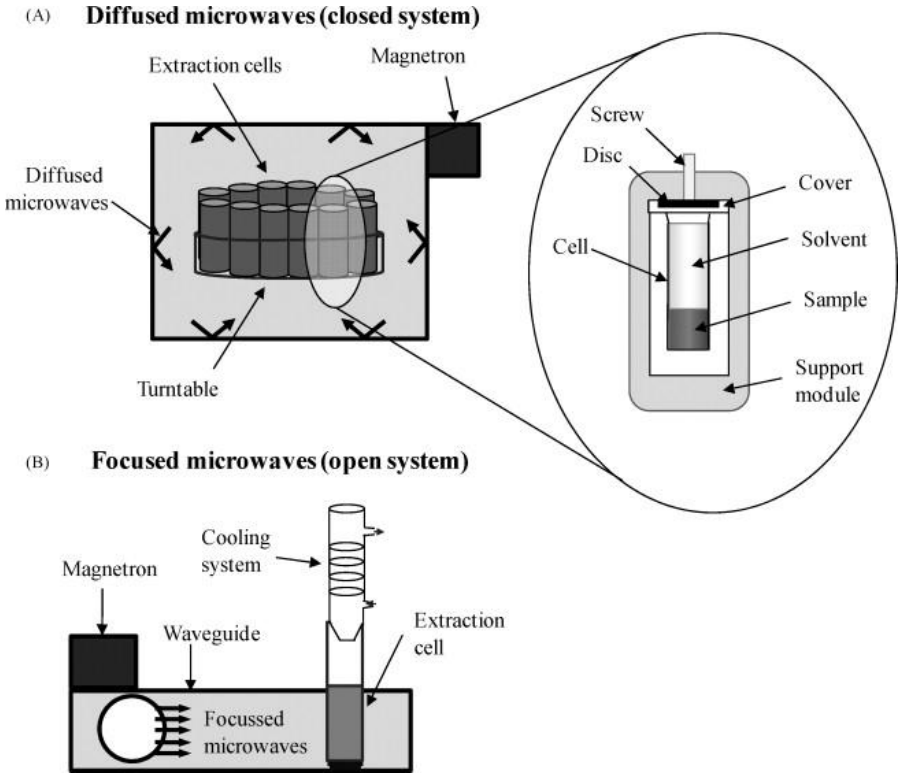


Figure 11. Modes of MAE : closed (A) and open (B) systems

Microwaves are electromagnetic waves that interact with matter, in particular with polar molecules to generate heat. They can therefore, penetrate water and biological matrices heating up the whole at a homogeneous rate. Radiation produces superheating of water within plant cells and causes the rupture of the cell wall, facilitating the transfer of desirable substances to the bulk phase inside the extraction vessel and the penetration of the solvent into the plant matrix. Microwaves can therefore improve the extraction yields of bioactive compounds. The volume of

133. Mandal V., Mohan Y., Hemalatha S. (2007). Microwave Assisted Extraction- An innovative and promising extraction tool for medicinal plant research. *Phcog. Rev. 1*:7-18.

solvent needed, and the extraction time are reduced. The effectiveness of MAE depends heavily on the polarity of the solvent, and on particle size and on the distribution of the plant material. It can be applied to extract polar components, but it is not suitable for dry materials or too wet matrices using non-polar solvents.

Solvents which are transparent to microwaves, do not heat up under microwave and those with good microwave-absorbing capacity get heated up faster and enhance the extraction process. Hexane is an example of microwave transparent solvent (see **Table 1**). To achieve optimum extraction yields, researchers even use mixtures of high and low microwave-absorbing solvents.

Table 2. The dielectric constant values of various solvents[134]

Solvent	Dielectric Constant (20°C)	Dipole moment (20°C) (Debeye)
Hexane	1.89	0.1
Tolune	2.4	0.36
Dichloromethane	8.9	1.14
Acetone	20.7	2.69
Ethanol	24.3	1.69
Methanol	32.6	2.87
Water	78.5	1.87

134. Kaufmann, B. and P. Christen. 2002. Recent extraction techniques for natural products: microwave assisted extraction and pressurized solvent extraction. *Phytochemical analysis* 13:105-113.

The MAE efficiency is influenced by the microwave power and irradiation time, which in turn greatly affect each other. To optimise a MAE procedure, a combination of low or moderate power with longer exposure is generally selected[135]. Although due to the use of high power, there is an associated risk of thermal degradation/deterioration, and reports also exist indicating that a change in power from 500 W to 1000 W had no significant effects on the flavonoid yield[136]. Matrix characteristics such as particle size and the nature of the material considerably affect the recoveries of the compounds. The finer the particle size of the sample the larger the surface area and hence the better the penetration of microwaves[137]. Some researchers apply the technique of pre-leaching extraction, (soaking of matrix with solvent prior to irradiation) to get better yields[138]. The higher temperature improves the extraction efficiency as desorption of analyte from active sites in the matrix increases. At higher temperatures, surface tension and solvent viscosity decreases and solvents have a greater capacity to solubilize the analytes by improving sample wetting and matrix penetration, respectively.

5.4. Pressurized liquid extraction (PLE)

Pressurized liquid extraction is a relatively new technology for extracting phytochemicals under high temperatures and pressures. This approach was described

135. Tatke P., Jaiswal Y. (2011). An Overview of Microwave Assisted Extraction and its Applications in Herbal Drug Research. *J. Med. Plants Res. 5*:21-31. DOI: 10.3923/rjmp.2011.21.31.
136. Raner K.D., Strauss C.R., Vyskoc F., Mokbel L., (1993). A comparison of reaction kinetics observed under microwave irradiation and conventional heating. *J. Org. Chem., 58*: 950-995.
137. Huie C.W., (2002). A review of modern sample preparation techniques for the extraction and analysis of medicinal plants. *Anal. Bioanal. Chem*. *373*: 23-30.
.138 Pan, X. Niu, G., Liu. H. (2001). Microwave assisted extraction of tanshinones from *Salvia miltiorrhiza* bunge with analysis by high performance liquid chromatography. *J. Cromatogr. A. 922*: 371-75.

for the first time in 1996[139]. During PLE, pressure is applied, allowing the use of temperatures above the boiling point of solvents.

The combined use of high pressure (3.3-20.3 MPa) and temperature (40-200°C) provides faster extraction processes that require small amounts of solvents. High temperature and pressure improves analyte solubility and the desorption kinetics from the matrices[139]. Thus, for high recoveries, several extraction conditions such as the extraction solvent, temperature, pressure, static time, and number of cycles can be optimised.

The PLE process can be conducted in both the dynamic and static mode. The static mode has been the most widely used and is the most frequent when commercial instruments are used. The dynamic mode, presumably, could improve the extraction rate by allowing a better contact between the matrix and fresh solvent pumped in a continuous way through the extraction cell and is used mainly with lab-made devices[131].

The schematic process of the PLE is represented in the figure below **(Figure 12)**.

139. Richter B.E., Jones B.A., Ezzell J.L., Porter N.L., Avdalovic N., Pohl C. (1996). Accelerated solvent extraction: a technique for sample preparation. *Anal. Chem. 68*: 1033-1039.

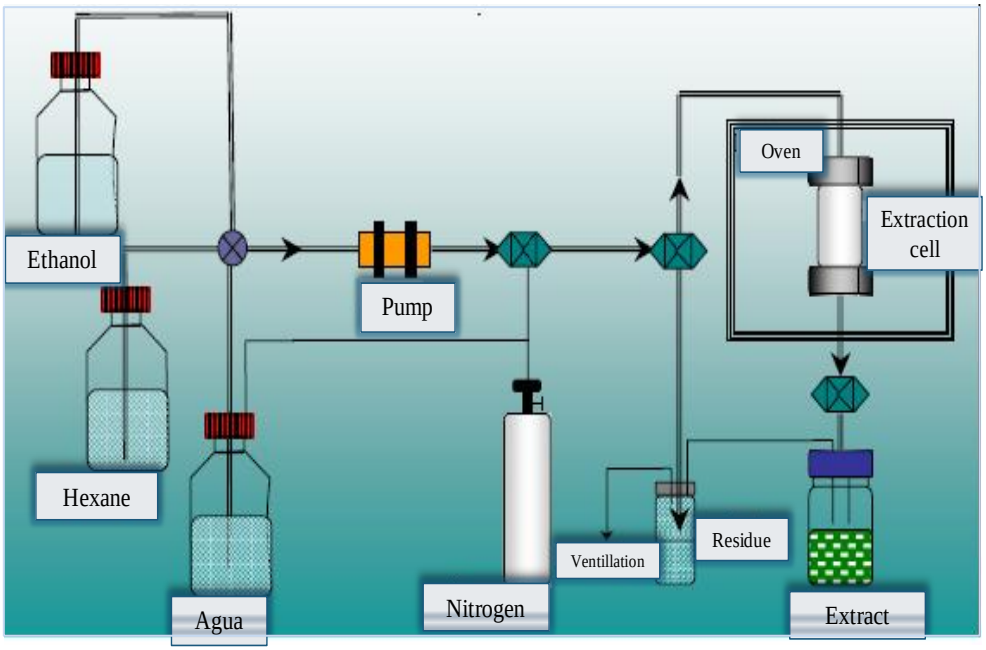


Figure 12. Schematic diagram of the PLE system

PLE is also known as pressurized solvent extraction (PSE), subcritical solvent extraction (SSE) or accelerated solvent extraction (ASE). When 100% water is used as a solvent, PLE is generally called superheated water extraction, subcritical water extraction, pressurized low-polarity water extraction or pressurized hot-water extraction[140]. Regarding the variable types of solvent that can be used in PLE, this technique is highly versatile and flexible in terms of bioactive compounds to be extracted. However, it does not offer high selectivity and requires expensive equipment.

5.5. Supercritical Fluid Extraction (SFE) SC-CO_2 extraction

Supercritical fluid extraction was first introduced as an alternative extraction method in 1879[131]. However, it was not until around 1960 that this extraction method started

140. Pronyk C., Mazza G. (2009). Design and scale-up of pressurized fluid extractors for food and bioproducts. *J. Food Eng. 95*: 215-226.

to be thoroughly investigated as an alternative to conventional extraction methods such as SLE and LLE[141]. Supercritical fluid extraction is based on the use of solvents at temperatures and pressures above their critical points. The supercritical state is reached by bringing the fluid to a temperature and pressure beyond its critical point **(Figure 13)**. Supercritical fluids present characteristics of both gases and liquids and properties that make them especially suitable for extraction processes.

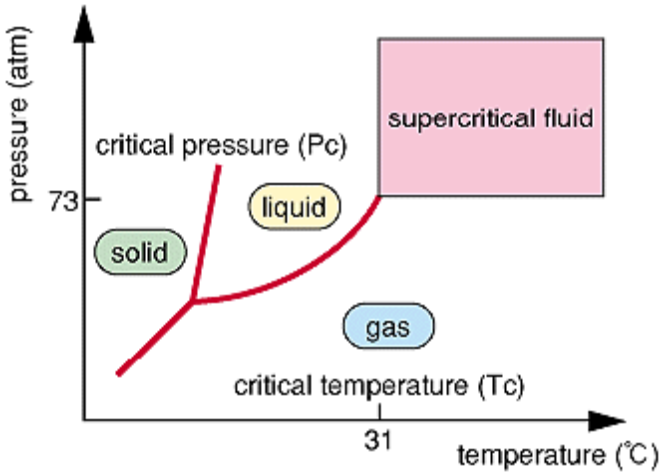


Figure 13. Phase diagram of supercritical CO_2

Extractions are made in dynamic mode, static mode, or a combination of the two. In a dynamic extraction, the supercritical fluid continuously flows through the sample in the extraction vessel and out of the restrictor to the trapping vessel. In static mode, the supercritical fluid circulates in a loop contained in the extraction vessel for some period of time before being released through the restrictor to the trapping vessel. In combination mode, a static extraction is performed for some period of time, followed by a dynamic extraction.

141. Hosikian A., Lim S., Halim R., Danquah M.K. (2010). Chlorophyll extraction from Microalgae: a Review on the process engineering aspects. *Inter. J. Chem. Eng*. Article ID 391632, 11. doi: 10.1155/2010/391632.

It is necessary to note that main variables such as fluid density (pressure and/or temperature), mode of extraction (static or dynamic), percentage and type of modifier addition, amount of sample to be extracted, type of restrictor, collection system, as well as its particle size and the use of dispersing agents influence the SFE. In some applications, the use of dispersing agents such as sand, glass beads, and diatomaceous earth or a hydromatrix to absorb liquid from the sample may be useful.

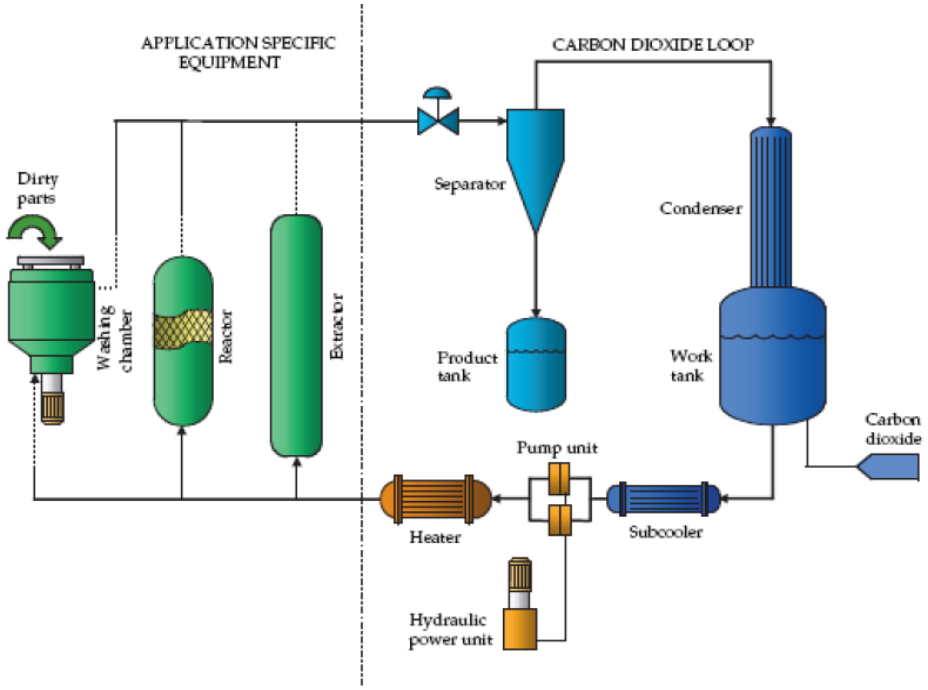


Figure 14. Schematic diagram of PLE system

The most used solvent is CO_2, which is cheap, safe, and non-toxic, and its supercritical conditions may be fairly easily reached. It can be used to extract polyphenols and other natural antioxidants. Advantages of using CO_2 under supercritical conditions for the extraction of bioactive compounds include its high diffusivity and the relative ease in tuning the temperature and pressures applied, so that solvent strength and density can be easily modified. Another advantage of SFE when using CO_2 is the possibility of attaining solvent-free extracts. Once the extraction procedure is

finished, the depressurization of the system converts CO_2 from a liquid to a gas, making it easier to recover the extract. These properties are responsible for the extended use of supercritical CO_2 for extracting bioactive compounds. The main drawback of supercritical CO_2 is its low polarity, a problem that can be overcome by employing polar modifiers or co-solvents to change the polarity of the supercritical fluid and to increase its solvating power towards the analyte of interest[131].

6. ANALYTICAL TECHNIQUES USED FOR SEPARATING PHENOLIC COMPOUNDS

Chromatography is a very special purification process because it can separate complex mixtures with great precision. In fact, chromatography can purify basically any soluble or volatile substance. It can be used to separate delicate products because the conditions are not typically hard. For these reasons, it can be used to separate mixtures of bioactive compounds from olive. Another advantage of this technique is that the separated compounds are immediately available for identification or quantification.

One of the most widely used chromatographic techniques for the determination of phenolic compounds in plant matrix is the high-performance liquid chromatography (HPLC) **(Figure 15)**.

Figure 15. HPLC system

6.1. High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is a separation technique where the matrix components are distributed between a mobile phase and a stationary phase. HPLC uses a liquid mobile phase to separate the components of a mixture. The stationary phase can be a liquid or a solid phase. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under a high pressure. In the column, the mixture separates into its components. The amount of resolution is important, depending upon the extent of interaction between the solute components and the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in

other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

6.1.1. Instrumentation of HPLC system

HPLC instrumentation includes a pump, injector, column, detector and data system **(Figure 16)**. Each of these units is essential for performing the analysis. The heart of the system is the column where separation occurs. Since the stationary phase is composed of micrometer-sized porous particles, a high-pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute into the top of the column. Separation of components occurs as the analytes and mobile phase are pumped through the column. The components interact with the stationary phase so that those having more affinity with the mobile phase are less retained by the stationary phase and are eluted first. By contrast, those having more affinity with the stationary phase move more slowly through the column and are eluted later. Eventually, each component is eluted from the column as a narrow band (or peak) on the recorder. The response of the detector to each detected component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyse the chromatographic data, a computer, integrator, and other data-processing equipment are frequently used.

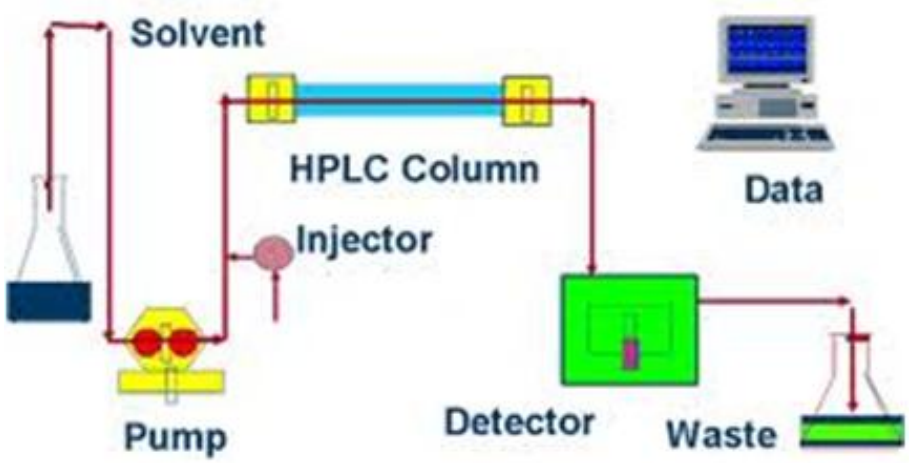


Figure 16. Schematic diagram of HPLC system

- Pumping unit

The eluent must be pumped at a constant flow rate and pressure through the system. Modern pumps have the capacity to pump more than one solvent with variable and programmable percentages, and dispose of a degasification system for the mobile phase.

- Sample-injection unit

A given volume of the sample is injected into the flow path for analysis. This is accomplished via a manual injector or an autosampler.

- Chromatographic column

The column contains the stationary phase where the separation of the components occurs. It is sometimes preceded by a “pre-column” to prevent the sample components, which may damage the stationary phase, from reaching the column. The temperature of the column is maintained constant by a thermostat-controlled oven.

- Detection unit

The components eluted from the column are detected, and the detection data are converted into an electrical signal. The detector is selected to suit the sample.

- Data-processing unit

The concentration of each detected component is calculated from the area or height of the corresponding peak, and reported. Although previously, easy-to-use integrators were mainly used, systems in which a PC performs both the operation of the units and the analysis of the results have recently played a central role.

6.1.2. Types of liquid chromatography

Different modes of liquid chromatography can be distinguished according to the nature of the stationary phase. The five basic modes are:

a) Adsorption chromatography: the stationary phase is a solid adsorbent (like silica gel or any other silica based packing) and the separation is based on repeated adsorption-desorption steps.

b) Ion-exchange chromatography: the stationary phase is solid and has ion-charged surface of opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionisable samples.

c) Size-exclusion chromatography: the column is filled with material having precisely controlled pore sizes, and the sample is simply screened or filtered according to its solvated molecular size. This technique is also called gel-filtration or gel-permeation chromatography.

d) Partition chromatography: the stationary phase is a liquid retained by a solid packing. This chromatography is based on the partition of the analytes between the mobile and stationary phases.

e) Affinity chromatography: the stationary phase is solid with biospecific retention properties.

Concerning the partition chromatography, two modes are defined depending on the relative polarity of the two phases: normal and reversed-phase chromatography. In

normal-phase chromatography, the stationary phase is strongly polar in nature and the mobile phase is non-polar or less polar. Polar samples are thus retained on the polar surface of the column packing for longer than are less polar ones. Reversed-phase chromatography is its opposite. The stationary phase is non-polar in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. Herein the more the non-polar the material is, the longer it will be retained.

Reverse-phase chromatography is used for almost 90% of all chromatographic applications. Eluent polarity plays the major role in all types of HPLC. There are two elution types: isocratic and gradient. In the first type, constant eluent composition is pumped through the column during the whole analysis. In the second type, the eluent composition is steadily changed during the run.

HPLC as compared with the classical LC technique is characterised by:

- High resolution
- Small diameter (4.6 mm), stainless steel, glass or titanium columns;
- Column packing with very small (3, 5, and 10 µm) particles;
- Relatively high inlet pressures and controlled flow of the mobile phase;
- Continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- Rapid analysis;

6.2. Detectors

By passing the column effluent through the detector, some chemical or physical property of the analyte is transduced to an electrical signal and the solutes are monitored as they are eluted from the column.

There are various types of detectors coupled to liquid chromatography. The choice of the detector for each application is based on the nature and properties of the

analytes to be determined, the required sensitivity and type of desired information (structural, quantitative, etc.). The ideal characteristics of an HPLC detector are high sensitivity, good stability, linearity, short response time, reliability, non-destructiveness, ease of use, and low dead volume.

The most commonly used detector in LC is the ultraviolet-absorption detector. Detectors include variable wavelength detector, photodiode Array UV detector (PAD), refractive index (RI), fluorescence (FLU), electrochemical (EC). The RI detector is universal but is also considered the least sensitive one. FLU and EC detectors are quite sensitive (up to 10-15 pmole) but also quite selective (**Table 3**).

Evaporative light-scattering detector (ELSD), Corona® Charged Aerosol Detector (CAD), and others are also used. In addition, the LC-MS system, in which the components separated by HPLC are further analysed using a mass spectrometer, is becoming widely used because of its high sensitivity and the possibility of specific detection.

Table 4. Major common types of detectors

UV detector	**The light source is a D2 lamp. This detector is used mainly to detect components having an absorption wavelength of 400 nm or less in the ultraviolet region.**
UV-VIS detector	A D2 lamp and a W lamp are used as the light source. This detector is effective for detecting colorants such as dyes and stains because of coverage of the visible light region.
Diode array detector (DAD)	Data on the spectrum from the ultraviolet to visible light range is also collected.
Fluorescence (FL) detector	Fluorescent substances can be detected specifically with high sensitivity.
Differential refractive index (RI) detector	Change in the refractive index is detected. Components absorbing non-ultraviolet light can also be detected despite low sensitivity.
Conductivity detector	Mainly inorganic ions are detected by monitoring the conductivity.

In the following sections, we give details for the different detectors used in the present study.

6.2.1. UV-Visible absorption detection

UV-Vis is the most common detection method. It detects solute analytes by their absorbance of light at various wavelengths.

In UV-Visible detectors, the mobile phase is passed through a small flow cell where the radiation beam of a UV-Vis photometer or spectrophotometer is located. As a UV-Visible absorbing solute passes through the flow cell, a signal is generated that is proportional to the solute concentration. The mobile-phase components should be selected carefully so that they absorb little or no radiation. Absorption of radiation is

a function of concentration (c) as described by the Beer-Lambert law: A=εbc, where A: absorbance, ε: molar extinction, and b: flow-cell path length[142].

There are many molecules which can absorb radiation in this region of electromagnetic spectrum, so that this detector can be considered universal. This behaviour has the advantage that it can be used to solve a large number of analytical problems, but the disadvantage of limited selectivity. The UV-Visible is a robust detector, so that it is one of the best for quantifications. Nevertheless, UV-Visible spectroscopy does not offer structural information and does not permit the unambiguous identification of compounds without using standards, but it can be useful in determining phenolic compounds to limit the family to which the analytes belong, since each family has characteristic absorption bands.

Three types of absorption detectors are available: fixed wavelength, variable wavelength and photo diode array.

- Fixed-wavelength detector. This detector uses a light source that emits maximum light intensity at one or more discrete wavelengths (e.g. 254, 280 and 365 for a mercury lamp) that are isolated by appropriate filters.
- Variable-wavelength detector. This detector works in the range of UV-Visible spectrum (190-650). This detector type has been equipped with two lamps, one of deuterium for measurements at 190-360 nm and the other of tungsten for the measurements in the visible spectrum from 360 to 650 nm, with a monochromator (a moveable grating controlled by stepper motor to select the

142. LaCourse W.R. (2009). Liquid chromatography : Instrumentation. In Handbook of methods and instrumentation in separation science (volume 1). Editors: Ian D Wilson and Colin Poole. P 478-484. Elsevier Ltd. Academic press in an imprint of Elsevier. 111-112.

wavelength through an exit slit). Nowadays, with only a deuterium lamp the entire UV-Visible spectrum is covered[143].

- Diode-array detector. This detector is the most modern of the three types. It conducts the light through a linear-diode system and avoids dispersion. A photodiode-array consists of a series of photodiodes in a chip of integrated circuits. For spectroscopy, photodiodes are placed in the image plan of the spectrometer in a way that allows the simultaneous detection of a wavelength range [143].

6.2.2. Mass spectrometry

Mass spectrometry (MS) is an analytical technique that measures the molecular masses of individual compounds and atoms precisely by converting them into charged ions. Quite often, the structure of a molecule can also be deduced. Mass spectrometry is also uniquely qualified to provide quantitative information of an analyte at extraordinary levels of structure specificity and sensitivity. In addition, mass spectrometry allows the study of reaction dynamics and chemistry of ions, to provide data on physical properties such as ionisation energy, appearance energy, enthalpy of a reaction, proton and ion affinities, etc., and to verify theoretical predictions based on molecular orbital calculations. Thus, mass spectrometry is probably the most versatile and comprehensive analytical technique currently available to chemists and biochemists[144].

143. Gomez-Caravaca A.M. (2009). Caracterización de alimentos funcionales mediante Metodologías separativas avanzadas y aplicaciones a Tecnología de alimentos. Doctoral thesis, Universidad de Granada. 112-113.
144. Dass C. (2007). Basics of mass spectrometry. In: Fundamentals of Contemporary Mass Spectrometry.John Wiley & Sons, Inc. 3-14.

In recent years applications of mass spectrometry as a detection system coupled with various separation techniques and in particular liquid chromatography have grown exponentially.

Principles and instruments

A mass spectrometer converts sample molecules into ions in the gas phase, separates them according to their mass-to-charge ratio *(m/z)* and sequentially records the individual ion current intensities at each mass - the mass spectrum. If these ion current intensities are drawn in histogram form taking the most intense ion current as 100%, the values of *m/z* vs. the percentage of relative intensity (%RI) is called a line diagram.

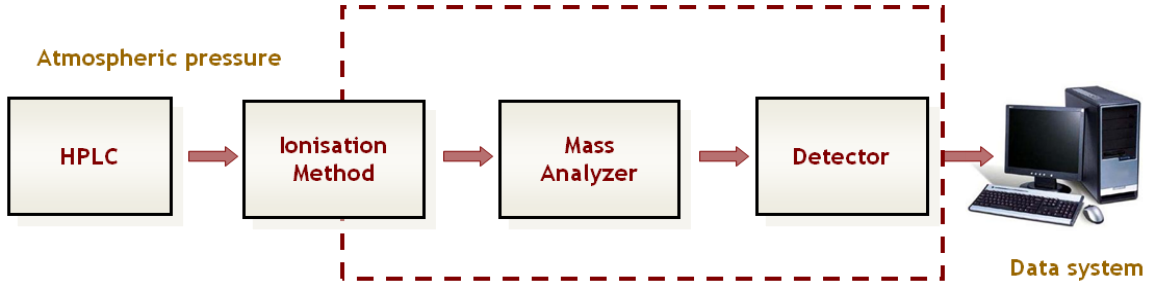


Figure 17. Combination of separative technique with mass spectrometry

Interface

The role of the interface is to eliminate the most of the matrix or medium (buffer or mobile phase separation), keeping the analytes. To that end, the interfaces tend to be very hot. For this reason, modifiers or employees must be volatile buffers to minimize interference in MS[145].

145. Rubinson K. A., Rubinson J. F. (2000). Contemporary Instrumental Analysis. Ed. Prentice Hall, 522-583.

The two main factors affecting the stability of the ionisation source are:

- Efficiency of the species. If the sample is not gaseous, the composition of the vaporised material may not reflect the composition of the sample.
- The efficacy of the ionisation. The fraction ionised through diverse ionisation methods varies according to the matrix composition.

Figure 18 presents a comparison between the different ionisation systems that have been most used in the combination of HPLC/MS. The comparison was based on the polarity and the molecular weight of the analytes under study.

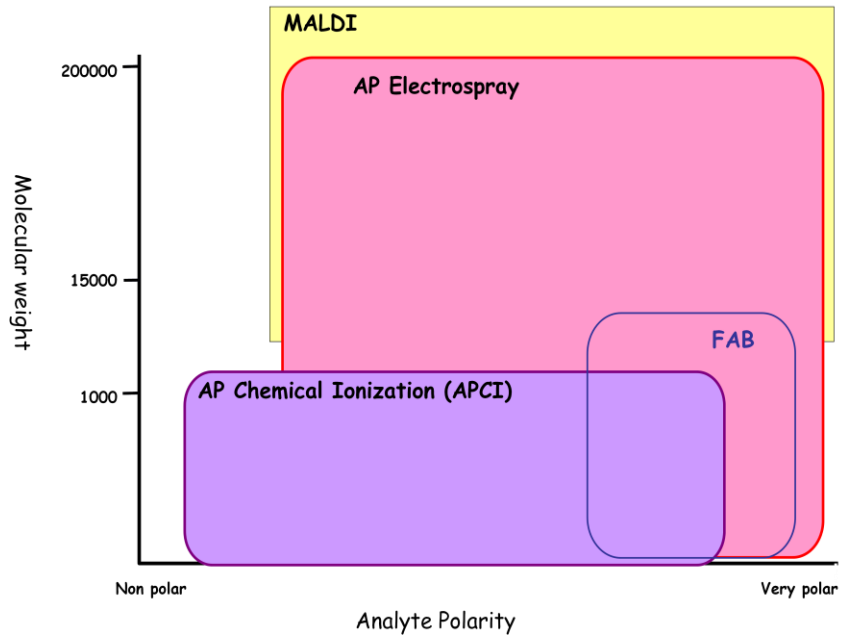


Figure 18. Ionisation systems

Electrospray ionisation

The basic electrospray process is simple to summarize. An aerosol spray consisting of fine droplets is created when a high electric potential is applied to a needle containing a solution with a polar solvent **(Figure 19)**. The spray process can be pneumatically or ultrasonically assisted. A drying bath gas or thermal desolvation method is used to eliminate clustering as the droplets are cooled by supersonic

expansion. The droplets are induced into the vacuum region through an orifice or skimmer. The vacuum interface consists of pumping stages and ion optics designed to maximize ion transmission; collision induced-dissociation (CID) in the vacuum interface aids in breaking up solvent clusters and providing a means for generating fragment ions, which are often structurally significant**.**

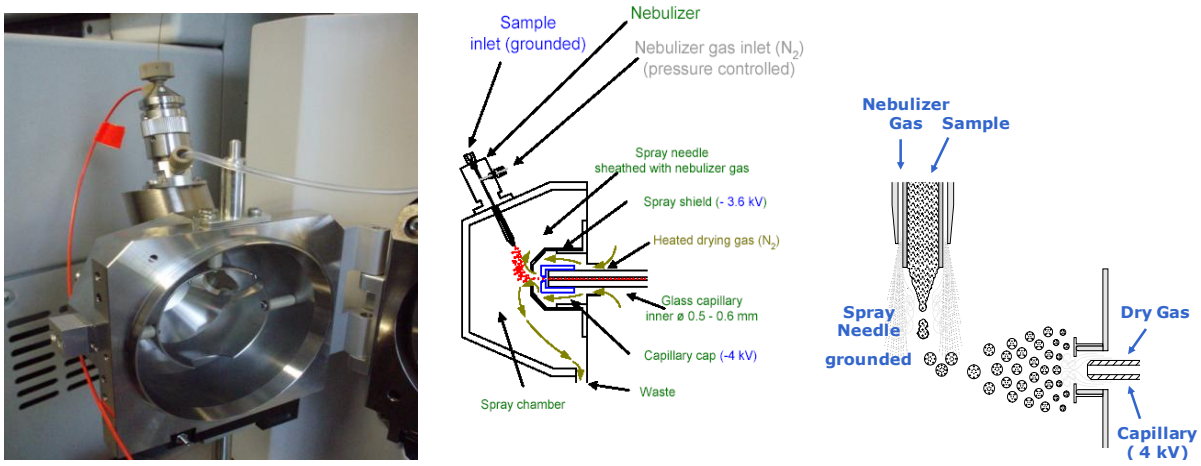


Figure 19. ESI ion source with its schematic diagram

Mass analysers

Once the gas-phase ions have been produced, they need to be separated according to their masses, which must be determined. The physical property of the ions that is measured by a mass analyser is their mass-to-charge ratio *(m/z)* rather than their mass alone.

