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MASTER THESIS

"Development and validation of a HPLC-ESI-QTOF-MS method for the determination of phenolic content in olive leaves"

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Development and validation of a HPLC-ESI-QTOF-MS method for the determination of phenolic content in olive leaves

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ΕΡΕΥΝΗΤΙΚΗ ΕΡΓΑΣΙΑ ΔΙΠΛΩΜΑΤΟΣ ΕΙΔΙΚΕΥΣΗΣ

Ανάπτυξη και επικύρωση μεθόδου για τον προσδιορισμό φαινολικού περιεχομένου σε δείγματα φύλλων ελιάς με HPLC-ESI-QTOF-MS

ΕΛΒΙΡΑ ΕΥΣΤΡΑΤΙΟΥ

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ABSTRACT

The olive tree (Olea europaea L.) is a small tree found mostly in the Mediterranean basin, and that has been since ancient years to produce table olives and olive oil. Also, olive leaves had been traditionally used in folk medicine since ancient times due to their several medicinal properties. The last decades, several studies have demonstrated the antihypertensive, anticarcinogenic, anti-inflammatory, antimicrobial, and hypocholesterolemic effects of olive leaf extract. Most of the health-related benefits of olive leaves have been associated to their high bioactive content. Many researchers and companies show an increased interest in characterizing olive leaves' phenolic profile to exploit them in the pharmaceutical, food, and cosmetic industry.

The aim of this study was the development and validation of an HPLC-ESI-QTOF-MS method for the determination of phenolic content in olive leaves. For method development, a generic sample preparation protocol was followed, and three different factors, including the drying method, the solid/solvent ratio and the extractant composition, were tested. The method selected included microwave drying of 0,25g initial olive leaf sample and extraction using MeOH: H2O (80:20). The developed method was then validated according to the requirements for new methods, including linearity, sensitivity, trueness, precision, and matrix effect. These characteristics were evaluated using spiked samples of a representative group of compounds, for which reference standards were available.

The method was then applied to 24 olive leaf samples collected in Lesvos Island from two different Greek varieties: Kolovi and Adramitiani, to identify and quantify the different phenolic compounds found. The target screening was based on specific identification criteria, while the quantification was performed with standard additions to the sample extract (matrix-matched calibration curves). The obtained results showed that both varieties have rich bioactive content. Moreover, eriodictyol and oleuropein were found in the highest concentrations in all samples. In conclusion, the results obtained in the present work illustrate the importance of exploitation of this natural product

SUBJECT AREA: Food Analytical Chemistry

KEYWORDS: Method Development, Phenolic content, Olive leaves, Exploitation, HPLC-ESI-QTOF-MS, Target screening

ΠΕΡΙΛΗΨΗ

Το ελαιόδεντρο (Olea europaea L.) είναι ένα μικρό δέντρο που συναντάται κυρίως σε χώρες της Μεσογειακής Λεκάνης και το οποίο καλλιεργείται από αρχαιοτάτων χρόνων με σκοπό την παραγωγή ελιών και ελαιόλαδου. Επίσης, τα φύλλα ελίας χρησιμοποιούνταν σαν γιατροσόφι στην αρχαιότητα λόγω των φαρμακευτικών ιδιοτήτων τους. Κατά τις τελευταίες δεκαετίες , σε πολλές έρευνες έχει αποδειχθεί η αντιυπερτασική, αντικαρκινική, αντιφλεγμονώδη, αντιμικροβιακή και υποχοληστερολαιμική δράση του εκχυλίσματος φύλλων ελιάς. Τα περισσότερα οφέλη των φύλλων ελιάς στην υγεία αποδίδονται κυρίως στο υψηλό βιοδραστικό τους περιεχόμενο. Το ενδιαφέρον πολλών ερευνητών και εταιρειών να χαρακτηρίσουν το φαινολικό προφίλ των φύλλων ελιάς αυξάνεται συνεχώς με σκοπό την περαιτέρω αξιοποίησή τους στην βιομηχανία φαρμάκων , τροφίμων και καλλυντικών.

Σκοπός αυτής της μελέτης είναι η ανάπτυξη και επικύρωση μιας μεθόδου για τον προσδιορισμό φαινολικού περιεχομένου σε φύλλα ελιάς με χρήση HPLC-ESI-QTOF-MS. Για την ανάπτυξη της μεθόδου, ακολουθήθηκε μια γενικευμένη προκατεργασία δείγματος και εξετάστηκαν τρεις διαφορετικοί παράγοντες : η μέθοδος ξήρανσης, η αναλογία δείγματος/εκχυλιστικού και η σύσταση του εκχυλιστικού. Η βελτιστοποιημένη μέθοδος περιλαμβάνει ζύγιση 0.25g δείγματος, ξήρανση με τη χρήση φούρνου μικροκυμάτων και εκχύλιση με μεθανόλη/νερό (80:20). Στη συνέχεια η ανεπτυγμένη μέθοδος, επικυρώθηκε με βάση τα χαρακτηριστικά ποιότητας των νέων μεθόδων και περιλαμβάνει την γραμμικότητα, την ευαισθησία, την ορθότητα, την ακρίβεια καθώς και την επίδραση της μήτρας. Τα χαρακτηριστικά της ανεπτυγμένης μεθόδου αξιολογήθηκαν με την χρήση εμβολιασμένων δειγμάτων με αντιπροσωπευτικούς αναλύτες από διάφορες ομάδες ενώσεων, για τα οποία υπήρχαν διαθέσιμα πρότυπα αναφοράς.

Στη συνέχεια η μέθοδος χρησιμοποιήθηκε για την ανάλυση 24 δειγμάτων φύλλων ελιάς που συλλέχθηκαν στο νησί της Λέσβου και ανήκουν σε δύο διαφορετικές ελληνικές ποικιλίες : την αδραμυτιανή και την κολοβή με σκοπό να πραγματοποιηθεί αναγνώριση και ποσοτικοποίηση των διαφορετικών φαινολικών ενώσεων. Η στοχευμένη σάρωση ενώσεων βασίστηκε σε συγκεκριμένα κριτήρια ταυτοποίησης ενώ η ποσοτικοποίηση έγινε με την μέθοδο προσθήκης γνωστής ποσότητας στην μήτρα(εκχύλισμα φύλλων ελίας).

Τα αποτελέσματα έδειξαν πως και οι δύο ποικιλίες έχουν πλούσιο βιοδραστικό περιεχόμενο. Επίσης το eriodictyol και το oleuropein βρέθηκαν στις μεγαλύτερες συγκεντρώσεις σε όλα τα δείγματα. Συμπερασματικά, τα αποτελέσματα της παρούσας διπλωματικής μεταπτυχιακής εργασίας αντανακλούν την σπουδαιότητα εκμετάλλευσης αυτού του φυσικού προϊόντος .

ΘΕΜΑΤΙΚΗ ΠΕΡΙΟΧΗ: Αναλυτική Χημεία Τροφίμων

ΛΕΞΕΙΣ ΚΛΕΙΔΙΑ: Ανάπτυξη μεθόδου, Φαινολικό περιεχόμενο, Φύλλα ελιάς, Εκμετάλλευση , HPLC-ESI-QTOF-MS, Στοχευμένη σάρωση

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PREFACE

This master thesis was performed at the Laboratory of Analytical Chemistry (Department of Chemistry, National and Kapodistrian University of Athens, Greece) under the supervision of Professor Nikolaos S. Thomaidis.

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CHAPTER 1

OLIVE LEAVES

1.1 Introduction

Olive (Olea europaea L.) is one of the oldest cultivated plants in the Mediterranean region and belongs to Oleaceae's family. It is a small tree flourishing best in regions with tropical and warm temperatures, such as the coastal countries of the eastern Mediterranean basin, the neighboring coastal areas of southeastern Europe, western Asia, Arabian Peninsula, India and Asia and northern Africa as well as northern Iran at the south end of the Caspian Sea [1]. Olive is considered to be one of the most important crops in the Mediterranean countries since olive oil and olives are essential components in the daily diet of a large part of the human population due to their high nutritional value. The cultivation of olive trees and olive oil extraction produces a considerable bulk of by-products [2]. These byproducts include enormous quantities of solid wastes and dark liquid effluents that have no practical applications. The most important byproducts of the olive tree culture and olive oil industry are olive leaves, olive pomace, olive mill wastewater (OMWW), and table olive processing wastewater (TOPW). These wastes include many valuable substances such as carbohydrates, organic acids, mineral nutrients, oils, fibers, and phenols. Because of these high added value compounds, many researchers focus on exploiting these byproducts [3] .

1.2 Olive leaves

The term “olive leaves” refers to a byproduct of olive tree cultivation and could also be found in the olive oil industry. Olive leaves are a mixture of leaves and branches accumulated during olive trees' pruning and the harvesting and cleaning of olives. During the tree pruning, olive leaves' productions are estimated to be ~25kg per olive tree [4]. Olive leaves also represent 10% of the harvested olives' total weight arriving at olive oil mills [5].

Olive leaves have been used ever since the ancient times as a remedy against fever and other diseases such as malaria. Nowadays, the number of scientists involved in studies on olive leaves increases due to the richness of phenolic compounds in olive leaves. These valuable phenolics show positive effects on human health due to their antioxidant and anti-inflammatory properties. Oleuropein is the main phenolic compound in olive leaves, followed by hydroxytyrosol. Many studies have demonstrated the antihypertensive, anticarcinogenic, hypoglycemic, antimicrobial, anti-HIV effects of these compounds. Because of these health benefits, olive leaves are used in cosmetics, medicine, pharmaceutical products, and the food industry [6].

1.3 Chemical composition

Different studies conducted on olive leaves show that their chemical composition varies depending on origin, the proportion of branches collected during the pruning, climatic conditions and storage conditions. More specifically, a study conducted by Ibrahim et al. (2016) shows the approximate chemical composition of whole and boiled olive leaves (Olea europaea L. Cv. Kalamata) presented in **Table 1**.

Table 1: Proximate chemical composition of whole and boiled olive leaves (on dry weight basis) *[7]*

Components %	Whole leaves	Boiled leaves
Moisture	50.5	55.9
Dry matter	49.5	44.1
Crude protein (N x 6.25)	10.6	10.7
Ether extract	7.9	8.1
Ash	6.8	6.7
Crude fiber	14.5	16.6
Total carbohydrates	74·7	74·5
Available carbohydrates	60·2	57·9

It could be observed that there are slight variations between the whole and boiled olive leaves regarding not only the moisture content (the moisture content of whole leaves

lower than this of boiled ones) but also their contents of crude protein, ether extract and ash contents. From the data presented in this Table, it could be observed that whole olive leaves have lower fiber content than that of boiled ones. The boiling process leads to the release of water-soluble compounds such as pigments and polyphenols, while other compounds that are insoluble in water like fibers concentrate in boiled leaves. Lastly, both boiled and whole olive leaves are high in protein, ash and carbohydrates [7] . Another study that evaluated the chemical composition of five different Southern Brazil varieties showed that protein, lipids, ash, and total carbohydrates contents in fresh leaves ranged from 10.5 to 13.1, 9.13 to 9.8, 4.37 to 6.0 and 8.74 to 32.63%, respectively [8]. Also, the analysis of four different varieties cultivated in Tunisia gave lower protein and lipid values than those of **Table 1** (ranging from 5.50 to 7.61%; and 1.05 to 1.30%, respectively) [9] . The difference between the obtained results published in literature is clear and may be attributed to variations of varieties and origins [7] .

1.4 High added value compounds and health benefits

Several published papers can be found in the available literature concerning the extraction and exploitation of high added value compounds found in olive leaves. Most of these compounds are linked to the numerous health benefits of olive leaves. Their complex composition encompasses flavonoids and their glycosylated derivatives, secoiridoids and their derivatives, simple phenols, phenolic acids and derivatives, terpenes, fatty acids, minerals, vitamins, and phytosterols. However, it is extremely difficult to obtain an overview of their composition because of these compounds' heterogeneity and the utilization of different analytical techniques [10]. The composition also differs according to olive variety, tree age, climatic conditions, genetics, and extraction procedures. The abovementioned compounds' chemical structures and other properties will be described thoroughly in the next paragraphs [11].

1.4.1 Phenolic compounds

By the term phenolic compounds, we mean the substituted derivatives of hydroxycinnamic acid (free from phenolics) and hydroxybenzoic acid (bound form phenolics). The chemical structure of phenols consists of an aromatic group that is the non-polar group and one or more hydroxyl groups attached to it. The hydroxyl groups are the polar part of phenols. The phenolic compounds are mostly found in plants as esters or glycosides and rather than as free molecules. They are grouped concerning major molecular characteristics as simple phenols and acids, secoiridoids and flavonoids. Polyphenols have low water solubility and are very sensitive to heat and light. Olive leaves may be considered a cheap and easily available natural source of phenolic compounds [5].

1.4.1.1 Flavonoids and glycosylated derivatives

Flavonoids are a large group of polyphenolic compounds found mostly in plants. More than 9000 different flavonoid compounds have been described, and this number is continuously increasing every year due to their health benefits. Flavonoids have anti-inflammatory, antiproliferative, antioxidant and anticancer activity, free-radical scavenging capacity, antihypertensive effects, coronary heart disease prevention and anti-human immunodeficiency virus functions.

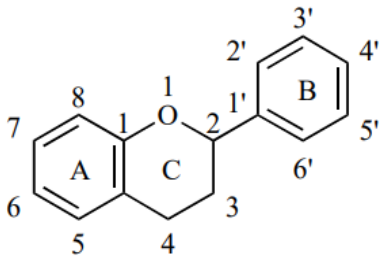


Figure 1: Chemical structure of the flavonoid nuclear *[12]* **.**

To begin with, all flavonoids are derivatives of a compound named 2-phenylchromone, which is composed of three phenolic rings named A, B, C rings, as shown in **Figure 1**.

Flavones, flavanones, flavonols, anthocyanidins, catechins (or flavanols), dihydroflavonols, isoflavones, dihydroflavonols, and chalcones [12].

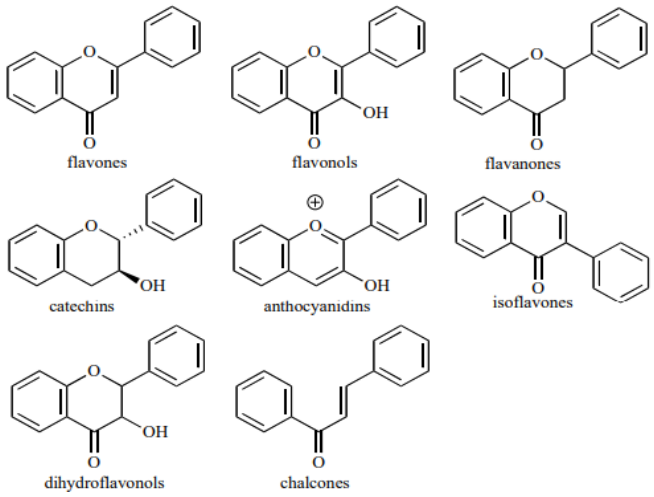


Figure 2: The major subclasses of flavonoids *[12]***.**

Flavonoids are compounds of great importance in higher plants because of their involvement in many ecological and physiological mechanisms. More precisely, bioflavonoids have a crucial role in plant-host relationships through defense mechanisms and as deterrents to fungal invasion and insect predation. Flavonoid aglycones are contained mostly in oil cells of many plants of arid and semiarid regions such as Citrus and olive. It seems that the role of flavonoids is multiple, ranging from operating as a UV screen that reduces the heat and, finally, as antimicrobial agents [13]. According to literature, the main flavonoids found in olive leaves are shown in **Table 2**.

Table 2: Flavonoids found in olive leaves [10]

Flavonoids
Catechin, rutin, luteolin-7-glucoside, apigenin-7-glucoside, diosmetin-7glucoside, luteolin, luteolin diglucoside, luteolin-7-rutinoside, apigenin-7rutinoside, taxifolin, diosmin, diosmetin, cirstimaritin, apigenin, quercetin, chryseriol-7-glucoside, eryodictiol.

1.4.1.2 Simple Phenols

Simple phenols are the most common and vital low-molecular weight phenolic compounds. The simple phenols found in olive leaves are mainly hydroxytyrosol, tyrosol and the derivatives of these two compounds. (**Table 3**)

Hydroxytyrosol is the second most important bioactive compound found in olive leaves after oleuropein. Hydroxytyrosol or 3,4-dihydroxyphenyl ethanol is a very bioactive alcoholic ortho-diphenol with potent antioxidant and antimicrobial activity. Many studies have also demonstrated that hydroxytyrosol has beneficial effects on the cardiovascular system and in several diseases. Native hydroxytyrosol is rarely in the free form in nature except for ripened olives, where it occurs through the hydrolysis of oleuropein. The production of hydroxytyrosol is the result of chemical or enzymatic hydrolysis of oleuropein. Free hydroxytyrosol occurs naturally when enzymatic hydrolysis occurs and, more specifically, native β-glycosidase and esterase are implicated. Acid hydrolysis is the most used mechanism in the laboratory and industry [14].

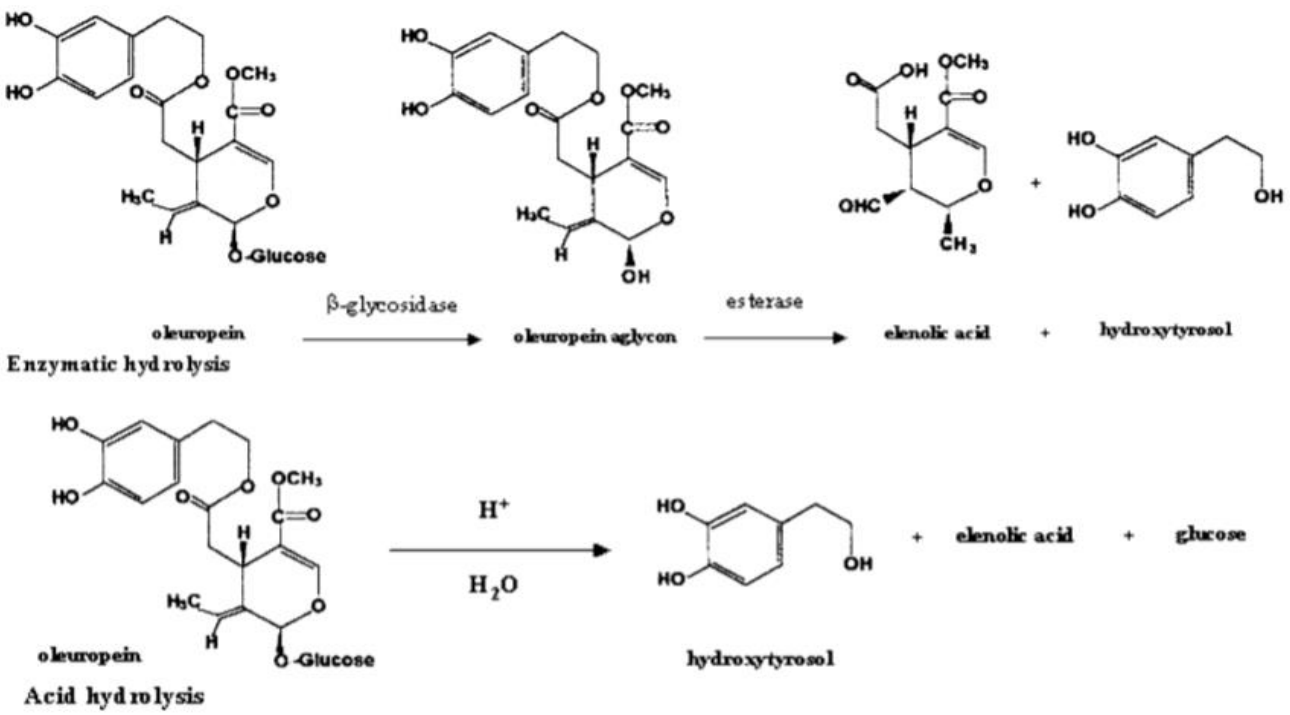


Figure 3: Hydroxytyrosol production by enzymatic and acid hydrolysis of oleuropein *[14]*

Hydroxytyrosol is a strong antioxidant because it is an easily oxidizing compound. The antioxidant activity of hydroxytyrosol is attributed to its hydrogen donation and the ability to scavenge free radicals by forming an intramolecular hydrogen bond between the free hydrogen of its hydroxyl group and the phenoxyl radicals. It generates a new radical that is stabilized by the aromatic structure's resonance effect. The propagation phase is therefore blocked, and the development of oxidation is delayed [5].

Table 3: Simple Phenols and derivatives found in olive leaves *[10]*

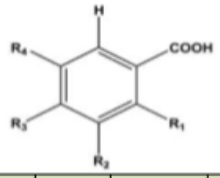
Simple Phenols and derivatives
Hydroxytyrosol, tyrosol, hydroxytyrosol hexoside, hydroxytyrosol acetate, tyrosol glucoside, vanillin, acteoside

1.4.1.3 Phenolic acids and derivatives

Phenolic acids are secondary aromatic metabolites extensively spread throughout the plant kingdom. They are responsible for the unique taste, flavor and health-promoting properties of most vegetables and fruits [15]. Therefore, increasing the phenolic content in these plants can enhance their quality. Phenolic acids and other phenolic compounds play a very significant role in the reproduction and growth of plants. They are produced to respond to environmental factors such as light, chilling, and pollution and defend injured plants [16].

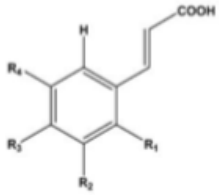
Phenolic acids are phenols that have one carboxylic acid functionality. They are part of the family of plant polyphenols because they are precursors of polyphenols and, more specifically, they are metabolites of biophenols. Their chemical structures of naturally occurring phenolic acids contain two different carbon frameworks: the hydroxycinnamic and hydroxybenzoic structures (**Figure 4**). Hydroxycinnamic acids are produced as simple esters with glucose or hydroxycarboxylic acids. Plant phenolic compounds have different molecular structures and are characterized by hydroxylated aromatic rings [15]. The function of these secondary metabolites in plants is poorly understood. Many phenolic acids are polymerized into larger molecules such as the proanthocyanidins (PA: condensed tannins) and lignins. Lastly, phenolic acids may arise in food plants as esters or glucosides with other natural compounds such as alcohols, sterols, glucosides and hydroxy fatty acids [16].

Hydroxybenzoic Acids



Position	R_1	R_2	R_3	R_4
Benzoic acid	H	H	H	H
Gallic acid	H	OH	OH	OH
Vaillinic acid	H	OCH_3	OH	H
Salicylic acid	OH	H	H	H

Hydroxycinnamic Acids



Position	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
Ferulic acid	H	OCH_3	OH	H
Sinapic acid	H	OCH_3	OH	OCH_3
Caffeic acid	H	OH	OH	H

Figure 4: Structures of the important naturally occurring phenolic acids *[15]*

It has been reported in the literature that phenolic acids play an essential role in the control of different human diseases through the consumption of plants that are good sources of natural antioxidants. They are natural compounds with scavenging free superoxide radicals, reducing the risk of cancer and protecting biological systems against the harmful effects of oxidative processes on macromolecules, such as carbohydrates, proteins, lipids and DNA. They protect the human cells from the damage caused by unstable molecules known as ROS (reactive oxygen species) and free radicals. In conclusion, phenolic acids have diverse biological activities, for instance, antiulcer and anti-inflammatory, antidiabetic, antiviral, antioxidant, cytotoxic and antitumor. The main phenolic acids found in olive leaves are shown below.

Table 4: Phenolic acids and derivatives found in olive leaves *[10]*

Phenolic acids and derivatives
Caffeic acid, vanillic acid, homovanillic acid, syringic acid, gallic acid, ferulic acid, caftaric acid, quinic acid, chlorogenic acid.

1.4.1.4 Secoiridoids and derivatives

Secoiridoids are a group of phenolic compounds that have been reported in olive leaves and other organs of the olive tree. They are produced from the secondary metabolism of terpenes. Secoiridoids are characterized by elenolic acid or its derivatives in its aglyconic or glycosidic form in their molecular structure [17]. The most important secoiridoid found in the olive tree and present in a high amount in unprocessed olive fruit and leaves is oleuropein. Oleuropein is responsible for the bitter taste of olive leaves and immature and unprocessed olives. During maturation of fruit or olive processing (such as oil production), oleuropein concentration is reduced because of chemical and enzyme reactions. It results from these reactions that the amount of hydroxytyrosol increases, which is the oleuropein's primary degradation product.

Oleuropein consists of three structural subunits: a polyphenol named 4-(2-hydroxyethyl) benzene-1, 2-diol, also known as hydroxytyrosol, a secoiridoid called elenolic acid and a glucose molecule (**Figure 5**). Oleuropein is the most prominent phenolic compound in olive cultivars and may reach concentrations of up to 140 mg g^{-1} on a dry matter basis in young olives and 60-90 mg g^{-1} of dry matter in leaves. Oleuropein is a bioactive compound with numerous health benefits. First of all, it has antioxidant, bactericidal and bacteriostatic activities and promotes blood platelet aggregation inhibition. Also, it has been reported that oleuropein has an antiatherogenic, anti-cancer, anti-inflammatory and neuroprotective effect. These properties make the oleuropein a phenolic compound with many potential applications in the pharmaceutical and food industry [18].

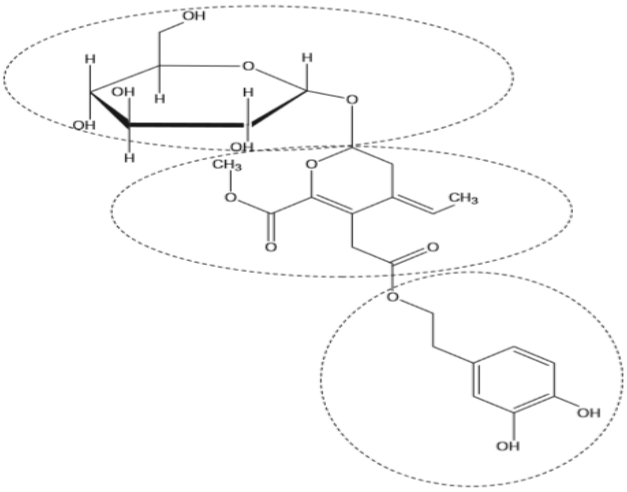


Figure 5: Molecular structure of oleuropein molecule *[18]*

Table 5: Secoiridoids found in olive leaves *[10]*

Secoiridoids and derivatives
Secologanoside, oleoside, oleuropein, 6′-O-[2,6-dimetyl-8-hydroxy-2octenoyloxi], secologanoside, syringaresinol, lucidomoside D, demethyloleuropein, oleuropein, oleuropein aglycon, oleoside methyl ester, oleuroside, oleuropein diglucoside, 2″-methoxyoleuropein, hydroxyoleuropein, elenolic-7O-glucoside, ligstroside, elenolic acid

1.4.2 Lignans

Lignans are polyphenols that belong to the phytoestrogen family, found in different sources in the plant kingdom. Polyphenols are secondary metabolites involved in the defense against ultraviolet radiation and pathogens [19]. The three lignans found in olive leaves are pinoresinol, acetoxypinoresinol and syringaresinol [10]. Pinoresinol ($C_{20}H_{22}O_6$) and 1-acetoxypinoresinol ($C_{22}H_{24}O_8$) possess two phenol groups in their chemical structure, forming a dimer. These phenol groups consist of an aromatic ring (phenyl or benzene group) bound to a hydroxyl group (OH). The phenol and benzene rings are

associated with several health benefits in humans, including antioxidant and/or anti-inflammatory effects [19].

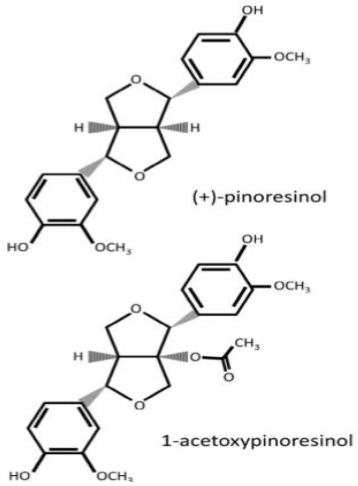


Figure 6: Chemical structures of (+)-pinoresinol and 1-acetoxypinoresinol *[19]*

1.4.3 Terpenes and derivatives

Triterpene compounds are one of the most important fractions of bioactive compounds isolated from plants. Olive leaves are a great source of these valuable compounds, containing great amounts of oleanolic acid, followed by significant concentrations of maslinic acid and minor erythrodiol, uvaol and ursolic acid levels. The amount of triterpenoids are higher in the olive leaf than in the fruit. In the fruit, triterpenes are exclusively located in the epicarp at concentrations 30-fold lower than that in the leaf, with maslinic and oleanolic acids as the significant triterpenes. Also, both olive leaves and fruit contain a great variety of triterpenic compounds in their epidermis. More precisely, triterpenic acids are found as free acids, while the pentacyclic triterpenoids can be free or esterified with fatty acids. Although, the development stage of the olive tree influences the concentration and profile of these triterpenoids.

It has been reported that oleanolic acid (3β-hydroxy-olean-12-en-28-oic acid) and its isomeric, ursolic acid (3β-hydroxy-ursan-12-en-28-oic acid) have not only strong antioxidant activity but also antiviral (including anti-HIV), antibacterial, antifungal,

antiallergic, anti-inflammatory, hepatoprotector, anticarcinogenic gastroprotector, antiatherosclerotic, antidiabetic and hypolipidemic effects. Other pentacyclic triterpenes seem to share similar pharmacological activities [20].

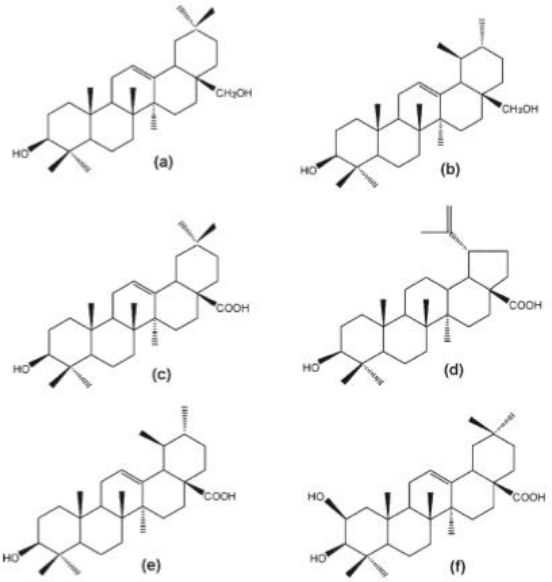


Figure 7: Chemical structures of the main triterpenic compounds found in olive fruits and leaves: (a) erythrodiol, (b) uvaol, (c) oleanolic acid, (d) betulinic acid, (e) ursolic acid, (f) maslinic acid *[20]*

1.4.4 Fatty Acids

Fatty acids are phytochemicals with many nutraceutical uses. One of the most important groups of fatty acids is the omega-3 and omega-6 fatty acids. They are polyunsaturated fatty acids that play an essential role in the human diet and human physiology.

The most common fatty acids found in olive leaves are linolenic acid (C18:3) [30.02–42.16%], oleic acid (C18:1) [18.28– 26.36%], linoleic acid (C18:2) and palmitic acid (C16:0) [18.22–22.42%]. The fatty acid composition varies between different varieties. Linolenic acid is the predominant fatty acid in olive leaves. It is a polyunsaturated omega-3 fatty acid that is metabolized to eicosapentaenoic acid, a compound with antithrombotic and anti-inflammatory activity. Furthermore, many researchers linked the consumption of linolenic acid with a lower risk of cardiovascular diseases. Another popular fatty acid known for its beneficial properties for the skin is linoleic acid. It has moisturizing, anti-

inflammatory activity and contributes to the reduction of acne. That is the reason it is often used in making creams, soaps and emulsifiers. Furthermore, olive leaf extracts containing polyunsaturated fatty acids constitute a potential source of fatty acids that can be used to manufacture dietary supplements. Other fatty acids of olive leaves found in minor levels are myristic acid (C14:0), palmitoleic acid (C16:1), and stearic acid (C18:0) [21].

1.4.5 Minerals

The mineral composition of olive leaves varies between different varieties and also depends on soil composition. More specifically, the soil's nutrient status and the environmental effects around the tree in which the olive tree grows affect olive leaves' mineral contents. Calcium, magnesium, aluminium and iron are the primary elements that their concentration depends on the soil composition, indicating a direct and important uptake of these elements from the soil. Nevertheless, some element contents in leaves do not show any relationship with the soil's element contents (Ce, Cr, Hg, Nd, Pb, and Zn). These differences have been attributed to the different availability of the elements in the soil. The mineral elements observed in higher concentrations in different olive leaves varieties are Al, Ca, Fe, K, Mg, P and S.

Most scientific publications show that calcium is the predominant mineral with concentrations ranged from 9.25 to 10.39 $mg \cdot g^{-1}$ dry mass. Calcium is an essential mineral because it helps to build and maintain strong bones and teeth. It also enhances the use of other nutrients.

Another mineral found in high concentrations in olive leaves is potassium, ranging from 4.47 to 9.14 $mg \cdot g^{-1}$ d.m. Potassium is a micronutrient that helps regulate fluid balance, muscle contractions and nerve signals. It also essential for heartbeat regulation. An adequate intake of calcium and potassium prevents cardiovascular diseases. Finally, calcium and potassium in olive leaf powder extracts could enhance the beneficial effects on other phytochemicals' health, such as phenolic compounds and tocopherols.

The magnesium contents usually vary from 1.5 to 3 $mg \cdot g^{-1}$ in most olive leave varieties, while sodium is found in minor concentrations [21].

1.4.6 Sugars

During photosynthesis in green plants, carbohydrates transform into monosaccharides, which are then transformed into disaccharides, trisaccharides, and sugar alcohols. Sugars play an essential role in plants since they are the main energy source for different metabolic changes. They are also the precursors in the biosynthesis of polysaccharides and lipids, proteins, and antioxidants. For example, some researches have reported a positive relationship between the oil content and the amount of sugars that act as precursors in lipid biosynthesis during olive fruit maturation. Another example refers to the processing of table olives, during which sugars act as a carbon source for microorganisms to release secondary metabolites responsible for the distinctive flavor and positive taste.

Sugars are also responsible for the cell-wall structure of olive leaves tissues. They act as osmoprotectants and osmoregulators of the tolerance response to abiotic stresses. In particular, water deficiency, saline water and soils with high salt content can lead to complex plant responses at the molecular level evidenced by biosynthesis, transport and accumulation of osmolytes. These osmolytes are mostly soluble sugar compounds, including alditols (myo-inositol, mannitol, sorbitol, dulcitol, galactinol, etc.) and saccharides (glucose, galactose, fructose, sucrose, raffinose, stachyose, etc.).