As there is a great variety of sources, several types of mass analysers have been developed. Indeed, the separation of ions according to their mass-to-charge ratio *(m/z)* can be based on different principles **(Table 3)**. All mass analysers use static or dynamic electric and magnetic fields that can be employed alone or combined. Most

of the basic differences between the various common types of mass analyser lie in the manner in which such fields are used to achieve separation[146].

Table 5. Types of analysers used in mass spectrometry

Type of mass analyser	Symbol	Principle of separation
Electric sector	E or ESA	Kinetic energy
Magnetic sector	B	Momentum
Quadrupole	Q	m/z (trajectory stability)
Ion trap	IT	m/z (resonance frequency)
Time of flight	TOF	Velocity (time of flight)
Fourier transform ion syclotron resonance	FTICR	m/z (resonance frequency)
Fourier transform orbitrap	FT-OT	m/z (resonance frequency)

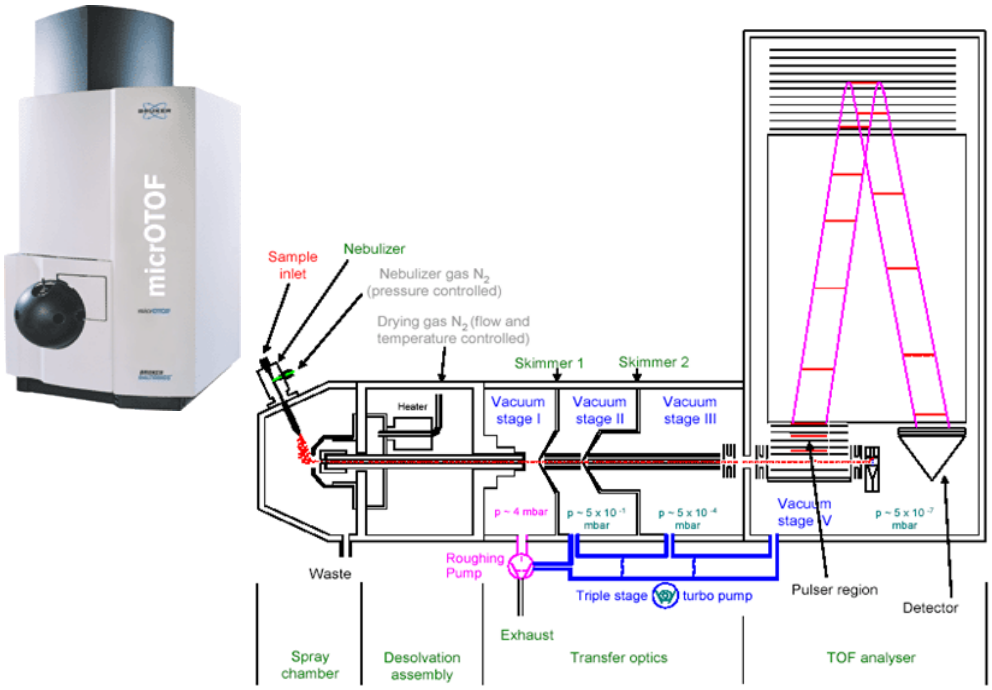
Time of flight (TOF)

Ion separation in a TOF analyser is based on Newton's third law. The underlying principle is that ions of different masses with equal kinetic energy have different velocities. If there is a fixed distance for the ions to travel, the time of travel is proportional to the square root of the mass-to-charge ratio (*m/z*) of the ions. For the measurement of the time of flight, ions are introduced into the mass spectrometer in

146. De Hoffmann E., Stroobant V. (2007). Mass analysers. In Mass spectrometry principles and applications. Edmond de Hoffmann, Vincent Stroobant (Eds), 3rd Ed, John Wiley & sons Ltd, England, pp: 85-171.

discrete packets so that a starting point for the timing process can be established. Ion packets are generated either through a pulsed ionisation process or through a gating system in which ions are produced continuously, but are introduced only at given times into the flight tube[147]. The resolution between the different *m/z* is better when the tube length is higher (there would be a major separation of ions in time) and when the energy dispersion of the formed ions in the source is lowert[148].

TOF technology presents numerous advantages such as high mass resolution, high mass accuracy, theoretically unlimited mass range, and relatively low cost. A representation of the TOF analyser and its schematic diagram are represented in the following figure **(Figure 20)**.



147. Dong M.W. (2005). Application of LC/MS in pharmaceutical analysis. In: Handbook of pharmaceutical analysis by HPLC. Ed. Satinder Ahuja and Mickael W. Dong. Printed in the UK. Elsevier Inc.

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

Figure 20. TOF analyser with its schematic diagram

In the first part of the diagram, the spray chamber, initiates the electrospray-formation process. The desolvation assembly separates the zones of atmospheric pressure from the first zone of high vacuum and is composed of a heater of the drying gas and a crystal capillary. Through this zone the transmission module or transfer optic, consisting of three units of high vacuum, is reached. The first one, as represented in the figure (stage I), is the only unit that is connected to an exterior rotator pump, while the three units (stages I, II and III) are connected to a turbo molecular pump that possesses different levels of vacuum. They are separated from each other by various skimmers. The hexapoles make the transfer the ions till the zone of high vacuum, while the lenses (1-5) determine the direction of those ions[143] **(Figure 21)**.

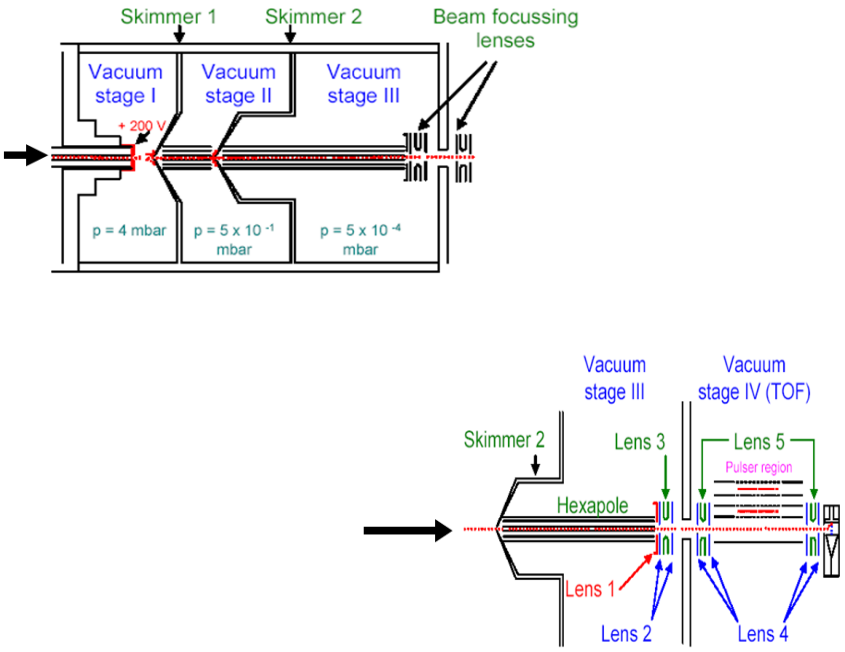


Figure 21. Schematic diagram of the transfer optic unit

The zone of orthogonal acceleration consists of two of the lenses, 4 and 5, and accelerates the ions to measure the time of flight **(Figure 22)**.

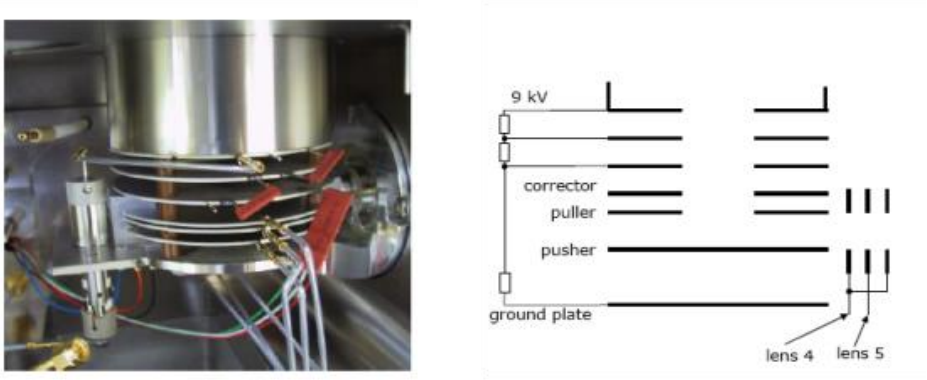


Figure 22. The zone of orthogonal acceleration of a MicroTOF with a schematic representation of its components

According to their mass, the ions enter in higher or lower measure in the reflector **(Figure 23)**, behind which there are zones of tension that repel arriving ions. The ions then fall into the detector.

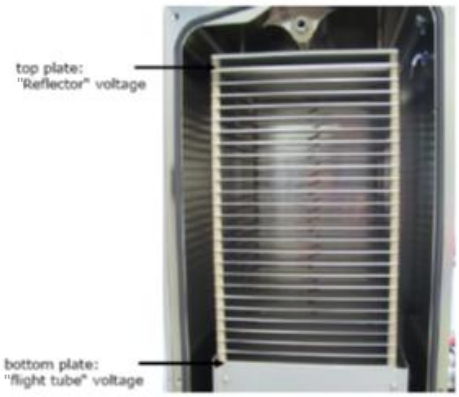


Figure 23. Image of the last part of the flight tube showing the reflector

Ion trap (IT)

The ion trap consists of a doughnut-shaped ring electrode and two endcap electrodes **(Figure 24)**.

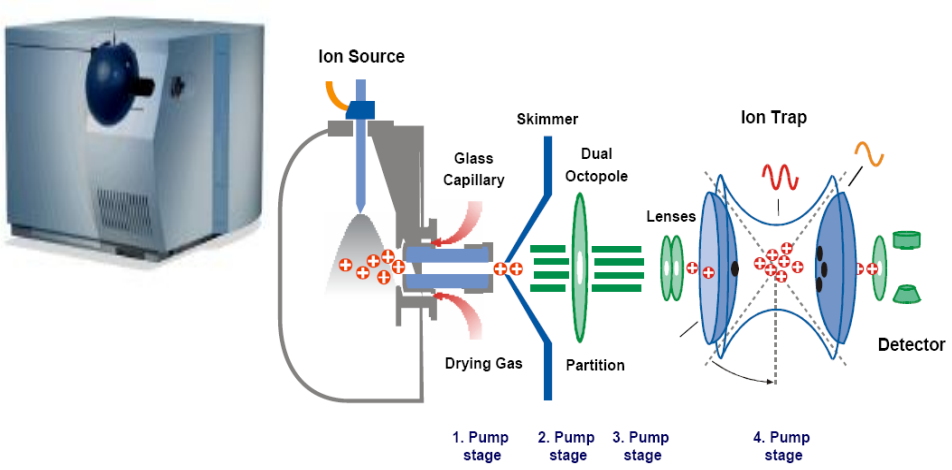


Figure 24. IT Mass analyser with its schematic diagram

A combination of radio frequency and direct current voltage is applied to the electrodes to create an electric field. This electric field traps ions in a potential energy well at the centre of the analyser. The mass spectrum is acquired by scanning the fields to destabilize low mass to charge ions. These destabilized ions are ejected through a hole in one endcap electrode and strike a detector. The mass spectrum is generated by scanning the fields so that ions of increasing *m/z* value are ejected from the cell and detected. The trap is then refilled with a new batch of ions to acquire the next mass spectrum[149] **(Figure 25)**.

149. Van Bramer S.E. 1998. An Introduction to Mass Spectrometry. 4-38. Available on http://science.widener.edu/~svanbram.

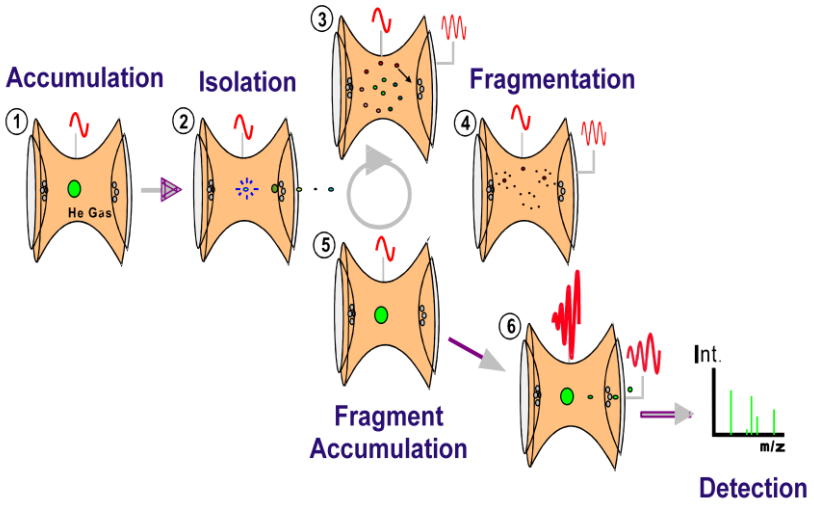


Figure 25. Schematic representation of the IT process

The advantages of IT include compact size, the ability to trap and accumulate ions to increase signal to noise ratio of a measurement and MS^n.

SECTION I

Olive oil

Chapter 1

The occurrence and bioactivity of polyphenols in Tunisian olive products and by-products: A review

This work is published in Journal of Food Science.

Taamalli A., Arráez-Román D., Zarrouk M., Segura-Carretero A., Fernández-Gutiérrez A. 2012. The Occurrence and Bioactivity of Polyphenols in Tunisian Olive Products and by-Products: A Review. Journal of Food Science, 77: R83–R92.

R: Concise Reviews in Food Science

The Occurrence and Bioactivity of Polyphenols in Tunisian Olive Products and by-Products: A Review

Abstract

Polyphenols have become a subject of intense research because of their perceived beneficial effects on health due to their anticarcinogenic, antiatherogenic, anti-inflammatory, and antimicrobial activities. It is well known that olives and their derivatives are rich in phenolic substances with pharmaceutical properties, some of which exert important antioxidant effects. The characterization and quantification of their polyphenol composition is one of the first steps to be taken in any evaluation of the putative contribution of the olive to human health. This review is concerned with polyphenols in Tunisian olive (*Olea europaea* L.) products (fruit and oil) and some by-products (leaves and olive-mill wastewater) with an emphasis on the analytical methods used, as well as the biological activities described in recent years.

Keywords: bioactive phenols, olive leaves, olive-mill wastewater, olive oil, Tunisian olive

Introduction

Olive-tree cultivation in the Mediterranean area goes back to ancient times. The Romans spread olive cultivation throughout the entire Mediterranean basin. This long-lived tree forms an integral part of the economy and culture of the inhabitants of this basin and also determines its rural landscape (1). Tunisia currently has about 66 million olive trees, covering 1685000 hectares, which represents a 3rd of its available agricultural land. Olive trees are cultivated in widely varied climatic conditions, thus from north to south they are situated as follows: 15% in the north, 66% in the center, and 19% in the south. Olive trees are mostly planted in monoculture and sometimes intercalated with other fruit trees (2).

The economic importance of the sector is reflected through its contribution of up to 44% of Tunisian agricultural exports. Tunisia occupies the second position in the world after the European Union in terms of olive-oil exportation (3). During the last decade, olive-oil production reached an average of about 150 thousand tons. The quantity exported represented more than 70% of the total production of olive oil and has achieved close to 111 thousand tons (3).

Besides its economic importance, the olive sector (olive growing and the olive-oil industry) provides a livelihood, directly or indirectly, for over 1 million people and generates 34 million workdays a year, equivalent to over 20% of the employment in agriculture (4). In addition to this important social role, the olive tree contributes amply to the valorization of the less favored areas (marginal regions) in the center and the south, and therefore helps to ensure the stability of rural populations as well as the preservation of the soil because it is a crop that can be grown in difficult conditions.

Virgin olive oil (VOO), the main olive product, which is extracted from olive fruit by mechanical means, is appreciated throughout the world by consumers attentive to both the healthy and nutritional aspects of food. Health-promoting effects may be

attributed to the antioxidant effect of the phenolic compounds present in olives and olive oil, and their pharmacological actions have often been reported in the literature (5, 6).

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 (7).

Due to the importance of polyphenols in food, this article reviews the polyphenols occurring in Tunisian olive products (olive fruit and olive oil) and some by-products (olive leaves and olive mill wastewater [OMWW]) together with the various methods used for their analysis and their bioactive properties, in the hope of opening up perspectives for further research.

Olive Products

Olive fruit

Olives attain their maximum weight 8 mo after the flowering period. This is followed by physiological modifications and changes in fruit color with the appearance of the purplish black fruit indicating the end of its morphological development (8). The olive ripeness index (RI) can be determined according to the method developed by the Agronomic Station of Jaen (9) based on the evaluation of olive skin and pulp colors. RI values range from 0 (100% intensely green skin) to 7 (100% purple flesh and black skin).

Olives are rarely consumed directly as a natural fruit due to their extreme bitterness but are used instead for the extraction of oil and to a lesser degree as table olives (after curing). Traditionally, polyphenols are extracted from Tunisian olives by solid–

liquid solvent extraction using methanol (10), ethyl acetate (11), or mixtures of methanol with water (80: 20 v/v) (11, 12).

In a typical extraction procedure, a volume of extractant is added to the olive paste, and this is left to stand overnight under stirring at room temperature. Subsequently, the solution is filtered using GF/F filter paper, and then the extract is washed with hexane in a separatory funnel. The extract is finally concentrated in a vacuum.

Spectrophotometric determination of total phenols.

For a simple and a rapid quantification of total phenols in olives, the traditional spectrophotometric method based on the Folin-Ciocalteu assay at 727 nm was used. Bouaziz and others (12) studied the total phenol content in the most important Tunisian olive cultivar, the Chemlali, and studied its variation during ripening. They found that the total phenol concentration of olive extracts increased from 6 to 16 g as pyrogallol equivalents per kilogram fresh weight during ripening. The highest amount was recorded in black olives from the harvest at the end of October to the end of February. Later, Ben Othman and others (10) studied the effect of ripening (green, varicolored, and black olives) on Chétoui olive flesh (the second most important cultivar in Tunisia) total phenol content at 760 nm. The results showed that green olives have the highest phenolic content of 2.558 g in the form of gallic acid per 100 g dry weight, followed by varicolored and black olives, which had phenolic contents of 2.233 and 1.760 g/100 g dry weight, respectively. The evolution of total phenol content in olive flesh runs inversely to that obtained for the whole olive by Bouaziz and others (12). To test the effect of fermentation on olive phenols, Ben Othman and others (10) used green, varicolored, and black olives of the Chetoui variety. Olives were placed in 8% w/v NaCl brine for 67 days and some were left to undergo spontaneous fermentation, while others were fermented with a selected

strain of *Lactobacillus plantarum*. They observed that the evolution of total phenols was the same in both the olives fermented spontaneously and those fermented with *L. plantarum*. A significant decrease was observed after 9 days' fermentation, which continued until day 23, after which the phenolic contents remained stable. Nevertheless, total phenolic reductions for the green, varicolored, and black olives were lower in the controlled than the spontaneous fermentation. This might be explained by the development of a biofilm that could possibly have acted as a barrier for phenolic diffusion (10). The popularity of this colorimetric assay can be attributed mainly to its simplicity and speed of analysis (13). The method is a conventional one, however, since any reducing substance may result in interference, besides which, the response of each phenol to the oxidizing agent is different (14). Furthermore, the method does not distinguish between individual compounds with different molar masses and structures. The need for profiling and identifying individual phenolic compounds requires the replacement of traditional methods by high-performance chromatographic analyses.

Chromatographic determination of the phenolic compounds

During ripening, several metabolic processes occur in olives that can lead to a variation of the profiles of some components. Thus, studying the changes in phenolic profile and contents during fruit ripening is of a great interest. Some Tunisian studies have dealt with this subject, focusing mainly upon the Chemlali and Chetoui varieties. Table 1 shows the different analytical methods that provided the highest number of identified phenolic compounds in Tunisian olives. Bouaziz and others (12) characterized the phenolic compounds in the Chemlali olive in order to examine their profile during ripening (from July 1 to February 20). Using reversedphase high-performance liquid chromatography with UV detection (RP-HPLC-UV), they identified 15 phenolic compounds: hydroxytyrosol (HyTy), tyrosol (Ty), *p*-hydroxybenzoic acid,

vanillic acid, caffeic acid, coumaric acid, vanillin, ferulic acid, oleuropein (Ol), rutin, quercetin 3-arabino-glucoside, luteolin 7-o-glucoside, quercetin, luteolin, and apigenin (Table 1). Quantification of phenolic compounds in Chemlali olives during ripening showed that the highest quantity was recorded for Ol, which reached a maximum value of 6.5 g/kg fresh weight for the sample harvested on August 30 (green fruit). The Ol concentration varied greatly during sampling, however, increasing from 2.5 g/kg until reaching a maximum value by the end of July. After that, its concentrations decreased until reaching a minimum value of 1.5 g/kg during the last stage of ripening. Similar results have been reported in other studies in which the Ol concentration was higher in green Chemlali olives (89.65% of dry weight extract) (11) and in green Ch´etoui olive flesh (266 mg gallic acid/100g dry weight) (10).

The HyTy and Ty concentrations in Chemlali olives increased during ripening and reached their maximum values at the last ripeness stage (12). The evolution of these two compounds is opposite to that found in olive flesh by Ben Othman and others (10). Ferulic acid remained relatively constant; *p*-coumaric acid showed a weak variation and decreased in the last phase of ripening. Caffeic acid, *p*-hydroxybenzoic acid, vanillic acid, and vanillin were present in low concentrations. With regard to flavonoids, luteolin-7-o-glucoside, quercetin-3-arbinoglycoside, and rutin increased during olive ripening until reaching maximum quantities in mid-September, November 4, and mid-November, respectively. After these dates, the quantities of each compound decreased. Quercetin concentrations showed very little change throughout ripening. Apigenin remained substantially unchanged and quite low throughout all the ripening stages.

In a further study, Fki and others (11) used a combination of HPLC and atmospheric-pressure chemical-ionization mass spectrometry (HPLC-APCI-MS) in positive ion mode. In addition to the flavonoids reported previously by Bouaziz and others (12),

they identified six flavonoids in Chemlali olive extract: luteolin 7-o-rutinoside, quercetin 3-o-glucoside, apigenin-7-o-rutinoside, chrysoeriol-7-o-glucoside, luteolin-4-o-glucoside, and chrysoeriol (Table 1). They also found that some of these flavonoids, quercetin, luteolin, apigenin, and chrysoeriol, were only present in the aqueous methanol extract from black olives.

Changes in phenolic compounds during olive processing (fermentation) were studied by Ben Othman and others (10). These authors studied olive-flesh phenolic compounds during spontaneous and controlled fermentations of Chétoui olives at 3 stages of ripeness (green, varicolored, and black olives) using the HPLC-UV technique. After fermentation, changes in the quantity of phenolic compounds were observed; phenolic content in flesh increased after the fermentation of varicolored and black olives, especially in the controlled fermentation (from 384 and 311 to 621 and 510 mg gallic acid/100 g dry weight, respectively). In contrast, the phenolic content decreased in green olives from 652 to 460 mg gallic acid/100 g dry weight and to 380 mg/100 g dry weight in spontaneous and controlled fermentations, respectively.

There was a decrease in the concentrations of protocatechuic acid, ferulic acid, and oleuropein, while HyTy concentration increased after fermentation, due to the acid and enzymatic hydrolysis of oleuropein. The concentrations of gallic, p-hydroxyphenylacetic, vanillic, and benzoic acids also decreased after the fermentation of green olives, although their concentrations increased for varicoloredand black olives.

Bioactivity

Fki and others (11) studied the lipid-lowering effect and the antioxidative activities of the Tunisian Chemlali green and black olive phenolic extracts (Table 2). Wistar rats fed on a standard laboratory diet or a cholesterol-rich diet for 16 wk were used. The results showed that the administration of aqueous methanol and ethyl acetate

extracts of green olives and ethyl acetate extract of black olives significantly lowered the serum levels of total cholesterol and low-density lipoprotein cholesterol while increasing the serum level of high-density lipoprotein cholesterol. Furthermore, the malondialdehyde content in the liver, heart, and kidney decreased significantly after oral administration of green and black olive extracts compared to those of rats fed on a cholesterol-rich diet. In addition, olive extracts increased catalase and superoxide dismutase activities in the liver (Table 2). The hypocholesterolemic and antioxidative effects of aqueous methanol and ethyl acetate extracts of green olives and ethyl acetate extract of black olives could be related to their HyTy- and Ol-rich contents (11).

Olive oil

In the kitchens of consumers, olive oil is often the fat of choice for health conscious people looking to enjoy the benefits of the Mediterranean diet. It is of great interest for its healthy virtues. Its chemical composition is principally triacylglycerols, which account for more than 98% of its total weight. Minor components amount to about 2% of the total weight and include, among others, aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, and several antioxidants (15). Phenolic compounds represent one of these families of antioxidants present in olive oil.

Two basic extraction techniques, liquid–liquid extraction (LLE) and solid-phase extraction (SPE), have been described for isolating polyphenols from Tunisian olive-oil samples (16-24). Table 1 shows the different analytical methods that have identified the highest number of phenolic compounds in Tunisian olive oil.

As far as the LLE is concerned, the phenolic fraction of olive oil is isolated by the extraction of an oil solution in hexane with several portions of methanol/water (at different ratios), followed by solvent evaporation of the aqueous extract. Some

authors have used methanol/water at a ratio of 60 : 40 v/v (17, 18, 20, 23) or a ratio of 80 : 20 v/v (22, 24).

With regards to the SPE, the polyphenols of Tunisian olive oil samples have been studied using a diol-bonded phase SPE cartridge (16, 19, 21). The experimental approach was as follows: a sample of filtered olive oil was dissolved in 6 mL of *n*-hexane. A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive volumes of methanol and hexane. The vacuum was then released to prevent drying of the column. The oil solution was applied to the column and the solvent was pulled through, leaving the sample and the standard on the solid phase. The sample container was washed with hexane, which was run out of the cartridge. The sample container was washed again with hexane/ethyl acetate, which were run out of the cartridge and discarded. Finally, the column was eluted with methanol and the solvent was evaporated in a rotary evaporator at room temperature and low speed under a vacuum until dry.

Spectrophotometric determination of total phenols.

The colorimetric assay based on the reaction of Folin–Ciocalteu reagent was used for the determination of total phenols. Total odiphenols were determined separately with a solution of sodium molybdate in ethanol/water (50 : 50 v/v). These colorimetric assays were used to evaluate the effects of genotype, geographic location, ripening, and irrigation on the total phenol and o-diphenol contents of VOO.

Zarrouk and others (25) analyzed the variation in total phenol contents among several olive-oil varieties grown in southern Tunisia. The quantities of total phenols and o-diphenols showed significant differences among cultivars. Zalmati oil contained the highest total phenol concentration (507 mg of caffeic acid/kg of oil), whereas Jemri Ben Guerdane oil recorded the lowest (364 mg/kg). The highest o-diphenol content was observed in Chemlali Zarzis oil (213 mg/kg), followed by “Jemri Ben

Guerdane" (about 199 mg/kg), while "Zalmati" showed the lowest value (188 mg/kg). Nevertheless, when exploring 10 different farms in the north of the country where the Chétoui variety is cultivated, Ben Temime and others (17) found that Chétoui olive oil from the region of Amdoun had very high levels of total phenols and o-diphenols, which exceeded 740 and 280 mg of caffeic acid/kg of oil, respectively.

Mahjoub-Haddada and others (20) studied total phenol contents in VOO from Tunisian olive varieties cultivated in the north of the country. They found that oils from Chetoui showed higher values for phenols and o-diphenols (321.68 and 33.17 mg of caffeic acid/kg of oil, respectively) than the other varieties from the same area studied.

Baccouri and others (18) evaluated the influence of an irrigation regime on the total phenols and o-diphenols in Chétoui VOO at 3 different stages of ripeness. They found that total phenol quantities were significantly affected by the irrigation regime. In fact, oils obtained from irrigated trees had lower levels of total phenols (which did not exceed 294 mg of gallic acid/kg of oil) than nonirrigated ones (which reached 852 mg/kg). In the rain-fed Chetoui variety, the total phenol contents increased to reach a maximum level at stage II of fruit ripening, then the total phenol content decreased and no evident losses in o-diphenol content were observed. The total phenol and o-diphenol contents decreased with ripeness, however, when the Chétoui cultivar was under irrigation. Similar behavior was found by Ben Youssef and others (21). These authors observed that the quantities of total phenol increased progressively during the ripening of Chétoui olives until they reached a maximum value (1043 mg of caffeic acid/kg of oil) at a ripeness index of between 2 and 3.5, after which it decreased to a minimum of 483 mg/kg.

Chromatographic determination of the phenolic compounds in virgin olive oil

HPLC has been the analytical technique most used for characterizing polyphenolic compounds. As can be seen in Table 1, the phenolic profile of olive oil is different from that of the fruit, where the glycosidic forms are predominant. In addition, the analytical method used has an influence on the determination of the phenolic compounds.

Using RP-HPLC-UV, Abaza and others (16) studied the phenolic composition of seven Tunisian olive-oil varieties. They identified the following phenolic compounds: HyTy, Ty, p-coumaric acid, ferulic acid, vanillic acid, vanillin, HyTy-acetate, Ty-acetate, dialdehydic forms of ligostroside and Ol aglycones, isomer derivated, aldehydic forms of ligostroside and oleuropein aglycones, apigenin, luteolin, pinoresinol (Pin) and acetoxypinoresinol (Ac-Pin) (Table 1).

Two of these seven varieties (Chétoui and Chemlali) were studied by Baccouri and others (26) and later by Guerfel and others (22). Baccouri and others (26) used HPLC coupled to a UV–Vis detector and a mass-spectrometer detector (MS) equipped with an electrospray ionisation (API-ES) interface operating in positive mode. They identified phenolic compounds in these two varieties that had not previously been identified by Abaza and others (14), such as vanillic and o-coumaric acids. In 2009, Guerfel and others (22) analysed the phenolic composition of VOO from the same varieties (Chétoui and Chemlali) using the HPLC-UV system. They identified three additional phenolic acids: ferulic, syringic and caffeic acids (Table 1). By using an HPLC system coupled to a time-of-flight mass spectrometer (TOF-MS) equipped with an ESI operating in negative ion mode, Taamalli and others (23) were able to identify other phenolic compounds that had not previously been reported in Chétoui olive oil: elenolic acid (EA), hydroxy-elenolic acid (H-EA), hydroxy-D-oleuropein aglycon (H-D-Ol agl), methyl-D-Ol agl, 10-H-Ol agl, syringaresinol, hydroxy-Pin (H-Pin), and sinapinic acid (Table 1).

Table 1. Identified phenolic compounds in olive products and the analytical methods used.

Source		**Olive fruit**		**Olive oil**			
Extraction method		SLE		SPE		LLE	
		Me-OH: H2O (80:20), ethyl acetate	Me-OH: H2O (80:20)	diol-bonded phase cartridge		Me-OH: H2O 80:40 (v/v)	Me-OH: H2O 60:40 (v/v)
Chromatographic technique		HPLC	HPLC	HPLC	HPLC	HPLC	HPLC
	Column	C-18 (4.6x250mm) Shim-pack VP–ODS Lichrosphere 100 RP-18(4 x250 mm)	C-18 (4.6x250mm) Shim-pack VP-ODS[b] C-8 (4.6x250 mm) Shim-pack CLC[d]	Lichrospher 100 RP-18, 5 µm (250x4 mm)	Phenomenex C18, 5 µm, (250x3.0 mm)	Lichrosphere 100 RP 18.5 µm (250x4 mm)	Zorbax C18 (4.6×150mm, 1.8 µm)
	Mobile phases	0.1% phosphoric acid in water (A) and 70% acetonitrile in water (B)	0.1% phosphoric acid in water (A) and 70% acetonitrile in water (B)	water/phosphoric acid (99.5:0.5, v/v) (A) and methanol/acetonitrile(50:50, v/v) (B)	0.5% acetic acid in water (A)and acetonitrile (B)	0.2% acetic acid in water (A) and Methanol (B)	0.5% acetic acid in water (A) and acetonitrile (B)

Table 1. Cont..

	Detector	UV and MS (APCI)	UV (280, 335 nm)	UV (280, 335 nm)	UV (280 , 335 nm) MS (ESI - TOF)	UV (278 nm)	UV (240, 280 nm) and MS (ESI-TOF)
Identified compounds		Ol[a], HyTy[a], Ty[a] lueolin 7-o-glucoside[a] luteolin 7-o-rutinoside[a], quercetin 3-o-glucoside[a], rutin[a], apigenin 7-o-rutinoside[a],chrysoeriol 7-o-glucoside[a], luteolin 4-o-glucoside[a], quercetin[b], luteolin[b], apigenin[b], chrysoeriol[b]	HyTy,Ty, hydroxybenzoic acid,vanillic acid ,caffeic acid, coumaric acid, vanillin, ferulic acid, Ol, rutin, quercetin 3-arabino-glucoside, , luteolin 7-o-glucoside, quercetin, , luteolin, apigenin	HyTy, Ty , vanillic acid, p-coumaric acid ,HyTy acetate ,dialdehydic form of Ol-agl,tyrosol acetate , isomer derivated, dialdehydic form of lig-agl, Pin, Ac-Pin, vanillin,aldehydic form of Ol-agl, aldehydic form of lig-agl, ferulic acid , luteolin, apigenin	HyTy, Ty, HyTy-Ac, EA, D-Ol-agl, Ac-Pin, Ol agl, lig agl,	HyTy, Tyr, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, dialdehydic form of Ol-Agl, o-Coumaric acid, Ol-Agl	HyTy,Tyr,HyTy-Ac ,D-Ol agl, D-Lig-agl, Ol-agl, H- D-Ol agl, methyl- D-Ol agl, 10- H-Ol agl, Lig-agl, vanillic acid, o-coumaric acid, ferulic acid, taxifolin,EA, H-EA, Pin,Ac-Pin, H-Pin,syringaresinol, sinapinic acid, luteolin, apigenin

Table 1. Cont..

Olive cultivars	Chemlali	Chemlali	Chetoui, Chemlali, Sayali, Oueslati, Gerboui, Chemchali, Zalmati	Chetoui	Chetoui, Chemlali	Chetoui, Chemlali, El Hor, Jarboui, Chemchali, Oueslati
References	(11)	(12)	(16)	(21)	(22)	(23)

Studies showed that the quantities of the phenolic compounds differ between olive-oil varieties (16, 19, 21, 23, 26). Abaza and others (16) found that the prevalent compounds were the aldehydic form of ligstroside and oleuropein aglycones and their dialdehydic forms with levels reaching 124.43, 211.39, 405.53 and 66.79 mg/kg respectively. The major simple phenol alcohols were HyTy and Ty, which varied from 0.81 to 23.73 mg/kg and from 2.66 to 33.17mg/kg respectively. The content of the two main flavonoids identified in the olive oils studied luteolin and apigenin, were very low and did not exceed 6 mg/kg. As far as the lignans are concerned, Ac-Pin was the most abundant and exceeded 13 mg/kg. Among the oils analysed, the Chétoui variety had the highest phenolic content (925.7 ± 24.3 mg/kg).

In a study by Taamalli and others (23) the Chemchali and El Hor varieties showed interesting levels of phenolic compounds compared to the Chetoui variety. The polyphenols reached 3564 mg/kg.

Baccouri and others (26) studied differences in the phenolic composition of the two main mono-variety Tunisian virgin olive oils, Chétoui and Chemlali at 5 different stages of ripeness. The Chétoui cultivar was also tested in an irrigation regime against a rain-fed control. The concentration of phenolic compounds gradually increased during ripening until reaching a maximum value at the ‘‘reddish” and ‘‘black” pigmentation stage (RI between 3 and 4), after which it decreased. The analysis showed that olive-oil samples contain low quantities of phenyl acids and phenyl alcohols and high quantities of secoiridoid derivatives such as oleuropein aglycon Ol agl, lig agl and D-Ol agl. The secoiridoid derivative levels behaved similarly during ripening in both varieties: they increased to reach a maximum level recorded at a RI ranging between 3.5 and 4.1, before rapidly decreasing again. Ol agl was the main secoiridoid derivative identified in all the VOOs analysed. The evolution of its levels was similar to the secoiridoid derivative content throughout the ripening process. D-Ol agl showed an important variation in Chemlali oils compared to those in the

Chetoui variety, its levels decreasing progressively from 12.6 mg of 3,4-dihydroxyphenylacetic acid/kg of oil down to 1.8 mg/kg. Lig agl was present at lower levels than other secoiridoids. In Chétoui oils its levels decreased slightly as ripening progressed from 10.9 to 6.4 mg/kg, whereas in the Chemlali samples the content of this compound increased to reach a maximum value of 19.75 mg/kg and then decreased to 3.56 mg/kg. In Chétoui VOOs the levels of simple phenols (sum of Ty, HyTy, vanillic and o-coumaric acids) increased as ripening progressed whereas this behaviour was not observed in Chemlali samples.

Phenolic compound levels in Chétoui oils were significantly affected by the irrigation regime applied to the olive trees during ripening (26). Chétoui oils obtained from unirrigated trees had higher quantities of secoiridoid derivatives, ranging from 263.8 to 510.1 mg/kg. In fact, in samples obtained under the irrigation system, rather than rain-fed conditions, secoiridoid-derivative contents decreased slightly as ripening progressed. The simple phenol levels were also affected by the irrigation regime throughout ripening; they increased markedly at the three first ripeness indexes to reach a maximum quantity of 109.4 mg/kg, and then decreased to 48.3 mg/kg. The authors concluded that the early harvest-date for the Chétoui cultivar (RI between 3 and 4) gave the best results, whilst, for the Chemlali cultivar, the data clearly showed higher values of phenolic contents between the second and the third harvest dates (RI between 2.8 and 4.5).

To determine the effect of geographic location on the quantities of phenolic compounds identified, Guerfel and others (22) analysed the Chétoui and Chemlali olive-oil varieties cultivated in different locations. They found that the Chétoui variety was subject to greater alteration than Chemlali according to geographic location. Chétoui cultivated in its traditional area produced oil richer in HyTy, Ty and the dialdehydic forms of Ol agl and Ol agl, which reached 17.82, 23.93, 42.86 and 744.9 mg/kg respectively. Other simple phenols such as vanillic acid, *o*-coumaric acid, caffeic acid, syringic acid and ferulic acid were found in low concentrations.

An investigation into the behaviour of the phenolic composition of VOO under medium-temperature accelerated storage conditions was undertaken by Krichene and others (27). The changes undergone by phenolic compounds were studied under medium temperature (50°C) accelerated oxidation conditions over a storage period of 8 months. Different oxygen availability (open and closed bottles) and four different mono-variety Tunisian virgin olive oils were used. The results showed that the content of both oleuropein-derived aglycons (aldehydic and dialdehydic forms of Ol-agl) and ligstroside-derived aglycons (aldehydic and dialdehydic forms of Lig-agl) were rapidly reduced under the average temperature (50°C) accelerated storage conditions of the assay. The initial degradation rate observed in the secoiridoid compounds was quite significant, even in the closed bottle samples, although 2–4 times lower than in open bottles. As a consequence, the residual quantity of complex phenolic compounds in the closed bottle samples after the eight months' accelerated storage at 50°C was significantly higher than in open-bottle samples, in particular when the initial concentration of oleuropein or ligstroside-derived compounds was high. The behaviour of both HyTy and Ty in closed-bottle stored samples during the eight-month assay was very similar whereas their behaviour was different in open-bottle stored samples, in which a greater stability of Ty could be detected, showing that the very different behaviours observed between these two compounds was mainly due to the antioxidant role of HyTy.

Bioactivity

Tunisian olive oils were investigated for their anti-allergic property. To this end, Yamada and others (28) studied five olive varieties grown in various regions of Tunisia. They found that among the varieties studied, Sayali olive oil presented the most potent inhibitory effect on β-hexosaminidase release by the IgE antibody-sensitized, BSA antigen-simulated RBL-2H3 cells at the antibody-antigen binding

stage. The results of the experiment showed that the anti-allergic effect of olive oils at this binding stage may depend upon their flavone content. At the antibody-receptor stage, among the varieties studied, Zarrazi olive oil showed a higher inhibitory effect on β-hexosaminidase release from RLB-2H3 cells. They investigated the effect of olive oil samples on histamine release and cytokine production by activated human basophilic (KU812) cells. Different dilutions of Zarrazi olive oil inhibited histamine release from A23187 plus phorbol 12-myristate 13-acetate (PMA)-stimulated KU812 cells in a dose-dependent way. Therefore, this study showed that the consumption of Tunisian olive oils may be beneficial for the prevention and even treatment of various types of allergy (Table 2).

Table 2. Cont.

Cell differentiation	promyelocytic leukemia HL-60 human cell	ethanol olive-leaf extract at 1/100 dilution	Gerboui extract	olive leaves: Chemchali, Chemlali, Chetoui, Gerboui, Sayali, Zarrazi and Zalmati	(32)
Inhibition of β-hexosaminidase release at antigen-antibody binding	rat basophilic leukemia (RBL-2H3) cells	olive-oil emulsions at 1/100-1/10000 dilutions	Sayali at 1/10000 dilution	olive-oil emulsions: Chemlali, Chemchali, Chetoui, Sayali, Zarrazi, Zalmati and Gerboui	(28)
Inhibition of β -hexosaminidase release at antibody-receptor binding	rat basophilic leukemia (RBL-2H3) cells	olive-oil emulsions at 1/100-1/10000 dilutions	zarrazi at 1/100 dilution	olive-oil emulsions: Chemlali, Chemchali, Chetoui, Sayali, Zarrazi, Zalmati and Gerboui	(28)
Inhibition of histamine release	human basophilic (KU812) cells	olive-oil emulsions at 1/100-1/10000 dilutions	Zarrazi at 1/100 dilution	olive-oil emulsions: Sayali and Zarrazi	(28)
Hypolipidimy (TC, LDL-C, TG)	serum of rats fed on a cholesterol-rich diet	Ol, ol agly and HyTy rich extracts 3 mg /kg of body weight	tested extracts	olive leaves: Chemlali	(31)

Table 2. Cont.

Increase of antioxidant enzyme activities (CAT, SOD)	liver of rats fed on a cholesterol-rich diet	Ol, ol agly and HyTy rich extracts 3 mg /kg of body weight	tested extracts leaves: Chemlali	olive	(31)
Serum antioxidant potential	serum of rats fed on a cholesterol-rich diet	Ol, ol agly and HyTy rich extracts 3 mg /kg of body weight	tested extracts leaves: Chemlali	olive	(31)
Lipid peroxidation reduction (TEBARS levels)	liver, heart and aorta homogenates of rats -rich diet	Ol, ol agly and HyTy rich extracts 3 mg /kg of body weight	tested extracts leaves: Chemlali	olive	(31)
Effect on histopathology	liver, heart and aorta tissues of rats fed on a cholesterol-rich diet	Ol, ol agly and HyTy rich extracts 3 mg /kg of body weight	tested extracts leaves: Chemlali	olive	(31)

Olive by-products

Both the cultivation of olive trees and olive oil extraction generate substantial quantities of products generally known as “olive by-products”. The different by-products considered in this review are defined as follows: olive leaves and olive-mill waste-water (OMWW). Table 3 shows the analytical methods that have identified the highest number of phenolic compounds in Tunisian olive by-products.

Olive leaves

Large amounts of olive leaves are one of the by-products of olive farming; they accumulate during the pruning of the olive trees and are also account for up to 10% of the total weight of the olives at olive-oil mills (29).

Two different solvent mixtures were used for the extraction of polyphenols from olive leaves: methanol/water (30, 31) and ethanol 70% (32).

Spectrophotometric determination of total phenols

Total phenols in olive leaves were also determined using the Folin-Ciocalteu reaction. Boudhrioua and others (33) showed a variation among the studied Tunisian cultivars from 1.4 to 2.32 mg as caffeic acid/100g of dry matter, Chetoui and Zarrazi being the richest ones.

Chromatographic determination of the phenolic compounds in olive leaves

Not enough information exists concerning the phenolic composition of Tunisian olive leaves, and studies that have been made have only focused on the major phenolic compounds.

Abaza and others (32) studied the phenolic composition of some Tunisian olive-leaf extracts by using reversed phase-HPLC-UV. They found that two main compounds were present in all the extracts: apigenin-7-o-glycoside and Ol. Their quantities varied

among the varieties studied from 1.31 to 2.68 and 0.91 to 2.81 g as o-coumaric acid/kg dry weight respectively. The highest quantity of apigenin-7-o-glycoside was found in the Zalmati cultivar whilst the Chemchali cultivar presented the highest quantity of Ol. A few minor compounds were found to be present but were not detailed.

With the aim of studying the evolution of the quantity of the major phenolic compounds found in Chemlali olive leaves, Bouaziz and others (30) used leaves collected from July 2003 to March 2004. Extract analysis was performed by HPLC, which revealed that Ol was always the major compound. This accords with the findings of Jemai and others (31), who found that the major phenolic compound in Chemlali olive-leaf extract, Ol, reached 4.32g/100g dry weight. Bouaziz and others (28) found low variations in Ol concentration during the whole period of harvest (from 12.4 to 14.2%). Besides Ol, they identified flavones (luteolin 7-o-glucoside, luteolin 7-o-rutinoside, apigenin 7-o-glucoside, luteolin and apigenin) and one flavonol (rutin) using a combination of HPLC-UV and APCI-MS but did not quantify them (Table 3).

Table 3. Phenolic compounds identified in olive by-products and the analytical methods used.

Source	**Olive leaf**	**OMWW**
Extraction method	SLE Methanol: water (80:20)	continuous counter current extraction Ethyl acetate
Chromatographic Technique	HPLC	HPLC
Column	C-18 (4.6x250 mm) Shim-pack VP-ODS[a] Lichrosphere 100 RP-18 (4x250 mm) [b]	4.6x 250mm (Shim-pack VP-ODS)
Mobile phases for HPLC	0.1% phosphoric acid in water (A) and 70% acetonitrile in water (B) [a] 2% acetic acid in water (A) and methanol, acetic acid and water (18:1:1) (B) [b]	0.1% phosphoric acid in water (A) and 70% acetonitrile in water (B)
Detection system	UV (at 280 nm) [a] photodiode array (APCI)-MS[b]	UV (at 280 nm)
Identified compounds	Ol, Luteolin 7-*O*-glucoside, Luteolin 7-*O*-rutinoside, Apigenin 7-*O*-glucoside, Rutin, Luteolin, Apigenin	HyTy, 3,4-Dihydroxyphenylacetic acid, HyTy-4-β-glucoside, *p*-Coumaric acid, Ferulic acid, Tyrosol, Ol
Olive cultivars	Chemlali (Sfax)	Chemlali
References	(30)	(36)

[a]*: for monomer identification;* [b]*: for flavonoid identification*

Bioactivity

Abaza and others (32) investigated the protective effects of seven olive- leaf extracts of Tunisian olive varieties against human leukemia (Table 2). The extracts showed an antiproliferative effect on HL-60 cells incubated for 48 h and the most potent was the Chemlali extract. The Gerboui extract showed the highest capacity to reduce nitroblue tetrazolium. Apigenin-7-glucoside was mainly responsible for the differentiation of HL-60 cells mediated by Gerboui extract.

Jemai and others (31) investigated the hypolypidemic and antioxidant activities of Ol and its hydrolysis-derivative-rich extracts in rats fed on a cholesterol-rich diet. The results showed that the administration of polyphenol-rich olive leaf extracts significantly lowered the serum levels of total cholesterol and triglycerides and low-density lipoprotein cholesterol whilst at the same time increasing the serum level of high-density lipoprotein cholesterol (HDL-C). In addition, these extracts lowered the content of thiobarbituric acid reactive substances in the liver, heart, kidneys and aorta compared with those of rats fed on a cholesterol-rich diet. In addition, they increased the serum antioxidant potential and hepatic superoxide dismutase and catalase activities (Table 2).

Olive-mill waste-water (OMWW)

Olive-mill waste-water is a by-product of the three-phase process of the extraction of oil from olives. This black waste-water is composed of the olive-fruit vegetation water, the water used for washing and treating the olives and a portion of the pulp and residual oil (34).

A continuous counter-current extraction procedure has been reported for the removal of polyphenols from Tunisian OMWW (35-37). Among several polar solvents

such as methyl isobutyl ketone, methyl ethyl ketone and diethyl ether, ethyl acetate was found to extract broadly the whole OMWW monomeric fraction (35*).*

Chromatographic determination of the phenolic compounds in OMWW

A RP-HPLC system coupled to a UV-detector was used to identify the major phenolic compounds of the OMWW extract (35). HyTy and Ty were found to be the major compounds in the OMWW extract at concentrations of 1,225 and 345 mg/L respectively; lower concentrations of *p*- hydroxyphenyl acetic acid, caffeic acid and p-coumaric acid were present. 3,4-dihydroxyphenylacetic acid and ferulic acid were also present at the same concentration, 70 mg/L. Protocatechuic acid, vanillic acid, syringic acids and other compounds were detected but not quantified.

In another study, also using RP-HPLC, Feki and others (36) studied the storage effect on the phenolic composition of OMWW (Table 3). Fresh and stored Chemlali OMWWs of two harvest periods were used. Two compounds, H-4-β-glucoside and Ol, were not cited by Fki and others (35). Among the compounds identified, HyTy was the most abundant phenolic monomer in both fresh and stored OMWW. Its concentration increased during storage for both harvest periods, varying over a 5-month period from 0.98 to 3.5 g/L for OMWW from the harvest period 2004-2005, and from 0.77 to 3.1 g/L after 4 months' storage from the harvest period 2005–2006. In contrast to the evolution of HyTy, the concentration of Ol decreased markedly in the extracts from both harvest periods. This evolution might be put down to some hydrolysis reactions of HyTy derivatives composed of HyTy units attached to other compounds *via* ester and/or glucosidic linkages. These hypotheses accord with those of Gómez-Caravaca and others (38).

Comparison between the Tunisian olive phenolic compounds and bioactivity and some Mediterranean varieties

As we have already mentioned in this review, several phenolic compounds from Tunisian olive tree derivatives (fruit, oil, leaf, OMWW) exert biological activity. In comparison to the biological activity of other Mediterranean olive derivatives, it is interesting to note that the anti-allergic property of olive oils has been studied for the first time in Tunisian olive oils. As far as the bioactive compounds are concerned, Ol, HyTy, Ol agl, apigenin-7-glucoside and other flavones such as apigenin and luteolin present in Tunisian olive derivatives exhibit different biological activities. It has been reported that Ol and HyTy inhibit inhibit the copper-sulphate-induced oxidation of LDL strongly and dose-dependently (39, 40). HyTy has also proved to be effective in a model of oxidative stress induced in intestinal epithelial cells (41) and in building up plasma antioxidant capacity (42). Identified in other oils have also demonstrated antimicrobial properties (43), antiproliferative effect against human promyoleocytic HL60 leukemia cells and human colon cancer lines (44-45).

On the basis of olive oil being the most important product of the olive tree, we have set out in Table 4 some olive oils produced in the Mediterranean basin together with their phenolic composition and bioactivity. According to the bibliography, the main phenolic compounds identified in Tunisian olive oils have also been reported in other olive oils from Mediterranean countries. As Table 4 shows, not many studies have been undertaken into the bioactivity of Tunisian olive oils and so it would be interesting to investigate other biological activities of Tunisian olive oils as some varieties showed interesting phenolic profiles.

Comparison of phenolic compounds present in olive oils from some Mediterranean olive varieties together with their bioactivity.

Country	Tunisia	Spain	Italy	Turkey	Greece
Studied cultivars	£Chetoui, Chemlali, Chemchali, Sayali, Zarrazi, Zalmati and Gerboui ¤ Chetoui, Chemlali, El Hor, Jarboui, Chemchali, Oueslati	¥Arbequina, Hojiblanca, Cornozuelo and Manzanilla # Picual, Manzanilla, Cornicabra and Hojiblanca	EVOOs on current Italian market	¤Memicik, Erkence, Nizip-yaglik, Gemlik and Ayvalik, * Ayvalik	Lianolia
Phenolic compounds	£HyTy, Ty, VA, *p*-Coumaric acid, HyTy-ac, Dialdehydic form of Ol agl, Ty-ac, Isomer derivatated, Dialdehydic form of Lig agl, Pinoresinol, Ac-pinoresinol, Vanillin, Aldehydic form of Ol agl, aldehydic form of Lig agl, FA, Luteolin, Apigenin ¤HyTy, Ty, HyTy-Ac ,D-Ol Agl, D-Lig-Agl, Ol-Agl, H- D-Ol Agl, Methyl- D-Ol Agl, 10- H- Ol Agl, Lig-Agl, Vanillic	¥ HyTy, Ty, vanillin, p-Coumaric acid, HyTy-ac, EA, Hy-EA, decarboxymethyl Ol agl, H-D-Ol agl, Syringaresinol, Pinoresinol, Decarboxymethyl Lig agl, H-D-Lig agl, 10-H-Ol agl, Ol agl, luteolin, Methyl D-Ol agl, Lig agl, apigenin, Methyl Ol agl	◊HyTy,Ty, Ol agl, Lig agl	¤HyTy, 2,3-Dihydroxybenzoic acid, Ty, 4-Hydroxybenzoic acid, 4-Hydroxyphenylacetic acid, Vanillic acid, Caffeic acid, Vanillin, *p*-Coumaric acid, Ferulic acid, Cinnamic acid, Luteolin, Apigenin	HyTy, Ty, Vanillic acid, Syringic acid, HyTy derivative, Ty derivative, Complex phenolic compounds

Table 4. Cont.

	coumaricd acid, ferulic acid, taxifolin,EA, H-EA, Pin,Ac-Pin, H-Pin,Syringaresinol, Sinapinic acid, Luteolin, Apigenin				
		# HyTy, Ty, HyTy-Ac, HyTy glycol, Lig agl, Ol agl, lignans and flavones not specified	² HyTy, Ty, dial dehyd D-Ol agl, dialdehydic D-Lig agl,(+)-1-Acetoxypinoresinol, (+)-pinoresinol, Ol agl, Lig agl	*Hyty, Ty, Vanillin, p-coumaric acid, luteolin, apigenin, dialdehyd Ol agl, pinoresinol, Aldehydic Ol agl, Aldehydic Lig agl	
Bioactivity	£anti-allergy	¥ anti-breast cancer # anti-microbial	◊anti-oxidative stress, ²anti-oxidant/anti-cancer		
Interesting varieties	£Sayali, Zarrazi	¥ Picual (Cordoba)			
References	£(28) ¤23	¥ (47) # (48)	◊ (49) ² (50)	¤ (51) *(52)	(53)

Conclusions

As can be seen from this review, the composition and quantity of phenolic compounds in olive products and by-products is the result of a complex interaction of various factors. From the existing literature concerning the characterization of polyphenols in theTunisian olive and its derivatives, it is clear that the main focus has been directed towards the two most important varieties: Chétoui and Chemlali. Numerous phenolic compounds have been identified and quantified and some of them show interesting biological activities. Nevertheless, bearing in mind the diversity of Tunisian olive germplasm, a widening of the study to other varieties is called for. Investigation of other olive-tree organs such as vegetative and flowering buds and flowers may also be of interest. Apart from this, research should be undertaken into the role of polyphenolics in the regulation of flower and fruit development in olives.

With regard to extraction procedures, modern technologies such as accelerated solvent extraction, supercritical or superheated fluid extraction and microwave-assisted solvent extraction, used for accelerating, automating the removal step and/or manipulating the extract characteristics are notable for their absence in this field of research. As far as the analytical methods are concerned, the method of choice tends to be HPLC due to its high resolution, high efficiency, high reproducibility and relatively short analysis time without restrictions on sample volatility. Moreover, HPLC has been coupled to a variety of detectors such as UV and MS.

The biological activities of olive derivatives may have a significant impact on the health population by reducing the development of chronic degenerative disease. Thus, in view of the growing importance of polyphenols, research in this field may provide more information about olive polyphenols and open up new possibilities for practical applications of their bioactivity and for adding value to olive by-products.

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Chapter 2

Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area

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ORIGINAL PAPER

Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area

Absract

A study on the characterization of Tunisian extra-virgin olive oil varieties produced in their place of origin has been carried out. Due to the influence of the genotype and environmental, agronomic and technological factors on the chemical composition of olive oil and its quality, all the olives studied were collected on the same season, and the oil was obtained under the same processing technique. Several analyses were performed to characterize the different olive oils: free acidity, peroxide value, fatty acid composition, radical scavenging activity, Rancimat assay, pigments content and phenolic compounds by HPLC–MS. In order to evaluate all the results obtained (36 parameters for each variety), different statistical analyses were used to discriminate the extra-virgin olive oil varieties: one-way analysis of variance was performed to check significant differences among cultivars (p B 0.05); PCA was applied to the data showing that variables such as oleic, linoleic, quinic and vanillic acids, apigenin, luteolin, taxifolin, oleuropein aglycon, pinoresinol acetate, elenolic acid and oxidative stability allowed discriminating among the different varieties of extra-virgin olive oil studied. Besides, LDA model was able to classify the samples depending on their geographical origin in Tunisia (North, Centre and South).

Keywords: Extra-virgin olive oil, Phenolic compounds, Fatty acids, Antioxidant activity- Geographical origin- Quality parameters

Introduction

The olive tree (Olea europaea L.) is one of the most important crops in Mediterranean countries. Agriculture is the mainstay of Tunisian economy, the olive tree being one

of its principal agricultural and economical sectors. Tunisia represents one of the biggest producers of olive oil worldwide; today, it is the fourth largest exporter after Spain, Italy and Greece (1). Its olive-growing areas are found all around the country, where a wide range of edapho-climatic conditions exists, from lower semi-arid to arid conditions. As it is widely known, the environmental factors, as well as genotype, agronomic and technological factors influence very much the chemical composition of olive oil, having a great impact on its quality (2–4). Furthermore, virgin olive oil, due to its use without refining, shows very interesting nutritional and sensorial properties, being one of the pillars of the Mediterranean diet. Its fatty acid composition, monounsaturated, and its natural antioxidants provide numerous advantages for health (5–7).

The genetic diversity in the olive sector of Tunisia is very wide; there are many different olive varieties in the various regions of the country. However, Chemlali and Chetoui are the two main olive cultivars in Tunisia. Chetoui represents more than 20% of the national olive oil production (8), while Chemlali is the most abundant olive variety, which represents two-thirds of olive plantation, principally used for oil extraction, and it is the major contributor of the national olive oil production (9). Despite the fact that these two cultivars are the most important from the economical point of view, there are other cultivars that are grown in restricted geographical localizations and that have a limited diffusion outside these areas. The study of these less-common cultivars appears of particular interest because they may have agronomic characteristics which can influence the quality and oxidative stability of the olive oil obtained. Many studies have been carried out about olive cultivars, and

their olive oils in Tunisia: comparison of different cultivars and growing areas of autochthonous olive oils (10, 11) and comparison of Tunisian and European varieties cultivated in Tunisia (2, 12–14). However, there is not much information about the characterization and comparison of the Tunisian autochthonous varieties of olive oil grown in their area of origin.

The present work was carried out on six monovarietal extra-virgin olive oils (EVOO) from the two main (Chemlali and Chetoui) and four minor Tunisian cultivars (Oueslati, Jarboui, El Hor and Chemchali) produced in their area of origin. Several analyses were performed to characterize the different olive oils: free acidity, peroxide value, fatty acid composition, radical scavenging activity, Rancimat assay, pigments content and phenolic compounds by HPLC–MS. This is a preliminary study with the aim of finding any variable able to discriminate among the monovarietal extra-virgin olive oils and evaluate the possibility to determine whether the parameters studied were able to classify the cultivars depending on their geographical origin. The Tukey's honest-test was used to determine the level of significance of each evaluated parameter characterizing the varieties. Pearson test allowed checking the correlations between different analyses. The classification of EVOO samples according to their geographical origin was performed by linear discriminant analysis (LDA). Finally, principal component analysis and agglomerative hierarchical clustering were performed to check the usefulness of chemical parameters as a tool to discriminate among the monovarietal EVOO.

Materials and methods

Chemicals

All solvents used were analytical or HPLC grade (Panreac, Barcelona, Spain) and used as received. Double-deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, o-coumaric acid, ferulic acid and quinic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland) and oleuropein from Extrasynthe`se (Lyon, France). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v).

Samples

Oils used in this study were obtained from six Tunisian olive varieties cultivated in their origin regions: North (Chetoui, Chemlali and Jarboui), Centre (Oueslati and El Hor) and South (Chemchali). Only healthy fruits, without any kind of infection or physical damage, were processed. Olives were harvested in the same season in 2008. The maturity index of all the olives was of 3 and was based on the degree of skin and pulp pigmentation according to the method developed by the Agronomic Station of Jaen (15). After harvesting, the olives were washed and deleafed. Then the fruits were crushed with a hammer crusher, and using an Abencor analyzer (MC2 Ingenierias y Sistemas, Sevilla, Spain) the paste was mixed at 25 °C for 30 min, centrifuged without addition of warm water and then transferred into dark glass bottles.

Oil yield

The determination of oil content was done as follows: 40 g of fruit samples was dried in an oven at 80 °C to constant weight. Then, the dry olives were extracted with

petroleum ether using a Soxhlet apparatus according to Donaire et al. (16). The results were expressed as percentage of dry matter (%DM).

Analytical indices

Determination of free acidity, peroxide value and specific ultraviolet absorbance were carried out following the analytical methods described in the EC Regulation (17, 18).

Fatty acid composition

The fatty acid composition of oil samples was determined as methyl esters by capillary gas chromatography analysis after alkaline treatment. The gas chromatograph (VARIAN CP-3800 Gas Chromatograph) was equipped with an autosampler (CP-8400), a capillary column HP Innowax (Agilent Technologies, USA) (30 m 9 0.53 mm, 1 lm), a split–splitless injector and a flame ionization detector (FID). Alkaline treatment was carried out by mixing 0.1 g of oil dissolved in 3 mL of n-hexane with 0.5 mL of 0.2 N methanolic potassium hydroxide solution according to the method of Reg EC 2568/91 (19). One microlitre of methyl esters was injected. Seven fatty acids including C16:0, C16:1, C18:0, C18:1, C18:2, C18:3 and C20:0 were identified from their retention times compared to those of standard compounds.

Radical scavenging activity

The olive oil samples were examined for their capacity to scavenge the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) according to Kalantzakis et al. (20). Olive oil

was solved in ethyl acetate (10%, w/v), 1 mL of this solution was added to 4 mL of a freshly prepared DPPH solution 10-4 M in a screw-capped 10-mL test tube. The reaction mixture was then shaken vigorously for 10 s in a Vortex apparatus, and the tube was maintained in the dark for 30 min, after which a steady state was reached. The absorbance of the mixture was measured at 515 nm against a blank solution using a UV–VIS dual beam spectrophotometer (UVS-2700 Labomed, Inc). A control sample was prepared and measured daily. The radical scavenging activity (RSA) towards DPPH was expressed as the % reduction in DPPH concentration by the constituents of the oils: %[DPPH]red = 100*(1-$[DPPH]_{30}$/$[DPPH]_0$), where $[DPPH]_0$ and $[DPPH]_{30}$ were the concentrations of DPPH in the control sample (t = 0) and in the test mixture after the 30-min reaction, respectively. The DPPH concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression: A515nm = 12.024 [DPPH]-0.0101 (r = 0.999), where [DPPH] was the concentration of DPPH, expressed as mol/L.

Rancimat assay

Oxidative stability was evaluated by Rancimat method [21). Stability was expressed as the oxidation induction time (h), measured with the Rancimat 743 apparatus (Metrohm, Switzerland), using an oil sample of 3.5 g warmed to 100 °C and an air flow of 10 L h-1.

Pigment contents

Oil (7.5 g) was accurately weighted and dissolved in 25 mL of cyclohexane. Chlorophyll and carotenoid contents were determined from the absorption spectra of the oils. Absorption at 670 nm is usually considered to be related to the chlorophyll fraction (pheophytin ‘a’ as its major component) and 470 nm to the absorption of the

carotenoid fraction (lutein). Chlorophyll and carotenoid amounts were calculated using the specific extinction values, by the method of Minguez-Mosquera et al. (22). The extinction coefficients applied were E0 = 613 for pheophytin 'a' and E0 = 2000 for lutein. Thus, pigment contents were calculated as follows:

[Chlorophyll] (mg/kg) = (A_{670} × 106)/(613 × 100 × d)

[Carotenoid] (mg/kg) = (A_{470} × 106)/(2000 × 100 × d)

Where A is the absorbance and d is the spectrophotometer cell thickness (1 cm). Chlorophyll and carotenoid contents were expressed as milligrams of pheophytin 'a' and lutein per kilogram of oil, respectively.

HPLC–MS analysis

Sample preparation

The polar fraction was extracted according to Tsimidou (23). Of the oil sample, 2.5 g was weighted and dissolved in 5 mL of n-hexane. After that 5 mL of methanol/water (60/40) were added, the mixture was vortexed and then centrifuged at 2,275 g during 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure and at a temperature of 35 °C. The residue was dissolved in 0.25 mL of methanol/water (50:50 v/v) and finally filtered through a 0.45-lm filter before the

HPLC analysis.

Chromatographic separation An Agilent 1200-RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump and a UV–vis detector was used for the chromatographic determination. Polyphenolic

compounds were separated by using a method previously described in bibliography (24). The compounds separated were monitored with DAD (240 and 280 nm) and with a mass spectrometry detector.