The predominant sugars found in olive leaves are mannitol, glucose, fructose and galactose. These sugars constitute >60% of the total soluble carbohydrates in olive leaves and fruit. This is not surprising because glucose and mannitol are the main transport sugars in olive trees and contribute significantly to osmotic adjustment. Other sugars such as xylitol, 1,6-anhydro-β-D-glucose, arabitol, adonitol, N-acetyl-D-glucosamine and lactose are usually found in low concentrations in most varieties [22].

The most important sugar extracted from olive leaves is mannitol. Mannitol is a sugar alcohol (polyol) used as a food additive due to its ability to reduce the postprandial rise in blood glucose and insulin response. This could be explained by the fact that mannitol has

slow enteric absorption, and also its metabolism is not dependent on insulin. Thus, mannitol and other sugar alcohols have been used safely in the diets of people with diabetes. Moreover, this polyol is a scavenger of hydroxyl radicals and a low-calorie sweetener. Owing to its numerous properties, mannitol is commonly used in the pharmaceutical formulation of chewable tablets and granulated powders. Since mannitol is found in a significant amount in the olive leaves, its production from this natural source can be considered an interesting alternative [23].

Table 6: Sugars found in olive leaves *[10]*

Sugars
D-(−)-arabinose, D-(+)-xylose, D-(+)-glucose, D-(+)-mannose, D-(−)-galactose, D-(−)-fructose, sedoheptulose, 1,6-anhidro-β-D-glucose, D-(+)-sucrose, D-(+)-lactose, D-(+)-raffinose, maltotriose, L-rhamnose, D-(+)-galacturonic acid, D-glucuronic acid, xylitol, L-(−)-arabitol, adonitol (ribitol), D-mannitol, D-(+)-chiro-inositol, myo-inositol, galactinol

1.4.7 Vitamins

Many researches have reported the presence of α-tocopherol and β-carotene in olive leaf extracts. These two vitamins are natural bioactive compounds found in the plant kingdom with many health benefits [10].

A-tocopherol is one of the eight naturally occurring forms of vitamin E synthesized by plants from homogentisic acid. The two major forms of vitamin E are alpha and gamma-tocopherols. Vitamin E is a strong anti-oxidizing agent playing a vital role in the cell antioxidant defense system. It is stored within the fatty tissues of animals and humans because of its fatty-soluble nature, and it does not have to be consumed every day. This vitamin is exclusively obtained from the diet and has numerous vital roles within the body. Researches have shown that vitamin E is effective against many conditions and diseases like cancer, ageing, cataracts and arthritis thanks to its potent antioxidant activity. It has

also been reported that platelet hyperaggregation, which leads to atherosclerosis, can be prevented with the adequate consumption of vitamin E [24].

B-carotene is one of the most common and studied carotenoids. Carotenoids are a group of phytonutrients responsible for the distinctive yellow, orange, and red color in most plants. It is a precursor of vitamin A and is present naturally as a mixture of various isomers (cis and trans). This important carotenoid has the highest provitamin A activity and offers an array of health benefits as it has potential anticancerous, antidiabetic, antioxidant properties and prevents many cardiovascular diseases. It also enhances the immune system and protects from age-related macular degeneration—the leading cause of irreversible blindness among adults [25].

1.4.8 Phytosterols

Phytosterols are steroid compounds present in plants with structure and functions similar to those of cholesterol. They all have a steroid nucleus, a hydroxyl group at carbon 3 in the b-position and a double bond mostly located between the C-atoms five and six in the B-ring. Despite this similar structure, phytosterols are not absorbed in significant quantities. The absorption for cholesterol is 30-60%, while phytosterols are absorbed in a rate of less than 2%. Several animal and human studies showed that phytosterols have various bioactive activities. One of the essential health benefits is their blood cholesterol-lowering effect due to the direct inhibition of cholesterol absorption through the displacement of cholesterol from mixed micelles. Other health benefits reported are anti-atherogenic effects as well as anti-inflammatory and immune-stimulating activities. Many phytosterols have a protective role against various cancer types like colorectal, prostate and breast cancers [26]. The concentration of phytosterols in olive leaves is between 39.2 and 369.1 μg/g. The predominant sterol in most study results is β-sitosterol, and minor compounds are campesterol, stigmasterol, Δ- avenasterol and brasicasterol. Other sterols found in olive leaves are shown in **Table 7** below [27].

Table 7:Phytosterols in olive leaves *[27].*

Phytosterols
β-sitosterol, cholesterol, brassicasterol, 24-methylene cholesterol, campesterol, campestanol, stigmasterol, clerosterol, sitostanol, avenasterol

1.5 The exploitation of bioactive content of olive leaves

In the past few decades, olive leaf extracts have been used in medicines, cosmetics, and pharmaceuticals because of their high concentrations in bioactive compounds. These extracts are also used in the food industry to improve foods' shelf life and produce functional foods such as dietetic bread for people with diabetes. Functional foods can modulate human physiological systems, such as the endocrinal, immunological, nervous, and digestive systems. Many techniques for obtaining these high added value compounds from olive leaves in high quantities have been proposed in the literature, including solid/liquid extraction and fractionation, biotransformation to obtain secondary metabolites (hydroxytyrosol) and supercritical fluid extraction [28].

1.5.1 Food industry

Olive leaves are by-products produced at the first stage of olive oil processing. The recovery and exploitation of their rich bioactive content are drawing food industry attention in the last decades. Different researches have been focused on finding innovative food applications of these compounds to improve the nutritional value of food, extend food products self-life, produce innovative food products, and ameliorate food additives' properties.

Many studies have shown that the enrichment of edible oils using olive leaf extracts increases the radical-scavenging capacity and improves the stability of the thermal degradation of edible oils. Several works used microwave-assisted methods to enrich olive, sunflower and soy oils. Achat et al. (2012) designed an ultrasound-assisted method that can be used to enrich olive oil using dried or ground olive leaves with high extraction yields. More specifically, the supplementation of frying oils with olive leaf extract reduces the development of off-flavors resultant from the oxidation of unsaturated fatty acids and improves the nutritive value of food. A survey conducted by Chiou et al. (2007) showed that potatoes fried in supplemented sunflower, palm and olive oils contained higher concentrations of total tocopherols, polyphenols, squalene and phytosterols. Bouaziz et al. (2010) observed that the addition of oleuropein, oleuropein aglycone and hydroxytyrosol to refined olive oils during storage under accelerated oxidation conditions prevented the oil oxidation. Jaber et al. (2012) experimented with the enrichment of olive oil with chlorophyll pigment extracted from Chemlali olive leaves. The results showed higher oxidative stability and higher concentrations of polyunsaturated fatty acids compared to non-enriched oil. Chatzidaki et al. proposed the encapsulation of these bioactive compounds to improve the unpleasant bitter and pungent taste of enriched oils. The encapsulation can also protect these compounds from degradation, control their release in the food matrix and improve their organoleptic properties. These supplemented oils are promising for the industry of functional foods.

Olive leaf extracts can be used for nutritional enrichment and preserving of many other foods like table olives, meat products and fruits. Lalas et al. (2011) treated debittered table olives with olive leaf extracts with high oleuropein and hydroxytyrosol concentrations for a whole week. The results showed high amounts of these two bioactive compounds, which means higher nutritional value. The only disadvantage was the increase in bitterness because of oleuropein. Moreover, some studies have shown that olive leaf extracts had antimicrobial activity and reduced lipid oxidation in products like raw minced beef, fresh and cooked pork sausages, cold-stored beef cubes and meatballs.

In most cases, the natural antioxidants found in olive leaf extracts improved these products' general quality. However, the protein-polyphenol binding phenomenon is a

parameter that should be considered by scientists. In food products rich in polyphenols and proteins, the complex created by this binding leads to gelation and meat products' emulsification. Lastly, olive leaf extracts have been used to increase the nutritional value of some fruit products. For instance, Ahmad-Qasem et al. (2015) treated dehydrated apple cubes with olive leaf extracts rich in polyphenolic compounds to increase oleuropein concentration. Kranz et al. (2010) developed a fruit smoothie enriched with olive leaf extracts. They experimented with different sodium chloride concentrations, sodium cyclamate, and sucrose to overshadow the extract's bitter taste. Sodium cyclamate showed the best bitterness reduction, followed by sucrose.

The enrichment of common food with olive leaf extracts is a technological challenge for the food industry that searches for innovative, healthier and safe new ingredients attending to consumer market demands [29].

1.5.2 Cosmetic industry

Olive leaves have the highest antioxidant and scavenging power among the different parts of the olive tree. Most of the olive leaf antioxidants are phenolic compounds, with oleuropein being the most abundant. These compounds can be used in many anti-aging skin products due to the limiting biochemical consequences of oxidation.

The exposure of skin to solar ultraviolet radiation and air pollutants results in reactive oxygen species (ROS) and other free radicals. In small concentrations, ROS are essential for intracellular functions. The imbalance between ROS and antioxidants, with ROS being in excess, is known as oxidative stress and is directly connected with aging. Aging is a natural process characterized by the loss of structural integrity and normal function of the skin.

There are many studies focused on the anti-aging properties of olive leaves. Chen et al. (2012) concluded that topical application is the best way to supply the skin with natural antioxidants. Diet and oral supplementation limit the amount of these low–molecular-weight antioxidants that can be delivered in skin because of the metabolic process. Kimura et al. (2009) researched olive leaf extract's effect on chronic UVB-induced skin

damage using hairless mice. These extracts protected the skin from the thickness and reduced elasticity.

Moreover, they reduced the incidence and growth of tumors in exposed skin. Katsiki et al. (2007) examined the anti-aging activity of oleuropein by enhancing proteasome activity. During aging, proteasome has impaired function. They also found that continuous treatment of early passage human embryonic fibroblasts with oleuropein reduces the amount of oxidized proteins through increased proteasome-mediated degradation rates and retains proteasome function during replicative senescence.

Many scientists achieved high extraction yields of phenolic compounds from olive leaves using clean techniques. The combination of pressurized liquid extraction with temperature and low amounts of non-toxic solvents or moderately toxic solvents like water and ethanol-water mixtures is efficient for extracting polyphenols in olive leaves. Furthermore, to guarantee that the compounds of olive leaf extracts used in cosmetic products are non-toxic, stability and toxicity assays should be performed to avoid irritant constituents. These compounds' cytotoxicity can be examined using MTS and LDH assays in different monolayer's skin cell lines. Also, many in vitro three-dimensional model tests have been developed to assess the potential skin or eye irritants by the cosmetic industry, such as the reconstructed human epidermis test (EpiSkin ^{TM}) or the Human Corneal Epithelial Model (SkinEthic^{TM} HCE) [30].

1.5.3 Pharmaceutical industry

In the past few decades, the numerous health benefits of olive leaf extracts have been investigated by scientists worldwide. Many industries invested in research, development and commercialization of dietary supplements and cosmetics from natural sources like olive leaves. Olive leaves are commercialized in the pharmaceutical market as herbal remedies at premium prices. Olive leaves exist on the market in the forms of brown-yellowish powder or liquid extract, and new patents appear daily. In most products, oleuropein is the primary compound representing more than 88-94% of total phenolic compounds. Also, the total polyphenols concentration found in olive leaf extracts showed

high variability. This can be explained by the interaction of the type of cultivar, climate and the geographic production zone. Moreover, the harvesting period, the elaboration process, the extraction process and the stability of bioactive content are factors that affect the levels of total polyphenols [31].

Figure 8: Production of olive leaf extract *[32]*

Most olive leaf extracts used in the pharmaceutical industry are produced by aqueous extraction followed by concentration, purification and vacuum drying. This is a typical method used in most industries because it is less likely to result in toxicological, nutritional, or microbiological hazards. Also, every product released in the market should meet appropriate specifications regarding its identity and potential contaminants. According to the European Pharmacopoeia and some other European Parliament regulations, many analyses indicate the manufacturing process's consistency and demonstrate that heavy metal, pesticide, and microbial levels are below specific limits. Usually, the analysis of olive leaf extracts for heavy metals includes the determination of lead, cadmium, mercury and total heavy metal amount. Microbiological testing is crucial in the industry to check pathogenic microorganisms like Escherichia coli, Staphylococcus aureus, Salmonella, Enterobacteria, yeast and mould that could be really dangerous for human health. Lastly, stability studies indicate that bioactive content concentrations stable for a specific period [33].

CHAPTER 2

DETERMINATION OF PHENOLIC COMPOUNDS IN OLIVE LEAVES

2.1 Introduction

In the last decades, there has been an increasing interest in medicinal and aromatic plants that provide high-added value compounds for the food, cosmetic and pharmaceutical industries. Among them, olive tree leaves used since the ancient years to treat many diseases are great sources of phenolic compounds. Nowadays, many researchers focus on optimizing and validating methods for determining phenolic content in olive leaves. The proper understanding of the different stages of analysis is crucial. These stages include the pretreatment of olive leaves, followed by the extraction and determination of phenolic compounds [5].

2.2 Sample pre-treatment techniques

After collecting olive leaves, the first step is to wash them with distilled water to remove any dust traces. After being thoroughly cleaned, leaves should be dried as soon as possible or used while still fresh [34]. This step is crucial for the stabilization of the by-product. Otherwise, storage and transportation could lead to the undesirable degradation of high-added value compounds. Also, olive leaves should be dried before the extraction of valuable compounds to improve extractability. The drying process should be undertaken in closed equipment with controlled conditions to ensure the final product's quality. The traditional drying methods still practiced are sun or shade drying, but these drying procedures cannot be controlled [2]. Researchers have explored many different drying approaches, but hot air oven drying (OD), freeze-drying (FD), vacuum drying (VD) and microwave drying (MD) have been mostly reported in the literature. [35]. Other methods found in the literature are infrared drying using an infrared dryer, heat pump drying using a pilot-scale heat pump conveyor dryer, hot air drying using a laboratory-

type tray dryer, drying with ultrasound power application using a pilot-scale convective dryer modified to apply power ultrasound and solar drying using a laboratory convective solar dryer [2]

Most researchers reported that microwave drying (MD) is superior to other drying methods (FD, OD, and VD). This method involves placing plant material in a microwave reactor at atmospheric pressure without adding any solvent or water. More specifically, Shahin et al. (2017) found that MD under optimum conditions (2.085 g sample at 459.257 W for 6 min of drying period) gave the best results regarding oleuropein's concentration, the total phenolic and flavonoid content, and antioxidant activity of olive leaves. The higher percentage of retained oleuropein and other phenolic compounds after MD's application was most probably due to the release of high-added value compounds from the plant matrix during the microwave drying.

Freeze drying is recommended as an alternative drying method that results in high bioactive compounds compared to OD and VD. For FD, leaf samples are dried using a freeze drier equipped with a vacuum chamber under reduced pressure for usually 24h. It is a way to avoid thermal degradation while removing the water from the leaves effectively. Moreover, ice crystals in the plant matrix result in greater disruption of plant tissues, thereby leading to better diffusion of low molecular substances like oleuropein. On the other hand, thawing frozen olive leaf samples can reduce oleuropein levels due to the cell membrane's breakage and, consequently, the release of active oleuropein-degrading enzymes. Also, FD is a very costly method because of the low drying speed and high vacuum required, uses very high energy. OD and VD show almost the same performance since drying time is 1-2h for OD and 1-4h for VD. Both drying methods require careful temperature selection because of the thermal degradation of phenolic compounds. OD might be accepted to be better when the energy consumption for vacuum is taken into account.

On the other hand, VD is superior if quality factors are considered. Elhussein et al. (2018) found that at 50 °C there was no significant difference between the OD and VD concerning phenolic, flavonoid and antioxidant activity results, but there was 38% decrease in

oleuropein content with OD. This decrease was owing to the nonenzymatic oxidation of this phenolic component.

Considering issues such as simplicity, short processing time and cost, microwave drying is considered the best selection for drying olive leaves to preserve phenolic compounds during the storage period. In most cases, dried olive leaves are grounded to obtain powder or crushed into smaller pieces (particle's diameter ≈1.0 mm) [35], [36], [37].

2.3 Sample extraction techniques

The selection of a proper extraction method is the most critical step in the utilization of bioactive content. Traditional extraction methods are based on heat and/or agitation to increase the mass transfer rate to a suitable leachant. These techniques are generally time-consuming and have low efficiency. In the last few years, "modern" sample-preparation techniques play an important role in the overall effort of ensuring and providing high-quality olive leaf extracts. In order to reduce extraction times and sample preparation costs, techniques like supercritical fluid extraction, superheated liquid extraction, pressurized liquid extraction, derivatized polar extraction, fractionation by solid-phase extraction, dynamic ultrasound-assisted extraction and microwave-assisted extraction techniques have all been used to extract oleuropein and other phenolic compounds from olive leaves. Researchers cannot develop a single method for optimum extraction of all phenolic compounds because phenolic compounds' polarities vary significantly. The selected method, the extraction temperature, solvent and time, are important parameters for the recovery of phenols. Different studies on olive leaves reported water, methanol, ethanol, acetone, and aqueous alcohol mixtures as the usual solvents for polyphenols' extraction [2], [5].

2.3.1 Solid-liquid extraction (SLE)

In the last decades, solid-liquid extraction is mostly used in industrial extraction processes to isolate intracellular compounds and liquids from plant cellular tissues. Solid-liquid

extraction or solvent extraction is a process that separates soluble phenolic compounds by diffusion from olive leaves (solid matrix) using a solvent (liquid matrix). Many factors affect the solvent extraction process's efficiency, such as solvent type, temperature, pH, the number of extraction steps, particle size of the solid matrix, and solvent/solid ratio [38]. Methanol mixtures are recommended for extracts with high levels of flavonoids. Bouaziz et al. (2008) concluded that extracting the sample in 80 % methanol has been the most efficient method for recovering olive leaves polyphenols.

On the other hand, Malik et al. (2008) found that the boiling of dried leaves is a more efficient method for extracting oleuropein and verbascoside that gave 96 and 94 % recoveries of these compounds, respectively, when compared with the methanol extract. In most laboratories, phenols are isolated with traditional methods that involve maceration and Soxhlet extraction with various extractants such as methanol-water mixtures and hexane. However, toxic extractants should be avoided when the extraction process is used on an industrial scale for human products. Also, the extraction times, in most cases, are time-consuming (24-48h). Different treatments like steam blanching or acid hydroxylation can improve the solid-liquid extraction of valuable phenolic compounds using water-based extractants [5].

2.3.2 Soxhlet extraction technique

A conventional Soxhlet system plant material is placed in a thimble-holder and filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the thimble holders' solution and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, the solute is separated from the solvent using distillation. The solute is left in the flask, and fresh solvent passes back into the plant solid bed. The operation is repeated until complete extraction is achieved [39].

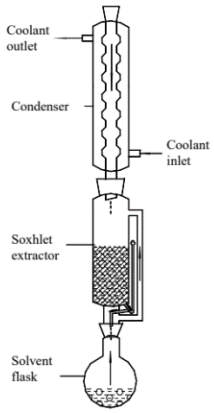


Figure 9: Experimental Soxhlet extraction apparatus [39]

Selecting a suitable extracting solvent is very important because different solvents will yield different extracts and extract compositions. The most widely used solvent used in the Soxhlet extraction technique is hexane. Although the use of alternative solvents such as isopropanol, ethanol, hydrocarbons, and even water, has increased because n-hexane is one of the most hazardous air pollutants. The main advantages of Soxhlet extraction include (1) the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix, (2) maintaining a relatively high extraction temperature with heat from the distillation flask, and (3) no filtration requirement after leaching. Also, this method is really cheap and straightforward. The main disadvantages are the long extraction time, a large amount of solvent used, the danger of thermal decomposition due to the high temperatures used and the fact that agitation cannot be provided in the Soxhlet device to accelerate the process [39]. Ghoreishi et al. (2009) used the Soxhlet extraction method to extract mannitol from olive leaves. The results indicated that mannitol's extraction yield as 57.34 % (w/w) [40].

2.3.3 Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) is a fully automated extraction technique that combines elevated temperature and pressure with conventional solvents. Furthermore,

the final extract is filtered automatically during its collection. Amongst the main advantages of this technique are the gain of extraction time, reduction of solvent volume and use of environmentally-friendly solvents such as water and ethanol. Water and ethanol can be efficiently used in PLE to extract polar to medium polarity analytes [41]. Other factors that affect the extraction efficiency in PLE are the solvent volume to sample mass ratio, the number of extraction cycles and the duration of each cycle [5]. Xynos et al. (2014) applied PLE to extract phenolic compounds from olive leaves using ethanol and water. They found that ethanolic extraction at 190 °C for three consecutive cycles is optimal concerning the extraction yield. Regarding the oleuropein content of the extract, a mixture of H_2O/EtOH (43:57) at 190 °C for one extraction cycle provided the optimal results [41].

2.3.4 Superheated Liquid extraction (SHLE)

The superheated liquid extraction (SHLE) technique is based on using aqueous or organic solvents at high pressure and temperature without reaching the critical point. These conditions accelerate the process of extraction. This method can be applied in three modes: in static mode (with a fixed volume of extractant), dynamic mode (where the extractant flows continuously through the sample) and static-dynamic mode (a combination of the above two modes). SHLE has demonstrated to reduce manipulation, improve selectivity, increase automatability and have an effortless experimental design. It is also an environmentally friendly technique because non-toxic or moderately toxic extractants such as water or ethanol-water mixtures can be used. Japon-Lujan et al. (2006) used a static–dynamic superheated extraction approach to extract phenolic compounds from olive leaves. Results showed that only 13 min is necessary to extract up to 23,000 mg/kg of oleuropein under the optimal working conditions. High amounts of oleuropein, verbascoside, apigenin-7-glucoside and luteolin-7-glucoside were extracted successfully with this technique [42].

2.3.5 Supercritical fluid extraction (SFE)

SFE is another sustainable green technology appropriate for the extraction of phenolic compounds. The most frequently used supercritical fluid is carbon dioxide because it does not contain free oxygen, and thus, the extracts undergo limited oxidative damage. Other important properties of supercritical fluids are their low viscosities and surface tensions and their high diffusion coefficients. Moreover, they provide high solubility and improved mass transfer rates. The operation of SFE can be manipulated by changing the temperature and pressure. The most important advantage of this extraction method in terms of its being used as an end-process is that it affects the quality of the products to a lesser extent than other techniques such as evaporation and distillation, usually applied for these purposes. All these advantages made SFE a standard extraction technique for studying herbal, food and agricultural samples [5]. An example of using countercurrent supercritical fluid extraction in olive leaf samples is an experiment performed by Floch et al. (1998). Olive leaf samples (30 mg) were subjected to SFE, using carbon dioxide modified with 10% methanol at 334 bar, 100°C (CO_2 density 0.70 g ml−1) at a liquid flow-rate of 2 ml min−1 for 140 min. SFE was found to produce higher phenol recoveries than sonication in liquid solvents such as n-hexane, diethyl ether and ethyl acetate [43].

2.3.6 Microwave-assisted extraction (MAE)

In MAE, microwave energy is used to heat the solvents in contact with solid samples and extract compounds of interest from sample to solvent. Microwaves increase the cell's internal pressure and subsequently facilitate the cellular wall's rupture and the release of active compounds to the solvent [44]. The appropriate choice of solvent is crucial for achieving an optimal extraction in MAE. The solvent choice for MAE depends on the solubility of the target analyte, the interaction between solvent and plant matrix, microwave absorbing properties of the solvent including dielectric constant (the ability of absorption of microwave energy), dielectric loss (the efficiency of converting microwave energy into heat) and dissipation factor. The MAE method has many advantages

compared to conventional methods like maceration due to its reduced extraction time, higher extraction efficiency, less labor and high extraction selectivity, making it a desirable method to extract phenolic compounds from olive leaves. Rafiee et al. (2011) found that the same phenolic content can be obtained from olive leaves using MAE with water as an extractant in the maceration method with alcoholic solvents [45].

2.3.7 Ultrasound-assisted extraction (USAE)

Ultrasonic-assisted extraction (USAE) is an effective extraction technique that can be used for a wide range of compounds found in different matrices. Ultrasounds can disrupt plant cell walls leading to improved release of the target compounds from several natural sources. Besides, there is also the oxidative energy of radicals created during sonolysis of the solvent (hydroxyl and hydrogen peroxide for water), which results in high extractive power of ultrasounds. There are many advantages and benefits of this technology for natural compounds extraction. More precisely, it offers greater penetration of the solvent into cellular material, short extraction time, higher yields of analytes and reproducibility, lower consumption of solvents, high processing throughput, a decrease of noise, significant savings in maintenance, less energy needed for processing, and finally, due to the mentioned facts, greener and cumulative cheaper processing [46].

2.4 Analytical techniques

The most used technique for determining phenolic compounds in olive leaves is high-performance liquid chromatography, mainly in the reversed-phase mode coupled to several detectors (UV-VIS, MS, NMR). Rare are the studies that reported the use of gas chromatography to characterize phenolics in olive leaves. Gas chromatography-MS techniques are preferred for determining minor leaf phenolics when identification is needed [34].

2.4.1 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) is a highly improved form of column liquid chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through up to 400 atmospheres under high pressures. That makes it much faster [47]. The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time "on-column." Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved [48].

There are the following variants of HPLC, depending upon the phase system (stationary) in the process:

Normal Phase HPLC: This method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and the non-polar mobile phase. Therefore, the stationary phase is usually silica, and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures.

Reverse Phase HPLC: The stationary phase is nonpolar (hydrophobic) in nature, while the mobile phase is a polar liquid, such as mixtures of water and methanol or acetonitrile. It works on the principle of hydrophobic interactions; hence the more nonpolar the material is, the longer it will be retained.

Size-exclusion HPLC: The column is filled with a material having precisely controlled pore sizes, and the particles are separated according to their molecular size. Larger molecules are rapidly washed through the column, while smaller molecules penetrate inside the porous of the packing particles and elute later.

Ion-Exchange HPLC: The stationary phase has an ionically charged surface of the opposite charge to the sample ions. This technique is used almost exclusively with ionic or ionizable samples. The stronger the charge on the sample, the stronger it will be

attracted to the ionic surface, and thus, the longer it will take to elute. The mobile phase is an aqueous buffer, where both pH and ionic strength are used to control elution time [47].

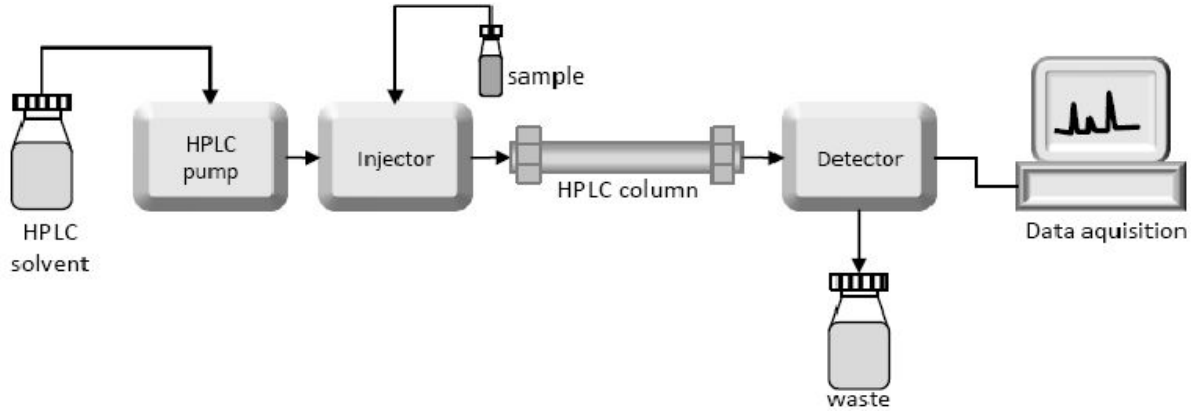


Figure 10: Instrumentation of HPLC [47]

As shown in **Figure 10** above, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs. The different parts of an HPLC system are:

1. Solvent Reservoir: Mobile phase contents are contained in a glass reservoir. In HPLC, the mobile phase, or solvent, is usually a mixture of polar and non-polar liquid components whose respective concentrations are varied depending on the composition of the sample.
2. Pump: A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on several factors, including column dimensions, the stationary phase's particle size, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.
3. Sample Injector**:** The injector can be a single injection or an automated injection system. An HPLC system injector should provide an injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi).

4. Columns: Columns are usually made of polished stainless steel, are between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 3–10 μm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns. Ideally, the temperature of the mobile phase and the column should be kept constant during an analysis.

5. Detector: The HPLC detector, located at the end of the column, detect the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

6. Data Collection Devices**:** Signals from the detector may be collected on chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the detector's response to each component and places it into a chromatograph that is easy to read and interpret **[47]**.

2.5 Detectors

2.5.1 UV-VIS detection systems

UV-Vis detection systems are the most used detection systems for phenolic compounds. Detection is based on ultraviolet spectra characteristics, so that photodiode array became an indispensable tool in leaf phenolic studies. The method's general use at a wavelength (280nm) is preferred in most works [34]. It should be considered that no single wavelength is appropriate for monitoring all target phenolics, as they display absorbance maxima at different wavelengths in the UV region (where they tend to show high absorption). Thus, in most publications, the chromatograph of oleuropein is monitored at 280 nm, verbascoside at 330 nm, apigenin-7-glucoside at 340 nm and luteolin-7-glucoside at 350

nm using a diode array detector. These analytes are the most abundant in olive leaves, so they were selected as response variables [49].

A DAD detects the absorption in UV to VIS region. A diode array detector is a piece of apparatus consisting of a two-dimensional diode pattern and a prism used to detect organic compounds. As the sample passes through a cell or cuvette, it is illuminated with light in the region 190--1100 nm and any light transmitted through the sample is dispersed by the prism so that light of different wavelengths falls on different diodes. The array's output is used to construct an absorption spectrum compared with standard spectra for identification purposes. Alternatively, a single diode can be used to monitor a specific wavelength at which there is maximum absorption [50].

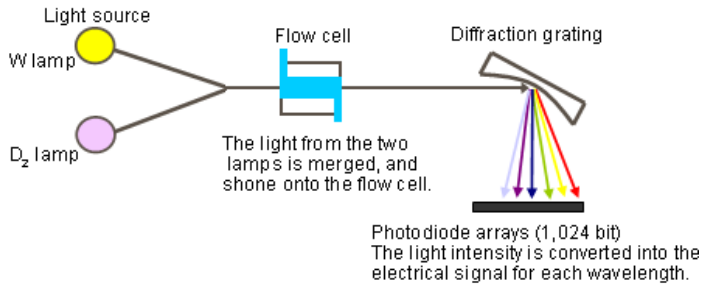


Figure 11: Diagrammatic illustration of a DAD optical system [50]

2.5.2 Mass spectrometry

Liquid chromatography (LC) hyphenated to mass spectrometry (MS) detection is one of the most important analytical techniques used to analyze phenolic compounds. The most employed tool to determine phenolic compounds from olive leaves is the on-line coupling of HPLC with MS using electrospray ionization (ESI) in negative mode. First of all, MS provides higher selectivity than spectrophotometric detection. It is also a really powerful method because of its high efficient resolution and characterization of a wide range of polar compounds. ESI is one of the most versatile ionization techniques and is the preferred one for detecting polar compounds separated by liquid chromatography. The advantages of MS detection include the ability to determine the molecular weight and to obtain structural information. Furthermore, high-resolution mass analyzers like TOF-MS

can provide excellent mass accuracy over a wide dynamic range and allow the isotopic pattern measurements to provide important additional information to determine the elemental composition [51].

2.5.2.1 Electrospray ionization (ESI)

ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. ESI-MS can thus analyze ionic species in solution with increased sensitivity. Neutral compounds can also be converted to an ionic form in solution or gaseous phase by protonation or cationisation (e.g., metal cationisation), and hence can be studied by ESI-MS.

The transfer of ionic species from solution into the gas phase by ESI involves three steps: (1) dispersal of a fine spray of charged droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplet tube, which is maintained at a high voltage (e.g., 2.5 – 6.0 kV) relative to the wall of the surrounding chamber. A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The application of a nebulizing gas (e.g., nitrogen), which shears around the eluted sample solution, enhances a higher sample flow rate. The charged droplets, generated at the exit of the electrospray tip, pass down a pressure gradient and potential gradient toward the mass spectrometer's analyzer region. With the aid of elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius. Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase. The emitted ions are sampled by a sampling skimmer cone and then accelerated into the mass analyzer to perform molecular mass and ion intensity measurement. The ESI mechanism is described in greater detail in a recent review. The precursor ions of interest can be mass selected and further fragmented in a collision cell to obtain structural information.

The fragment ions can then be mass analyzed by a second mass analyzer of a tandem mass spectrometer system described below [52].

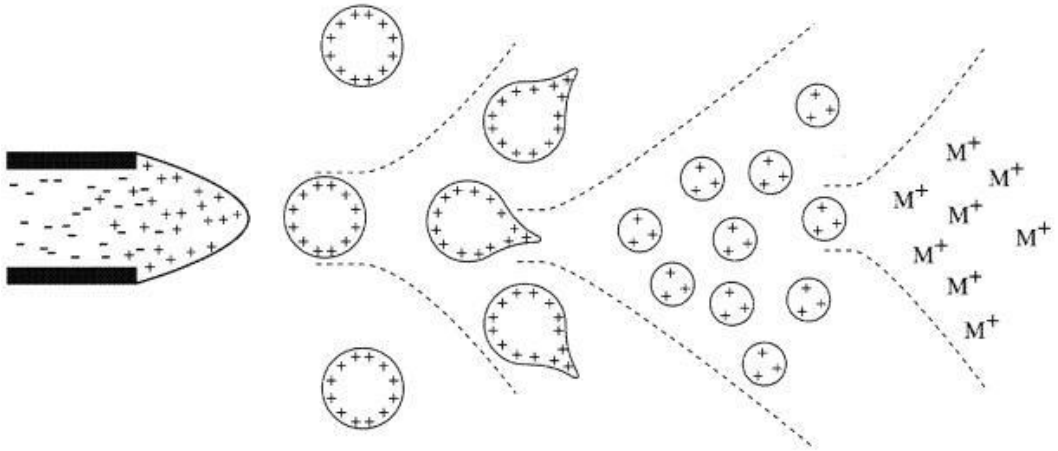


Figure 12: Mechanism of electrospray ionization [52]

2.5.3 Low-Resolution Mass Spectrometry

LRMS measurements provide information about the analyte's nominal mass, i.e., the m/z for each ion is measured to single-digit mass units. The most common LRMS instruments that have been used for the analysis of phenolic compounds are Quadrupole, Triple Quadrupole, and Ion Trap.