Mass spectrometry

The RRLC system was coupled to a Bruker Daltonik microTOF (time of flight) mass spectrometer (Bruker Daltonik, Bremen, Germany) using an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) equipped with an ESI interface. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50–1000. The optimum values of the ESI–MS parameters were as follows: capillary voltage, -4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; and nebulizing gas pressure, 2 bar. External calibration was performed using a sodium formiate solution injected at the beginning of the run, and all the spectra were calibrated prior to the polyphenol identification.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulas by using the Generate Molecular Formula^{TM} editor. The Generate Molecular Formula^{TM} editor uses the sigma Fit^{TM} algorithm, a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring plus doublebond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value^{TM}) for increased confidence in the suggested molecular formula.

Statistical analysis

One-way analysis of variance (ANOVA, Tukey's honest significant difference multiple comparison) and Pearson's linear correlations were evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

Agglomerative hierarchical clustering (AHC) was performed using XLSTAT 2008.

The standardized variables were used to perform principal component analysis (PCA) and cluster analyses using XLSTAT software (v. 2010.4.01, Addinsoft, NY, USA). To group the accessions based on chemical dissimilarity, agglomerative hierarchical clustering (AHC) was conducted on the Euclidean distance matrix with the Ward method. LDA statistical data treatment was performed using SPSS (v. 15.0, Statistical Package for the Social Sciences, Chicago, IL, USA).

Results and discussion

Oil yield of olives

As reported in Table 1, all the studied varieties are characterized by a high oil yield according to the classification of Tous et al. (25). Expressed as percentage of dry matter, the oil yield presented significant differences between the six olive varieties; the content of oil in the samples was in the range of 48–57%, the Chetoui olives being the richest in oil with a mean value of 56.9%, and the Oueslati being the one with the lowest content in oil (47.7%).

VOOs samples included in our study were produced using Abencor system which is suitable for processing small quantities of olives, especially for typical productions such as monovarietal olive oils. However, it is important to mention that the mini and microcondition of olive oil production could lead to obtain an oil different from that produced in industrial conditions as previously reported by Cerretani et al. (26) and Inarejos-Garcia et al. (27).

Free acidity, peroxide value and specific ultraviolet absorbance

For all analysed oils, the mean values of studied quality parameters fell within the range allowed by the regulation EC (17, 18) for the extra-virgin olive oil category (free acidity B 0.8%; peroxide value B 20 Meq O2 kg-1; K270 B 0.22; K232 B 2.5) (Table 1). Free acidity of the oils studied was in a range from 0.25 to 0.60%. This fact means that, taking into account the acidity of olive samples, all olive oils could be classified as ‘‘extra-virgin olive oils’’.

Concerning the peroxide values, samples ranged from 2.03 meqO2/kg of Oueslati to 4.0 meqO2/kg of Chetoui and Chemchali. These low values are a measure of the high freshness of the oils analysed (28, 29).

The specific ultraviolet absorbance K232 varied from 1.70 to 2.17 having the highest values in Oueslati, Chetoui and El Hor varieties, while K270 ranged from 0.20 of Oueslati to 0.13 of Chemlali.

Fatty acid composition

Methyl ester fatty acid composition and their levels in the analysed oils are shown in Table 1. As it can be observed, oleic (C18:1), linoleic (C18:2) and palmitic (C16:0) acids are the major fatty acids present in the studied samples. The fatty acid composition of olive oils varies widely depending on the cultivar. These findings are in good agreement with those of other authors working on Tunisian olive oil varieties (12, 30).

Among studied samples, the Oueslati olive oil showed the highest percentage of oleic acid (C18:1) and the lowest percentage of linoleic acid (C18:2) (69.9 and 12.1%, respectively), and the Chetoui olive oil showed the lowest percentage of palmitic acid which did not exceed 11.5%.

Concerning palmitoleic (C16:1), stearic (C18:0), linolenic (C18:3) and arachidic (C20:0) acids, the studied olive oil varieties presented low amounts of all of them. The

highest percentage of palmitoleic acid was found in Chemlali cultivar, stearic acid presented the highest amount in Chetoui variety, while Jarboui olive oil was the richest in linolenic acid.

Other interesting points for the chemical characterization of studied oils are the proportions of some classes of free fatty acids. The monounsaturated fatty acids have great importance because of their nutritional implication and effect on oxidative stability of oils (5). Table 1 shows that the proportion of monounsaturated fatty acids also changed according to the cultivar. It reached a maximum value of 71.3% for the Oueslati olive oil, which was characterized, among the studied oils, by the highest MUFAs/PUFAs and C18:1/C18:2 ratios (5.6 and 5.8, respectively). The C18:1/C18:2 ratio has the most marked relationship with stability, and it is said that an oil presents a good stability index if this value is over 7. Nevertheless, Tunisian olive oils are described in bibliography to present lower C18:1/C18:2 ratios compared to most of the European ones (12, 31).

Table 1. Chemical characteristics of the different olive oil varieties

	Cultivars					
	Oueslati	Chetoui	Chemlali	**El Hor**	**Chemchali**	**Jarboui**
% Humidity	53,905 c	50,650 e	54,300 b	60,600 a	51,345 d	47,285 f
% oil yield/DM	47,670 e	56,875 a	51,790 c	49,090 d	52,645 b	51,415 c

Free acidity (as %C18:1)	0,250 d	0,310 c,d	0,350 b,c	0,420 b	0,300 c,d	0,603 a
Peroxid value (MéqO2/kg)	2,033 b	3,167 a	4,000 a	3,667 a	4,000 a	3,667 a
K 232	2,2 a	1,9 a,b	1,7 a,b	2,0 a,b	1,7 b	2,1 b
K 270	0,2 a	0,2 a-c	0,1 c	0,2 a,b	0,2 a,c	0,2 b,c
% C16:0	13,6 b	11,5 b	17,8 a	12,3 b	15,9 a	16,4 a
% C16:1	1,3 b	0,3 c	2,6 a	0,5 c	1,3 b	0,6 c
% C18:0	1,9 c	2,8 a	2,0 b	2,5 b	2,4 c	1,6 d
% C18:1	69,9 a	63,3 b,c	60,0 d	61,8 c,d	65,0 b	54,5 e
% C18:2	12,1 e	20,3 b	16,0 c	20,9 b	13,7 d	25,7 a
% C18:3	0,7b	0,6 b,c	0,6 b,c	0,9 a	0,6 c	0,9 a
% C20:0	0,4 a	0,4 a	0,4 a	0,4 a	0,5 a	0,3 a
%DPPH red	86,6 c	97,1 a	80,8 d	93,2 a,b	95,9 a,b	91,3 b,c
Oxidative stability (h)	48,455 b	49,485 b	40,995 d	46,870 c	58,845 a	46,315 c
Chlorophyll (mg/kg)	4,774 a,b	3,502 b,c	2,790 c	2,172 c	2,425 c	5,421 a
Carotenoids (mg/kg)	1,573 b	1,487 b	1,553 b	0,868 c	1,503 b	2,412 a
∑MUFAs	71,252 a	63,595 b	62,556 b	62,296 b	66,360 b	54,981 c
∑PUFAs	12,836 f	20,909 c	16,652 d	21,810 b	14,247 e	26,629 a
MUFAs/PUFAs	5,551 a	3,041 d	3,757 c	2,856 d	4,658 b	2,065 e
C18:1/C18:2	5,760 a	3,118 d	3,746 c	2,951 d	4,758 b	2,117 e

Pigment contents

The olive oil colour is directly related to the chlorophyll and carotenoid contents, and it has been proposed as a characterizing factor and as a quality index related to the oil extraction method and to the olive variety (22). Besides, the colour is the first attribute of virgin olive oil evaluated by consumers. In analysed oils and according to the cultivar, chlorophyll and carotenoid contents ranged from 2.17 and 0.87 mg/kg to 5.42 and 2.41 mg/kg, respectively (Table 1), Jarboui olive oil being the richest in terms of both of them.

Identification and quantification of phenolic compounds

The identification of phenolic compounds was carried out comparing their migration times, UV data 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 using Generate Molecular Formula Editor. In this way, 24 phenolic compounds could be identified. Table 2 includes the identified compounds, migration time, molecular formula, calculated and experimental m/z, sigma value and tolerance (ppm) in generated molecular formula. Major phenolic compounds previously observed in bibliography (32, 33) were also detected in the present study.

Table 2. Phenolic compound identified in an olive oil extract by HPLC-ESI-TOF-MS including retention time, m/z experimental and calculated, molecular formula and sigma value.

Compound	Retention time (min)	m/z experimental	m/z calculated	Molecular formula	Sigma
Quinic acid	2.1	191.0574	191.0561	C7H11O6	0.0010
HYTY	7.9	153.0582	153.0557	C8H9O3	0.0014
TY	9.7	137.0623	137.0608	C8H9O2	0.0040
Vanillic acid	11.1	167.0361	167.035	C8H7O4	0.0080
o-Coumaric acid	13.2	163.0424	163.0401	C9H7O3	0.0313
Taxifolin	13.6	303.0504	303.051	C15H11O7	
Ferulic acid	13.7	193.0525	193.0506	C10H9O4	0.620
HYTY-Ac	13.8	195.0681	195.0663	C10H11O4	0.0107

EA	14.7	241.0755	241.0718	C11H13O6	0.0075
D OA	15.9	319.1252	319.1187	C17H19O6	0.048
HY EA	15.3	257.0689	257.0667	C11H13O7	0.0089
HY D-OL Agl	16.29	335.1148	335.1136	C17H19O7	0.0052
Syringaresinol	17.8	417.1547	417.1555	C22H25O8	0.0156
Pin	18.5	357.135	357.1344	C20H21O6	0.0048
Decarbox-Lig Agl	18.8	303.1258	303.1238	C17H19O5	0.0070
Ac Pin	19.0	415.1409	415.1398	C22H23O8	0.0031
Methyl D OL agl	19.9	333.1355	333.1344	C18H21O6	0.0293
Sinapinic acid	21.3	223.0640	223.0612	C11H11O5	0.0107
10-H-OL AGL	22.6	393.1188	393.1191	C19H21O9	0.0183
OL Agl	22.8	377.1248	377.1242	C19H21O8	0.0002
Lut	23.3	285.0420	285.0405	C15H9O6	0.0026
H Pin	24.4	373.1309	373.1293	C20H21O7	0.0258
Lig Agl	25.3	361.1308	361.1293	C19H21O7	0.0095
Apig	25.6	269.0481	269.0455	C15H9O5	0.0097

To build the calibration curves and carry out the quantification of the identified phenolic compounds, eleven standards were used: hydroxytyrosol (HYTY), tyrosol (TY), oleuropein (Ol), pinoresinol (Pin), luteolin (Lut), apigenin (Apig), taxifolin, vanillic acid, o-coumaric acid, quinic acid and ferulic acid. All of them presented good linearity between different concentrations, and regression coefficients were higher than 0.990 in most cases. HYTY, TY, Pin, Lut, Apig, taxifolin, vanillic acid, ferulic acid, quinic acid, and o-coumaric acid were quantified using their own commercial standards. HYTY-acetate (HYTY-Ac) was quantified using HYTY calibration curve; elenolic acid (EA), H-elenolic acid (H-EA), Ol aglycon (Ol Agl), decarboxylated Ol Agl (D-Ol Agl), 10-H-OL Agl, ligtroside aglycon (Lig Agl), decarboxylated Lig Agl (D-Lig Agl), H-D-Ol Agl, methyl D-Ol Agl were quantified with oleuropein calibration curve; acetoxy Pin (Ac-Pin), H-Pin and syringaresinol with Pin calibration curve and sinapinic acid using Ferulic acid calibration curve. Table 3 summarizes the concentrations of the phenolic compounds identified in the different olive oil varieties expressed as mg/kg of oil. It could be observed that the distribution of phenolic compounds varied significantly in the different cultivars (p B 0.05). Among major secoiridoids, Ol Agl, Lig

Agl and D-Ol Agl were found in higher concentrations in Chetoui/El Hor (1839.9/1915.9 mg/kg), Chemlali (303.15 mg/kg) and Chemchali (2455.3 mg/kg), respectively. Meanwhile, Oueslati and Jarboui presented the lowest content in Ol Agl (222.6 mg/kg) and D-Ol Agl (146.1 mg/kg), respectively.

As regards simple phenols, El Hor cultivar was the richest in terms of HYTY and TY (10.9 mg/kg). The rest of minor simple phenols identified were present in small amounts for all the samples. Five phenolic acids occurred in the studied samples. Quinic acid presented the highest levels (0.5–5.1 mg/kg), o-coumaric acid ranged from 0.1 up to 0.7 mg/kg, ferulic acid 0.0–0.1 mg/kg, sinapinic acid 0.1–0.6 mg/kg and vanillic acid which was present as traces in Oueslati, Chemlali, El Hor and Jarboui EVOOs was only quantified in Chetoui and Chemchali samples (0.9 and 1.5 mg/kg, respectively). (+)-Ac-Pin was the lignan which presented higher amounts ranging from 0.46 to 4.45 mg/kg, followed by syringaresinol (0.5–2.3 mg/kg), (+)-Pin (0.3–1.8

Table 4. Phenolic compounds of the studied olive oils by HPLC-ESI-TOF-MS

VARIETY	Oueslati	Chetoui	Chemlali	El Hor	Chemchali	Jarboui
HYTY	7.18±0.27a.b	7.26±0.6b	6.80±0.15b	7.68±0.31a	4.32±0.04c	1.81±0.23d
TY	2.39±0.04c	3.53±0.54a,b	3.48±0.05a	3.25±0.01a,b	3.01±0.17b	2.27±0.1c
Elenolic acid	638.61±15.93c	182.99±44.57d	806.28±26.11a	743.39±20.88b	12.13±0.52e	143.98±32.38d
HYTY-Acetate	0.68±0.04d	1.68±0.21c	2.94±0.26b	2.67±0.12b	7.11±0.06a	1.05±0.06d
Decarbox Ol Agl	1834.12±58.74b	679.01±75.93c,d	808.01±8.84c	538.21±37.87d	2455.25±102.46a	146.07±44.33[e]
Ol Aglycone	222.62±14.34d	1839.87±217.1a	578.19±101.26c	1915.86±81.74a	971.91±68.59b	1042.85±168.85b
10-H-Ol Agl	0.95±0.02d	2.27±0.45c	3.62±0.05a	2.40±0.07b	2.28±0.17b	3.61±0.16a
Decarbox-Lig Agl	16.11±0.55b	6.83±0.46c,d	12.67±0.05b,c	3.67±0.16e	60.77±6.34a	4.70±0.32[e]
Ligstroside Aglycone	9.89±0.3c	258.28±59.10b	303.15±59.81a	80.55±13.15c	17.36±0.82c	26.21±6.97c
Pinoresinol	1.23±0.02c	1.34±0.05b	0.73±0.02d	1.79±0.03a	0.70±0.02d	0.25±0.01[e]
Luteolin	0.74±0.04e	4.18±0.53b,c	2.93±0.09d	4.38±0.00b	4.28±0.33b	10.20±0.59a
Ac Pinoresinol	4.45±0.08a	0.46±0.02e	2.51±0.1c	0.74±0.01d	0.87±0.05d	2.92±0.18b
Apigenin	0.09±0.01b	0.82±0.22b	0.52±0.01b	0.45±0.00b	0.53±0.06b	2.86±0.82a
H-Pinoresinol	0.51±0.01c	0.56±0.06b	0.65±0.02a	0.35±0.01e	0.32±0.01e	0.44±0.02d
Taxifolin	-	-	-	-	-	0.147±0.002
Vanillic acid	-	0.93±0.34b	-	-	1.51±0.27a	-
o-Coumaric acid	0.10±0.03e	0.41±0.09c	0.33±0.00c	0.15±0.00e	0.48±0.03b	0.66±0.12a
Ferulic acid	0.08±0.01b	0.11±0.01a	-	0.02±0.00e	0.03±0.01d	0.06±0.00c
Quinic acid	0.48±0.02d	-	1.74±0.01b	5.05±0.3a	1.63±0.13b	0.87±0.08c
Sinapinic acid	0.59±0.03a	0.10±0.01d	0.21±0.01b	0.13±0.01c	0.05±0.01e	0.12±0.00c,d
H- Elenolic acid	0.58±0.05c	0.26±0.05e	2.44±0.13a	0.78±0.06b	0.47±0.01c,d	0.39±0.08d
H-Decarbox-Ol	8.41±0.2b	0.55±0.09d	3.08±0.03c	0.23±0.05d	16.12±1.36a	1.02±0.07d

Results are expressed as mg/kg of oil. Different letters for the same phenolic compound indicate significant differences among varieties ($p<0.05$).

mg/kg) and (+)-H-Pin (0.3–0.7 mg/kg), Oueslati olive oil being the richest in terms of lignans.

Concerning the flavonoid composition, luteolin was the most abundant flavonoid in studied samples, and the Jarboui EVOO was characterized by the highest amounts of both flavones Lut and Apig (10.20 and 2.86 mg/kg, respectively) and by the presence of the flavonol (+)-taxifolin which was not detected for the rest of studied oils.

It is known that the oxidative stability variations are affected by some minor compounds such as phenols and tocopherols (34) and as reported by other authors, oxidative stability was positively correlated to secoiridoids amounts ($r = 0.940$, $p<0.001$) (35). In general, our results agree with those previously reported. In fact, positive correlations were found between major secoiridoids and Ol Agl and DPPH radical scavenging capacity ($r = 0.640$ and $r = 0.715$; $p\leq0.05$, respectively). The correlations were even higher between oxidative stability and D-Lig Agl and major secoiridoids ($r = 0.822$ and $r = 0.814$; $p\leq 0.05$, respectively).

Oxidative stability and radical scavenging activity

Oxidation stability is an important property of olive oil quality and is affected by lipid composition and different antioxidant compounds whose levels may be influenced by cultivar, year and place of production (3).

The oxidative stability of the extra-virgin olive oils was measured as the induction time determined using the Rancimat method. The highest oxidative stability was presented by Chemchali oil with a mean value of 58.8 h. This characteristic may be partially attributed to the low level of rainfall in the south region where it was cultivated as demonstrated by Tovar et al. (36), since oxidative stability is affected significantly by water regime that determines the phenol content of the olive fruit. Furthermore, the stability is the result of the oxidant–antioxidant balance. The weight of the antioxidants as phenolic compounds could be greater than this of fatty acid

profile. In fact, Chemchali olive oil did not present the best MUFA/PUFA ratio (4.7 ± 0.0) however presented the highest content in phenolic compounds (3564.1 ± 176.3 mg/kg), and as the result it is the oil with the highest oxidative stability value. Oueslati was the oil with the highest MUFA/PUFA ratio (5.5 ± 0.3); however, a lower concentration of phenolic compounds (2752.3 ± 79.5 mg/kg) provides a lower oxidative stability value (48.5 h).

It is known that especially antioxidant properties are very important due to the scavenging activity of free radicals in foods and in biological systems (37). When an antioxidant reacts with a free radical, it yields an electron, is oxidized, and becomes a weak, non-toxic free radical that is stable and unable to propagate the reaction. An exogenous supply of antioxidants through the diet is necessary because of the incomplete efficiency of endogenous human antioxidant defences and the occurrence of situations in which excessive free radicals are produced (38).

Olive oils were examined for their radical scavenging activity towards the stable DPPH free radical. As reported in Table 1, the Chetoui, El Horr and Chemchali were the cultivars which showed higher percentage of DPPH reduced and thus presented higher antioxidant capacity to scavenge the free DPPH radical. A positive correlation was found between the oxidative stability and the radical scavenging capacity ($r = 0.718$; $p < 0.05$).

Principal component analysis and cluster analysis

PCA was applied to all VOO samples. Except C20:0, which showed no significant variation among studied VOOs ($p = 0.117$, $\alpha = 0.05$), all variables were submitted to a PCA. The first three PCs explained 73.9% of variance. The first PC accounted for 30.4% of total variance and was highly correlated with luteolin ($r = 0.919$ at $\alpha = 0.05$), apigenin ($r = 0.911$ at $\alpha = 0.05$), taxifolin ($r = 0.938$ at a = 0.05), oleic ($r = -0.883$ at $\alpha = 0.05$) and linoleic ($r = 0.873$ at $\alpha = 0.05$) acids. The second PC (22.4% of variance) was correlated to OL Agl ($r = 0.888$ at $\alpha = 0.05$), quinic acid ($r = 0.844$ at $\alpha = 0.05$) and Ac

Pin (r = -0.814 at α = 0.05), while the third PC was correlated to oxidative stability (r = 0.925 at α = 0.05), EA (r = -0.898 at α = 0.05) and vanillic acid (r = 0.850 at α = 0.05). Figure 1 shows a projection of the six cultivars in the space defined by the first three principal components. We can distinguish the presence of four principal groups. Group 1 consists of Chemlali and Oueslati varieties which produce oils rich in EA and with fewer amounts of Ol Agl and Lut. Group 2 composed of the variety Chemchali, which is the one that presented the highest oxidative stability, was richer in D-Ol Agl, H-D-Ol Agl and presented low amounts of EA. Group 3 consists of the Chetoui and El Hor olive oil varieties that are characterized by higher amounts of Ol Agl and quinic acid and low amount of Ac-Pin. The last group was the Jarboui variety characterized by the presence of taxifolin, higher amounts of linoleic acid, Lut and Apig and lower amounts of oleic acid.

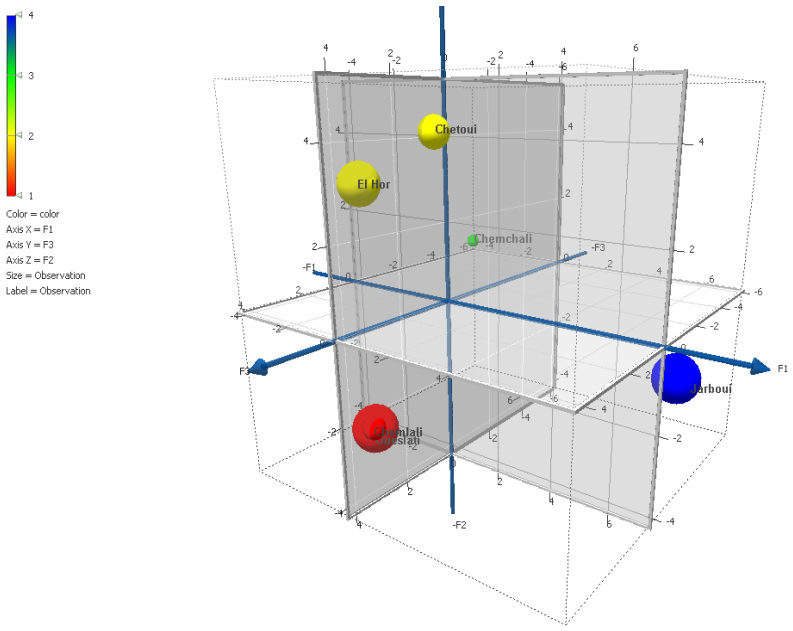

Figure 1. Principal Component Analysis (PCA) of the six varieties composed of the three first components**.**

The results obtained with HCA are analogous to those from PCA: 'Jarboui' is the most dissimilar of the varieties, and low similarity exists between 'Chétoui, and 'El Hor' and the rest of varieties (Fig. 2).

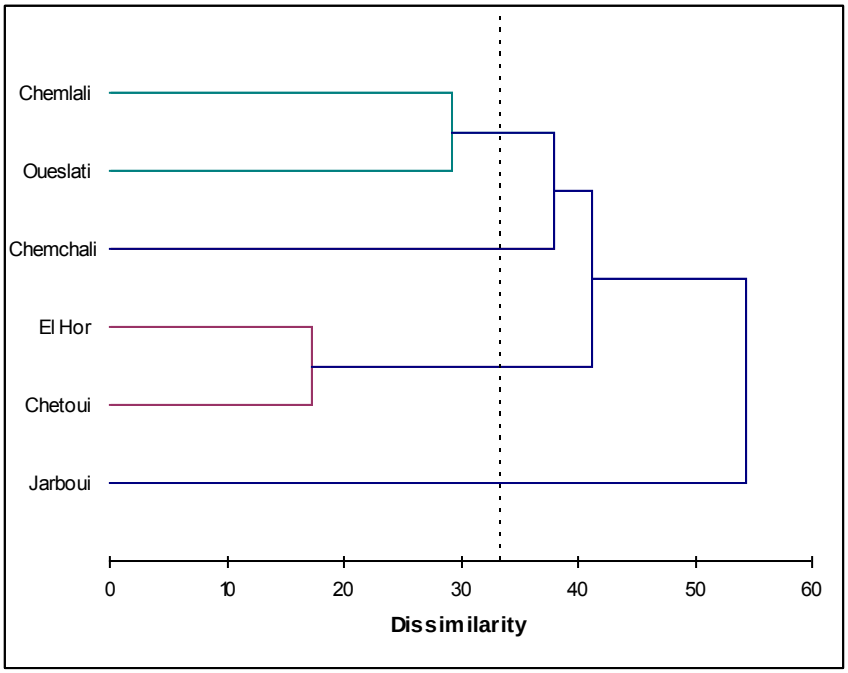

Figure 2. Dendrogram of the Tunisian varieties.

LDA analysis

Linear discriminant analysis (LDA) is one of the most commonly used classification techniques. LDA is a supervised classificatory technique widely recognized as an excellent tool to obtain vectors showing the maximal resolution between a set of previously defined categories. In LDA, vectors minimizing the Wilks' lambda, λ_w, are obtained (39). This parameter is calculated as the sum of squares of the distances between points belonging to the same category divided by the total sum of squares.

Using the normalized variables, an LDA model capable of classifying the EVOO samples according to their respective geographical origin was constructed (North, Centre and South). When the LDA model was constructed, a good resolution between all the category pairs was achieved (λ_w = 0.001) as it can be seen in Fig. 3. For this model, and using leave-one-out validation, all the points of the training set were correctly classified. The corresponding evaluation set was then used to check the prediction capability of the model. Using a 95% probability, all the objects were correctly assigned.

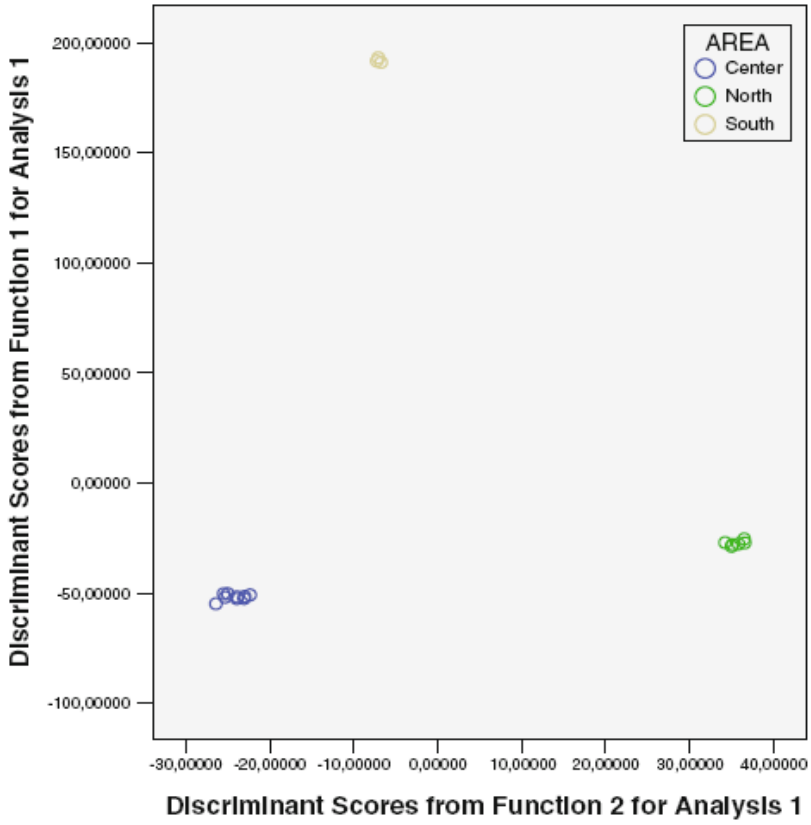

Figure 3. Two dimensional score plot of linear discriminant analysis LDA for classification of the EVOO samples according to their respective geographical origin.

Table 4 shows the variables selected by the SPSS stepwise algorithm (the predictors with large discriminant capabilities) and the corresponding standardized coefficients of this model.

Table 4. Standardized canonical discriminant function coefficients

	Function	
	1	**2**
% C18:1	-0,325	5,106
Carotenoids	-3,703	-3,825
EA	-10,660	2,902
HYTY-Ac	15,136	3,928
D-Lig Agl	4,675	-2,358
H-Pin	-11,886	-15,269
Sinapinic acid	3,954	17,556
H-D-OL Agl	12,519	-4,027

Variables such as oleic acid and EA were also among the variables that explained the largest portion of the variance in PCA. Besides, oleic acid is the most abundant fatty acid present in olive oil, and the rest of the variables constitute some of the most representative phenolic compounds of olive oil.

Conclusion

After the study of the phenolic profile and other chemical parameters for every of the six Tunisian extra-virgin olive oils it could be observed that there were significant differences among studied varieties. The statistical analysis also allowed distinguishing the monovarietal olive oils depending on some of these parameters such as the concentrations of oleic and linoleic acids, apigenin, luteolin, taxifolin, oleuropein aglycone, pinoresinol acetate, elenolic acid and oxidative stability. Furthermore, it was possible to classify the varieties of olive oil depending on their geographical origin by using LDA analysis as long as each variety had been grown in its own place of origin. In the case of LDA analysis also variables as oleic acid and elenolic acid play an important role as discriminating functions in the differentiation of the geographical origin.

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Chapter 3

Classification of ‘Chemlali’ accessions according to the geographical area using chemometric methods of phenolic profiles analysed by HPLC–ESI-TOF–MS

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Analytical Methods

Classification of 'Chemlali' accessions according to the geographical area using chemometric methods of phenolic profiles analysed by HPLC–ESI-TOF–MS

Abstract

The present work describes a classification method of Tunisian 'Chemlali' olive oils based on their phenolic composition and geographical area. For this purpose, the data obtained by HPLC-ESI-TOF-MS from thirteen samples of extra virgin olive oils, obtained from different production area throughout the country, were used for this study focusing in twenty three phenolics compounds detected. The quantitative results showed a significant variability among the analysed oil samples. Factor analysis method using principal component was applied to the data in order to reduce the number of factors which explain the variability of the selected compounds. The data matrix constructed was subjected to a canonical discriminant analysis (CDA) in order to classify the oil samples. These results showed that 100% of cross-validated original group cases were correctly classified, which proves the usefulness of the selected variables.

Key words: monovarietal virgin olive oil, phenolic compounds, geographic area, liquid chromatography, mass spectrometry, discriminant classification.

1. Introduction

Virgin olive oil (VOO) is unique among other vegetable oils due to its high levels of monounsaturated fatty acids (mainly oleic acid) and to the presence of minor components, such as phenolic compounds (1). Recent epidemiological studies have proved that the consumption of olive oil rich in phenolic compounds has a beneficial effect for the treatment or prevention of various types of allergic diseases (2) and leads to a reduced risk of cardiovascular disease (3,4), neurodegenerative disease (5,6), certain types of cancer (7-9), and HIV-1 infection (10).

In Tunisia, the second country after the European Union in the olive oil exportation scale, the variety 'Chemlali' contributes to 80% of the national olive oil production and covers a wide geographical area where a wide range of edapho-climatic conditions are prevailing. The quality and peculiarity of the olive oil depends on several factors and the cultivar is the main one. Thus, monovarietal olive oils are influenced by different factors clustered into four groups: environmental (soil, climate), agronomic (irrigation, fertilization) cultivation (harvesting, ripeness) and technological factors (post-harvest storage, extraction system) (11). Therefore, it is of great importance to find parameters that allow olive classification according to these variables. Furthermore, olive oil classification according to its chemical parameters has been considered to be an important tool for the monitoring of adulteration (12) and mislabelling (13) without forgetting that a complete farm-fork traceability of virgin olive oil involves the chemical characterization of the oils obtained from the main cultivars in each producer zone. Several researches have tried to correlate the chemical composition of olive oil to fruit varieties, geographic origin, denomination of origin, year of harvest or different qualities. Countless techniques of different complexity with numerous chemometric treatments have been employed for this purpose with promising results (14). Despite being the main Tunisian olive variety, we have a lack of information about the behaviour of 'Chemlali' olive oil phenolic

compunds according to the production area. For this reason, a complete study of the phenolic profile variation is needed.

The purpose of the current work has been to study the influence of geographical area on the behaviour of the phenolic profile of oils from 13 accessions belonging to the variety 'Chemlali', and to develop a method for oil classification according to their geographical area by using the obtained HPLC-ESI-TOF-MS data. The obtained results would contribute to a future traceability of Tunisian virgin olive oils.

2. Materials and methods

2.1. Chemicals and reagents

All solvents used were analytical or HPLC grade (Panreac, Barcelona, Spain) and used as received. Double-deionized water was obtained with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, *o*-coumaric acid, ferulic acid and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland) and oleuropein from Extrasynthèse (Lyon, France). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v).