2.5.3.1 Quadrupole

A quadrupole analyzer uses radiofrequency alternating current (AC) and direct current (DC) voltages as a mass filter for separating ions. The quadrupole consists of four parallel rods. The positive DC voltage is applied on two opposite rods, and the same value of the negative DC voltage is applied on the remaining two rods. The AC is connected to all four rods. Combined DC and RF potentials on the quadrupole rods can be set to pass only a selected m/z ratio. All other ions do not have a stable trajectory through the quadrupole mass analyzer and will collide with the quadrupole rods, never reaching the detector. The single quadrupole is undoubtedly the most straightforward, cheapest, most robust, and

ubiquitous mass analyzer in research and development laboratories, but it suffers from a limited sensitivity, resolving power and mass accuracy [53].

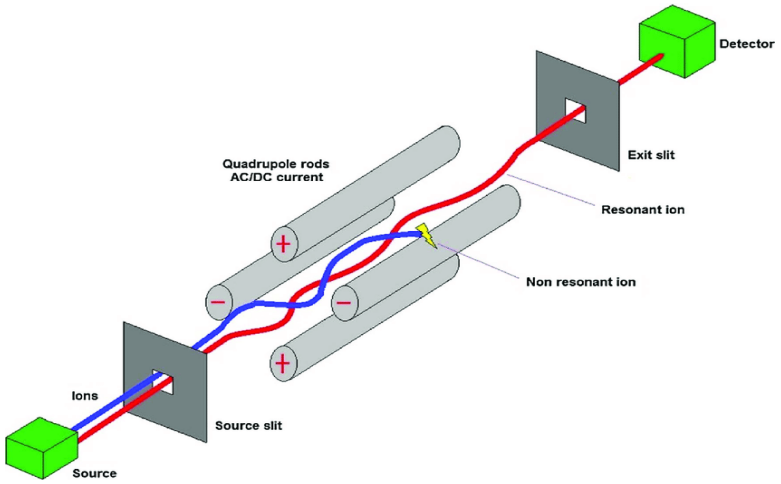


Figure 13: Quadrupole mass spectrometer [54]

2.5.3.2 Triple Quadrupole (QqQ)

A tandem quadrupole mass spectrometer, often called a triple quadrupole, consists of two quadrupole mass analyzers separated by a collision cell. Precursor ions are selected in the first quadrupole mass analyzer. The selected precursor ion is then fragmented in the collision cell through collision-induced dissociation (CID). CID results from collisions of the analyte of interest with an inert gas, such as nitrogen or argon. The specific product ions produced by CID are a function of the bond energies inherent in the molecular structure of the precursor ion, as well as the collision gas and energy used. Product ion patterns and relative ion abundance can be highly reproducible if the CID conditions are stable and robust. The product ions are analyzed or selected by the final quadrupole mass analyzer and then passed to the detector. These pairs of precursor and product ions are called mass transitions [55]. The triple quadrupole mass spectrometer is designed to work under different scan modes: product ion, precursor ion, neutral loss, single reaction monitoring (SRM), multiple reaction monitoring (MRM) and MSn scans [56]. The main benefits of analysis in MS/MS mode are increased selectivity, improved S/N, lower limits of quantitation (LOQ), wider linear range, and improved accuracy.

In the advanced QqQ instruments, the basic linear quadrupole structure is modified with the curved quadrupoles, which offer longer flight paths, and thus, these systems could be used for more accurate (higher-resolution) selection of m/z. The typical quadrupole instruments' unit mass resolution corresponds to 0.7Da (full width at half maximum (FWHM)). However, resolution up to 0.1Da (ultraselective) could be obtained with advanced quadrupole instruments [57].

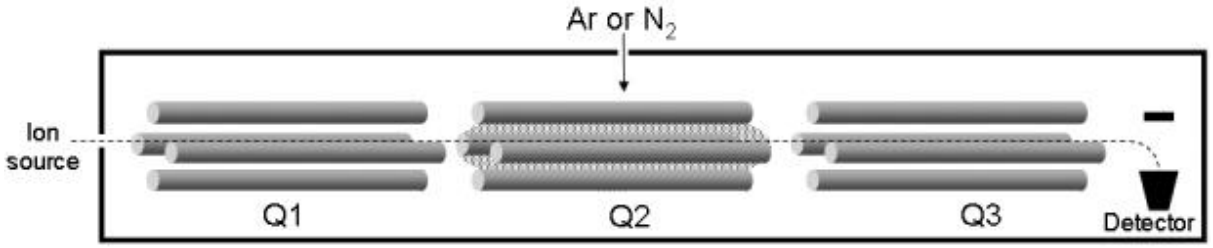


Figure 14: Triple Quadrupole Mass Spectrometer [57]

2.5.3.3 Ion Trap (IT)

Ion trap analyzers use three hyperbolic electrodes to trap ions in a three-dimensional space using static and radio frequency voltages. Ions are then sequentially ejected from the trap based on their m/z values to create a mass spectrum. Alternatively, a specific ion can be isolated in the trap by applying an exciting voltage while other ions are ejected. Inert gas can also be introduced into the trap to induce fragmentation. An interesting feature of these ion trap analyzers is the ability to fragment and isolate ions several times in succession before the final mass spectrum is obtained, resulting in so-called MSn capabilities [57].

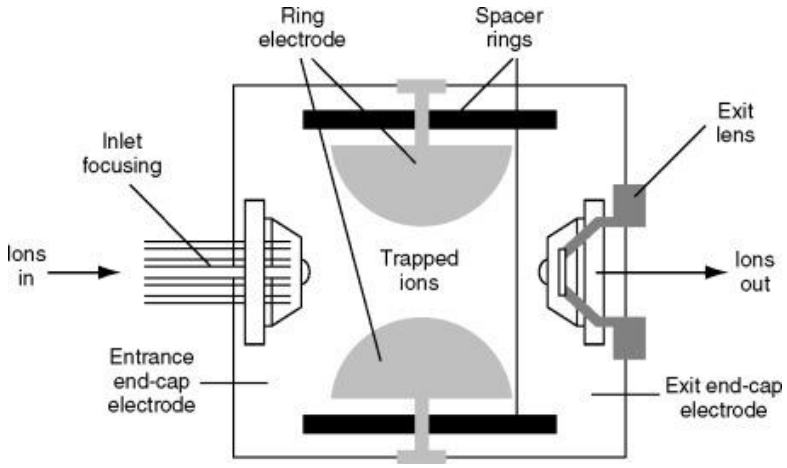


Figure 15: Ion Trap mass analyzer *[58]*

2.5.4 High-Resolution Mass Spectrometry

HRMS provides information about the analyte's exact mass, i.e., each ion's m/z is measured from four to six decimal points. As a result, co-eluting molecules with the same nominal mass can be identified. The HRMS instrument that has been used mostly for the analysis of phenolic compounds in olive leaves is the Time of Flight (TOF) mass analyzer.

2.5.4.1 Time-of-Flight (TOF)

TOF-MS is based on the fact that ions with the same energy but different masses travel with different velocities. Ions formed by a short ionization event are accelerated by an electrostatic field obtaining the same kinetic energy and then travel over a drift path to the detector. The lighter ions arrive before, the heavier ones, and a mass spectrum are recorded. Measuring the flight time for each ion allows for determining their m/z ratios [59]. This cycle is repeated with a repetition rate that depends on the highest mass flight time to be recorded. The enhancement in the mass resolution is obtained by using a reflectron (ion mirror). The reflectron is a series of ring electrodes with an increasing voltage that creates retarding fields. The higher-energy ions reaching the reflectron area penetrate more deeply inside, resulting in an extension of the time until they are reflected. Due to this phenomenon, the same m/z value ions with different initial energies hit the detector simultaneously. The flight times of the ions separated in a field-free region are proportional to the square root of the respective m/z value [60].

TOF mass analyzers' advantages include their simplicity, ruggedness, high scan speed, a virtually unlimited mass range, and a higher resolution than Q and IT mass analyzers. However, TOF mass analyzers suffer from limitations in resolution related to the relatively large distribution in flight times among identical ions [59].

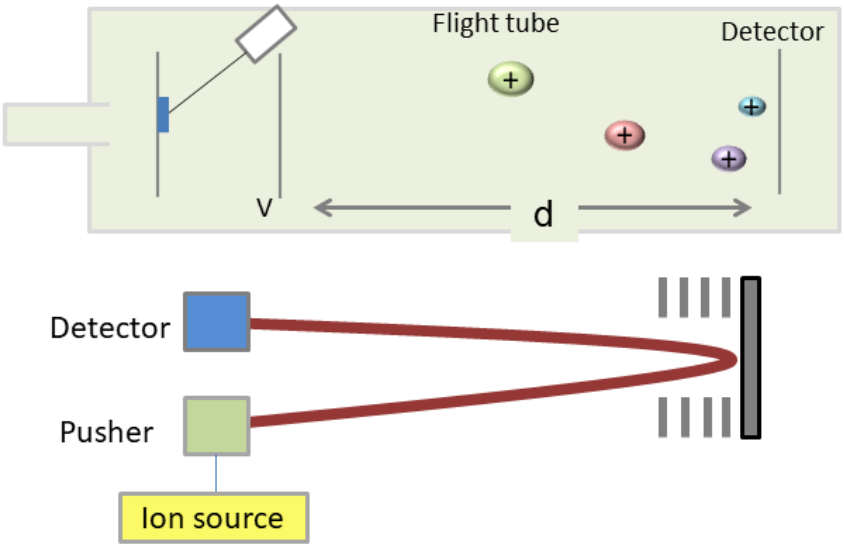


Figure 16: Figure 17: Scheme of TOF instrument *[61]*

2.5.4.2 Hybrid Instruments

The coupling of two different analyzers is known as a hybrid instrument. An example of a hybrid instrument used for the analysis of phenolic compounds is QqTOF. The Q-TOF MS combines the simplicity of a quadrupole MS with a TOF mass analyzer's ultra-high efficiency. In a Q-TOF instrument, the sample is introduced through the interface, and ions are focused using the hexapole ion bridge into the quadrupole MS. Here, the precursor ion is selected for later fragmentation and analysis with a mass window of approximately 3 mass units, a typical window to preserve the isotope envelopes in the product ion spectra. The ions are ejected into the hexapole collision cell, where argon is used for fragmentation. From this point, the ions are collected into the TOF region of the MS/MS. The introduction of ions is such that the ions' flight path changes 90º, which is

called an orthogonal TOF. The purpose of the change in direction is to focus optically on the ions' kinetic energy so that their kinetic energies are as similar as possible. The ions are then accelerated by the pusher and travel about 1 m down the reflectron's flight tube. The purpose of the reflectron is to slow down ions of equal mass but higher kinetic energy and then focus this beam of ions at the detector such that ions of the same mass but slightly different energies arrive at the detector at the same moment. This process results in the mass accuracy of the Q-TOF MS/MS.

Thus, the TOF side of the Q-TOF MS achieves simultaneous detection of ions across the full mass range at all times. This continuous full-scan mass spectrum contrasts with the tandem quadrupoles that must scan over one mass at a time. That is the reason why Q-TOF MS/ MS is more sensitive when scanning the TOF side of the instrument (estimates are 10^100 times in product literature) in scan mode than the third quadrupole of the triple quadrupole MS/MS. However, it is essential to remember that the TOF side of the Q-TOF MS/MS has the same sensitivity in scan mode and selected-ion mode. On the other hand, this is not true for the triple quadrupole MS/MS, which has increased sensitivity in MRM compared to scan mode. The Q-TOF MS/MS system is considered a high-resolving power instrument capable of 10,000 resolving power expressed at FWHM. The Q-TOF MS/MS unique capability compared to the triple quadrupole and ion-trap MS/MS instruments lie in determining accurate mass on the fragment ions generated in the collision cell. Because the quadrupole allows ions of nominal mass to pass through the quadrupole, masses may interfere with the molecular ion's determination. Interfering ions are much less likely for the fragment ions, which help determine accurate mass by lowering mass interferences and increasing accuracy with the same resolving power [60].

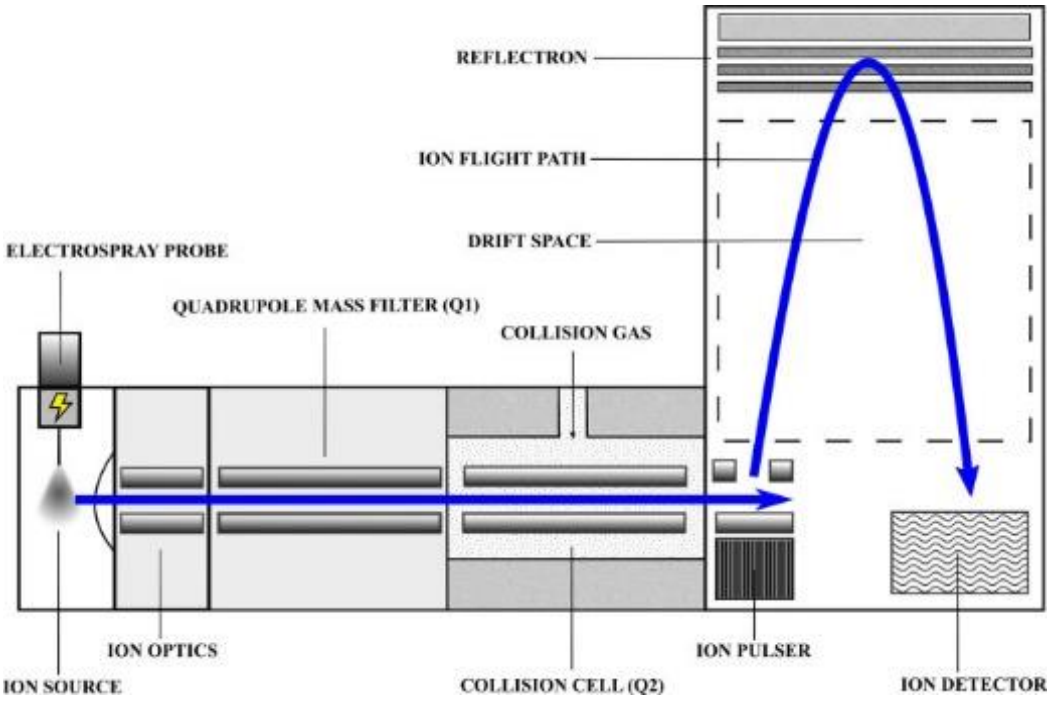


Figure 18: Scheme of QqTOF instrument *[62]*

2.6.1 Data Dependent Acquisition (DDA)

In this acquisition, a full scan is defined as the survey scan and data are processed “on-the-fly” to determine the candidates of interest based on predefined selection criteria, such as intensity threshold or suspect inclusion list. If the selection criteria are met, MS/MS analysis is then triggered, and MS/MS scans (data-dependent) are performed. With this acquisition, ‘clean’ spectra with structural information are obtained in one injection. However, if the number of candidates is enormous, the number of scans decreases, so fewer data points affect the sensitivity of the chromatographic peak [63].

2.6.2 Data Independent Acquisition (DIA)

With this acquisition, there is no need to pre-select the precursor ion. Full-scan spectra at different collision energies are obtained in one injection. This acquisition provides

simultaneously accurate mass data of parent compounds and fragment ions in a single run using two scans, one at low and one at high collision energy. By applying low energy (LE) in the collision cell, no fragmentation is performed. A full-scan spectrum is obtained that provides information for the parent ion (the (de)-protonated molecule) and, in some cases, the adduct ions and the in-source fragments. By applying high energy (HE) in the collision cell, fragmentation is performed, and a spectrum similar to MS/MS experiments is obtained. This approach is called all-ions MS/MS, MSE or bbCID, according to the QTOF manufacturer [63].

2.7 Determination of phenolic compounds in olive leaves-Literature review

An overview of the main analytical procedures based on liquid chromatographic methods hyphenated with mass spectrometry to determine phenolic compounds in olive leaves was performed. Details on the variety of olive leaves used, methods of determination and different sample preparations are presented in **Table 8.**

Table 8: Literature review of LC-MS methods for determination of phenolic compounds in olive leaves

Variety	Sample preparation	Technique	Reference
Sikitita olive leaves (Spanish cultivar)	Outdoor drying -Stored at -80ºC -Crushing and extraction of dry leaves via Ultra- Turrax IKA T_{18} with 10mL MeOH/H_2O (80/20). -Ultrasonic bath for 10 min. -Centrifuged at 1000g for 10min. -Supernatant removed and extraction x2. -Supernatants collected. -Extracts evaporated and reconstituted with 2mL of MeOH/H2O (50/50)	HPLC-DAD-ESI-TOF-MS	[64]
Olea europaea L., variety Hojiblanca	-Leaves were dried in the dark at ambient temperature for 50 days. -the dried leaves were ground up under liquid nitrogen -stored in a dark room at 4ºC until use. -PLE : Ethanol (Obtained at 150 °C) Water (Obtained at 200 °C) Extraction time: 20 min	HPLC–ESI–QTOF–MS	[65]
Tunisian varieties of olive leaves	-washed with distilled water -ground under liquid Nitrogen. -Dried with many methods: MAE, Conventional extraction(MeOH/$H_2$0), PLE and SFE.	HPLC-ESI-TOF/IT-MS	[66]

Buža, Istarska bjelica Rosinjola	-Leaves were left to dry on pruned branches in the air, and under cover, for 2 months -1 g of milled ground plant material and 100 mLpure methanol for 1h, T=40ºC, atm pressure. -Evaporation using a rotary evaporator -dry extracts (50 mg) were dissolved in 5 mL of methanol. - Aliquots (2 mL) were mixed with 2 mL of H2O and 1 mL of trifluoroacetic acid. -vials with reaction mixtures sealed and heated in a water bath for 3 h. -cooling, and evaporation to dryness -Reconstitution in 2 mL of methanol. -solutions were filtered and injected in HPLC/DAD or LC/MS system.	HPLC/DAD LC/MS	[67]
Hardy's Mammoth olive leaves	-Immediately frozen in liquid nitrogen -Freeze drying - Dried leaves cut into small piece - 18ºC before analysis -Dried olive leaves blended with methanol: water (5 mL; 50:50 v/v) for 20 s using an Ultra Turrax blender	HPLC-DAD-ESI-TOF- MS	[68]

	-solution was left for 30 min at ambient temperature and filtered using a Buchner funnel apparatus.		
	-The solid mass was recovered and reextracted as before -stand for 15 min before filtering. The filtrates were combined and washed with hexane (5 mL) -the aqueous phase was filtered with GF/F filter paper using a Buchner funnel apparatus		
10 Greek cultivars	-Boiling of leaves for 3min -blended using an electric blender - 50.0g of the blended material was added to water (250 mL) and boiled for 3 min. -the hot mixture was filtered through a filter paper -the filtrate was extracted once with EtOAc (250 mL). -50.0mL of the EtOAc layer was obtained and evaporated to dryness under reduced pressure to give a solid residue. -(5.0 mg) of solid residues obtained, - added under sonication to a mixture of MeOH (500 mL) and ammonium acetate 0.05 M (500 mL) -mixture was centrifuged at 10,000 rpm for 10 min.	HPLC-DAD-MS	[69]

Tunisian olive variety “El Hor”	-fresh leaves washed with distilled water and ground under liquid nitrogen - samples were stored at −20 °C until use - 1.25 g of fresh-milled olive leaves transferred into the microwave extraction vessels -suspended in 10 mL of the extraction solvent. After extraction, the vessels were cooled to room temperature before opening, using the system's ventilation option. - The obtained extracts were filtered through a 0.45 μm syringe filter before analysis.	HPLC-ESI-TOF-MS/IT-MS^2	[70]
Arbosana Arbequina Picual Sikitita Changlot Real Koroneiki	-Leaves dried outdoors and stored at -80 ºC. -Dry leaves (0.5 g) crushed and extracted via Ultra-Turrax IKA® T18 basic using 30 mL of MeOH/H2O (80/20). -After solvent evaporation, the extracts were reconstituted with 2 mL of 99 MeOH/H2O (50/50).	HPLC-DAD-ESI-TOF- MS	[71]
the olive cultivar ‘El Hor’ from the Center of Tunisia.	-After collection, leaves were washed with distilled water -Portions of leaves immediately stored at −80 ◦C, and other portions were dried either at room	HPLC-ESI-TOF-MS	[17]

	temperature in a programmable mechanical convection oven (Binder Gmbh) or various temperatures of 40, 60, 80, 100, and 120 ◦C. -Leaves were ground using an Ultra Centrifugal Mill ZM 200 (Retsch Gmbh, Germany). -10 g of ground olive leaves were homogenized with 15 g of sea sand -SFE(150bar, 40 ºC, CO2) -The obtained extract was collected, and the solvent was evaporated under vacuum at 38 ◦C -A minimum of three replicate extractions was performed for each plant sample, -HPLC analysis.		
Olive leaves from Novi Vinodolski, Primorje-Gorski Kotar County, Croatia	-0.100 g lyophilized, pulverized and homogenized bark and leaf samples were suspended in 10 ml methanol -Suspensions were stirred at 200 rpm for 4 h at room temperature -extraction (x3) -The extracts were filtered and evaporated to dryness at a temperature not exceeding 40 ◦C. -The residues were redissolved in HPLC grade Merck methanol and filtered through a 0.45 m syringe filters	LC-TOF-MS LC–ESI/MS/MS	[72]

CHAPTER 3

SCOPE

Olive leaves, a by-product derived from the cultivation of olive trees, are rich in phenolic compounds and can be found in high amounts in the olive oil industry (leaves represent around 10% of the total weight of olives arriving at the mill). Over the last decades, the interest in recovering polyphenols from olive leaves has increased due to the numerous health benefits associated with these compounds. Olive leave is an inexpensive, renewable and abundant source of biophenols, so the olive extract has already been used in dietary supplements, nutraceuticals, functional food ingredients and cosmetic products. The extraction process of phenolic compounds from the olive leaf matrix is complicated due to their low stability. An efficient extraction protocol is of paramount importance for exploiting olive leaves' bioactive content in different applications. The olive leaf composition can be affected by several factors such as the variety, sampling period, cultivar, age of the olive tree and climatic changes.

This study aimed to develop and validate a novel methodology for determining phenolic compounds in olive leaves and applying the method in different samples from Lesvos Island. Finally, a method for the targeted screening of many phenolic compounds and assessing the olive leave samples' phenolic profile was created.

Firstly, a generic extraction protocol for the determination of polyphenols in olive leaf samples was applied. However, this method's optimization was followed since various operational variables can affect the studied analytes' extraction efficiency. The optimization step included 3 significant parameters: the need for a drying step, selecting the appropriate solid to solvent ratio and the best extractant composition. After the method development, validation ensued in order to test the reliability and reproducibility of the method. Several performance characteristics (linearity, sensitivity, trueness, matrix effect and precision) were evaluated. Finally, 15 phenolic compounds were determined in the samples.

CHAPTER 4

MATERIALS AND METHODS

4.1 Reagents, standards and solvents

For the UHPLC-ESI-QToF analysis, the solvents used were:

- Methanol (MeOH hypergrade for LC-MS, Sigma-Aldrich)
- Ultrapure water (18.2 MΩ cm^{-1}, produced by a Milli-Q water purification system)
- Ammonium acetate (Fluka, Sigma-Aldrich).
- Sodium formate (Sigma Aldrich)

For the extraction protocol, the solvents used were:

- Methanol (MeOH HPLC- grade, Fischer Scientific (Geel, Belgium).
- Ethanol (EtOH HPLC-grade, Fischer Scientific (Geel, Belgium).

The standards used for the analysis of phenolic compounds using LC-QTOF-MS were the following:

Syringic acid (purity 95 %), eriodictyol (purity 99%) and taxifolin (purity 99%) were purchased from Extrasynthèse (Genay, France), 4-hydroxybenzoic acid (purity 99%), 3,4-dihydroxybenzoic acid (purity 97%), 2,5-dihydroxybenzoic acid (purity 99%), salicylic acid (purity 99%), vanillic acid (purity 97%), gallic acid (purity 98 %), ferulic acid (purity 98 %), p-coumaric acid (4-hydroxycinnamic acid; purity 98 %), quercetin (purity 98 %), pinoresinol (purity 95%), rutin (purity 90%), eudesmic acid (95%), quinic acid (purity 98%), verbascoside (purity 86%), diosmetin (purity 98%), homovanillic acid (purity98%), sinapic acid (purity 98%), oleuropein (purity 98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Whereas hydroxytyrosol (purity 98%), luteolin (purity 98 %) and abscisic acid (purity 97%) were purchased from Santa Cruz Biotechnologies. Also, caffeic acid (purity 99 %), vanillin (purity 99 %), apigenin (4,5,7-trihydroxyflavone; purity 97 %), tyrosol [2-(4-hydroxyphenyl) ethanol, purity 98 %] and naringenin (4',5,7-Trihydroxyflavanone; 97%) were acquired from Alfa Aesar (Karlsruhe, Germany). Cinnamic acid (purity 99%) was

purchased from Merck (Hohenbrunn, Germany). Finally, the analytes ligstroside aglycone and oleocanthalic acid were isolated from olive oil samples in the lab of Pharmacognosy and Natural Products Chemistry in the Department of Pharmacy of the National and Kapodistrian University of Athens.

- Stock solutions

Standard stock solutions for each analyte were prepared at the concentration of 1000 mg/L. So, 0.01g of each analyte was weighed and diluted in MeOH in a 10 mL volumetric flask. The solutions were stored at −20 °C in amber glass bottles to prevent photodegradation.

- Working solution

An intermediate working solution of 20 mg/L consisting of 32 analytes was prepared by dilution of standard stock solution in MeOH. This working solution was diluted in MeOH:H2O 50:50 in order to construct a calibration curve at 5 concentration levels (0.5, 1, 2, 5 and 10 mg/L. All working solutions were stored in the refrigerator.

4.2 Sampling and storage

Twenty-four olive leaf samples were collected from Lesvos Island in Greece. Sampling took place in different places of Lesvos, and samples belonged to Adramitiani and Kolovi varieties, which are the most important varieties cultivated in this region. Samples were stored in the dark and preserved in a cold room before analysis. Details considering geographical origin and variety of samples are presented in the following Table 9.

Table 9: Characterization of greek olive leaf samples

a/a	Code	Variety	Region	Country
1	F1	Adramitiani	Lesvos	GREECE
2	F2	Kolovi	Lesvos	GREECE
3	F3	Adramitiani	Lesvos	GREECE
4	F4	Kolovi	Lesvos	GREECE
5	F5	Kolovi	Lesvos	GREECE
6	F6	Adramitiani	Lesvos	GREECE
7	F7	Kolovi	Lesvos	GREECE
8	F8	Adramitiani	Lesvos	GREECE
9	F9	Kolovi	Lesvos	GREECE
10	F10	Kolovi	Lesvos	GREECE
11	F11	Adramitiani	Lesvos	GREECE
12	F12	Kolovi	Lesvos	GREECE
13	F13	Kolovi	Lesvos	GREECE
14	F14	Adramitiani	Lesvos	GREECE
15	F15	Adramitiani	Lesvos	GREECE
16	F16	Adramitiani	Lesvos	GREECE
17	F17	Adramitiani	Lesvos	GREECE
18	F18	Kolovi	Lesvos	GREECE
19	F19	Kolovi	Lesvos	GREECE
20	F20	Adramitiani	Lesvos	GREECE
21	F21	Kolovi	Lesvos	GREECE
22	F22	Adramitiani	Lesvos	GREECE
23	F23	Adramitiani	Lesvos	GREECE
24	F24	Kolovi	Lesvos	GREECE

4.3 Sample preparation

Initially, olive leaf samples were cut into smaller pieces (particle's diameter ≈1.0 mm). Then they were dried using a microwave oven for 5 minutes at 300W. For the extraction process, 10 ml of methanol/water (v/v 80:20) was added in a sample of 0.25g. The sample

was shaken for 15 minutes. To enhance the extraction efficiency, the solution was placed in an ultrasonic bath in the darkness for 15 min at 40°C. After that, the sample was centrifuged at 4000 rpm for 5 min, and the supernatant was collected. The extraction process was repeated once again, and the two extracts were combined. Subsequently, the extract was evaporated till dryness under vacuum employing a rotary evaporator set at 40°C. Finally, it was reconstituted in 1 ml of MeOH:H_2O 50:50 and filtered through a 0.2 μm RC syringe filter. Injection of the sample extract to the RP Chromatographic system ensued.

4.4 UHPLC-HRMS/MS system and analysis

The analysis of olive leaf samples was carried out using an UHPLC-QToF-MS system composed of:

- A UHPLC rapid separation pump system, Dionex UltiMate 3000 (Thermo Fisher Scientific)
- Autosampler
- QToF mass spectrometer, Maxis Impact (Bruker Daltonics)

HRMS Data were processed using Data Analysis 4.4 and TASQ 1.4 software (Bruker Daltonics, Bremen, Germany). The QToF-MS system is equipped with an ESI source operating in negative ionization mode. The chromatographic separation was performed on a reversed-phase (RP) chromatographic system. Specifically, an Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 μm) from Thermo Fisher Scientific, connected to an ACQUITY UPLC BEH C18 1.7 μm, VanGuard Pre-Column from Waters, and thermostated at 30 °C, was used.

Figure 19: The UHPLC-QToF-MS system

For negative ionization mode, the aqueous phase consisted of H_2O: MeOH 90:10 with 5 mM ammonium acetate and the organic phase comprised of MeOH with 5 mM ammonium acetate.

The elution gradient program started with 1% of the organic phase (flow rate 0.2 mL min^{-1}) for one minute, increasing to 39 % in 3 min (flow rate 0.2 mL min^{-1}), and then to 99.9 % (flow rate 0.4 mL min^{-1}) in the following 11 min. These almost pure organic conditions were kept constant for 2 min (flow rate 0.48 mL min^{-1}), and then initial conditions were restored within 0.1 min, kept for 3 min, and then the flow rate decreased to 0.2 mL min^{-1} for the last minute. The injection volume was set to 5 µL.

The operation parameters of ESI were as follows: capillary voltage, 3000 V; endplate offset, 500 V; nebulizer pressure, 2 L min−1 (N_2); drying gas, 8 L min−1 (N_2); and drying temperature, 200 °C

All the samples were analyzed in full scan mode. The QTOF-MS system was operating in broadband collision-induced dissociation (bbCID) acquisition mode and recorded

spectra over the range m/z 50−1000 with a scan rate of 2 Hz. The Bruker bbCID mode provides MS and MS/MS spectra at the same time working at two different collision energies; at low collision energy (4 eV), MS spectra were acquired, where all ions from the preselected mass range are heading towards the flight tube without isolation at the quadrupole, and there is no collision-induced dissociation at the collision cell. At high collision energy (25 eV), the ions from the preselected mass range are fragmented at the collision cell.

A QTOF-MS external calibration was performed daily with a sodium formate solution, and a segment (0.1−0.25 min) in every chromatogram was used for internal calibration, using a calibrant injection at the beginning of each run. The sodium formate calibration mixture consists of 10 mM sodium formate in a mixture of water/isopropanol (1:1). The exact theoretical masses of calibration ions with formulas $HCOO(NaCOOH)_{1\text{-}14}$ in the range of 50−1000 Da were used for calibration.

CHAPTER 5

METHOD DEVELOPMENT AND VALIDATION

5.1 Method development

Olive leaves are a plant matrix with a complex composition that encompasses many different groups of high-added value compounds such as polyphenols. The recovery of phenolic compounds from olive leaves is an important challenge for food and pharmaceutical industrial applications. That is why it is essential to develop the optimal pretreatment and extraction conditions to obtain the desired phenolic compounds efficiently [38]. In this study, the method development was based on a generic extraction protocol, found in a research paper published by Talhaoui et al. (2014) [64]. The tested factors, including different drying methods of olive leaf samples, initial sample weight (solvent/solid ratio) and extractant composition, to achieve the best recovery. The analytes used during optimization experiments were: apigenin, elenolic acid, hydroxytyrosol, luteolin, oleokoronal, oleuropein, eriodictyol, oleuropein aglycone, quercetin, oleacein, oleocanthal, oleomissional, p-coumaric acid, rutin, salicylic acid, taxifolin, verbascoside, 3.4 dihydroxybenzoic acid, 4-hydroxybenzoic acid, caffeic acid, cinnamic acid, ferulic acid, gallic acid, homovanillic acid, ligstroside aglycone, syringic acid, tyrosol, vanillic acid, vanillin and naringenin.

5.1.1 Selection of the appropriate drying method

Drying is the most critical parameter employed before the extraction of high added-value compounds from plant matrices. This technique's application prevents enzymatic degradation and microbial spoilage by reducing the water content of plants [35]. Four different experiments were conducted in an olive leaf sample to evaluate the effect of different drying methods on extraction yield. In the first test, olive leaves were oven dried at 40^{o}C for 48h (2 days). The second test was freeze-dried using a freeze drier equipped

with a vacuum chamber under a reduced pressure of 0.05 mbar at -50oC for 24 hours. In the third experiment, they were microwave dried at 300W for 5 min. In the last test, olive leaves were extracted without any pretreatment step (raw). The selection of the appropriate drying method was based on the absolute response of analytes naturally exist in olive leaves. All determinations were carried out in triplicate, so the average values of response (area) and the relative standard deviation (%RSD) were calculated.

5.1.2 Selection of the appropriate solid to solvent ratio

The assessment of the preferred solid to solvent ratio ensued. The same extraction protocol was applied to three different initial sample weights: 0.25g, 0.5g and 1g of an olive leaf. This study aimed to determine whether a lower solid to solvent ratio leads to a higher diffusion of analytes [73]. The selection was based on each analyte's absolute response after multiplying each test with a suitable factor to compare equalized measurements (for 0.25g, 2 for 0.5g, nothing for 1g). Three replicates for each experiment were conducted, and the average values of response (area) along with the relative standard deviation (%RSD) were calculated.

5.1.3 Selection of the appropriate extractant composition

In the last section, the extraction media's effect was investigated based on the recovery of phenolic compounds and each analyte's matrix effect. Olive leaves were extracted using three different extractants such as ethanol, ethanol/water and methanol/water solvents. These solvents were studied because they are widely used in literature for the extraction of polyphenols. The analytes of interest have a high hydrophilic character due to hydroxyl groups contained in their moiety. Thus they can be extracted much better with high polarity solvents such as water, methanol and ethanol [74], [75].