2.2. Samples

Olives from the variety 'Chemlali' were harvested at the same ripeness stage from different farms in the north, center and south of Tunisia. P1 (Zaghouan), P2 (Siliana), P3 (Oueslatia), P4 (Ain Zena), P5 (Chebika), P6 (El Hajeb), P7 (Sidi Bou Zid), P8 (Chott Meriem), P9 (Teboulba), P10 (Lamta), P11 (Bir Ali Ben Khelifa), P12 (Ghraba), and P13 (Kettana). Only healthy fruits in the same ripeness stage, without any kind of

infection or physical damage, were processed. The olives were washed and deleafed, then crushed with a hammer crusher using an Abencor analyzer (MC2 Ingenierias y Sistemas, Sevilla, Spain). The paste was mixed at 25°C for 30 min, centrifuged without addition of warm water and the oil was transferred into dark glass bottles.

2.3. Sample preparation

2.5 g of oil sample was weighted and dissolved in 5 mL of hexane. After, 5 mL of methanol: water (60/40) were added, the mixture was vortexed and then centrifuged at 3500 rpm during 10 min (15). The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure and a temperature of 35°C. The residue was dissolved in 0.25 mL of methanol/water (50:50 v/v) and finally filtered through a 0.45 µm filter before the HPLC analysis.

2.4. HPLC-MS Analysis

HPLC analyses were carried out using an Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Palo Alto, CA, USA), equipped with a vacuum degasser, an autosampler, a binary pump and a DAD. The column used for the chromatographic separation was a Zorbax Eclipse Plus C_{18} (1.8 µm, 150 x 4.6 mm) (Agilent Technologies, Palo Alto, CA, USA).

In order to obtain the separation of the compounds from the olive oil polar extracts, the flow rate used was 0.80 mL/min and the analysis was carried out at room temperature. The mobile phases used were water with 0.25 % acetic acid as eluent A and methanol as eluent B. The optimal chromatographic method consisted in the following multistep linear gradient 0 min, 5%B; 7 min, 35%B; 12min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5%B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis (16). The injection volume in the HPLC system was 10 µL. The compounds separated were monitored with DAD, peak spectra were recorded between 190 and 450 nm.

Besides, the HPLC system was coupled with a microTOF™ (Bruker Daltonik, Bremen, Germany) instrument, an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), using an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in both ionization modes. When this interface is used, in order to obtain a stable spray and consequently reproducible results, the effluent from the HPLC must be splitted because the work flow in HPLC is too high. In this work, a "T" with a split ratio 1:3 was employed, so the flow was reduced from 0.8 to 0.2 ml min^{-1}.

The optimum values of the ESI-TOF source parameters were: capillary voltage, + 4 kV, drying gas temperature, 190 ºC; drying gas flow, 9 L min^{-1}, nebulizing gas pressure, 2 bar and end plate offset, - 0.5 kV. On the other hand the optimum values of transfer parameters were: capillary exit, - 120 V; skimmer 1, - 40 V; hexapole 1, - 23 V; RF hexapole, 50 Vpp and skimmer 2, - 22.5 V. The detection of the compounds of interest was carried out considering a mass range 50-1000 *m/z*.

External mass spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA) containing sodium formiate clusters solution (5 mM sodium hydroxide and water:2-propanol 1:1 (v/v) with 0.2% of formic acid). The calibration solution was injected at the beginning of the run and all the spectra were calibrated prior to the identification for obtaining accurate mass values due to the compensation of temperature drift in the mass analyzer.

The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulae by using the Generate Molecular Formula™ Editor.

2.4. Statistical analysis

All the statistical analyses (Anova test, factor analysis and canonical discriminant analysis) were performed by means of SPSS 13.0 for Windows. Analyses were performed in triplicate and the data are given as individual values ± standard deviation (S.D.). The mean values obtained in the different groups were compared by One-way ANOVA (ANOVA, Duncan significant difference multiple comparison). In multivariate analysis each sample was considered to be a data vector represented by the concentration of the selected phenolic compounds.

3. Results and discussion

3.1. Characterization and quantification of olive oil phenolic compounds

The characterization of phenolic compounds was carried out comparing their migration times, DAD data and mass spectra provided by TOF–MS with those of authentic standards when available. Remaining compounds, for which no commercial standards were available, were characterized by the interpretation of their mass spectral provided by the TOF-MS and the information previously reported in the literature. The analysis of the true isotopic pattern by ESI-TOF-MS in combination with excellent mass resolution and mass accuracy is the perfect choice for molecular formula determination using the Generate Molecular Formula Editor. To characterize the phenolic compounds, a low tolerance of 0.05 and a low error ($\leq$ 5 ppm) were chosen. The position of the generated molecular formula in the table of possible compounds was also considered. In this sense, most of the characterized compounds were in the first position in the list of possibilities. For compounds quantification, eleven standards were used for calibration curves: hydroxytyrosol (HyTy), tyrosol (Ty), oleuropein (Ol), pinoresinol (Pin), luteolin (Lut), apigenin (Apig), taxifolin, vanillic acid (VA), o-coumaric acid (CA), quinic acid (QA) and ferulic acid (FA). All of them presented good linearity between different concentrations, and regression coefficients were higher than 0.990 in most cases. HyTy, Ty, Pin, Lut, Apig, taxifolin,

vanillic acid, ferulic acid, quinic acid, and o-coumaric acid were quantified using their own commercial standards. HyTy -acetate (HyTy-Ac) was quantified using HyTy calibration curve; elenolic acid (EA), Hydroxy-elenolic acid (H-EA), Ol aglycon (OA), decarboxylated OA (D-OA), 10-HOA, ligtroside aglycon (LA), decarboxylated LA (D-LA), H-D-OA, methyl D-OA were quantified with Ol calibration curve; acetoxy Pin (Ac-Pin), H-Pin and syringaresinol with Pin calibration curve and sinapinic acid (SA) using FA calibration curve. It has to be taken into account that the response of the standards can be different from the one of the analytes present in the oil samples, and consequently the quantification of these compounds is only an estimation of their actual concentrations.

Table 1. Quantification of the identified phenolic compounds in the oils from different locations.

PA / Cpd[a]	P1 *Zaghouan*	P2 *Siliana*	P3 *Oueslatia*	P4 *Ain Zena*	P5 *Chebika*	P6 *El Hajeb*	P7 *Sidi Bou Zid*	P8 *Chott Meriem*	P9 *Teboulba*	P10 *Lamta*	P11 *Bir Ali Ben Khelifa*	P12 *Ghraba*	P13 *Kettana*
HyTy	0.97±0.02	6.17±0.36	12.01±0.6	5.62±0.17	Nq	4.50±0.08	9.93±0.13	2.32±0.10	9.70±0.99	Nq	13.32±0.53	3.76±0.14	6.18±0.27
Ty	3.10±0.20	5.59±0.08	7.83±0.01	2.05±0.02	7.18±0.11	6.05±0.06	6.91±0.05	3.07±0.12	10.36±10	3.36±0.04	11.69±0.88	5.52±0.13	3.39±0.10
EA	85.23±1.10	332.25±15	435.18±8	341.19±5.00	94.71±1.70	268.58±3.00	99.34±1.53	119.37±5.00	59.75±0.10	83.18±1.70	445.19±26	97.85±5.60	84.27±0.10
HyTy-Ac	Nd	Nd	1.25±0.07	Nd	Nd	Nd	0.70±0.03	Nd	Nd	Nd	1.05±0.06	Nd	0.72±0.05
D- OA	117.36±2.00	116.15±2.0	117.67±1.1	153.74±0.00	20.38±0.30	84.68±0.03	270.76±12	96.01±1.20	45.58±0.41	23.109±1.00	46.50±0.12	45.90±0.30	159.78±5.00
OA	130.83±0.80	332.15±24.00	496.36±5.50	370.64±5.10	40.55±0.10	286.98±6.00	292.45±5.20	89.18±0.90	121.49±0.70	23.10±1.40	345.21±35	82.50±1.00	45.75±0.10
10-H- OA	2.10±0.12	17.88±0.10	23.48±0.12	13.61±0.00	Nd	6.48±1.54	7.60±0.08	1.67±0.08	3.76±0.20	Nd	9.83±1.07	0.75±0.00	2.10±0.05
D-LA	10.39±0.49	4.73±0.05	3.09±0.08	1.88±0.00	5.73±1.39	1.80±0.18	8.29±0.26	3.97±0.56	1.73±0.14	3.07±0.07	Nq	Nq	0.11±0.00
LA	96.68±1.16	233.82±54.1	123.10±0.57	120.54±0.30	59.47±0.29	83.99±0.64	82.33±0.07	81.75±0.20	48.74±0.06	33.28±0.01	82.43±0.62	45.86±0.16	34.024±0.20
Pin	1.18±0.01	0.53±0.01	0.73±0.02	0.41±0.00	1.08±0.03	1.043±0.03	0.75±0.01	0.84±0.03	1.49±0.05	1.40±0.00	1.00±0.03	1.61±0.07	1.28±0.00
Lut	2.96±0.18	0.52±0.30	3.52±0.22	3.62±0.00	1.50±0.14	3.25±0.090	1.22±0.00	3.46±0.19	4.33±0.09	2.96±0.14	6.50±0.12	3.54±0.07	1.02±0.13
Ac-Pin	4.17±0.21	1.43±0.00	3.41±0.33	1.72±0.00	7.65±0.14	6.34±0.14	4.51±0.03	3.71±0.20	9.04±0.20	12.17±0.10	8.39±0.85	13.11±0.11	6.77±0.21
Apig	1.46±0.28	0.16±0.00	0.62±0.27	0.69±0.00	0.20±0.11	0.50±0.15	0.49±0.00	1.55±0.63	1.59±0.04	2.1±0.08	3.09±0.15	1.80±0.17	0.43±0.02
H-pin	3.84±0.21	0.75±0.03	0.85±0.85	1.06±0.00	3.29±0.2	1.09±0.00	1.78±0.03	1.98±0.09	3.69±0.09	3.64±0.19	1.78±0.04	2.27±0.01	0.63±0.02
VA	0.17±0.04	0.06±0.05	0.60±0.02	Nq	0.57±0.08	Nq	0.29±0.01	0.55±0.41	Nq	1.12±0.01	Nq	1.82±0.11	Nq
oC A	0.26±0.02	0.15±0.01	0.49±0.03	0.18±0.00	0.24±0.02	0.36±0.02	0.25±0.01	0.27±0.01	0.17±0.00	0.31±0.01	0.37±0.02	0.57±0.010	0.13±0.00
F A	0.01±0.00	Nq	0.01±0.00	Nq	0.02±0.00	0.004±0.06	0.03±0.007	0.01±0.01	0.01±0.006	0.02±0.002	0.005±0.001	0.057±0.01	Nq
Q A	0.22±0.01	0.01±0.01	0.01±0.00	0.13±0.00	0.02±0.03	Nq	Nq	0.04±0.01	Nq	0.05±0.02	0.24±0.00	0.009±0.00	Nq
S A	Nd	0.10±0.00	0.05±0.00	0.95±0.00	Nd	Nd	Nd	Nd	Nd	Nd	Nd	Nd	
H-EA	0.193±0.00	4.00±0.02	5.02±0.31	4.12±0.00	1.83±0.03	1.47±0.02	0.88±0.01	1.28±0.31	0.180±0.03	Nq	2.11±0.30	0.15±0.01	0.79±0.15
H-D-OA	0.83±0.06	0.94±0.82	1.72±0.15	2.64±0.00	Nd	0.24±0.05	6.32±0.09	0.44±0.20	Nd	Nq	Nq	Nq	0.65±0.05
Syr	2.13±0.05	0.47±0.00	0.77±0.01	0.61±0.00	2.37±0.11	1.27±0.06	1.63±0.01	1.26±0.06	1.97±0.03	2.71±0.02	0.86±0.02	2.049±0.005	0.83±0.01
M-D- OA	3.572±0.20	5.10±0.07	5.08±0.00	4.20±0.00	0.60±0.00	Nd	1.64±0.00	1.51±0.10	2.50±0.04	Nq	Nd	0.26±0.02	0.78±0.02

PA production area, Cpd compound. [a] expressed as mg standard equivalent/kg of oil. Nq: not quantified. Nd: not detected. HyTy hydroxytyrosol. Ty tyrosol. O oleuropein. Pin pinoresinol. Lut luteolin. Apig apigenin. HyTy-Ac HyTy-acetate. EA elenolic acid. H-EA H-elenolic acid. OA O aglycon.

As table 1 shows, 23 phenolic compounds were characterized for this purpose. The analysis of the profiles of the different oils showed significant differences in quantitative distribution of the different phenolic compounds detected. Among the mentioned compounds, the highest content values were registered for the secoiridoids that reached 98 % of the total phenolic content. Ol agl, D-Ol agl, Lig agl and EA which varied from 23.11 (Teboulba) to 496.37 (Oueslatia), from 20.38 (Chebika) to 259.79 (Sidi Bou Zid), from 33.29 (kettana) to 233.82 (Siliana) and from 59.76 (Lamta) to 445.2 as mg of Ol/kg of oil (Bir Ali Ben khelifa); repectively. These compounds were also found to be major in previous works (17-19). Hy Ty and Ty were the major simple phenols being oils from Bir Ali Ben Khelifa the richest in terms of these phenolic alcohols that reached 13.33 as mg of HyTy/kg of oil for HyTy and 11.7 as mg of Ty/kg of oil for Ty. The rest of simple phenols were present in small amounts for oils from different provenances which is in accordance with previous findings reported by Krichene et al. (20) and Ouni et al. (18). The lignans amounts also showed a variability among studied oils and, among them, higher values were registered for Ac-Pin which reached 13.12 mg/kg for Ghraba oil. Concerning the flavonoids, luteolin concentrations were higher than those of apigenin, being oil from 'Bir Ali Ben Khelifa' the richest in both of them with 8 mg as luteolin/kg of oil and 3 mg as apigenin/kg of oil.

3.2. Chemometrics

3.2.1. Factor analysis using principal component

The objective of this factor analysis (FtA) is to obtain a reduced number of principal components which would explain the variability of the selected compounds. When this data set was used, the first six principal components were chosen (89.63% of the total variance) because the eigenvalues were higher than 1, and therefore, they explain higher percentage of variance than each original variable. All variables

presented a communality higher than 0.750, which indicates that they are well represented by the six factors. A Varimax rotation was carried out to minimise the number of variables that influence each factor, and then, to facilitate the interpretation of the results. The first principal component that explained the higher percentage of variance (30.48%) was mainly associated with OA, 10-H OA, H-EA, EA and LA. The second principal component that explained the 13.07 % of the total variance was related to D-OA and H-DOA. The third principal component explained 12.8 % of the total variance and was correlated to FA, CA and VA. The fourth principal component which explained 12.04 % of the total variance was related with the phenolic alcohols Ty and HyTy. The remaining selected factors, with 21.18% of the total variance explained, are related to QA and Lut (fifth principal component) and D-LA (sixth principal component). The figure 1 shows the Projection of the variables in the rotated space defined by the three first principle components.

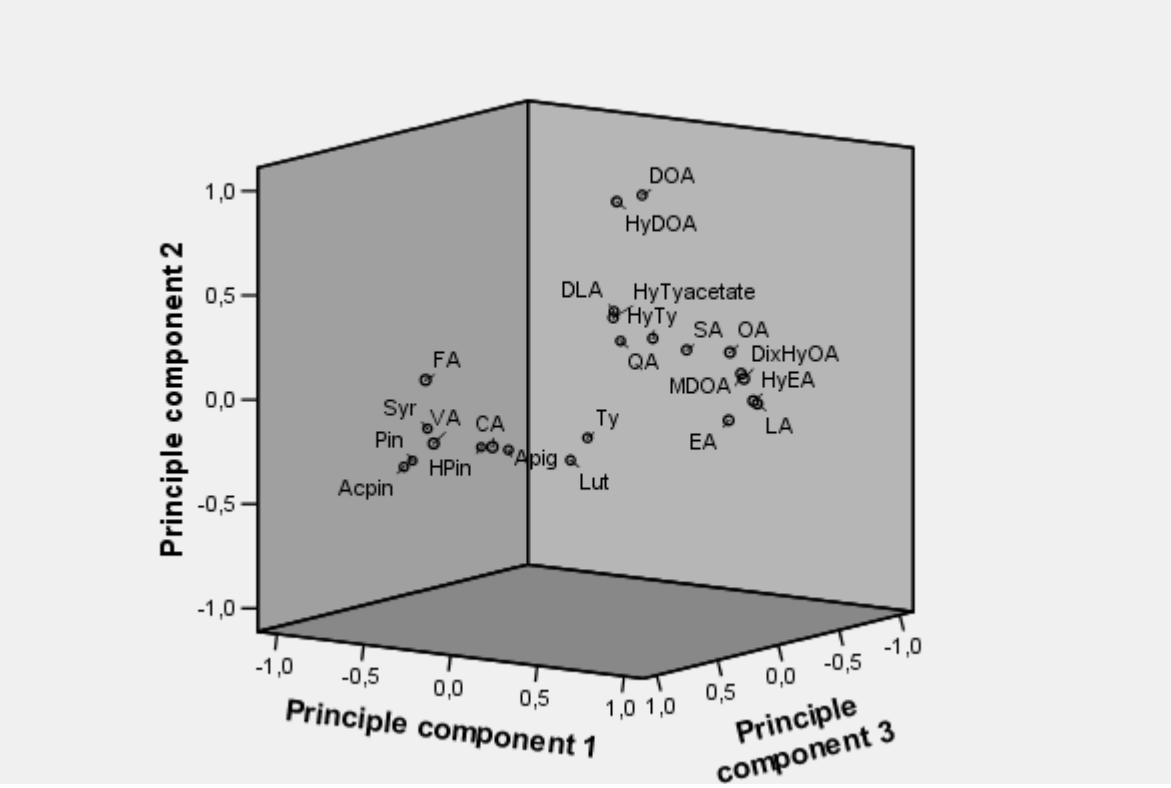

Figure 1. Projection of the variables in the rotated space defined by the three first principle components

3.2.2. Discriminant analysis

Given a set of independent variables, discriminant analysis attempts to find linear combinations of those variables that best separate the groups of cases. These combinations are called discriminant functions. This statistical technique is based on the extraction of discriminant functions of the independent variable by means of a quantitative dependent variable and the quantitative independent variables. The procedure automatically chooses a first function that will separate the groups as much as possible. It then chooses a second function that is both uncorrelated with the first function, and provides as much further separation as possible. The procedure continues adding functions in this way until reaching the maximum number of functions as determined by the number of predictors and categories in the dependent variable.

Using new matrix of data integrated by the standardized reduced original variables, which were selected from the previous FtA, a CDA was developed (Fig.2).

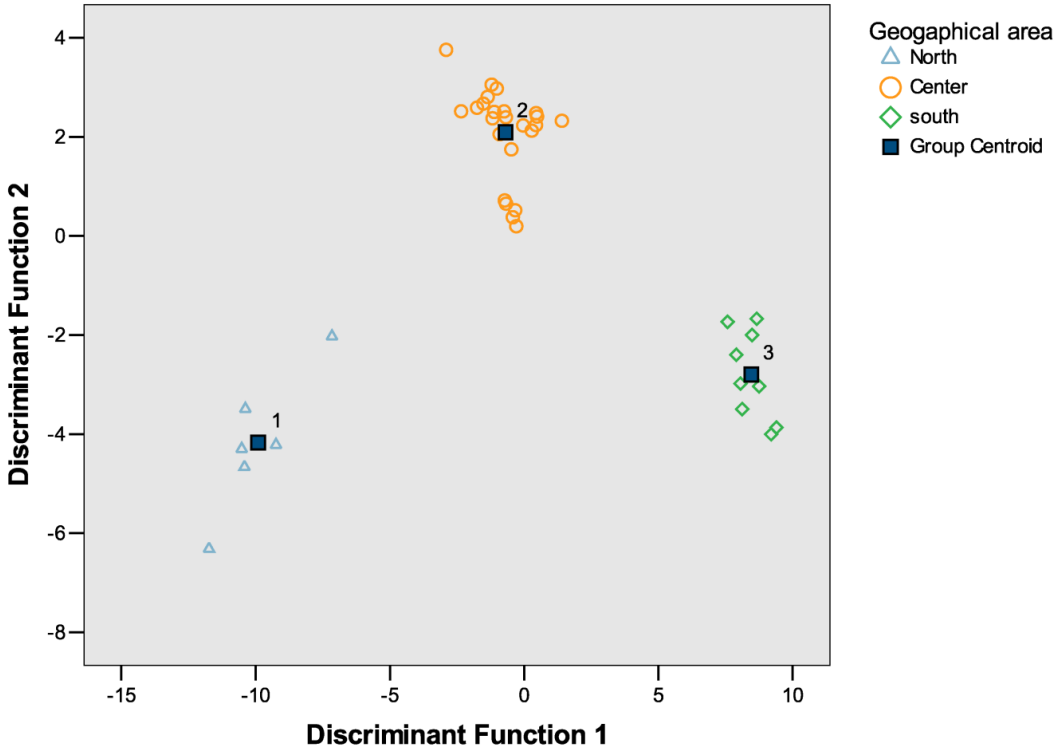

Figure2. Score plot on the plane of the two CDA discriminant functions obtained to predict the geographical origin of EVOOs.

This data set was subjected to the CDA according to the geographical origin (North, Center and South). The leave-on-out cross validation was used to validate the results.

Table 2. CDA classification parameters and results

Data set (15 phenolic compounds chose FtA)
after FtA)

Eigenvalues

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	34.589[a]	81.644	81.644	0.986
2	7.777 [a]	18.356	100.000	0.941

[a] *First 2 canonical discriminant functions were used in the analysis.*

Wilks' Lambda

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1 through 2	0.003	166.580	30	0.000
2	0.114	62.991	14	0.000

Classification Results([b], [c])

		Geogaraphical area	Predicted Group Membership			Total
			North	Center	South	
Original	Count	North	6	0	0	6
		Center	0	24	0	24
		South	0	0	9	9
	%	North	100.0	0	0	100.0
		Center	0	100.0	0	100.0
		South	0	0	100	100.0
Cross-validated([a])	Count	North	6	0	0	6
		Center	0	24	0	24
		South	0	0	9	9
	%	North	100.0	0	0	100.0
		Center	0	100	0	100.0
		South	0	0	100	100.0

[a] Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
[b] 100.0% of original grouped cases correctly classified.
[c] 100.0% of cross-validated grouped cases correctly classified.

The table 2 shows classification parameters such as number of eigenvalue, canonical correlation, Wilk's lambda and Chi square coefficients. The Wilks' lambda, which is a measurement of how well each function separates objects (oil samples) into groups, and which corresponds to the proportion of the total variance in the discriminant scores not explained by differences among the groups, showed small values indicating the great discriminatory ability of the functions. These classification results revealed a correct aggregation for the two first discriminant factors defined. The predicted results provided a percentage of predicted membership values according to the geographical area of 100% which means that all the objects were correctly classified. The 'Chemlali' oils from the same geographical area were clustered together. When the cross-validation procedure was applied, the same percentage (100 %) was obtained (Table 2).

Conclusion

According to the results obtained, we can say that the production area has significantly influenced the phenolic composition of the 'Chemlali' olive oil variety being oils produced from 'Oueslatia', 'Ain Zena', 'Siliana' and 'Bir Ali Ben Khelifa' the richest in polyphenols. It should be noted that HPLC-ESI-TOF-MS technique provides an adequate pathway to classify the oils according to their geographical area. A correct classification pathway with 100% of the correct predicted membership is achieved by selecting only a reduced number of phenolic compounds, such as secoiridoids (OA, 10-H OA, H-EA, EA, LA, D-OA, H-DOA, and D-LA), phenolic acids (FA, CA, VA and QA) and flavonoids (Lut). Hence, the results can be considered satisfactory and acceptable being the selected variables useful to classify and differentiate the 'Chemlali' VOOs according to the geographical origin. Furthermore, these results contribute to the development of new methods to detect adulterations in olive oils.

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SECTION II

Olive leaves

Chapter 4

Optimisation of Microwave-assisted extraction for the characterization of olive-leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS^2

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Article

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Optimization of Microwave-Assisted Extraction for the Characterization of Olive Leaf Phenolic Compounds by Using HPLC-ESI-TOF-MS/IT-MS^2

Abstract

In the present work a simple and rapid method for the extraction of phenolic compounds from olive leaves, using microwave-assisted extraction (MAE) technique, has been developed. The experimental variables that affect the MAE process, such as the solvent type and composition, microwave temperature and extraction time were optimized using a univariate method. The obtained extracts were analyzed by using high-performance liquid chromatography (HPLC) coupled to electrospray time of flight mass spectrometry (ESI-TOF-MS) and electrospray ion trap tandem mass spectrometry (ESI-IT-MS^2) in order to prove the MAE extraction efficiency. The optimal MAE conditions were methanol: water (80:20, v/v) as extracting solvent, at a temperature equal to 80 °C for 6 min. Under these conditions several phenolic compounds could be characterized by HPLC-ESI-MS/MS^2. Compared to the conventional method, MAE can be used as an alternative extraction method for the characterization of phenolic compounds from olive leaves due to its efficiency and speed.

Keywords: Tunisian olive leaves, phenolic compounds, Microwave-Assisted Extraction, high-performance liquid chromatography, time-of-flight mass spectrometry, tandem mass spectrometry

INTRODUCTION

Polyphenols are found in plant tissues and are needed for pigmentation, growth, reproduction, resistance to pathogens and for many other functions (1). These compounds form one of the main classes of secondary metabolites and have received a great deal of attention in recent years for their ability to act as powerful antioxidants. Olive leaves present an easily available natural material of low cost; their extract has been used by native people of the Mediterranean basin in folk medicine. This property can be linked to the fact that the leaves are rich in polyphenols, especially in oleuropein, rutin, verbacoside, apigenin-7-glucoside and luteolin-7-glucoside (2, 3). Recently, several studies have been focused on contents of the olive leaves and extraction of their high-added value compounds. The traditional solid-liquid extraction technique is based on the correct choice of solvents and the use of heat or/and agitation to improve the extraction efficiency; however, this technique requires longer extraction time and large amounts of solvents. In recent years, much attention has been given to the application of microwave heating in analytical and biological chemistry (4-6). Major advantages of MAE include short-extraction time, low-energy requirement, high extraction efficiency, and minimum degradation of target components (7). Furthermore, MAE has demonstrated its promising application in the extraction of phenolic compounds, especially thermo-sensitive ones (8). Nevertheless, the use of microwaves for extracting phyto-constituents is still in infancy (9).

Microwaves are electromagnetic fields in the frequency range 300 MHz to 300 GHz or between wavelengths of 1 cm and 1m (10). MAE is the process by which microwave energy is used to heat solvents in contact with solid samples and to partition compounds of interest from the sample into the solvent (11). Using microwave irradiation, the thermal degradation effects can be avoided while favouring the rapid desorption from matrices (12). Sample preparation before chromatographic

separation is the most time-consuming and error-prone part of the analytical procedure (13). Thus, optimizing an appropriate sample-preparation technique with significant advantages over conventional methods for the extraction and analysis of medicinal plants is a key factor in the overall effort of ensuring and providing high-quality herbal products. Regarding the great significance of olive leaves in obtaining high added value compounds, the purpose of this study was to obtain a new rapid and reliable extraction method based on MAE technique for the analysis of phenolic compounds present in olive leaf by using a combination of HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2.

MATERIALS AND METHODS

Samples. Leaves used in this study were obtained from Tunisian olive variety 'El Hor'. Olive leaves were collected from different parts of the tree, so as to minimize the sun exposure effect. After collection, fresh leaves were immediately transferred to the laboratory, washed with distilled water and ground under liquid nitrogen. Finally samples were stored at -20 °C until use.

Chemicals and Reagents. HPLC-grade acetonitrile (ACN) methanol and ethanol were purchased from Labscan (Dublin, Ireland). Acetic acid was of an analytical grade (assay>99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore, Bedford, USA).

Standards compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, o-coumaric acid, ferulic acid and quinic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA), (+)- pinoresinol was acquired from Arbo Nova (Turku, Finland) and oleuropein and rutin from Extrasynthèse (Lyon, France).

MAE apparatus

MAE experiments were carried out with a START E Milestone Microwave Laboratory System (Milestone S.r.l, Sorisole (BG) Italy). The apparatus is equipped with a single magnetron system with rotating diffuser for homogeneous microwave distribution in the cavity, delivered microwave power is 1.200 Watts, controlled via microprocessor, allowing rapid heating of high-throughput rotors, output power up to 1200 Watts in 1 Watt increments, a Fiber-Optic Automatic Temperature Control (ATC-FO) System which allows direct continuous monitoring and control of a reference vessel up to 300 °C, a MPR-600/12S medium pressure segmented rotor containing 12 vessels for operating pressure up to 30 bar (435psi). The microwave is operated via a compact Control Terminal 260 Interface with bright, touch-screen display.

HPLC apparatus

Separation of phenolic compounds from olive leave extracts was performed on an Agilent 1200 series Rapid Resolution Liquid Chromatographer (Agilent Technologies, CA, USA) consisting of vacuum degasser, autosampler, and a binary pump equipped with a C18 Eclipse Plus analytical column (4.6×150 mm, 1.8 µm) from Agilent Technologies. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: from 0 to 10 min, 95% (A):5% (B) to 70% (A):30% (B); from 10 to 12 min, 70% (A):30% (B) 67% (A):33% (B); from 12 to 17 min, 67% (A):33% (B) to 62% (A): 38% (B); from 17 to 20 min, 62% (A):38% (B) to 50% (A):50% (B); from 20 to 23 min, 50% (A):50% (B) to 5% (A):95% (B); from 23 a 25 min, 5% (A):95% (B) to 95% (A):5% (B); from 25 to 35 min, 95% (A):5% (B) to 95% (A):5% (B). The flow rate used was set at 0.80 mL/min throughout the gradient. The column temperature was maintained at 25 °C and the injection volume was 10 µL.

ESI-TOF-MS detection

The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:3). Thus in this study the flow which arrived into the ESI-TOF-MS/IT-MS^2 detectors was 0.2 mL/min. Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bar.

The accurate mass data of the molecular ions were processed through the newest software Data Analysis 4.0 (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas by using the Smart Formula ™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, passing a solution of sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v). Using this method, an exact calibration curve based on numerous cluster masses each differing by 68 Da ($NaCHO_2$) was obtained. Due to the compensation of temperature drift in the microTOF, this external calibration provided accurate mass

values for a complete run without the need for a dual sprayer setup for internal mass calibration.

IT-MS² detection

The identical HPLC system was coupled to a Bruker Daltonics Esquire 2000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, CA, USA) in negative ion mode. The ion trap scanned at the 50–1,000-*m/z* range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count at 20,000 and compound stability was set at 50%. The optimum values of the ESI-MS parameters were: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas pressure, 21.7 psi. The instrument was controlled by Esquire NT software from Bruker Daltonics.

Conventional solvent-extraction method

10 mL of a mixture of methanol and water (80:20, v/v) was added to 1 g of fresh milled olive leaves and the sample was maintained 24 h in the dark at room temperature. The extracts were centrifuged at 5000 g for 10minutes and then filtered through a 0.45µm syringe filter prior analysis (14).

Microwave-Assisted Extraction Method

1.25 g of fresh milled olive leaves were transferred into the microwave extraction vessels and suspended in 10 mL of the extraction solvent. After extraction, the vessels were cooled down to room temperature before opening, using the ventilation option of the system. The obtained extracts were filtered through a 0.45 µm syringe filter prior analysis. Extraction optimization was carried out according to a univariate optimization procedure under different MAE conditions. Solvent optimization is of primary importance in MAE (15). It is common practice to use a mixture of organic

solvent and water at varying ratios to improve recovery of phenolic compounds with MAE. For this reason, different solvents such as methanol, ethanol and their aqueous forms (40-100%, v/v) were investigated to determine the effective extraction of phenolic compounds. The extraction time was set at 8 min and the temperature at 40 °C. As in other extraction techniques, time is another parameter whose influence needs to be taken into account. The extraction time must be optimized to ensure maximum recovery in the minimum analysis time. For the extraction time optimization, samples were extracted with the most efficient solvent, optimized in the previous step, at 40 °C, and changing the irradiation time from 4 to 16 min. The high temperatures that can be achieved with microwave heating increase the solvating power of most solvents by decreasing surface tension and solvent viscosity, which improves sample wetting and matrix penetration (16). In our study, extraction at different temperatures from 10 to 120 ºC, using the optimised extraction time and solvent, were tested. The extraction efficiency is represented by a number of known phenolic compounds present in olive leaves (oleuropein (Ol), oleuropein aglycon (Ol agl), luteolin, apigenin, rutin, quercetin, apigenin-7-o-glucoside, luteolin diglucoside and luteolin glucoside) expressed as the peak area of each one. For precision study, repeatability of the optimized method was measured as relative standard deviation (RSD %). Thus, 1.25 g sample was extracted under the optimized MAE conditions (n= 2) on the same day (intra-day precision) and on 7 consecutive days (inter-days precision, n=14) and then analyzed by HPLC-MS. Each analyte was expressed as percentage of the total peak area of the identified phenolic compounds. Finally, the olive leaf extracts obtained under the optimal MAE conditions were analyzed by using HPLC coupled to ESI-TOF-MS and ESI-IT-MS^2. Peak identification was performed based on their relative retention time values, TOF-MS and IT-MS^2 data, comparison with authentic standard solutions when available, and using the information previously reported in the literature (17-20).