More precisely, five tests were conducted using 5 different extractant compositions

- Test 1: EtOH:H_2O (60:40)
- Test 2: EtOH:H_2O (80:20)

- Test 3: EtOH 100%
- Test 4: For the first extraction EtOH:H_2O (60:40) and for the second EtOH:H_2O (80:20)
- Test 5: MeOH:H_2O (80:20)

In this case, recovery experiments were conducted in the olive leaf sample after studying its bioactive profile, which was conducted in the previous experiments. Hence the compounds which were not found in the analyzed sample were spiked. An intermediate working solution composed of 15 compounds was prepared and spiked in the olive leaf extract at a final concentration of 20 ppm. The mix was composed of: 4-hydroxybenzoic acid, caffeic acid, catechin, cinnamic acid, ferulic acid, gallic acid, homovanillic acid, ligstroside aglycone, syringic acid, tyrosol, vanillic acid, vanillin, naringenin, oleocanthalic acid and 3,4-dihydroxybenzoic acid. All the experiments were performed in triplicate. The average recovery for each spiked analyte, followed by the relative standard deviation were calculated. On the other hand, some analytes were found in significant amounts in the analyzed olive leaf sample, so the selection, in this case, was based on the mean absolute response of each analyte along with the relative standard deviation as described in the previous sections.

5.2 Validation

Validation of a method is a crucial step after developing a new methodology to assess its performance. It is an essential process as it ensures the reliability of reproducible and comparable analytical data. For this reason, several performance parameters should be tested and evaluated, including linearity, sensitivity, repeatability (precision), trueness and selectivity (matrix effect). For the validation, an olive leaf sample with low phenolic content was fortified with a mixture of analytes. This mixture was composed of: syringic acid, salicylic acid, naringenin, pinoresinol, eriodictyol, 2,5 dihydroxybenzoic acid, cinnamic acid, rutin, gallic acid, vanillin, vanillic acid, luteolin, quercetin, tyrosol, eudesmic acid, quinic acid, verbascoside, p-coumaric acid, diosmetin, apigenin, caffeic acid, ferulic acid, abscisic acid, sinapic acid, oleuropein, hydroxytyrosol and taxifolin. It has to be

noticed that the great majority of the analytes used in the validation process have been cited to exist in olive leaf samples according to literature data. Spiking was performed in 6-fold at three concentration levels: 2, 8 and 40 mg/Kg. Together with the control samples, the fortified samples were processed using the extraction procedure described above. More precisely, the validated parameters were the following.

5.2.1 Linearity

Linearity is the ability of a method to elicit test results that are directly proportional to the analyte concentration within a given range. The instrument's linearity was studied for each compound by analyzing standard solutions in five different levels (0.5, 1, 2, 5 and 10 mg/L). These calibration standards were prepared in methanol: water (50:50). To test the linearity of the method for each compound, matrix standards were prepared in the same concentration levels: 2, 4, 5, 20 and 40 mg/Kg (0.5, 1, 2, 5, 10 mg/L at the final extract) and analyzed in order to create a matrix match calibration curve. Calibration and standard addition curves were estimated using linear regression analysis.

5.2.2 Sensitivity

Concerning the evaluation of the sensitivity, limits of Detection (LOD) and Quantitation (LOQ) for instrument and method were determined theoretically. The term LOD is defined as the lowest concentration at which the instrument can detect but not quantify, and the noise to signal ratio for LOD should be 1:3. The term LOQ is defined as the lowest concentration at which the instrument can detect and quantify. The noise to signal ratio for LOQ should be 1:10. The estimation of LOD and LOQ for each phenolic compound tested were calculated using the standard deviation of intercept from the linear regression analysis of both external and matrix-matched calibration curve according to the following equations:

$$LOD = \frac{SDintercept}{slope}\ x\ 3.3 \qquad (1)$$

$$LOQ = \frac{SDintercept}{slope}\ x\ 10 \qquad (2)$$

5.2.3 Trueness

Trueness is one of the most critical parameters that should be assessed for method validation. Trueness is the closeness of agreement between the average value obtained from an extensive series of test results and an accepted reference value and involves common systematic errors (bias) [76]. In this work, trueness was assessed by measuring each analyte's recovery rate at three concentration levels: 2, 8 and 40 mg/Kg.

In this work, the recovery is calculated by the following equation:

$$\%\boldsymbol{R} = \frac{\boldsymbol{Area}_{(\boldsymbol{spiked\ sample})} - \boldsymbol{Area}_{(\boldsymbol{sample})}}{\boldsymbol{Area}_{(\boldsymbol{matrix\ matched\ \ sample})} - \boldsymbol{Area}_{(\boldsymbol{sample})}} \times \mathbf{100} \quad (3)$$

5.2.4 Matrix effect

Matrix effects occur when compounds that are coeluted with the analyte interfere with the MS detector's ionization process. The mechanism and the origin of the matrix effect are not fully understood, but it may originate from the competition between an analyte and a co-eluting compound or undetected matrix components reacting with primary ions formed in the interface. Depending on the environment in which the ionization and ion evaporation processes occur, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the efficiency of forming the desired analyte ions present at the same concentrations in the interface. It is intuitively clear that the efficiency of forming the desired ions is matrix-dependent due to the competition between the molecule of interest and several other undetected but co-eluting molecules present in the system, which is capable of reacting with primary ions [76], [77]. The matrix effect was determined by comparing the analytes' response between matrix-based standards and standard solutions at three different concentration levels (2, 8 and 40 mg/Kg). The equations used for the calculation of ME% are given below.

$$MF = \frac{Area\ matrix\ matched - Area\ sample}{Area\ standard} \quad (4)$$

$$ME\ \% = (1 - MF)\ x\ 100 \quad (5)$$

5.2.5 Precision

Two types of precision were examined: repeatability and intermediate precision. Repeatability expresses the closeness of the results obtained with the same sample (or subsamples of the same sample) using the same measurement procedure, same operator, same measuring system, same operating conditions and same laboratory over a short period of time. These conditions are called repeatability conditions. The short period is typically one day. The method's repeatability was evaluated by calculating the Relative Standard Deviation (%RSDr) of the area derived from the analysis of three different spiked samples at each of the 3 concentration levels: 2, 8 and 40 mg/Kg. Intermediate precision (or within-lab reproducibility) is obtained within a single laboratory over a more extended period, and it takes into account more changes than repeatability [76]. The method's intermediate precision was evaluated by calculating the $\%RSDr_w$ obtained with the same method, on the same sample, in the same laboratory, by the same operator but on different analysis days. Thus, the $\%RSDr_w$ was estimated by the area derived from the analysis of triplicates of spiked samples at each of the 3 concentration levels: 2, 8 and 40 mg/Kg in two consecutive analysis days.

5.3 Application of target screening approach to olive leaf samples

A total of 24 olive leaf samples from two different varieties (Adramitiani and Kolovi), collected from Lesvos island, were analyzed to measure their phenolic content. The characterization of olive leaf samples is extensively presented in section 4.2. Quantitative analysis was performed in order to provide a comprehensive overview of the phenolic composition of olive leaves. All samples were screened using the software TASQ CLIENT 1.4 from Bruker in order to confirm the presence or absence of the 54 target analytes. A database contained all necessary information (retention time, precursor and qualifier ions) for all target analytes was used to create a TASQ method. The identification criteria on which the screening of samples was based are retention time closeness, mass accuracy, isotopic fitting and the presence of qualifier ions. A score, namely MRSQ that visualizes all parameters' tolerance fit, can measure the confidence of the identification based on

the quality of the retention time, m/z error, mSigma, and qualifier ion results. The parameters set for screening and scoring was:

- RT Scoring – Narrow 0.2 min /Wide 0.4 min
- m/z Scoring – Narrow 2 mDa /Wide 5 mDa
- mSigma Scoring – Narrow 20 /Wide 200
- presence or absence of qualifier ions
- The S/N ratio should be better than 3, and the intensity threshold is higher than 1000.

Moreover, the quantification of target analytes was performed using matrix match calibration curves. Each analyte's concentration was determined using the corresponding equation from the matrix match curve using absolute areas.

The procedure of target screening of oleuropein in olive leaf samples is demonstrated in the next section. The upper window displays the identification points of oleuropein, which are useful to assess the presence or absence of this analyte to the samples. According to this, oleuropein successfully fulfills the identification criteria exhibiting a good MRSQ score. In the second window, we can see information about the pseudo molecular ion and the qualifier ions. Finally, the lower windows depict the chromatogram of pseudomolecular and diagnostic ions and the isotopic pattern (not seen in this figure) of oleuropein in the sample F10.

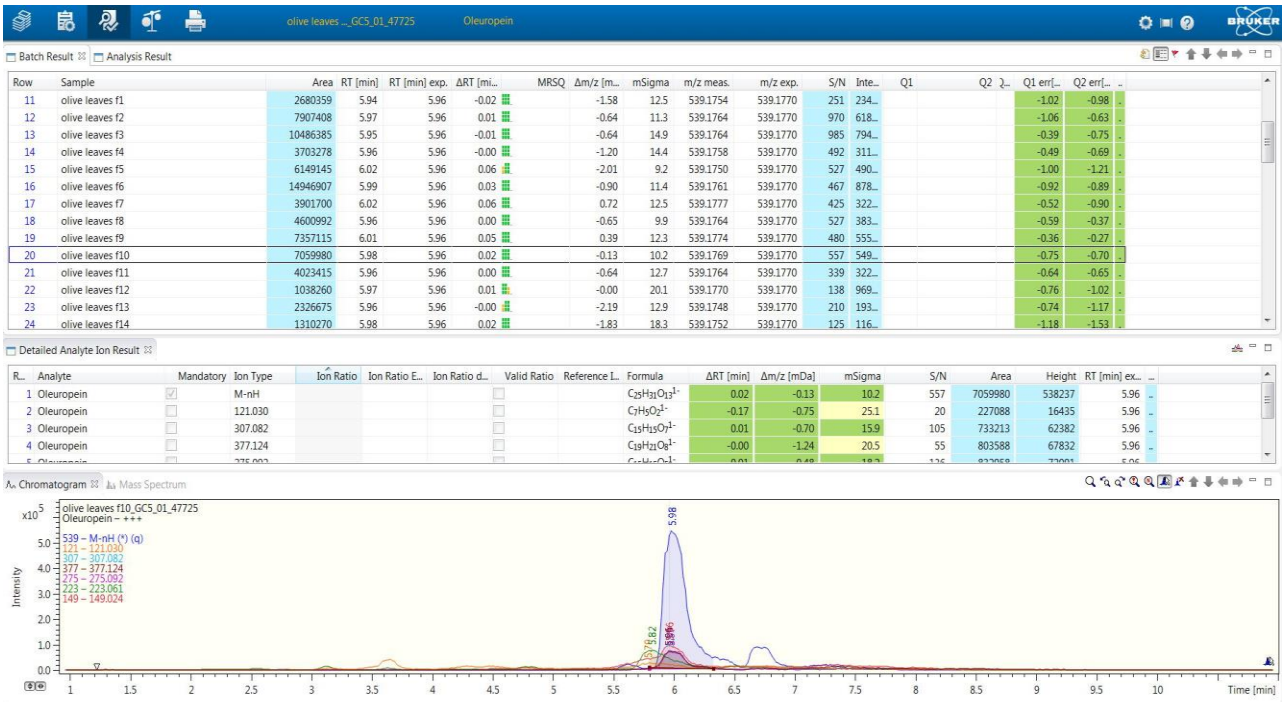


Figure 20: Target screening of samples for the determination of oleuropein

CHAPTER 6

RESULTS

6.1 Method development

6.1.1 Selection of the appropriate drying method

In this section, the effect of different drying methods on olive leaves' phenolic content was studied. So, the average responses of analytes found in olive leaves, followed by the relative standard deviation (%RSD), are presented in the following table (**Table 10**).

Table 10: Average Area and %RSD for each compound with different drying methods

	Oven drying		Microwave drying		Freeze drying		Raw olive leaves	
Compounds	Average	%RSD	Average	%RSD	Average	%RSD	Average	%RSD
Apigenin	30178229	7.0	21702779	18	9321627	19	10171181	8.3
Elenolic acid	1116279	3.1	3266440	29	2558246	5.6	2066440	14
Eriodictyol	182396	19	123259	13	70638	13	40949	16
Hydroxytyrosol	271994	42	2231253	17	1084652	13	536242	41
Luteolin	29708662	5.3	62482719	14	53240233	11	45241708	19
Oleokoronal	83590	49	134932	36	714492	13	1713570	39
Oleuropein	76441578	59	599205783	15	672356833	2.3	25714126	44
Oleuropein aglycone	6683940	50	4390928	45	149889990	83	213830567	27
Quercetin	888734	30	1920676	5.1	409528	8.1	296010	28
oleacein	10539366	38	31598376	21	186361108	13	27442948	52
oleocanthal	165575	36	69694	57	530072	11	-	-
oleomissional	22043842	44	4011672	17	173742833	11	349644917	29
p-coumaric acid	81486	28	61304	16	-	-	-	-
rutin	1017571	14	6737158	30	9887771	8.6	2977452	22
salicylic acid	321854	34	197473	16	117861	15	75768	35
taxifolin	92517	14	107489	12	78573	24	50422	15

As shown above, in table 10, it can be concluded that microwave and freeze-drying are the best drying methods for most of the tested analytes and almost for all important compounds found in olive leaves. The discrepancy among the compounds found more

abundant in these two tests is small except for Oleuropein aglycone, which shows a much higher abundance in the freeze-drying test, but it also has a double RSD value. Analytes found in olive leaves in a significant amount such as elenolic acid, hydroxytyrosol, quercetin, luteolin, taxifolin, and eriodictyol have shown better results microwave than the other pretreatments as can be depicted for some of them in the following bar charts. Furthermore, some other significant compounds of olive leaves, such as oleuropein and rutin, have shown similar results with microwave and freeze-drying pretreatments. It should be highlighted that although the freeze-drying test gave good responses not only for the before mentioned analytes but also for some others such as oleuropein aglycone, oleomissional and oleokoronal, the microwave was selected for this study due to low cost, simplicity and time required. Also, the superior effect of microwave drying has been reported in the literature by different scientists [35], [37].