Statistical analysis

In order to see the difference between the optimized MAE and conventional methods, One-way ANOVA test at a confidence level of 95% was performed using SPSS 13.0 for windows software.

RESULTS AND DISCUSSION

Optimization of MAE conditions

Solvent optimization

By comparing the extraction efficiency of both, ethanol and methanol (Figures 1 (A) and (B), it can be seen that at fixed time and temperature increasing the concentration of the solvent has a benefit in term of increasing the extraction efficiency for the majority of the phenolic compounds.

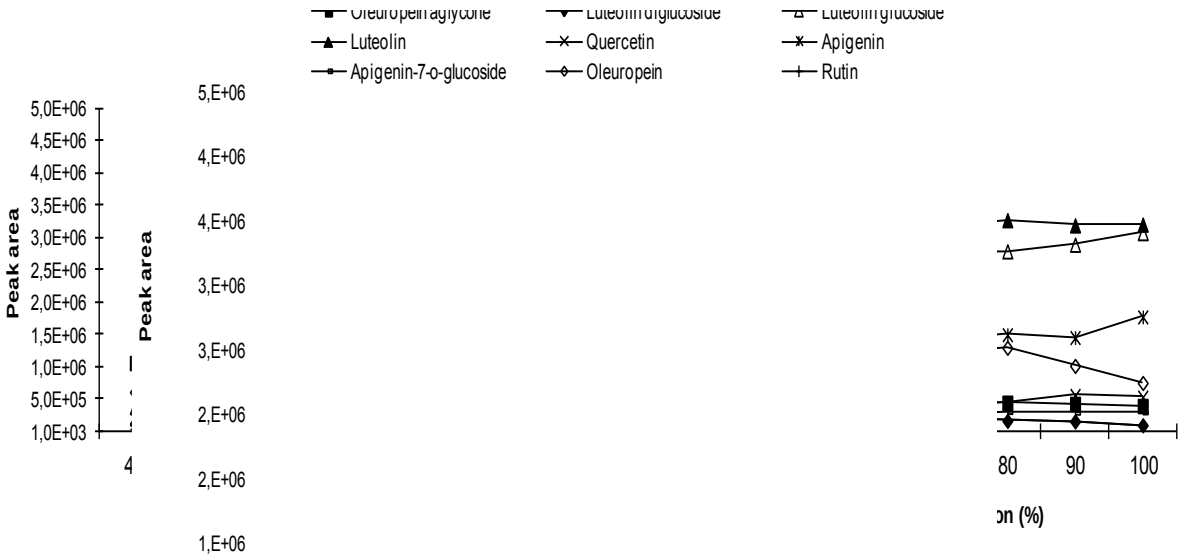

Figure 1. Influence of solvent composition (ethanol and methanol) on the recovery of the main phenols in olive leaves (Figure 1 (A) and (B).

The highest recovery of phenolic compounds using methanol or ethanol was obtained at a mixture solvent: water (80:20, v/v). Nevertheless, ethanol did not provide as much yields as methanol-based extraction solvents. Thus a mixture methanol: water (80:20) was selected as the most efficient solvent composition. It has been reported that aqueous methanol is often the solvent of choice for recovery of a wide range of phenolic compounds from diverse types of samples including fruit, vegetables and olive oil (21). Small amount of water in the extracting solvent can penetrate easily into the cells of the plant matrix and facilitate better heating of the plant matrix. This in turn increases the mass transfer of the active constituents into the extracting solvent (15). A recent study carried out on the optimization of solvent type in the solid-liquid extraction showed that a mixture of methanol-water (80:20) was found to be the best solvent for olive leaf extracts with high levels of flavonoids and important antioxidant activity (14).

Extraction time optimization

The obtained results showed (see Fig. 2 (A)) that after 6 min there was no remarkable increase of phenolic compound extraction with the increase in extraction time; therefore 6 min was selected as an appropriate extraction time.

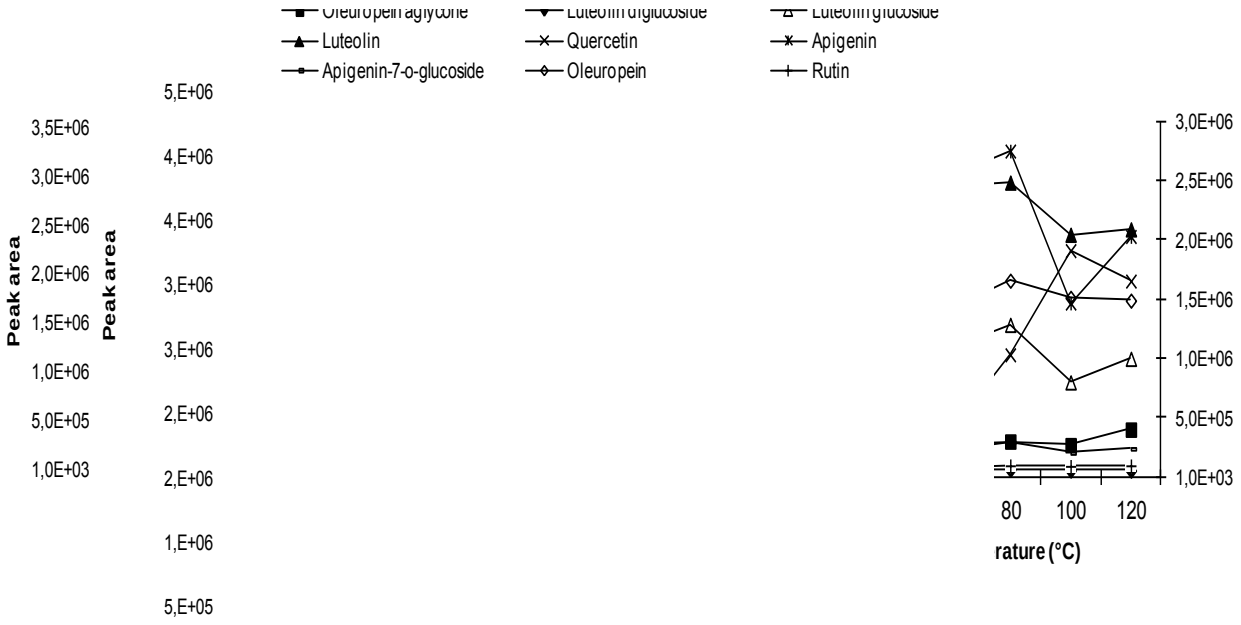

Figure 2. Influence of extraction time and temperature when using methanol/water (80/20) on the recovery of the main phenols in olive leaves (Figure 2 (A) and (B). In Figure 2 (B): right axis: apigenin, oleuropein, apigenin-7-o-glucoside and left axis: luteolin, quercetin, luteolin glucoside, oleuropein aglycon, rutin and luteolin diglucoside.

Temperature optimization

The obtained results are shown in Figure 2 (B). The extraction efficiency of the phenolic compounds increased with the rise of temperature. Nevertheless, up to 80 °C, the extraction efficiency began to decrease for most of the phenolic compounds under study. It can be explained by the thermal degradation of some of the selected phenolics. Thus, the optimal temperature was chosen at 80 °C.

Precision study.

The RSD % for the yield and the major and well-known phenolic compounds of the extract are represented in Table 1. Intra-day repeatability of the developed method

was between 1.30 and 2.49%, whereas the inter-day repeatability was from 3.01 to 8.47%.

Table 1. Precision study of the optimized method results expressed as relative standard devation (RSD%) for yield and each analyte

Compound	RSD[a] %	RSD[b] %
Yield	0.99	3.76
Quinic acid	1.75	4.49
HyTy	1.86	5.48
Ol agl derivative	1.48	7.59
10-Hy-Ol	1.73	3.01
Ol	1.52	4.57
Apigenin-7-o-glucoside	1.30	4.69
Apigenin rutinoside	1.89	8.47
Luteolin glucoside isomer 1	1.77	6.42
Luteolin glucoside Isomer 3	1.73	5.02
Diosmetin	2.49	7.82
Chryseriol-7-o-glucoside	2.39	5.57

[a] *intra-day precision,* [b] *inter-day precision*

Intra-day precision was higher than the inter-day precision and the method showed a good overall repeatability.

Analysis of olive leaf extracts by HPLC-ESI-TOF-MS/IT-MS^2

TOF-MS instrumentation can provide excellent mass resolution and mass accuracy and in combination with measurement of true isotopic pattern, is the perfect choice for molecular formula determination using the Smart Formula editor^{TM}. Furthermore, IT-MS^2 is suitable for obtaining fragments ions of structural relevance for identifying target compounds in complex matrix.

The HPLC-ESI-TOF-MS profiles of the analyzed extracts showed several peaks that could be characterized as showed in Figure 3.

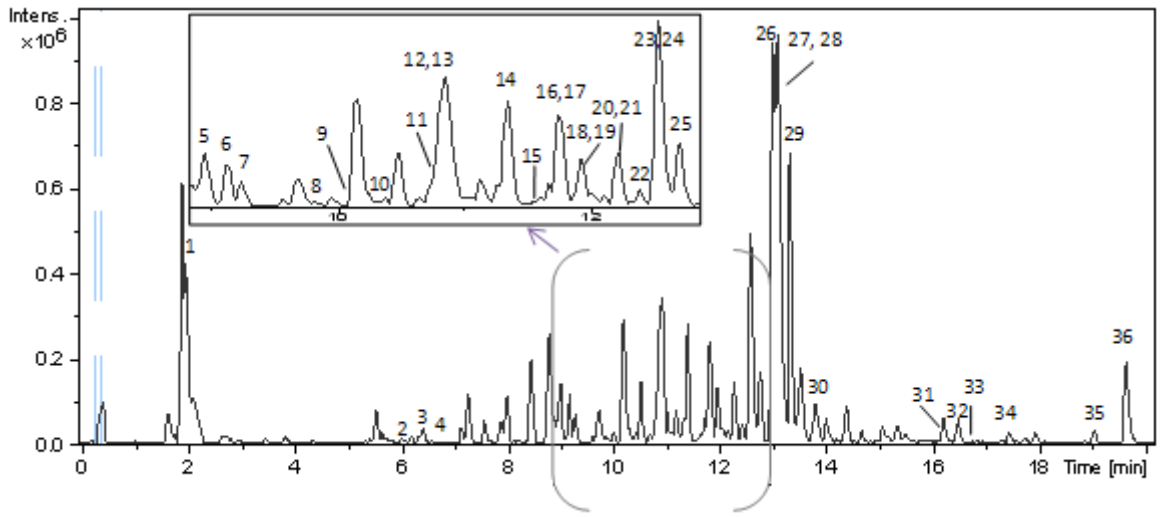

Figure 3. BPC of an olive leaf extract obtained under the optimized condition of MAE.

The identified compounds are summarized in Table 2 along with their retention time, molecular formula, *m/z* experimental and calculated, tolerance, sigma value, error (ppm), and classification order in the list of possibilities (sorted with respect to sigma value).

Table 2. Characterization of olive leaf extract obtained under optimal conditions of MAE by HPLC-ESI-TOF/IT-MS (Selected ion: $[M—H]^-$)

peak	Compound	R.T (min)	Molecular formula	*m/z* experimental	*m/z* calculated	Tolerance (ppm)	Sigma value	Error (ppm)	classification order in GMF
1	Quinic acid	2.00	C7H11O6	191.0562	191.0561	4	0.0098	-0.7	1st (1)
2	Secologanoside	6.02	C16H21O11	389.1089	389.1089	5	0.0034	0.2	1st (1)
3	Vanillin	6.45	C8H7O3	151.0402	151.0401	4	0.0052	-0.7	1st (1)
4	HyTy	6.62	C8H9O3	153.0561	153.0557	5	0.0515	-2.2	1st (1)
5	E A glucoside isomer 1	8.90	C17H23O11	403.1241	403.1246	4	0.0139	1.3	1st (1)
6	Ol agl derivative	9.14	C16H25O10	377.1453	377.1453	4	0.0062	0.2	1st (1)
7	Luteolin diglucoside isomer 1	9.20	C27H29O16	609.1463	609.1461	4	0.0060	-0.3	1st (2)
8	E A glucoside isomer 2	9.80	C17H23O11	403.1249	403.1246	4	0.0544	-0.7	1st (1)
9	Luteolin diglucoside isomer 2	10.13	C27H29O16	609.1465	609.1461	4	0.0317	-0.6	1st (3)
10	2-(2-Ethyl-3-Hydroxy-6-Propionylcydohexyl)Ac Ac glucoside	10.27	C19H31O9	403.1965	403.1974	4	0.0056	2.1	1st (1)
11	Rutin	10.70	C27H29O16	609.1438	609.1461	4	0.0414	3.7	1st (2)
12	Luteolin rutinoside isomer 1	10.80	C27H29O15	593.1469	593.1512	8	0.0127	7.2	1st (3)
13	10-Hy-Ol	10.87	C25H31O14	555.1703	555.1719	4	0.0090	2.9	1st (2)
14	Luteolin glucoside isomer 1	11.37	C21H19O11	447.0930	447.0933	4	0.0096	0.7	1st (1)
15	Ol glucoside	11.50	C31H41O18	701.2282	701.2298	4	0.0495	2.3	1st (1)
16	Apig rutinoside	11.79	C27H29O14	577.1534	577.1563	5	0.0064	4.9	1st (2)
17	Syringaresinol	11.80	C22H25O8	417.1548	417.1555	4	0.0061	1.8	1st (1)
18	Diosmin isomer 1	11.90	C28H31O15	607.1674	607.1668	4	0.0150	-0.9	1st (2)
19	Luteolin rutinoside isomer 2	11.94	C27H29O15	593.1518	593.1512	4	0.0309	_1	1st (2)
20	Diosmin isomer 2	12.24	C28H31O15	607.1684	607.1668	4	0.0227	-2.6	1st (3)
21	Taxifolin	12.30	C15H11O7	303.0491	303.051	4	0.0145	3.6	1st (1)
22	Luteolin glucoside isomer 2	12.42	C21H19O11	447.0936	447.0933	4	0.0066	-0.6	1st (1)
23	Apigenin-7-glucoside	12.51	C21H19O10	431.0974	431.0984	4	0.006	2.2	1st (1)
24	Luteolin glucoside isomer 3	12.56	C21H19O11	447.0920	447.0933	4	0.0049	2.9	1st (1)
25	Chryseriol-7-o-glucoside	12.72	C22H21O11	461.1090	461.1089	4	0.0113	2.2	1st (1)
26	2"-Methoxyoleuropein isomer 1	12.96	C26H33O14	569.1902	569.1876	5	0.4840	-4.6	2nd (4)
27	Luteolin glucoside isomer 4	13.03	C21H19O11	447.0928	447.0933	4	0.0010	1.2	1st (1)
28	2"-Methoxyoleuropein isomer 2	13.04	C26H33O14	569.1912	569.1876	10	0.4900	-6.4	3rd (4)
29	Ol	13.28	C25H31O13	539.1745	539.177	5	0.0076	4.7	1st (2)
30	Ol isomer	13.74	C25H31O13	539.1754	539.177	4	0.0067	_3	1st (2)
31	Luteolin	16.15	C15H9O6	285.0410	285.0405	4	0.0004	-2.7	1st (1)
32	Quercetin	16.40	C15H9O7	301.0351	301.0354	4	0.0083	1.1	1st (1)
33	Pinoresinol	16.87	C20H21O6	357.1332	357.1344	4	0.0206	3.1	1st (1)
34	Ac-Pinoresinol	17.51	C22H23O8	415.1397	415.1398	5	0.0212	0.2	1st (1)
35	Apigenin	19.02	C15H9O5	269.0451	269.0455	4	0.0098	1.7	1st (1)
36	Diosmetin	19.61	C16H11O6	299.0551	299.0561	4	0.0059	3.4	1st (1)

Retention time (R.T), oleuropein (Ol), hydroxytyrosol (HyTy), elenolic acid (EA), oleuropein aglycon (Ol agl), 10-hydroxy-oleuropein (10-Hy-Ol), acetoxypinoresinol (Ac-pinoresinol), Generated molecular formula (GMF).

As shown in Table 2, the phenolic compounds identified were from different families: simple phenols (vanillin and hydroxytyrosol (HyTy)), secoiridoids (2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl) acetic acid glucoside, elenolic acid glucoside (EA glucoside isomer 1), EA glucoside isomer 2, Ol agl derivative, 10-hydroxy-oleuropein (10-Hy-Ol), Ol, 2”-methoxyoleuropein isomer, Ol, Ol isomer, Ol glucoside and secologanoside), flavonoids in aglycone form (luteolin, quercetin, apigenin, diosmetin and taxifolin) and in glycosylated form (luteolin rutinoside isomer 1, luteolin rutinoside isomer 2, apigenin-7-*o*-glucoside, rutin, luteolin glucoside with 4 isomers, luteolin diglucoside isomer 1, luteolin diglucoside isomer 2, chryseriol-7-o-glucoside, apigenin rutinoside, diosmin isomer 1 and diosmin isomer 2) and lignans (syringaresinol, pinoresinol and acetoxypinoresinol (Ac-pinoresinol)). Another polar compound has been identified: quinic acid.

In this work, the ESI-IT-MS² was focused on some phenolic compound showing peaks at *m/z* 701, 607, 609, 593 577 and 569 when their TOF-MS spectra were not enough to confirm their identity (Figure 4).

The ESI-IT-MS² spectrum showed a peak at *m/z* 701 with fragments (539, 377, 307, and 275 *m/z*) (Figure 4 (A)). The ion at *m/z* 539 arises from the loss of a glucosyl unit (162 Da), which, by the loss of another glucosyl moiety, produces the fragment at *m/z* 377. The fragment ion at *m/z* 307 is explained by the loss of a C4H6O from the latter fragment while the fragment at *m/z* 275 derives from the loss of CH3OH from the fragment at *m/z* 307. Thus, the peak was identified as oleuropein glucoside.

The ion at *m/z* 607.1668 obtained by ESI-TOF-MS showed two peaks (RT 11.9 and 12.24 min) with identical molecular formula (Table 2). The ESI-IT-MS² spectra showed fragments at *m/z* 299 and 284 (Figure 4 (B)). The fragment ion at *m/z* 299, typical mass in the negative mode of diosmetin, indicates the loss of rutinose (*m/z* 308) and the fragment ion at *m/z* 299 showed a loss of a methyl group (15 Da) producing the fragment ion at *m/z* 284. Therefore, these two peaks were identified as diosmin and

its isomer. To our knowledge, this is the first time that this compound has been identified in olive leaves.

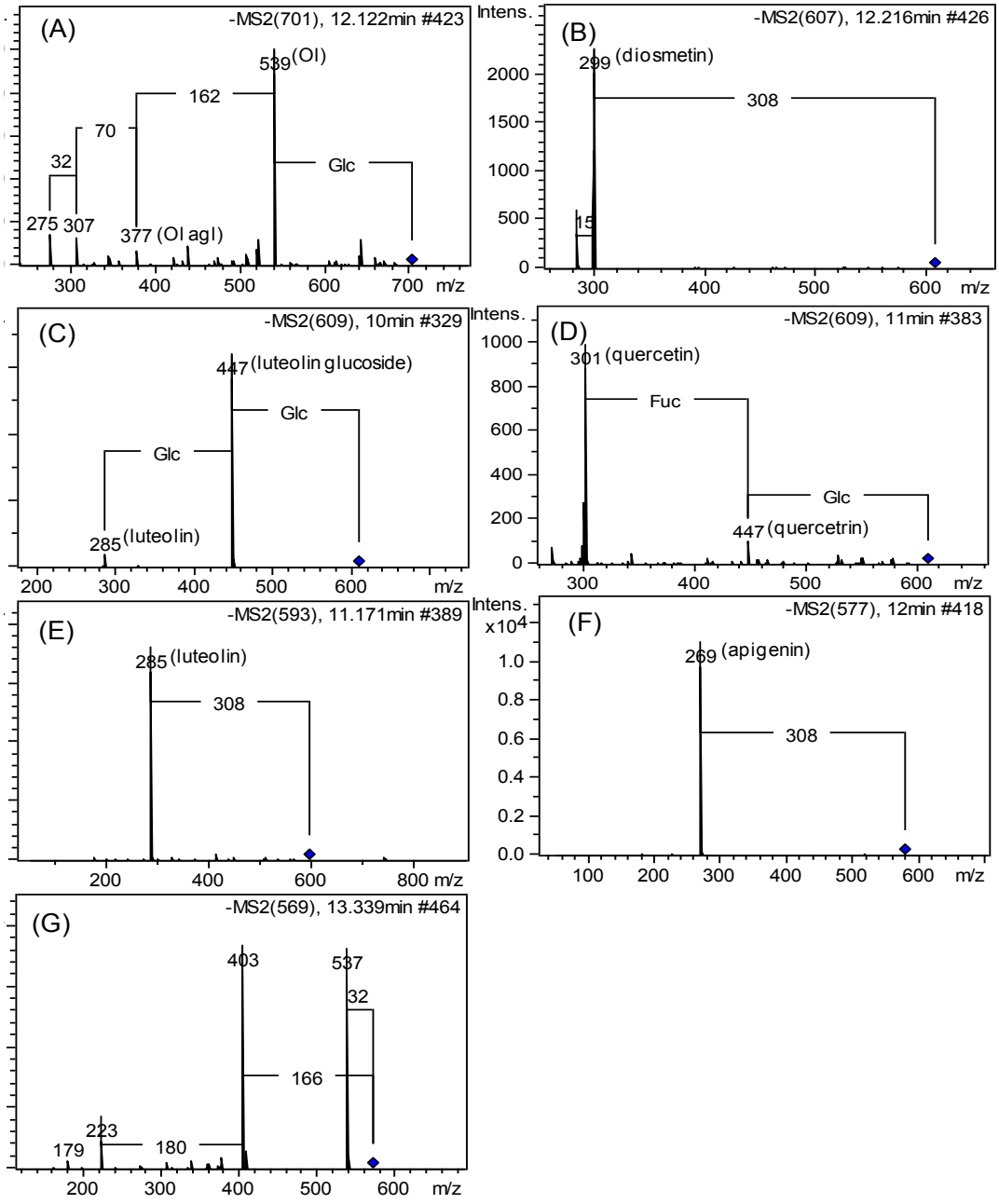

Figure 4. ESI-IT-MS² spectra of *m/z*: 701 (A), 607 (B), 609 (C), 609 (D), 593 (E), 577 (F) and 569 (G).

Several peaks were detected with *m/z* 609.1461 obtained by ESI-TOF-MS, among which two peaks (RT 9.2 and 10.13 min) had identical molecular formula and yielded the same fragments (*m/z* 285 and 447) by ESI-IT-MS² (Figure 4(C)). Diagnostic fragments at *m/z* 447 and 285 suggested the removal of one and two glucosyl units respectively, with *m/z* 285 representing the aglycon form. Thus, these peaks were identified as luteolin diglucoside and its isomer.

With the same *m/z* at 609.1461, a peak with different molecular formula was detected at 10.7 min. Its ESI-IT-MS² yielded fragments 301 and 447 (Figure 4 (D)). Fragment at *m/z* 301 is diagnostic of quercetin derivatives resulting from the loss of a rutinosyl moiety (*m/z* 308) and the fragment at *m/z* 447 could correspond to the pseudomolecular ion of quercetin-3-rhamnoside (quercetrin) indicating the loss of a rhamnose unit. Therefore, this peak was identified as rutin (quercetin 3-o-rutinoside).

The *m/z* at 593.1512 showed the presence of two peaks with identical molecular formula (RT 10.8 and 11.94 min). The ESI-IT-MS² applied to these ions showed the presence of the fragment at *m/z* 285 (Figure 4 (E)) which corresponds to the aglycon luteolin, indicating the loss of a rutinosyl moiety. Thus, these peaks were identified as luteolin rutinoside and its isomer. To our knowledge, the luteolin rutinoside isomer is reported for the first time in olive leaf extract.

The ESI-IT-MS² spectrum of the ion at *m/z* 577 showed the presence of a fragment at *m/z* 269 (Figure 4 (F)) that corresponds to the aglycon form apigenin which indicates the loss of a rutinose unit (308 Da). Thus, this peak was identified as apigenin rutinoside.

Two peaks were detected at 12.96 and 13.04 min showing the same *m/z* at 569. The Smart Formula ™ editor provided the same molecular formula for these two peaks which showed the same fragmentation pattern. These compounds were tentatively identified as 2”-methoxyoleuropein and its isomer. This secoiridoid glycoside was

reported in other specie of the Oleaceae family as *Jasminum officinale* (22). The ESI-IT-MS² spectrum is represented in the figure 4 (G) and a proposed fragmentation pathway for 2”-methoxyoleuropein is represented in the figure 5. The fragment at *m/z* 537 could be attributed to the loss of a methoxyl group while the fragment at *m/z* 403 is due to the cleavage of the phenolic moiety and it could undergo an elimination of the glucose moiety (fragment at *m/z* 223) or methoxyl group (fragment at *m/z* 179). The fragment at *m/z* 337 could be due to the cleavage of the elenolic ring from the precursor ion.

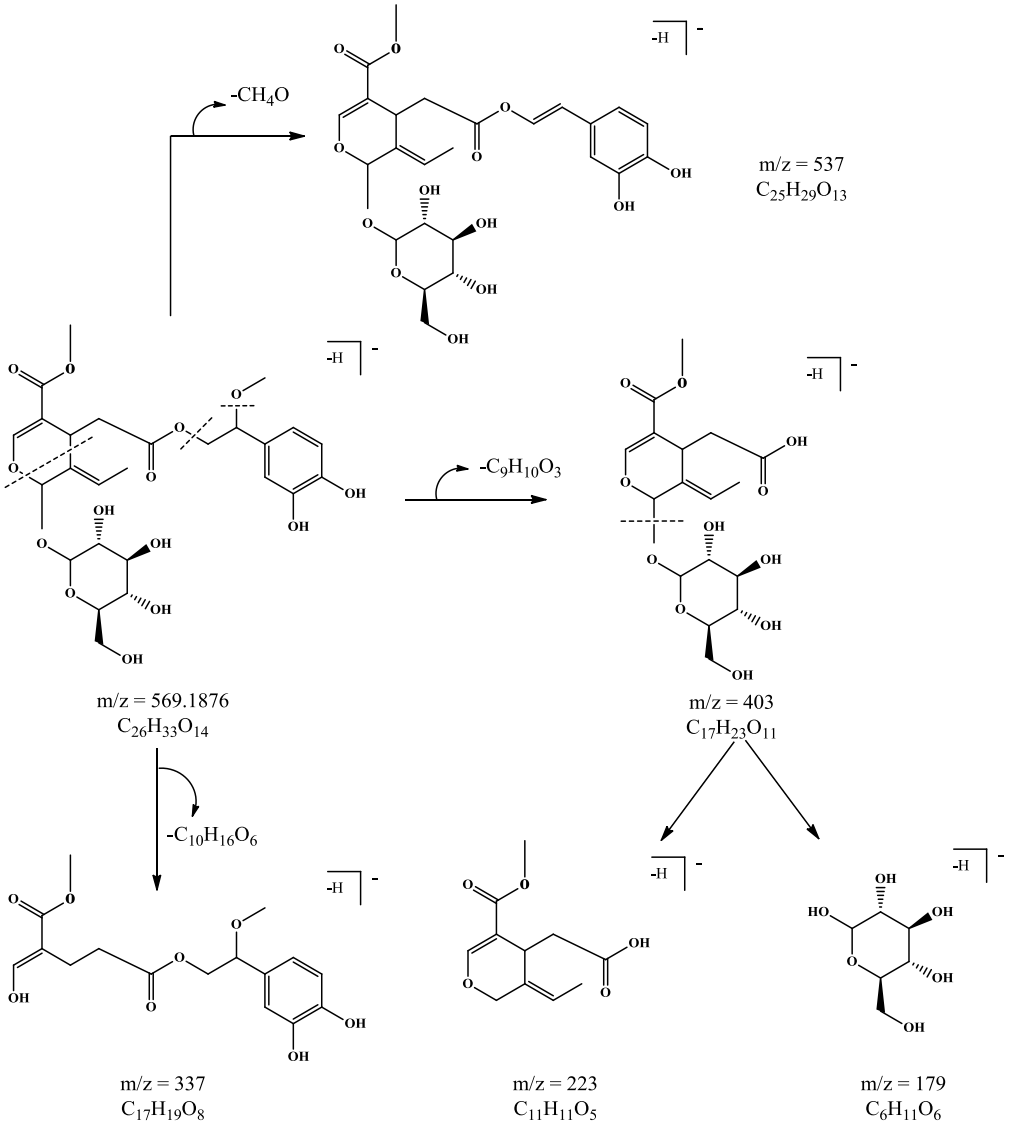

Figure 5. Fragmentation pathway for the secoiridoid 2”-methoxyoleuropein.

Efficency of the optimized MAE method

To improve the efficiency of the optimized MAE method, it was compared with the conventional extraction method. With respect to the extraction time, MAE was the fastest, requiring just 6 minutes giving the highest yield (16.7 % of fresh weight) whereas in the extract obtained by the conventional extraction the yield did not exceed 10% (Table 3).

Table 3. Comparison between the optimized microwave-assisted extraction and the conventional method

Extraction process (conditions: solvent, T, time)	**Yield[a] (%±SD)**	**10-Hy-Ol[b] (%±SD)**	**Ol and isomers[b] (%±SD)**	**Apigenin -7-o-glucoside[b] (%±SD)**	**2"-Methoxyol europein and isomers[b]**	**Luteolin glucoside and isomers[b] (%±SD)**
MAE (methanol:water 80:20, 80 °C, 6 min)	16.70±0.24	7.35±0.11	12.51±0.07	7.42±0.11	30.23±0.13	12.63±0.09
Conventional (methanol:water 80:20, room T, 24 h)	9.40±0.41	4.16±0.06	6.30±0.38	1.42±0.09	26.08±0.12	12.05±0.11

[a] Expressed as % of leaf fresh weight, [b]expressed as % of the total peak areas, SD standard deviation

Qualitatively, the examination of the profiles of the extracts obtained under the optimized conditions of MAE and the conventional method revealed that three compounds were not detected in the extracts obtained by conventional method which were HyTy, secologanoside and luteolin glucoside isomer 2. Quantitatively, the statistical analysis showed significant differences between both methods (p<0.05). The main significant observation was that the major detected secoiridoids and

flavonoids in the extracts (2”-methoxyoleuropein, oleuropein, 10-Hy-Ol, apigenin-7-o-glucoside and luteolin glucoside) showed better recoveries with MAE. Being the major compounds identified in the extract under study, these compounds are represented in table 3 and expressed as percentage of the total peak areas. 2”-Methoxyoleuropein and its isomer represented the highest percentages (30.23 and 26.08 %, for MAE and conventional extraction; respectively). Apigenin-7-glucoside was much better extracted using MAE (7.42%) than using the conventional one (1.42%). Ol and its isomers also showed important percentage of 12.51% with MAE whereas they presented 6.30% in the extract obtained by the conventional method. It has been frequently reported that oleuropein is of the major phenolic compounds in olive leaf (23, 24). Nevertheless the phenolic composition of olive leaf would be affected by several factors.

In this study, a new precise and effective time-saving extraction method, based on the use of microwave energy, has been optimized for the analysis of phenolic compounds from Tunisian olive leaves. The characterization of the extracts obtained under the optimized MAE conditions, by using a combination of HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2, revealed the existence of a large number of phenolic compounds from different classes; among them, the secoiridoid 2”-methoxyoleuropein, the flavonoids diosmin and its isomer, luteolin diglucoside isomer, luteolin rutinoside isomer are reported for the first time in olive leaves. The proposed MAE method allows the extraction of these compounds in a very shorter time (6 min) with higher efficiency when compared to the conventional solvent method. Therefore, MAE proved to be an attractive alternative to conventional extraction methods for the extraction of phenolic compounds from olive leaves.

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Chapter 5

Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast-cancer cells

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Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells

Abstract

A comparison among different advanced extraction techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurizedliquid extraction (PLE), together with traditional solid liquid extraction, was performed to test their efficiency towards the extraction of phenolic compounds from leaves of six Tunisian olive varieties. Extractions were carried out at the best selected conditions for each technique; the obtained extracts were chemically characterized using high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (ESI-TOF-MS) and electrospray ion trap tandem mass spectrometry (ESI-IT-MS^2). As expected, higher extraction yields were obtained for PLE while phenolic profiles were mainly influenced by the solvent used as optimum in the different extraction methods. A larger number of phenolic compounds, mostly of a polar character, were found in the extracts obtained by using MAE. Best extraction yields do not correlate with highest cytotoxic activity against breast cancer cells, indicating that cytotoxicity is highly dependent on the presence of certain compounds in the extracts, although not exclusively on a single compound. Therefore, a multifactorial behavior is proposed for the anticancer activity of olive leaf compounds.

Keywords: phenolic compounds, supercritical-fluid extraction, pressurized liquid extraction, microwave-assisted extraction, breast cancer cells, HPLC-ESI-TOF-MS/IT-MS^2

1. Introduction

In the search of new bioactive compounds from natural raw materials, food by-products have gained a considerable attention for their high potential as a source of phytochemicals, low cost and high environmental impact of such residues. For instance, in the olive oil industry, one of the most promising source of bioactives are olive leaves obtained as biomass after pruning of olive trees (1). This residue is a very abundant vegetable material and it supposes a potential source of polyphenols (2). Constituents of olive leaves have shown antiviral (3), antimicrobial (4), antioxidant, anti-inflammatory (5, 6), and anti-carcinogenic (7, 8) activities.

Different extraction techniques have been used to extract bioactives from olive leaves; among them, conventional solid-liquid extraction with ethanol (9) or methanol: water (5) and ultrasound assisted extraction (10). Considering the importance of the extraction process as a way to isolate and purify interesting compounds from natural raw materials, testing different extraction procedures is mandatory. One of the main needs in the development of extraction processes is to substitute inefficient and long extraction processes, usually requiring high volumes of toxic organic solvents, for non-conventional extraction procedures such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) or pressurized-liquid extraction (PLE), that require considerably less amounts of toxic solvents while providing higher extraction efficiencies and lower environmental impact (11-13). These modern extraction techniques can be regarded as a possible tool not only from a laboratory point of view but also for the natural products and food industries. In fact, industrial applications of SFE have experienced a strong development since the early 1990s in terms of patents (14). It was reported that a large-scale commercial (3 tonne/hour) MAE is available for industrial use (15). Although few reports can be found considering pilot scale units (16, 17), in view of the advantages of MAE and the development of equipment for large-scale

commercial operation, MAE has a bright future. Thus lab scale studies can be used to determine factors required for scale-up the extraction process and equipments.

Recently, olive compounds have shown significant anti-carcinogenic effects by directly modulating the activities of various types of receptor tyrosine kinases, including the human epidermal growth factor receptor (HER2) (18-20). Although secoiridoids seem to contribute importantly to such activity, the main responsible compounds have not been identified yet (21, 22). A recent review has highlighted the chemoprevention of doxorubicin toxicity (doxorubicin is a key of chemotherapeutic agent in different types of cancer treatment with chronic and acute associated toxic side effect) making use of natural antioxidants among them antioxidants from virgin olive oil (23).

Therefore, the goal of the present study was to compare different extraction processes (conventional extraction, MAE, SFE and PLE), performed under certain conditions reported in the literature, towards the selective extraction of phenolic components from olive leaves deriving from six Tunisian olive varieties. To fully characterize their phenolic composition, a new method was used based on HPLC coupled to ESI-TOF-MS and ESI-IT-MS^2. Moreover, the cytotoxicity of the different extracts against the JIMT-1 breast cancer cell line, a trastuzumab-resistant human cell line, was assayed. The possible correlation between the phenolic composition of the extracts and their cytotoxic activity was also studied.

2. Experimental

2.1. Chemicals and Reagents

HPLC-grade acetonitrile (ACN) methanol and ethanol were purchased from Labscan (Dublin, Ireland). Acetic acid was of an analytical grade (assay>99.5%) and purchased from Fluka (Switzerland). Water was purified by using a Milli-Q system (Millipore,

Bedford, USA). The carbon dioxide liquefied at high pressure used in supercritical extraction was supplied by Praxair Inc. (Danbury, CT, USA).

Standard compounds such as hydroxytyrosol, luteolin, apigenin, quercetin, taxifolin, vanillin and quinic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), oleuropein and rutin from Extrasynthèse (Lyon, France).

2.2. Plant material and Treatment

Leaves used in this study were obtained from six Tunisian olive varieties: 'Oueslati' (1), 'Chetoui' (2), 'Chemlali' (3), 'El Hor' (4), 'Jarboui' (5) and 'Chemchali' (6). Olive leaves were collected from different parts of the tree, so as to minimize the sun exposure effect. After collection, fresh leaves were immediately transferred to the laboratory, washed with distilled water and ground under liquid Nitrogen.

2.3. Apparatus and instruments

2.3.1. MAE apparatus

MAE experiments were carried out with a START E Milestone Microwave Laboratory System (Milestone S.r.l, Sorisole (BG) Italy). The apparatus was equipped with a single magnetron system with rotating diffuser for homogeneous microwave distribution in the cavity, delivered microwave power was 1.200 Watts, controlled via microprocessor, allowing rapid heating of high-throughput rotors, output power up to 1200 Watts in 1 Watt increments, a Fiber-Optic Automatic Temperature Control (ATC-FO) System which allows direct continuous monitoring and control of a reference vessel up to 300°C, a MPR-600/12S medium pressure segmented rotor containing 12 vessels for operating pressure up to 30 bar (435 psi). The microwave was operated via a compact Control Terminal 260 Interface with bright, touch-screen display.

2.3.2. SFE apparatus

The SFE system was based on a Suprex Prep Master (Suprex Corporation, Pittsburg, PA, USA) with several modifications. A thermostatic oven heated by air convection was used to set the extraction cell (8 mL) containing the sample. An HPLC pump Waters 510 (Waters Corporation, Milford, MA, USA) was used to introduce the modifier in the extraction system. A pre-heater system was employed by placing a heating coil inside a glycerine bath (JP Selecta Agimatic N, JP Selecta S.A., Abrera, Spain) to guarantee that the fluid employed in all the experiences reaches the extraction cell at the target temperature. After the modifier pump, a check valve (Swagelok SS-CHS2-BU-10, Swagelok Corporation, Solon, OH, USA) was used. A micrometering valve (Hoke SS-SS4-BU-VH, Hoke Incorporated, Spartanburg, SC, USA) was placed after the extraction cell to manually control the flow. A computer-controlled mass flowmeter (EL-FLOW® Mass Flow Meter/Controller F-111C, Bronkhorst High-Tech BV, AK Ruurlo, The Netherlands) was used to adjust the carbon dioxide flow rate at the values selected for each experiment.After depressurization, the extracts were collected in a collection vessel previously described (24).Inside the collection vessel, 30 mL volume glass vials wereplaced to recover the extracts.

2.3.3. PLE apparatus

The PLE system consisted in a home-made device described elsewhere (25). Basically, it consisted of an extraction cell housed in an oven provided with temperature control and regulation, a Hewlett-Packard 1050 series isocratic pump (Palo Alto, USA) to deliver and pressurize the solvent in the extraction cell and two six-port Rheodyne valves (model 7000, Rheodyne L.P., Rohnert Park, CA, USA) connected to the inlet and outlet ends of the extraction cell. The temperature and the heating rate were set by varying the energy applied to the heating resistances. The temperature programme was manually started at the beginning of each experiment and stopped at the selected extraction time. The extraction cell (8 mL) consisted in a stainless steel

holder (100 mm x 4.6 mm i.d. x 6.6 mm o.d.) sealed with 5 µm stainless steel frits (Supelco, Bellefonte, USA).

2.3.4. HPLC apparatus

Separation of phenolic compounds from olive leaf extracts was performed on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of vacuum degasser, autosampler, and a binary pump equipped with a C18 Eclipse Plus analytical column (4.6×150 mm, 1.8 µm) from Agilent Technologies. The mobile phases used were water with acetic acid (0.5%) (mobile phase A) and acetonitrile (mobile phase B) and the solvent gradient changed according to the following conditions: from 0 to 10 min, 95% A to 70% A; from 10 to 12 min, 70% A to 67% A; from 12 to 17 min, 67% A to 62% A; from 17 to 20 min, 62% A to 50% A; from 20 to 23 min, 50% A to 5% A; from 23 a 25 min, 5% A to 95% A; from 25 to 35 min, 95% A. The flow rate used was set at 0.80 mL/min throughout the gradient. The effluent from the HPLC column was splitted using a T-type phase separator before being introduced into the mass spectrometer. Flow entering into the ESI-TOF-MS or ESI-IT-MS detector was 0.2 mL/min. The column temperature was maintained at 25 °C and the injection volume was 10 µL.

2.3.5. ESI-TOF-MS analysis

The HPLC system was coupled to a micrOTOF (BrukerDaltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer (oaTOFMS), using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). Parameters for analysis were set using negative ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bar.

The accurate mass data of the molecular ions were processed through the newest software Data Analysis 3.4 (BrukerDaltonics, Bremen, Germany), which provided a list

of possible elemental formulae by using the Generate Molecular Formula ™ editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm.

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

2.3.6. IT-MS² analysis

An identical HPLC system was coupled to a BrukerDaltonics Esquire 2000 ion trap mass spectrometer (BrukerDaltonics, Bremen, Germany) equipped with an electrospray interface (Agilent Technologies, CA, USA) operating in the negative ionization mode. The ion trap scanned at the 50–1,000-m/z range at 13,000 u/s during the separation and detection. The maximum accumulation time for the ion trap was set at 200 ms, the target count at 20,000 and compound stability was set at 50%. The optimum values of the ESI-MS parameters were: capillary voltage, +3.0 kV; drying gas temperature, 300 °C; drying gas flow, 7.0 L/min; and nebulizing gas

pressure, 21.7 psi. The instrument was controlled by Esquire NT software from BrukerDaltonics.

2.4. Extraction methods and conditions

2.4.1. Conventional solvent extraction (CM)

10 ml of a mixture of methanol and water (80:20, v/v) was added to 1 g of fresh milled olive leaves and the sample was maintained 24h in the dark at room temperature. The extracts were filtered through a 0.45µm syringe filter prior to analysis (26).

2.4.2. Microwave-assisted extraction procedure (MAE)

1.25 g of milled fresh olive leaves were transferred into the microwave extraction vessel and suspended in 10mL of a mixture of methanol and water (80:20, v/v). Then extraction was carried out for 6 min, considering an irradiation temperature equal to 80 °C. After extraction, the vessel was cooled down to room temperature before opening, using the ventilation option of the system. The extracts were filtered through a 0.45µm syringe filter prior to analysis.

2.4.3. Supercritical fluid extraction procedure (SFE)

Prior to the extraction process, 1 g of milled olive leaves was homogenized with 1 g of sea sand, that was selected as inert material to hold the sample inside the extraction cell and to improve efficiency while avoiding formation of preferential flow paths. This mixture was introduced into the extraction cell and packed with glass wool. Extractions were carried out at 150 bar and 40 °C; once reached the experimental conditions, the extraction solvent (consisting on a mixture of CO_2 plus 6.6% of ethanol as modifier) passed through the extraction cell for 2 h.

2.4.4. Pressurized liquid extraction procedure (PLE)

One g of grinded olive leaves was placed in the extraction cell that was subsequently filled with 5 g of sea sand. Once the cell was mounted in the device, the selected solvent (either ethanol or water) was pumped into the cell and the lines from the pump to the outer valve. Then, the solvent was pressurized up to the selected pressure (ca. 100 bar), which was controlled via the pump recorder. Simultaneously, the temperature programme was started to heat both, sample and extraction solvent at the selected temperature (150 °C) for a given time (20 min). After the static time, the upper valve was switched to allow the pressurized solvent leave the cell. Blank samples were run after each extraction to avoid any contamination or memory effect.

2.4.5. Cytotoxicity assays

The human breast carcinoma cell line JIMT-1, which derives from a breast cancer clinically resistant to trastuzumab (27) was kindly provided by Institut Català d'Oncologia (Girona, Spain). Cells were routinely grown in DMEM + GlutaMAX medium supplemented with 10% of heat-inactivated foetal bovine serum (GIBCO) containing 50 U/ml penicillin and 50 mg/mL of streptomycin (GIBCO). Cells were incubated at 37º C in a humified 5% CO_2 air atmosphere. Cell viability was determined through the MTT assay (Fu et al. 2010). Briefly, cells were plated in 96-well plates at a density yielding 70-80 % confluence when the cytotoxicity assay was performed. Complete medium was refreshed and cultures were treated with different doses of the extracts for 72 hours. All the results corresponding to MTT experiments were expressed as the mean of a minimum of 6-8 replicates ± SD. The 50% cytotoxic concentration values (CC50) were determined from the survival plots using GraphPad PRISM 5 (GraphPad Software).

2.5. Statistical analysis

Two-way ANOVA test at a confidence level of 95% was performed using SPSS 13.0 for windows software.

3. Results and discussion

Extraction conditions were established for each extraction process considering previous results published in the literature for phenolics extraction using SFE, PLE and MAE in different matrices, including rosemary and olive leaves (11, 12, 28, 29). PLE and SFE are considered green technologies to produce bioactives from natural sources such as plants and algae (11, 12, 30), as GRAS-qualified solvents such as CO_2, ethanol or water are frequently used in these technologies. In this study, PLE and SFE extraction processes were carried out under optimum conditions using only GRAS-qualified solvents. These have been compared to MAE and conventional solid-liquid extraction, which used a mixture of methanol and water (80:20). Hydroalcoholic extraction is the choice of solvent for optimum phenolic's extraction due to the high extraction yield of a wide range of phenolic compounds from diverse types of samples including fruit, vegetables and olive oil (31). Our main target was to find out advantages and drawbacks between the different extraction processes in relation to the extraction of phenolics from olive leaves.

First of all, it is worth to mention that extraction processes assisted by temperature, such as PLE and MAE, have shorter extraction times than conventional technologies and SFE; this is mainly due to the increase of the analytes solubility in the extraction media when surface tension and solvent viscosity decreases, which, at the end, improves extraction efficiency. MAE extraction temperature was selected at 80 °C because, as previously reported (29), higher temperatures provide lower extraction efficiencies. Regarding PLE, 150°C was selected as optimum temperature for phenolics extraction when ethanol was used as extracting solvent, since this condition has been reported to achieve the highest phenolic content and antioxidant capacity (28). Although previous results obtained in our research group suggested the use of 200 °C for PLE water extractions, in this study we selected 150°C in order to

avoid the generation of compounds deriving from thermal dehydration of saccharides such as 5-hydroxymethylfurfural (32). On the other hand, these conditions were also optimum in terms of phenolics content, antioxidant activity and extraction yield (28). Table 1 shows the yield obtained for the different extraction processes using 6 different varieties of Tunisian olive leaves. A two-way analysis of variance has been carried out and the results showed that the yield was significantly influenced by the extraction method (p<0.05), the olive variety (p<0.05). The interaction of extraction method and the olive variety was also significant (p<0.05).

Table 1. Values of extraction yield (expressed as % of fresh weight) obtained for the six different Tunisian olive leaf varieties using different extraction processes (SFE, PLE, MAE, conventional) performed at the mentioned conditions

Extraction process (conditions: solvent, T, time, pressure)	**Oueslati (1) Yield (%)**	**Chetoui (2) Yield (%)**	**Chemlali (3) Yield (%)**	**El Hor (4) Yield(%)**	**Jarboui (5) Yield (%)**	**Chemchali (6) Yield (%)**
SFE (CO_2 + ethanol, 40°C, 1 h, 150 bar)	9.5	8.9	5.8	8.2	9.7	5.8
PLE (ethanol, 150°C, 20 min, 100 bar)	19.9	19.5	14.8	19.6	16.7	22.4
PLE (water, 150°C, 20 min, 100 bar)	10.4	7.5	8.4	8.9	11.2	11.0
MAE (methanol:water 80:20, 80°C, 6 min)	16.7	10.6	11.2	11.6	5.2	12.1
Conventional (methanol:water 80:20, room T, 24 h)	9.4	8.1	8.2	8.2	9.1	16.8

As observed, the use of PLE with ethanol as extraction solvent produced the highest yield for all the studied varieties. In this sense, the highest yield was obtained from the Chemchali (6) variety. MAE also produced high extraction yields, but significantly lower than those obtained by PLE using ethanol. The only exception was the case of Jarboui (5) variety for which MAE was the less efficient extraction technique in terms

of total extracted yield. Lastly, PLE using water as solvent, conventional extraction (MeOH: H_2O), as well as SFE using CO_2 and ethanol as cosolvent produced comparable yields. It is important to remark that the extraction yield strongly depends on the solvent employed. Nevertheless, extracts with similar yields, but produced under different extraction techniques, would show completely different chemical composition. Therefore, the phenolic composition of the extracts was studied in detail to evaluate the potential of the different extraction techniques.

In a recent study, we have optimized the MAE extraction conditions for phenolics' extraction from olive leaves and also identified, via HPLC-ESI-TOF-MS/IT-MS^2, the main phenolic compounds present in MAE extracts (29). This information and the data previously reported in literature (21, 33, 34, 35) have been used as a basis for identifying the compounds detected in the different olive leaf extracts in the present study through the comparison of their relative retention time values, TOF-MS and IT-MS^2 data in addition to the comparison with authentic standard solutions when available. Representative base peak chromatograms (BPCs) of a mass range (50–1000 *m/z*), for the extracts under the optimum extraction conditions for MAE, PLE, SFE and conventional extraction are presented in figure 1 (only BPCs of samples showing the highest cytotoxic activity in each extraction method are shown). Peak identification is shown in Table 2.

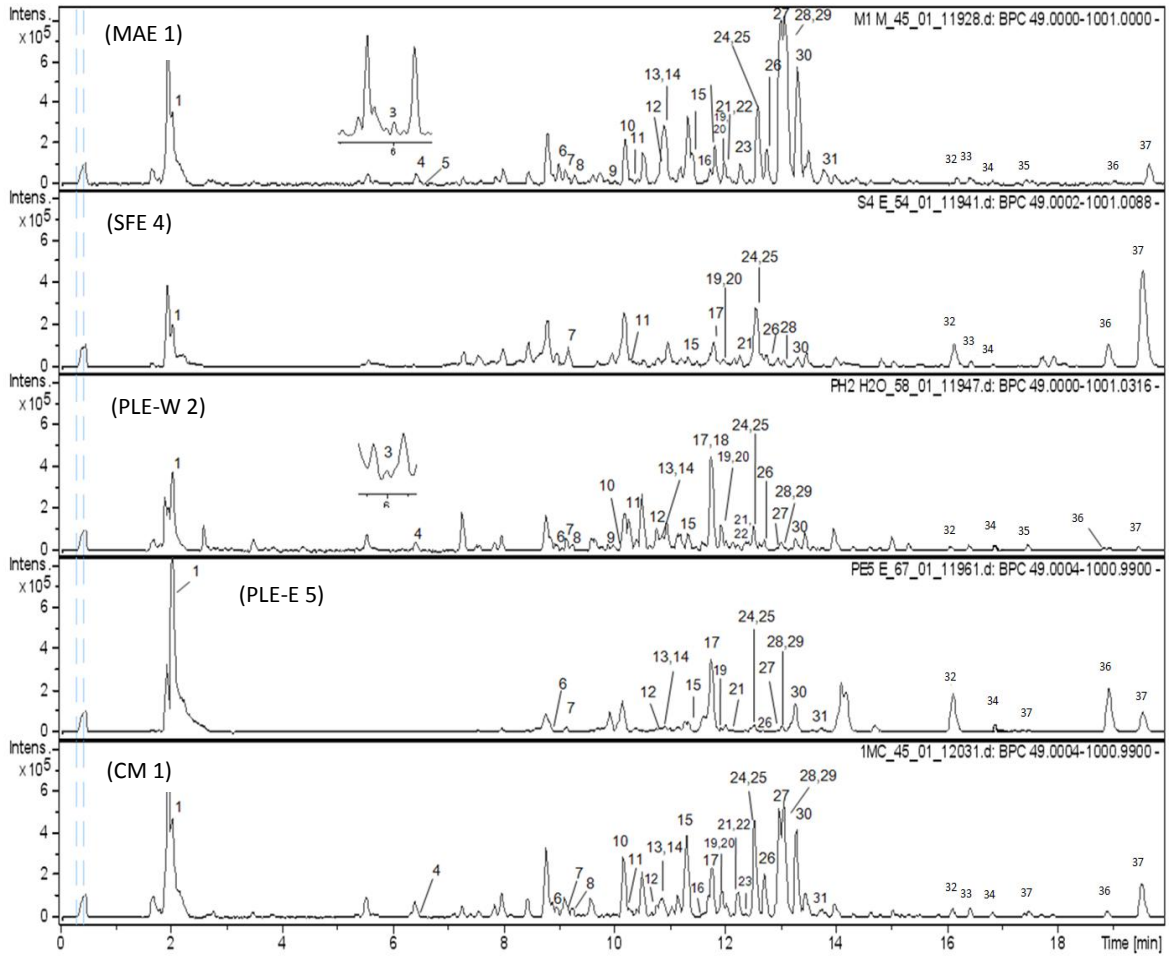

Figure 1. Base Peak chromatograms (BPCs) of olive leaf extracts obtained with different extraction methods that showed highest cytotoxic activity on human breast cancer cells

As can be seen in Table 2, hydroxytyrosol glucoside was not detected in 'Oueslati', 'Chetoui', 'Chemlali' and 'El Hor' varieties whereas its aglycon form (hydroxytyrosol), in addition to elenolic acid glucoside isomer 2, and luteolin rutinoside isomer 2, were not found in 'Chemchali' variety. On the other hand, secologanoside, luteolin rutinoside isomer, syringaresinol and luteolin diglucoside isomer 2 were not detected in the 'Jarboui' extract. Among the different extraction techniques used, extracts obtained under MAE conditions showed the largest number of identified phenolic compounds (Table 2). Hydroxytyrosol glucoside, secologanoside, hydroxytyrosol, elenolic acid glucoside isomer 2, vanillin and taxifolin were not detected in SFE

samples from different olive leaf varieties, although non-polar compounds were extracted in a higher extent, as expected. Vanillin was not detected in the extracts obtained by PLE using ethanol as extracting solvent (PLE-E), whereas quercetin was not detected in PLE using water as solvent (PLE-W).

Table 2. Characterized compounds in olive leaf extracts obtained by the different extraction methods using HPLC-ESI-TOF-MS/IT-MS^2 (selected ion: $[M–H]^-$).

peak	Compound	MAE						SFE						PLE-W						PLE-E						Conventional extraction						References
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	
1	Quinic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29)
2	Hydroxytyrosol-glucoside	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	(21)
3	Secologanoside	+	-	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	+	(21, 29)
4	Vanillin	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	(29, 36)
5	Hydroxytyrosol	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-		(6, 21, 33, 36)
6	Elenolic acid glucoside isomer 1	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
7	Oleuropein aglycone derivative	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
8	Luteolin diglucoside isomer 1	+	+	+	+	-	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	(21, 29)
9	Elenolic acid glucoside isomer 2	+	-	+	+	+	-	-	-	-	-	-	-	+	+	+	-	+	-	-	+	-	-	-	-	-	+	+	+	+	-	(21, 29)
10	Luteolin diglucoside isomer 2	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	+	(21, 29)
11	2-(2-Et-Hy-6-propionylcyclohexyl) acetic acid glucoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	(21, 29)
12	Rutin	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29, 35, 36)
13	Luteolin-rutinoside isomer 1	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	(29, 35)
14	10-hydroxy-oleuropein	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
15	Luteolin glucoside isomer 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
16	Oleuropein diglucoside	+	+	+	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	-	-	+	+	+	-	+	+	+	(21, 29)
17	Apigenin rutinoside	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29, 34, 35)
18	Syringaresinol	+	+	+	+	-	+	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	+	-	+	+	+	-	+	(21, 29)
19	Diosmin isomer 1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29, 37)
20	Luteolin rutinoside isomer 2	+	+	+	+	-	-	+	+	+	+	-	-	+	+	-	+	-	-	+	+	-	+	-	-	+	+	+	+	-	-	(29, 35)
21	Diosmin isomer 2	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	(29, 37)
22	Taxifolin	+	+	+	+	+	+	-	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	-	(21, 29)
23	Luteolin glucoside isomer 2	+	+	-	+	+	+	-	-	-	-	+	-	+	-	-	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+	(21, 29)

24	**Apigenin-7-*o*- glucoside**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29, 34, 36)
25	**Luteolin glucoside isomer 3**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
26	**Chryseriol-7-*o*-glucoside**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
27	**2’’-MethoxyOleuropein isomer 1**	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	(29, 38)
28	**Luteolin glucoside isomer 4**	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
29	**2’’-Methoxyoleuropein isomer 2**	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+	+	(29, 38)
30	**Oleuropein**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(6, 29, 36)
31	**Oleurpein isomer**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 29)
32	**Luteolin**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(21, 33, 34, 36)
33	**Quercetin**	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	+	-	(21, 29)
34	**Pinoresinol**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29)
35	**Acetoxy-pinoresinol**	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29)
36	**Apigenin**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29, 33, 34, 36)
37	**Diosmetin**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(29, 33, 34, 36)

Quantitatively, the total phenolic contents (TPCs), expressed as the total peak areas of the identified compounds, showed variation among the different extracts according to the variety and the extraction method employed. As Figure 2 (A) shows, MAE samples showed the highest TPCs in comparison to the other extraction methods, being 'Chemlali' (MAE 3) and 'El Hor' (MAE 4) the varieties having the highest TPCs. MAE technique was followed by CM and PLE techniques, being PLE-W and SFE those achieving the lowest TPC values.

Figure 2 (B, C and D) shows the content of selected secoiridoid and flavonoid compounds, expressed as peak area, of the samples obtained with different processes and olive varieties.

Results showed that 2"-methoxyoleuropein was not well extracted with either PLE (using water or ethanol as solvent) or SFE (using CO_2 + 6.6% ethanol) as compared to the other extraction methods (Figure 2 B). SFE and PLE (using water as solvent) did not show a good efficiency either for extracting oleuropein. Besides, SFE was the best extraction procedure for apigenin and diosmetin isolation. In contrast, MAE showed the best recoveries for oleuropein, 10-hydroxy-oleuropein and 2"-methoxyoleuropein (isomer 1 and 2), being these values higher than those of 10-hydroxy-oleuropein in all the extracts, except for those extracted with PLE (Figure 2 B). Therefore, most oleuropein derivatives seemed to be more efficiently extracted with the use of MAE or CM, being SFE the worst behaving technique. It was generally observed that considering the great chemical variability of the samples, each technique seemed to be more adequate than others for the extraction of each particular class of compounds.

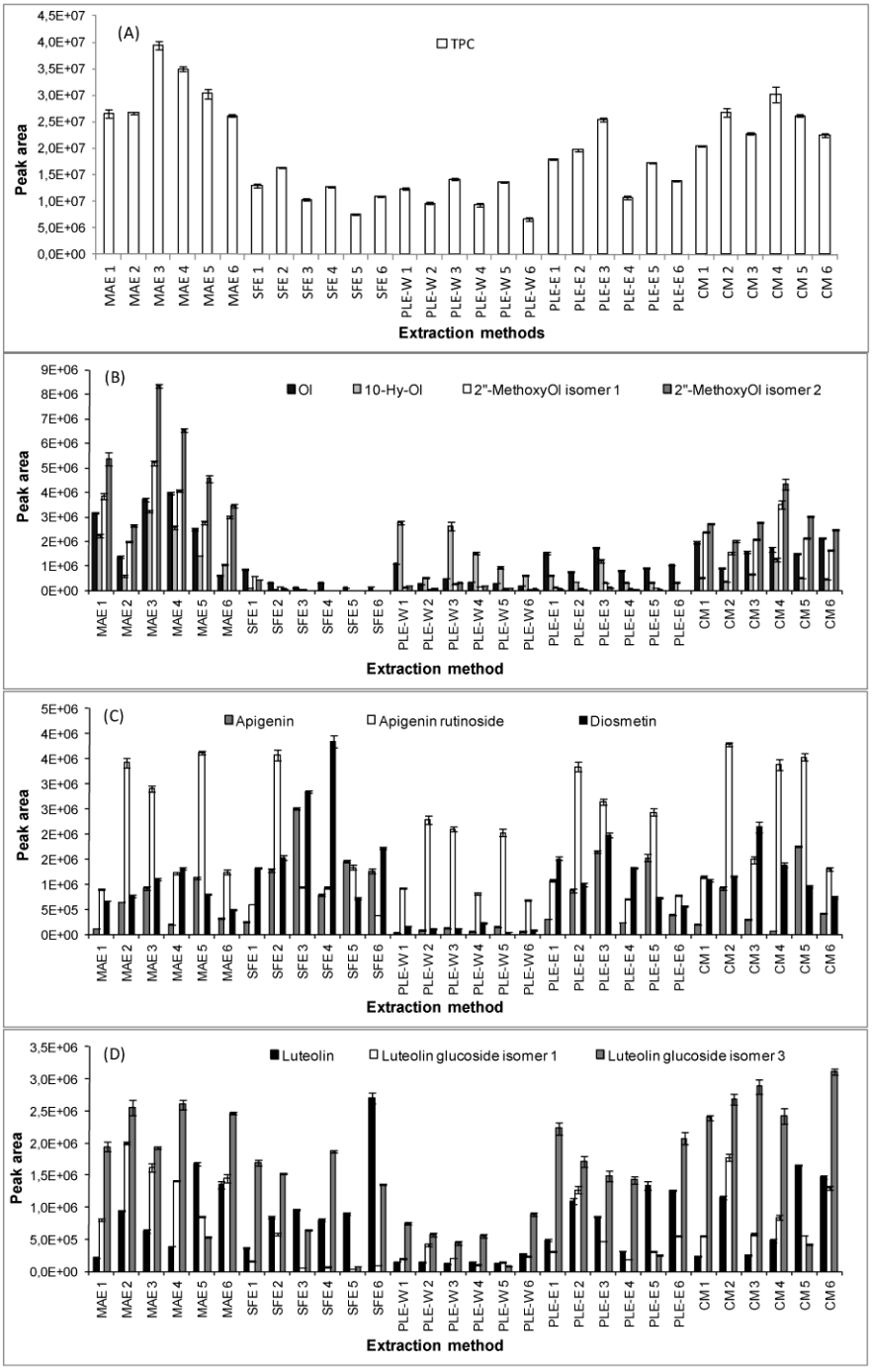

Figure 2. Extraction efficiency of the different extraction methods for total phenolic content (TPC) (A), for main identified secoiridoids (B) and flavonoids (C, D): microwave-assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized liquid extraction using water as solvent (PLE-W), pressurized liquid extraction using

ethanol as solvent (PLE-E) and conventional extraction method (CM), numbers correspond to the different varieties according to Table 1.

Abundance of the phenolic compounds was also dependent on the variety. Among all the analyzed samples, 'El Hor' olive leaf extract obtained by SFE (SFE 4) was the richest in diosmetin (Fig. 2 C), whereas, when extracted by MAE, 'El Hor' olive leaf extract (MAE 4) was the richest in oleuropein. 'Chemlali' and 'Chemchali' extracts obtained by SFE (SFE 3 and SFE 6, respectively) were the richest in apigenin (Fig. 2 C) and luteolin (Fig. 2 D), respectively. The 'Chetoui' olive leaf extract (sample 2) was the richest in apigenin rutinoside regardless the extraction technique utilized, indicating that this variety may be especially enriched in this flavone. Besides 'Chetoui' extracts, 'El Hor' extracts obtained by using CM extraction and 'Jarboui' extracts obtained by MAE and CM were also rich in apigenin rutinoside.

Olive leaf derived compounds have demonstrated their cytotoxic activity against different cancers such as leukemia, colon and breast cancer (Abaza et al. 2007; Fu et al. 2010; Hashim et al. 2008). Their cytotoxic activity is especially relevant in HER2 positive breast cancers where olive extracts reduce the overexpression of HER2 and diminish the resistence to trastuzumab, a monoclonal anti-HER2 antibody clinically used in the therapy of these cancers (Menendez et al. 2007). JIMT-1 cells derive from a HER2 positive breast carcinoma but resistant to trastuzumab and then, constitute an adequate model to test the cytotoxic activity of olive extracts. A recent study on *Cistaceae* extracts showed a notorious capacity to inhibit the proliferation of JIMT-1 breast cancer cells (Barrajón-Catalána et al. 2010). In our study, all the extracts obtained through the different techniques were tested for their cytotoxicity on JIMT-1 cells after incubation during 72 h. Table 3 shows the CC50 values obtained. Olive leaf extracts showing CC50 values higher than 800 µg/mL were considered as non active (NA). Most of the extracts obtained by PLE (water and ethanol) or CM extraction methods showed a low cytotoxic activity with the exception of PLE-E 5. In

contrast, MAE extracts showed a significant cytotoxic activity against JIMT-1 cells, especially that one deriving from 'Oueslati' variety (MAE 1). In general, the highest potency was obtained with extracts obtained by SFE. The highest cytotoxic activity corresponded to 'El Hor' olive leaf extract obtained by SFE (SFE 4), which showed a CC 50 as low as 7 µg/mL. Only in a few cases, higher cytoxicity correlated to higher extraction yield (MAE 1 and PLE-E 5) (Table 1). In general, extraction yield did not correlate with the total phenolic content, revealing that other compounds different than phenolics were extracted in the processes. Whereas highest yield was obtained using PLE followed by MAE, highest phenolic content was obtained with MAE followed by CM. Therefore, cytotoxicity seemed to be more related with phenolic compounds than with total yield. Nevertheless, most SFE extracts, which showed the lowest extraction yields i.e. < 10%, and a low total phenolic content, presented high cytotoxic activity. Thus, this activity might be due to a particular class of phenolic compounds.

Table 3. CC50 values obtained after treating JIMT-1 breast cancer cells with the different phenolic extracts deriving from six Tunisian olive leaf varieties NA: CC50 higher than 800 µg/mL

Olive leaf sample	MAE CC50 (µg/mL)	SFE CC50 (µg/mL)	PLE-W CC50 (µg/mL)	PLE-E CC50 (µg/mL)	Conventional extraction CC50 (µg/mL)
Oueslati (1)	188 ± 24.5	633.5 ± 260.3	NA	NA	550.5 ± 92.2
Chetoui (2)	415.9 ± 14.2	NA	729.2 ± 86.5	NA	796.1 ± 395.4
Chemlali (3)	481.1 ± 49.5	560.1 ± 66.6	NA	NA	NA
El Hor (4)	515.8 ± 69.1	7.0 ± 5.5	NA	NA	NA
Jarboui (5)	435.8 ± 42.6	364.0 ± 25.0	NA	135.6 ± 31.9	NA
Chemchali (6)	711.9 ± 178.9	284.9 ± 78.7	NA	NA	636.7 ± 57.6

As mentioned, SFE extracts were poor in oleuropein derivatives and especially abundant in flavones such as luteolin and diosmetin. Consequently, the possible influence of these compounds on cancer cell cytotoxicity deserves further attention in future studies. Anyhow, the strong cytotoxic activity observed for some extracts (SFE 4, MAE 1, and PLE-E 5) does not rely on the abundance of a single compound so the interaction of different compounds with different cellular targets is postulated to exist. In this regard, several authors have recently pointed out that dietary polyphenols may exert their pharmacological effect through their synergistic interactions by interacting with multiple targets (Efferth et al. 2011; Wagner et al. 2011). It is also plausible that the cytotoxic effect is dependent on additional compounds that were not identified in this study.

4. Conclusion

The development of new extraction processes to obtain bioactives, such as phenolic compounds, from food by-products is raising the attention of researchers and industries. A comparison has been carried out among non-conventional extraction techniques, such as MAE, SFE and PLE, and traditional solid-liquid extraction. Although the non-conventional techniques have shown important advantages, it is worth to mention that the main compositional differences among techniques depend on the type of solvent used to carry out the extraction. Thus, fast processes could be obtained using MAE and PLE, temperature-driven extraction processes, while greener processes could be achieved using SFE and PLE with water. Therefore, the optimum conditions for each process should be selected depending on the target compound to be isolated and other considerations (such as environmental impact, bioactivity, final use, etc.). In general, MAE and CM seem to be the choice for extracting more polar compounds such as oleuropein derivatives, apigenin rutinoside and luteolin glucoside isomer 3. As expected, SFE or PLE were more efficient to extract compounds with less

polarity such as apigenin, luteolin, or diosmetin. The cytotoxic activity of the different olive leaf extracts against breast cancer cells does not correlate either with olive variety, process extraction yield, or amount of phenolic compounds. Highest cytotoxic effect was observed with SFE extracts, which were richer in flavones such as diosmetin or luteolin, but this biological activity does not rely on the abundance of a single compound or a family of compounds. Anyhow, the potential anticancer activity of these compounds and their extracts, especially that of the 'El Hor' variety, deserves further attention.

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Chapter 6

Characterization of phenolic-compound metabolites in JIMT-1 human breast cancer cells treated with olive leaf extract using HPLC-TOF-MS

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Abstract

In the present work, a metabol-*omic* study at *in-vitro* level has been carried out by using a bioactive olive leaf extract of Tunisian variety in human breast cancer cells. The extract was obtained by using supercritical fluid extraction (SFE) and it has been used according to its richness in flavonoid, such as luteolin and diosmetin, and its important cytotoxic activity against human breast cancer cells. Thus, by using high-performance liquid chromatography (HPLC) coupled to electrospray ionization time-of-flight-mass spectrometry (ESI-TOF-MS), three flavonoid metabolites were detected in cytoplasm of JIMT-1 breast cancer cell lines treated with the olive leaf polar extract, among them diosmetin (methyl luteolin) was the major one.

Key words: HPLC, TOF-MS, metabolites, olive leaves, JIMT-1 breast cancer cells

1. Introduction

Historically, leaves from olive tree (*Olea europaea*) have been widely used as a remedy for the treatment of fever and other diseases like malaria (1-3). In recent years, there has been great interest in their potential health benefits and their phenolic copounds. These compounds constitute an important group in olive leaves. The evidence for their bioactivity is emerging and becoming an important issue. Several reports showed their antioxidant (4), anti-inflammatory, anti-microbial (5), hypocholesterolemic (6), anti-viral (7), anti-tumoral (8), antithrombotic, and even skin photoprotective properties (9). Several researches have demonstrated the potential activity of olive leaf phenolic compounds against different types of cancer (10-12). Nevertheless, knowledge of the bioavailability and metabolism of the various polyphenols is necessary to evaluate their biological activity within target tissues. In a recent study on olive leaf extracts from Tunisian varieties extracted using advanced

and non-conventional extraction methods, the polar extract from ‘El Hor’ variety obtained by supercritical fluid extraction (SFE) showed an important cytotoxic activity against the JIMT-1 human breast cancer cell line (12). The extract was characterized by its richness in flavones such as diosmetin and luteolin but the compounds responsible of the highest cytotoxic activity of the extract have not been determined. Thus in this study, we aimed to characterize the metabolites present in the cytoplasm of the JIMT-1 human breast cancer cell after incubation with ‘El Hor’ extract using HPLC–ESI-TOF-MS. For complex biological samples, this analytical technique provides the high degrees of sensitivity, selectivity, matrix independence and universality required for metabolomic-scale experiments

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade. Acetic acid, methanol and acetonitrile for were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Lab-Scan (Gliwice, Sowinskiego, Poland) respectively. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Plant material and treatment of biological samples

Samples

Olive leaf extracts were obtained from Tunisian olive variety ‘El Hor’ using supercritical fluid extraction method (SFE) and its phenolic profile was characterized as described in our previous work (12). This extract showed interesting, and the highest among the other studied extracts, cytotoxic capacity against human breast cancer JIMT-1 cell line.

Treatment of biological samples

The cytosolic fraction of control (untreated JIMT-1 cells) and JIMT-1 cells treated with olive-leaf extracts was obtained upon cell disruption with a Polytron homogenizer at 4 °C. First, cells were washed with phosphate-buffered saline (PBS) solution (Sigma–Aldrich Madrid, Spain) then distilled water was added. The cells were submitted to three cycles of freezing-thawing followed by two cycles of sonication for 20 sec at power 4. Finally they were centrifuged at 14,000 × g for 14 min at 4 °C. The supernatant (cytosolic fraction) was stored at −80 °C until the metabolite-extraction procedure was performed.

The extraction procedure was a liquid–liquid extraction using methanol and was as follows: 50 µL of the cytoplasm was mixed with 2 fold of MeOH: EtOH (50:50), the mixture was maintained in the freezer for 2 h at −20 °C. After the samples reached room temperature, they were centrifuged at 18,800 ×g for 10 min at 4 °C and the supernatant was evaporated to dryness. The dried sample was reconstituted in 50 µL of MeOH.

2.3. HPLC–ESI-TOF-MS analysis

Separation of metabolites was performed on an Agilent high performance liquid chromatographer (Waters, USA) consisting of a vacuum degasser, autosampler, and a binary pump equipped with a Zorbax Eclipse Plus C18 analytical column (150 mm x 4.6 mm, 1.8 µm particle size). The mobile phases used were water with acetic acid (0.5%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the following conditions: from 0 to 5 min, 95% (A):5% (B) to 85% (A):15% (B); from 5 to 25 min, 85% (A):15% (B) to 70% (A):30% (B); from 25 to 30 min, 70% (A):30% (B) to 5% (A): 95% (B); from 35 to 40 min, 5% (A):95% (B) to 95% (A):5% (B); initial conditions are held for 5 minutes. The flow rate used was set at 0.20 mL/min throughout the gradient. The column temperature was maintained at 25 °C, and the injection volume was 10 µL.

The HPLC system was coupled to a micrOTOF mass spectrometer (Bruker Daltoniks, Bremen, Germany) equipped with an ESI interface (Bruker Daltoniks, Bremen, Germany) operating in negative ion mode using a capillary voltage of +4 kV. The other optimum values of the ESI-TOF parameters were drying gas temperature, 210ºC; drying gas flow, 8 L/min, and nebulizing gas pressure, 2 bar. The detection was carried out considering a mass range of 50-1100 m/z.

The accurate mass data of the molecular ions were processed through the software DataAnalysis 4.0 (Bruker Daltoniks), which provided a list of possible elemental formulas by using GenerateMolecularFormula Editor. The Generate Molecular Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions has been established at 5 ppm. The use of isotopic abundance patterns as a single further constraint removes > 95% of false candidates. This orthogonal filter can condense several thousand candidates down to only a small number of molecular formulas. During the development of the HPLC method, external instrument calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, with a sodium acetate cluster solution passing through containing 5 mM sodium hydroxide and 0.2% acetic acid in water: isopropanol (1:1, v/v). The calibration solution was injected at the beginning of each run and all the spectra were calibrated prior to the compound identification. By using this method, an exact calibration curve based on numerous cluster masses each differing by Da ($NaC_2H_3O_2$) was obtained.

2.4. Statistical analysis

Statistical analysis permitting the comparison between the metabolite profiles of the cytoplasm in the control and the treated JIMT-1 cells was carried out using MATLAB R2008b version 7.7.0. The used algorithm provided the candidate metabolites that were detected only in the treated samples.

3. Results and discussion

The introduction of various data analysis procedures, computational tools and methodologies permit to accurately analyse large amounts of data. Matlab is a very flexible mathematical environment that allows applying many statistical and pattern recognition algorithms that are starting to be used in metabolomics and/or other -*omic* sciences.

The sample datasets were converted to the binary form and calibrated according to the introduced mass clusters of the calibrant used in the HPLC-MS analysis. The results of differential analyses are visualized using density plots. Peaks appear as color-coded spots characteristic of the signal of the m/z of the corresponding ion. In the **figure 1** we represent the plot of a candidate metabolite at m/z 299. The horizontal axe represents the migration time and the vertical axe represents the m/z values. The red colored spots correspond to the metabolites detected in the cytoplasm of the treated cells.

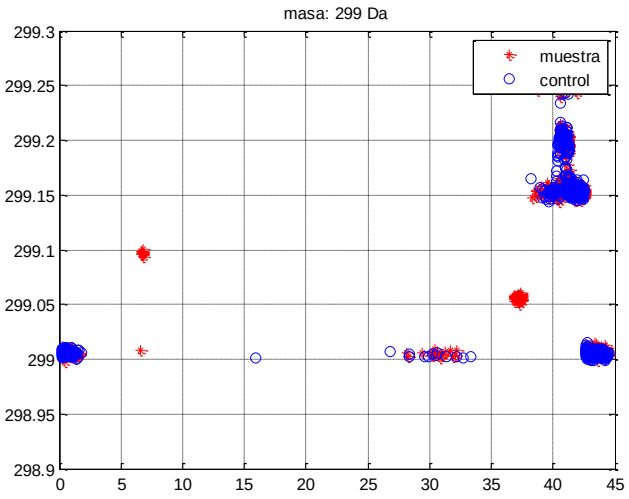

Figure 1. Density plot of the metabolite at m/z 299
as detected using the MATLAB algorithm

In order to identify the metabolites, the cell plasma profiles were studied using the ESI-TOF-MS spectra. To identify the phenolic compounds in their free form and their possible metabolites formed after incubation, we used the information available in literature and the valuable information provided by the TOF analyzer. This provided a list of possible molecular formulas using the information on mass accuracy and the isotopic pattern of the compounds.

The compounds identified as candidates present in the cytoplasm of treated JIMT-1 cell and not in the control ones are presented in the **table 1**.

Table 1. Metabolites identified in the cytoplasm of treated JIMT-1 cells (Selected ion: $(M-H]^-$

R.T (min)	Molecular Formula	m/z experimental	m/z calculated	Tolerance (ppm)	Error (ppm)	Sigma value	Possible metabolites
35.5	C15H9O6	285.0403	285.0405	5	0.7	0.0500	luteolin/apigenin +OH
36.8	C15H9O5	269.0460	269.0455	4	-1.5	0.0282	apigenin
37	C16H11O6	299.0556	299.0561	5	1.8	0.0097	diosmetin/ luteolin+CH3

The extracted ion chromatograms (EICs) of these compounds are presented in **figure 2**. The ion at m/z 285.0403 at RT 35.5 min was tentatively identified as luteolin which was already identified in the olive leaf extract under study**Erreur ! Signet non défini.** or as phase I metabolism compound resulting from the hydroxylation of apigenin. The ion at m/z 269.0460 detected at RT 36.8 min was identified as apigenin. This compound was identified in the olive leaf extract in our previous study (12). Concerning the ion at m/z 299 eluted at RT 37 min, it was tentatively identified as diosmetin or methylated luteolin (phase II metabolite).

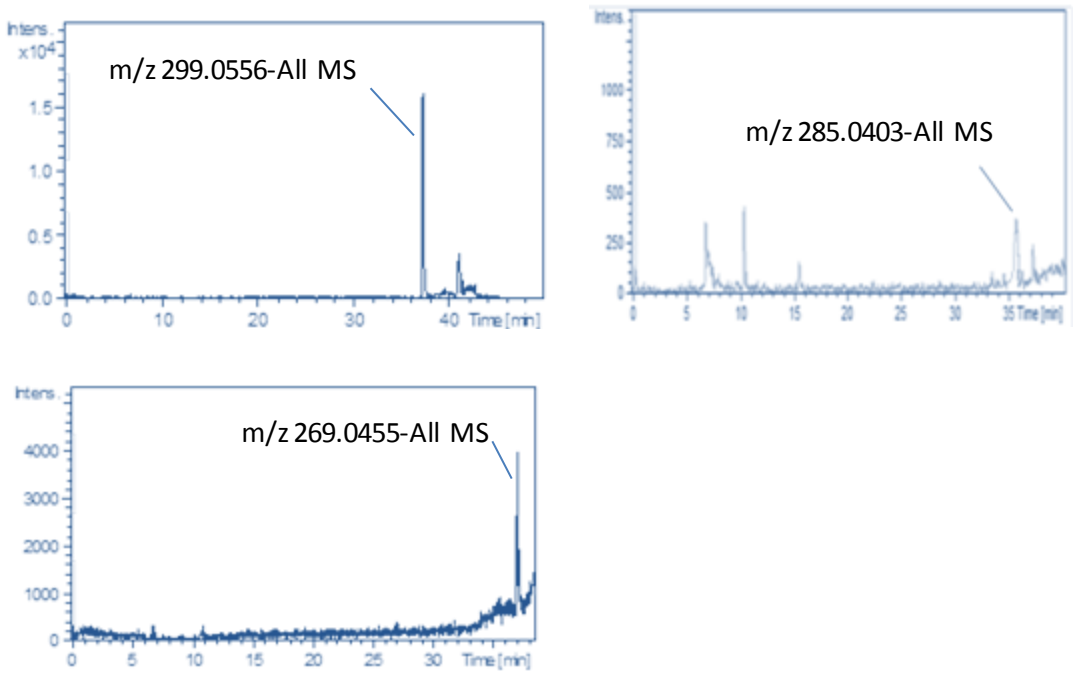

Figure 2. Extracted ion chromatograms (EICs) of the metabolites identified in the cytoplasm of treated JIMT-1 cells

Among the identified metabolites the methyl luteolin (diosmetin) was found to be present at the highest intensity. In our previous research, the diosmetin was found to be the major phenolic compound identified in the corresponding olive leaf extract as well**Erreur ! Signet non défini.**. The methyl luteolin metabolite was also reported to be present in the cytoplasm of colon cancer cells treated with phenolic extracts of virgin olive oils (13). No absorption of secoiridoid compounds was observed in the cytoplasm of the treated JIMT-1 cells. In fact the olive leaf extract when obtained by the SFE method was poor in secoiridoid compounds but rich in flavonoids especially in diosmetin. Some studies seem to indicate that some cells may readily incorporate polyphenols by specific mechanisms (14-16).

These findings are interesting and suggesting that the identified compounds may be responsible for the cytotoxic activity against the JIMT-1 cancer cell line and may influence some signaling pathways causing the cell apoptosis. Further investigation of possible synergism enhancing the activity of the metabolites would be interesting in

order to improve the bioactivity of the compounds. The reported data about the bioactivity of the parent phenolic compounds and their conjugated metabolites are controversial. It was reported that polyphenol conjugated metabolites were found to be as bioactive as the parent polyphenols or even more (17). Some studies have suggested that methylation can increase the bioactivity of polyphenols, whereas other studies have shown the opposite biological effect of methylated polyphenols (18).

4. Conclusion

Flavonoids such as luteolin, apigenin and diosmetin are worthy for consideration for further studies as effective chemoprotective metabolites. Further investigation is needed to clarify the molecular and cellular mechanisms underlying the bioactivity of olive leaf phenolic compounds with respect to human breast cancer.

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General Conclusions

1. The review of the studies on the polyphenols occurring in Tunisian olive products (olive, olive oil) and some by-products (olive leaves and olive mill waste-water) indicates that the phenolic composition variation is the result of a complex interaction of various factors such as cultivar, geographic location and environmental conditions, agronomic practices, and technological conditions. In the reviewed studies, total phenolic contents were determined using the colorimetric assay based on the reaction of Folin-Ciocalteu reagent. Besides that, for identifying the individual phenolic compounds, high performance liquid chromatography coupled to several detectors such as UV and mass spectrometry was the separative technique used. Thus, numerous phenolic compounds have been identified and quantified. The biological activity of the olive derivatives was also reviewed and revealed interesting healthy effects such as hypocholesterolemic, lipid peroxidation reduction, anti-cancer and antiallergic properties.

2. The study of the phenolic profile and other chemical parameters for the characterization of the six Tunisian extra-virgin olive oils showed a significant influence of the genetic factor. Some secondary varieties showed noteworthy phenolic profiles and oxidative stability. The statistical analysis revealed that parameters such as concentrations of oleic and linoleic acids, apigenin, luteolin, taxifolin, oleuropein aglycone, pinoresinol acetate, elenolic acid, and oxidative stability distinguished the monovarietal olive oils. Furthermore, it was possible to classify the olive-oil varieties based on their geographical origin (northern, central, or southern Tunisia) through the LDA model. Variables such as oleic acid, H-D-Ol Agl, H-Pin, HyTY-Ac, EA and sinapinic acid played an important role as discriminating variables in the differentiation of the olive oils according to their geographical origin. The varieties which showed a noteworthy phenolic profile would be more

investigated through the amplification of the sampling spectra in order to evaluate their variation according to other factors such as the climate, production zone, extraction system, and storage conditions.

3. The production area significantly influenced the phenolic composition of the 'Chemlali' olive-oil variety. The oils produced from 'Oueslatia', 'Ain Zena', 'Siliana', and 'Bir Ali Ben Khelifa' were the richest in polyphenols, among others. The HPLC-ESI-TOF-MS data provided an appropriate pathway to classify the oils according to their geographical area. A correct classification pathway with 100% of the correct predicted membership was achieved by selecting only a reduced number of phenolic compounds, namely oleuropein aglycon, 10-hydroxy-oleuropein aglycon, hydroxy-elenolic acid, elenolic acid, ligstroside aglycon, decarboxylated oleuropein aglycon, hydroxy- decarboxylated oleuropein aglycon, decarboxylated ligstroside aglycon, ferulic acid, coumaric acid, quinic acid, and luteolin.

4. For the characterisation of phenolic compounds from olive leaf extract, a simple and rapid microwave-assisted extraction (MAE) method has been optimized and combined to HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2. The optimized MAE showed time-saving (6 min) higher efficiency and automatisation compared to the traditional extraction method. The analysis of the extract profile showed the presence of phenolic compounds from different classes. As far as we know, compounds such as the secoiridoid 2″-methoxyoleuropein, the flavonoids diosmin and its isomer, luteolin diglucoside isomer, and luteolin rutinoside isomer are reported for the first time in olive leaves. Thus, the MAE technique proved its efficiency towards the extraction of phenolic compounds from olive leaf extracts.

5. The comparison among non-conventional extraction techniques, such as MAE, SFE and PLE, and traditional solid-liquid extraction together with the conventional method showed that the extraction method influences the phenolic profile of the

cultivars studied, which was also influenced by the olive-leaf variety. The main compositional differences among techniques depend on the type of solvent used.

MAE samples showed the highest TPCs in comparison to the other extraction methods, being 'Chemlali' and 'El Hor' the varieties having the highest total phenolic contents. MAE technique was followed by CM and PLE techniques, being PLE-W and SFE those achieving the lowest total phenolic contents values. In general, MAE and CM seem to be the method of choice for extracting more polar compounds such as oleuropein derivatives, apigenin rutinoside, and luteolin glucoside. In contrast, SFE or PLE were more efficient for extracting compounds with less polarity such as apigenin, luteolin, or diosmetin.

The cytotoxic capacity assay of the different olive-leaf extracts against breast-cancer cells did not show correlation either with olive variety, extraction process, yield, or the amount of phenolic compounds. The highest cytotoxic capacity was registered for SFE extracts from "El Hor" variety, the richest in diosmetin, which showed a CC50 of 7 µg/mL.

6. The analysis of metabolites detected in the cytoplasm of the JIMT-1 breast cancer cells treated with 'El Hor' olive leaf extract obtained by SFE showed the presence of three flavonoids: luteolin (hydroxylated apigenin), apigenin and diosmetin (methylated luteolin). Among them, diosmetin was present at the highest intensity. These metabolites are worthy for consideration for further studies as effective chemoprotective metabolites. Further investigation is needed to clarify the molecular and cellular mechanisms underlying the bioactivity of olive leaf phenolic compounds with respect to human breast cancer.

Conclusiones Generales

1. En la revisión llevada a cabo sobre los compuestos fenólicos presentes en productos oleícolas tunecinos (aceite de oliva, aceite de oliva) así como en algunos subproductos (hojas de olivo y aguas residuales) se pudo observar que la variación de la composición fenólica es el resultado de una compleja interacción de diversos factores tales como el tipo de cultivo, la ubicación geográfica, las condiciones ambientales, las prácticas agronómicas y las condiciones tecnológicas. En los estudios realizados hasta la fecha, el contenido de fenoles totales se determinó mediante ensayos colorimétricos basados en la reacción de Folin-Ciocalteu. Sin embargo, la identificación y la cuantificación pormenorizada de cada uno de los compuestos fenólicos presentes en las diferentes matrices se llevó a cabo mediante el uso de la Cromatografía Líquida de Alta Resolución (HPLC) acoplada a diferentes sistemas de detección tales como UV-visible y espectrometría de masas (MS). En cuanto a la bioactividad de éstos compuestos, numerosos estudios revelaron que dichos compuestos poseen importantes propiedades anti-alergénicas y anti-cancerígenas además de importantes efectos frente a la peroxidación lipídica.

2. El estudio del perfil fenólico de seis aceites de oliva virgen extra Tunecinos, conjuntamente con otros parámetros químicos, mostraron una influencia significativa en el factor genético de dichos aceites. Algunas variedades secundarias mostraron excelentes perfiles fenólicos además de una notable estabilidad oxidativa. El análisis estadístico reveló que parámetros tales como las concentraciones de los ácidos oleico y linoleico, la apigenina, luteolina, taxifolina, oleuropeína aglicona, acetato de pinoresinol, ácido elenólico, y la estabilidad oxidativa diferenció los aceites de oliva monovarietales. Además, era posible clasificar las variedades de aceite de oliva en función de su procedencia geográfica (norte, centro o sur de Túnez) a través de la LDA. Variables como el ácido oleico, H-D-Ol Agl, H-Pin, HyTY-Ac, ácido elenolico, y ácido sinapinico jugaron un papel importante en la discriminación de los aceites según su origin geográfica. Las variedades que mostraron un perfil fenólico notable se

investigaron en mayor profundidad a través de la ampliación de las zonas de muestreo con el fin de evaluar su variación en función de factores tales como el clima, la zona de producción, el sistema de extracción, y las condiciones de almacenamiento.

3. La zona de producción influyó significativamente en la composición fenólica del aceite de oliva de variedad "Chemlali'. Así, los aceites producidos a partir de 'Oueslatia', 'Ain Zena', 'Siliana', y 'Bir Ali Ben Khelifa' fueron los más ricos en compuestos fenolicos. Los datos de HPLC-ESI-TOF-MS se utilizaron como una vía apropiada para clasificar los aceites según su área geográfica. Una vía correcta clasificación con 100% de los miembros correcta predijo se logró mediante la selección de un número reducido de compuestos fenólicos, a saber, la oleuropeína aglicona, aglicona 10-hidroxi-oleuropeína, el ácido hidroxi-elenólico, ácido elenólico, aglicona ligustrósido, descarboxila aglicona oleuropeína, hidroxi-descarboxila oleuropeína aglicona, aglicona ligustrósido descarboxila, el ácido ferúlico, ácido cumárico, ácido quínico, y la luteolina.

4. Para llevar a cabo la caracterización de los compuestos fenólicos presentes en extracto de hojas de olivo tunecinas, un nuevo proceso de extracción por microondas (MAE) simple y rápido ha sido optimizado y combinado con la HPLC-ESI-TOF-MS y HPLC-ESI-IT-MS2. Así, el método (MAE) propuesto permitió el ahorro de tiempo (6 min) y mostró mayor eficiencia y automatización en comparación con el método de extracción tradicional. El análisis del perfil del extrato de hojas mostró la presencia de compuestos fenólicos de diferentes familias. Entre ellos, el secoiridoide 2''-metoxioleuropeina y los flavonoides, diosmina y su isómero, el isómero de la luteolina diglucósido y el isómero de la luteolina rutinósido han sido por primera vez

descritos en hoja de olivo. Por lo tanto, MAE ha demostrado su eficiencia para la extracción de compuestos fenólicos en hojas de olivo.

5. La comparación entre las técnicas de extracción no convencionales, como MAE, SFE y PLE y la extracción convencional sólido-líquido (CM), demostraron que el método de extracción influye de manera importante en el perfil polifenólico de las variedades de hoja de olivo. Las principales diferencias de composición entre las técnicas dependen del tipo de disolvente utilizado para la extracción. Los mayores contenidos en fenoles totales se registraron por los extractos MAE. Los extractos MAE de las variedades "Chemlali" y "El Hor" fueron las más ricas en fenoles totales.
En general, MAE y CM parecen ser la opción más adecuada para la extracción de los compuestos más polares tales como derivados de la oleuropeína, apigenina rutinósido, y luteolina glucósido. Por el contrario, SFE o PLE fueron más eficientes para la extracción de compuestos menos polaridad, como apigenina, luteolina o diosmetina.
El ensayo de la capacidad citotóxica de los diferentes extractos de hoja de olivo frente a la línea celular JIMT-1 de cáncer de mama no se correlaciona bien con la variedad de hoja, el proceso de extracción o la cantidad de compuestos fenólicos. La mayor capacidad citotóxica se registró por el extracto SFE de la variedad 'El Hor", ricos en diosmetina, que ha mostrado una CC50 de 7 μg/mL.

6. El extracto de la variedad "El Hor" fue utilizado para llevar a cabo estudios de metabolómica en citoplasmas de células de la línea JIMT-1 de cáncer de mama, consiguiendo caracterizar de 3 metabolitos los cuales serán unos compuestos a considerar a la hora de llevar a cabo otros estudios a nivel *in-vitro* o *in-vivo* que permitan esclarecer los mecanismos moleculares y celulares que subyacen La bioactividad de estos metabolitos con respecto al cáncer de mama.

Conclusions Générales

1. La revue des études portées sur les polyphénols présents dans les produits de l'olivier Tunisien (olive, huile d'olive) et certains sous-produits (feuilles d'olivier et margines) indique que la variation de la composition phénolique est le résultat d'une interaction complexe de divers facteurs tels que le cultivar, le site géographique et les conditions environnementales, les pratiques agronomiques, ainsi que les conditions technologiques. Dans les études revues, le contenu en phénols totaux a été déterminé en utilisant le test colorimétrique basé sur la réaction de Folin-Ciocalteu. En outre, pour identifier les composés phénoliques individuels, la chromatographie liquide à haute performance couplée à différents détecteurs tels que l'UV et la spectrométrie de masse était la technique de séparation utilisée. Ainsi, de nombreux composés phénoliques ont été identifiés et quantifiés. L'activité biologique des dérivés de l'olivier a été également revue et a révélé leurs propriétés bénéfiques à la santé telles que l'hypocholestérolémie, la réduction de la peroxydation lipidique, la capacité anticanceureuse et antiallergique.

2. L'étude du profil phénolique et d'autres parameters chimiques pour la caractérisation de six variétés Tunisiennes d'huile d'olive a montré l'effet significatif du facteur génétique. Certaines variétés secondaires ont montré un profil phénolique et une stabilité à l'oxydation intéressants. L'analyse statistique a révélé que des paramètres tels que les concentrations des acides gras oleique, linoleique, des phénols luteoline, taxifolin, oleuropeine aglycone, pinoresinol acétate, acide elenolique et la stabilité oxydative ont permis la distinction entre huiles d'olive monovariétales étudiées. Grace au modèle déterminé par l'analyse discriminante linéaire la classification des huiles suivant leur origine géographique a été établie. Des variables tels que l'acide oleique, H-D-Ol Agl, H-Pin, HyTY-Ac, acide elenolique et acide sinapinique ont joué un role important comme des variables discriminatives dans la differentiation des différentes huiles selon leur origine geographique. Les variétés d'olivier qui ont montré une composition et un profil phénolique intéressant peuvent être plus étudiées en augmentant le spectre d'échantillonnage dans le but

d'évaluer leur comportement sous l'effet d'autres facteurs tels que climat, site géographique, système d'extraction, conditions de stockage, etc…

3. La zone de production a un effet significatif sur la composition phénolique de la variété 'Chemlali'. Les huiles produites à 'Oueslatia', 'Ain Zena', 'Siliana' et 'Bir Ali Ben Khelifa' ont été les plus riches en polyphénols parmi les huiles analysées. Les résultats obtenus par HPLC–ESI-TOF–MS ont permis la classification des huiles selon leurs zones géographiques. Une classification correcte à 100% de membres prédits a été obtenue en sélectionnant seulement treize composés phénoliques, à savoir oleuropein aglycon, 10-hydroxy-oleuropein aglycon, hydroxy- acid elenolique, acid elenolique, ligstroside aglycon, oleuropein aglycon decarboxylée, hydroxy- oleuropein aglycon decarboxylaée, ligstroside aglycon decarboxylé, les acides ferulique, coumarique et quinique, et la luteolin).

4. Pour la characterisation des composés phénoliques des feuilles d'olivier, une extraction assistée aux microondes (MAE) simple et rapide a été optimisée et combinée à HPLC-ESI-TOF-MS and HPLC-ESI-IT-MS^2. La méthode d'extraction optimisée a montré un gain de temps (temps d'extraction: 6 min), une meilleure éfficacité et automatisation du processus en comparaison avec la méthode d'extraction conventionnelle. L'analyse de profil de l'extrait phénolique a montré la présence de composés phénoliques de différentes familles. Des composés comme le sécoiridoide 2″-methoxyoleuropeine et son isomère, les flavonoides diosmine et son isomère, l'isomère de la luteoline diglucoside et l'isomère de la lutéoline rutinoside ont été décrit pour la première fois dans les feuilles d'olivier. Ainsi, la technique de MAE a prouve son efficacité pour l'extraction des composés phénoliques des feuilles d'olivier.

5. La comparaison entre les techniques d'extraction non conventionnelles, telles que MAE, SFE et PLE, et la technique traditionnelle solide-liquide a montré que la méthode d'extraction influe le profil phénolique, qui a son tour a été influencée par la variété d'olivier. Les principales différences de la composition phénolique entre les techniques utilisées dépendent du type de solvant utilisé pour effectuer l'extraction. Les extraits de la MAE ont montré les teneurs en phénols totaux les plus élevées dont les teneurs les plus grandes valeurs ont été enregistrées pour les variétés “Chemlali” et “El Hor”. Après la MAE viennent les techniques de CM et PLE, celles de PLE-W et SFE ont montré les valeurs de phénols totaux les plus faibles.

En général, MAE et CM semblent être le choix pour l'extraction des composés plus polaires comme les dérivés de l'oleuropéine, l'apigénine rutinoside et la lutéoline glucoside. En revanche, SFE ou PLE étaient plus efficaces à extraire des composés moins polaires comme l'apigénine, la lutéoline et la diosmétine.

L'activité cytotoxique des différents extraits de feuilles d'olivier contre les cellules humaines de cancer de sein ne semble corréler ni avec la variété d'olive ni avec la technique d’extraction. La capacité cytotoxique la plus importante a été trouvée chez l’extrait SFE de la variété “El Hor”, le plus riche en diosmétine, qui a montré une CC50 de 7 µg/mL.

6. L’analyse des metabolites détectés dans le cytoplasme des cellules de cancer de sein JIMT-1 traitées par l’extrait phénolique des feuilles d’olivier de la variété ‘El Hor’ obtenu par SFE a montré la présence de trois flavonoides à savoir luteolin (hydroxy-apigenine), apigenine et diosmetine (methyl-luteoline). Parmi ces composés identifiés, la diosmetin était la majoritaire. Ces métabolites méritent plus de consideration pour des études futures *in vitro* ou *in vivo*. Des recherches futures sur les mécanismes moléculaires et cellulaires sont nécessaires pour comprendre la bioactivité des extraits phénoliques des feuilles d’olivier.