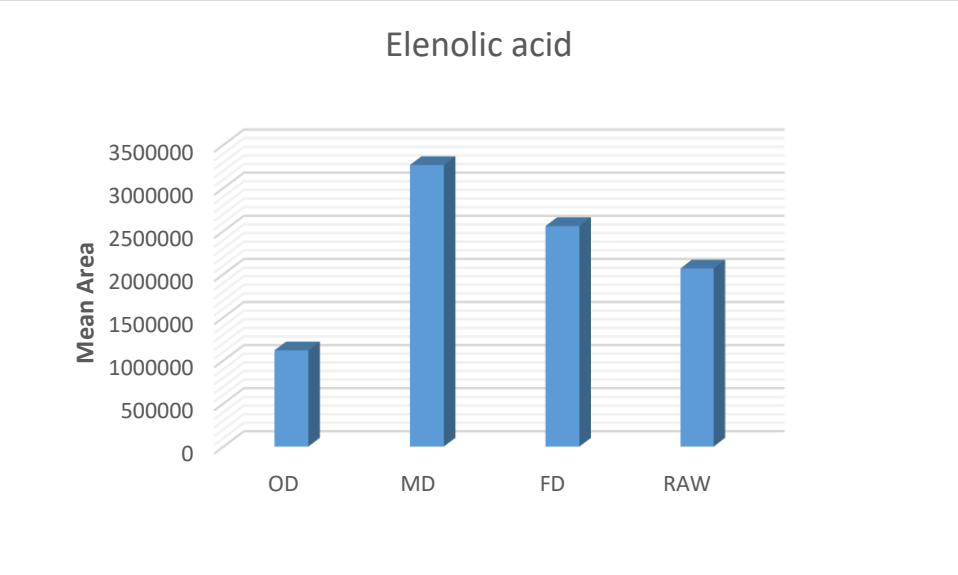


Figure 21: Bar chart of Elenolic acid

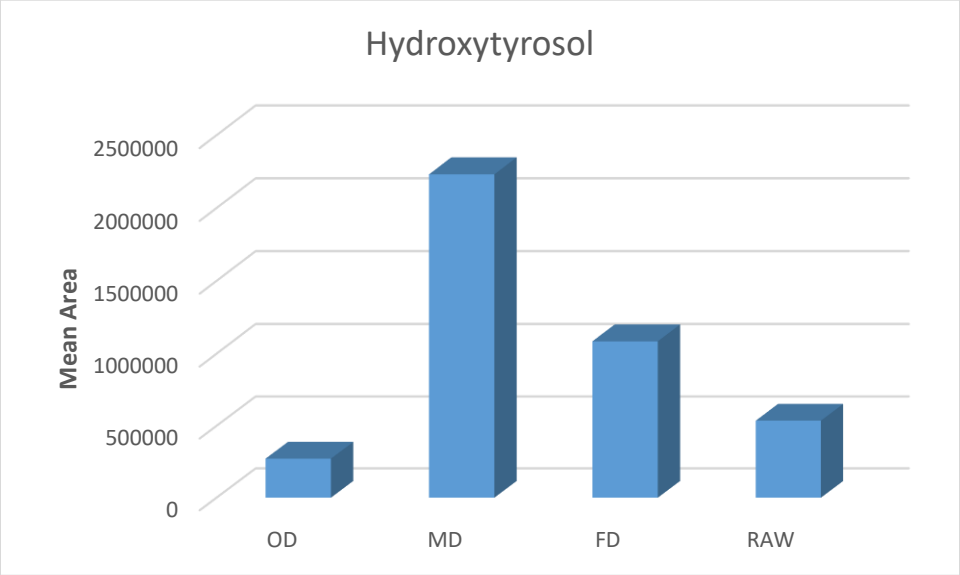


Figure 22: Bar chart of Hydroxytyrosol

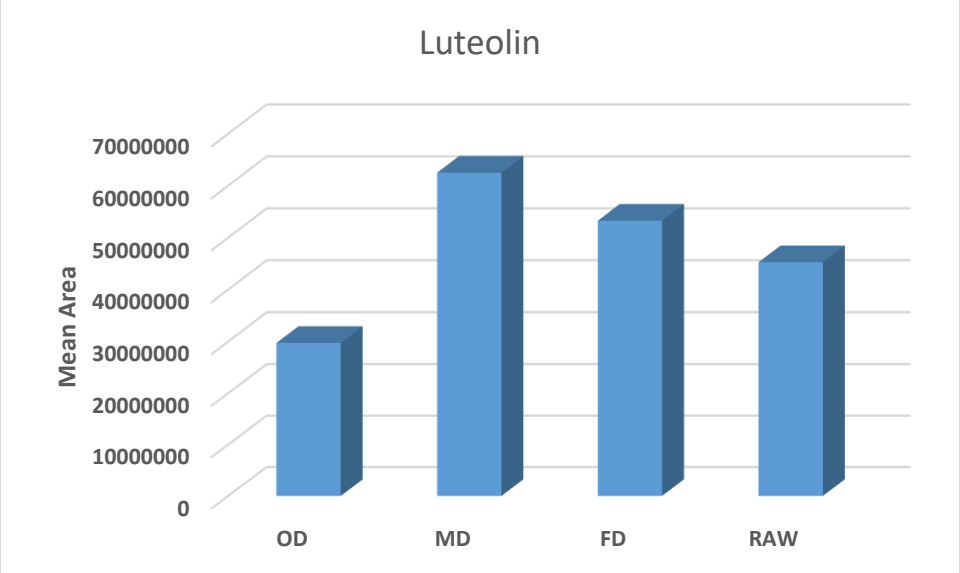


Figure 23: Bar chart of Luteolin

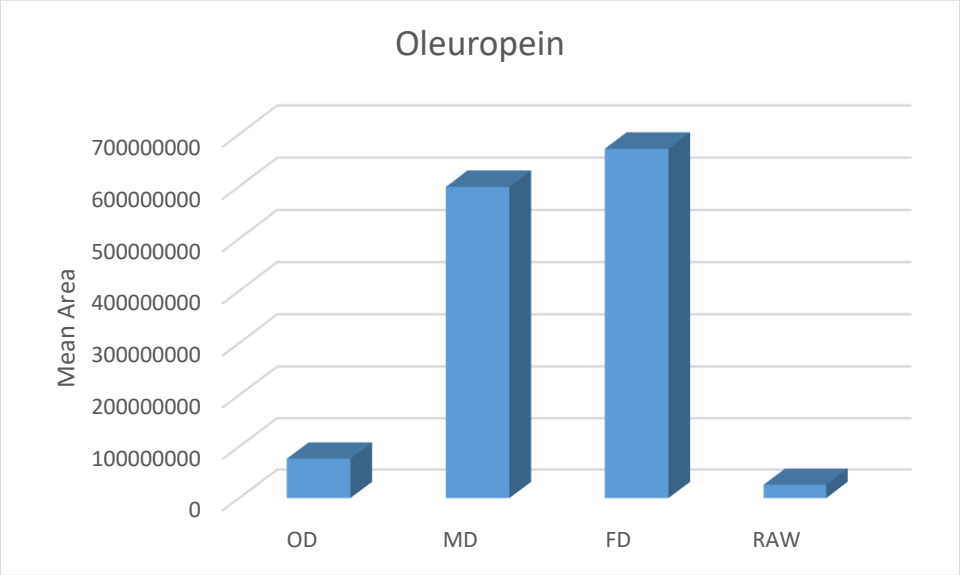


Figure 24: Bar chart of Oleuropein

6.1.2 Selection of the appropriate solid/solvent ratio

The same extraction process was carried out using three different initial sample weights (0.25g, 0.5g and 1g) of olive leaves in order to investigate the effect of solid/solvent ratio on the recovery of phenolic compounds. So, the average areas of analytes, followed by the relative standard deviation (%RSD), are presented in the following table (**Table 11**). For some analytes like oleuropein and luteolin, the diluted areas (50 times diluted) are presented in the table because the undiluted samples' responses reach a plateau. Also, the value of all responses found at 0.25g and 0.5g were normalized to 1g so that the results would be comparable.

Table 11*:* **Mean Area and %RSD for each compound with different initial sample weights**

	m=0.25 g		m=0.5 g		m=1.0 g	
Analytes	Average	%RSD	Average	%RSD	Average	%RSD
Apigenin	1323666	13	768040	11	810889	19
Elenolic acid	8408945	57	7221662	27	9684534	37
Eriodictyol	454907	27	258176	18	295932	44
Hydroxytyrosol	243428	45	185299	20	232674	15
Luteolin	3081653	6.9	2170675	19	2225761	21

Oleokoronal	300751	30	263800	15	101694	15
Oleuropein	32747061	3.0	31470591	8.0	11675446	73
Quercetin	5804467	30	4211036	17	5579522	43
Oleacein	2300117	40	1461770	19	1246198	8.4
Oleomissional	247252	11	219179	7.6	112777	64
Rutin	4745687	4.5	3428064	20	1463163	24
salicylic acid	1193394	48	566433	31	1952559	74
taxifolin	486857	29	306408	6.7	367256	39

According to these results, the extraction efficiency using 0.25 g olive leaf sample was higher for most analytes, including major phenolic compounds such as eriodictyol, hydroxytyrosol, and lute oleuropein, quercetin and taxifolin. The following bar charts show clearly that a lower solid/solvent ratio can lead to better extractability of phenolic compounds. A low solid to solvent ratio was selected in this study, achieving a better diffusion of analytes from the olive leaf matrix. Moreover, a low solid to solvent ratio means lower matrix effects, avoiding carry-over phenomena in the column and signal suppression.

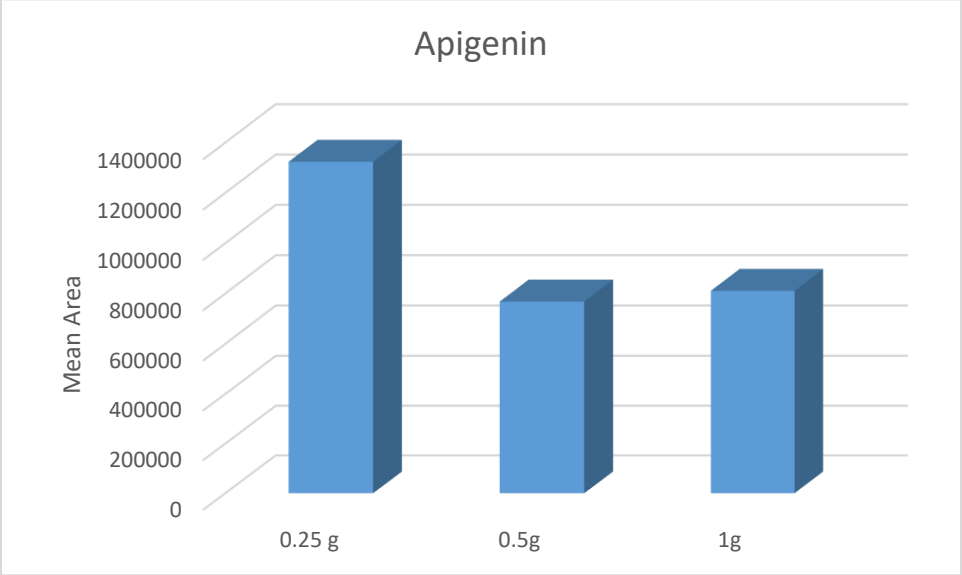


Figure 25: Bar chart of Apigenin

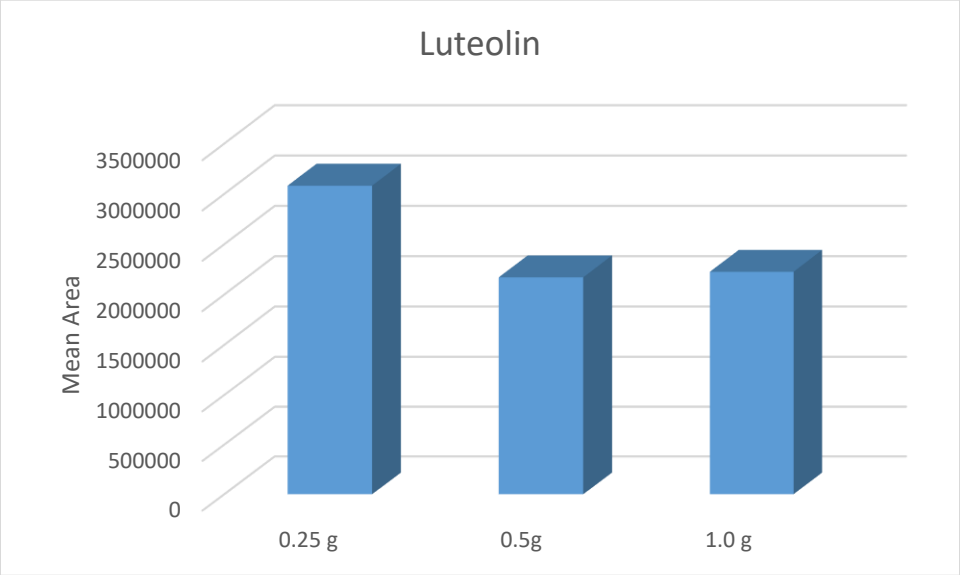


Figure 26: Bar chart of Luteolin

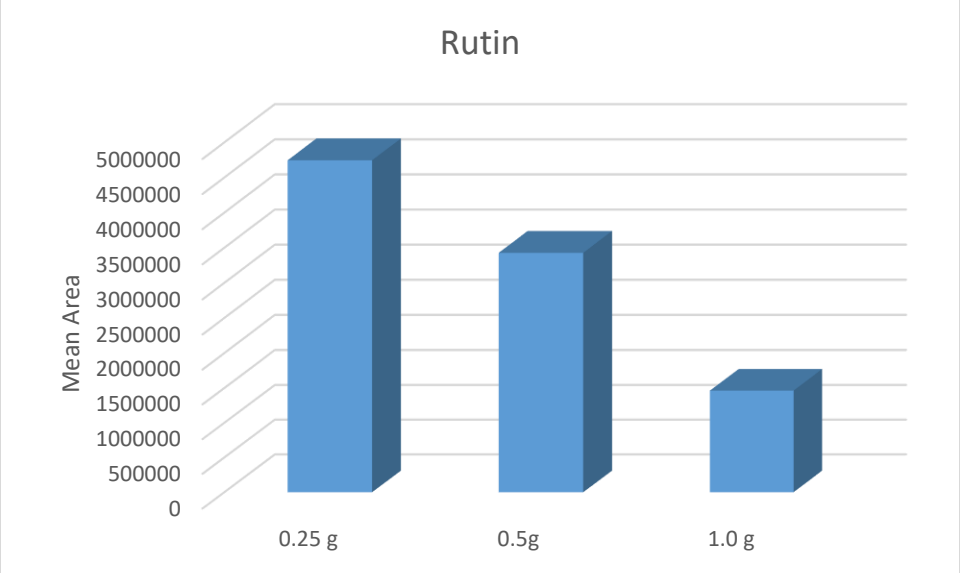


Figure 27: Bar chart of Rutin

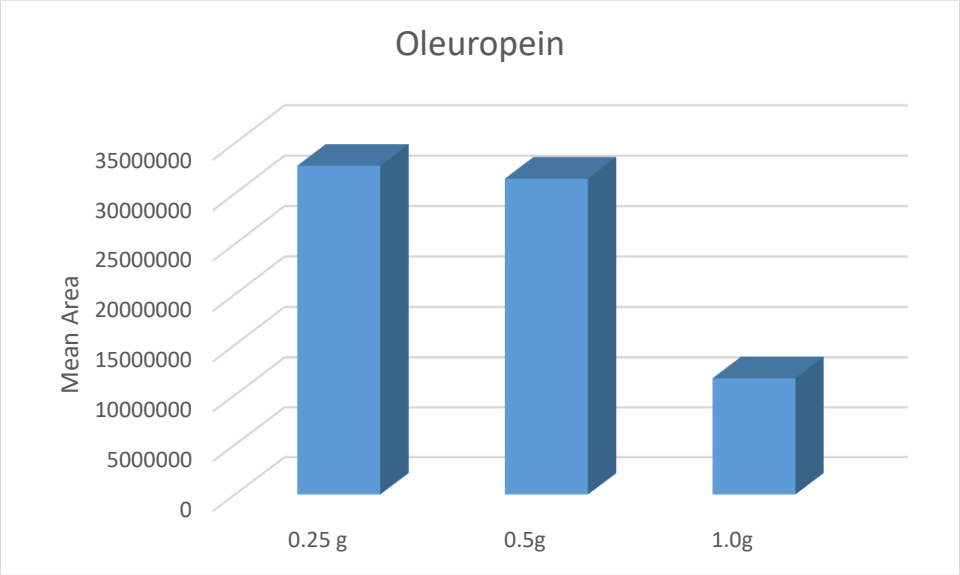


Figure 28: Bar chart of Oleuropein

6.1.3 Selection of the appropriate extractant composition

At this stage, five different extraction media were examined and evaluated based on the recovery of spiked analytes and endogenous compounds' response. The average areas and recoveries of analytes, followed by the relative standard deviation (%RSD), are presented in the following table (**Table 12**). It has to be mentioned that there were some compounds (eriodictyol, hydroxytyrosol and oleuropein) for which the diluted responses were used for the selection of the appropriate extractant in order to have more representative results.

Concerning the recovery of endogenous analytes of olive leaves, MeOH:H_2O (80:20) presented the higher extraction yield for several analytes such as elenolic acid, hydroxytyrosol, oleuropein and rutin. In contrast, other important analytes like quercetin and luteolin were extracted better with EtOH:H_2O (80:20). Regarding the recovery of spiked analytes, similar results were obtained using the two different extractant compositions with an extraction efficiency of more than 90% for all studied compounds. In this study, MeOH:H_2O (80:20) was selected because, for most analytes, mean recovery values were higher, and the method had better reproducibility than that produced by EtOH:H_2O (80:20) because the value of %RSD was lower. Also, Won-Young

Cho et al. (2020) concluded that olive leaf extracts using 90:10 methanol:water had higher phenolic content in contrast to 90% ethanol [75].

Table 12: Recovery rate %, Mean Area and %RSD for each phenolic compound

Endogenous compounds										
	100% EtOH		EtOH:H2O (60:40 +80:20)		EtOH:H2O (60:40)		MeOH:H2O(80:20)		EtOH:H2O (80:20)	
Analytes	Mean Area	%RSD	Mean Area	%RSD	Mean Area	%RSD	Mean Area	%RSD	Mean Area	%RSD
Apigenin	454822	62	354985	39	407070	30	458328	43	490550	17
Elenolic acid	79748	32	246668	49	184564	26	332024	51	204745	38
Eriodictyol	44841	23	50719	19	63501	21	61441	22	62803	6.3
Hydroxytyrosol	18942	14	21455	19	23213	13	37402	37	27225	26
Luteolin	1267828	33	1062917	22	983090	29	1304661	36	1482013	12
Oleokoronal	88921	29	70631	36	44690	25	67368	26	54510	14
Oleuropein	9882340	19	10861688	8.8	10817021	3.7	12482120	6.7	11081125	10
Quercetin	609911	45	820739	15	979351	21	930229	46	1051356	31
oleacin	132543	60	-	-	103734	25	241910	67	-	-
oleomissional	322872	19	313554	3.5	316305	7.7	326882	14	350515	5.3
rutin	82769	13	151602	16	168848	8.4	172243	6.2	-	-
salicylic acid	18406	59	30222	41	33689	51	37374	32	31335	34
taxifolin	464729	31	406135	2.1	405182	16	383612	24	443412	19
Spiked compounds										
	100% EtOH		EtOH:H2O (60:40 +80:20)		EtOH:H2O (60:40)		MeOH:H2O(80:20)		EtOH:H2O (80:20)	
Spiked Analytes	R% mean	%RSD	R% mean	%RSD	R% mean	%RSD	R% mean	%RSD	R% mean	%RSD
4-hydroxybenzoic acid	86	3.4	95	13	115	6.6	114	19	102	4.9
Caffeic acid	76	12	77	7.5	80	14	90	7.6	116	9.6
Catechin	84	19	79	5.8	55	14	99	4.8	123	9.6
Cinnamic acid	87	3.8	92	5.2	86	5.5	96	4.5	103	2.9
Ferulic acid	86	13	86	9.1	86	13	101	8.0	112	1.3
Gallic acid	68	14	99	12	64	17	98	1.9	118	7.9

Homovanillic acid	98	8.2	110	8.6	112	13	95	11	126	20
Ligstroside aglycone	80	12	74	11	69	5.6	81	8.9	100	2.5
Syringic acid	97	9.4	84	4.7	85	6.2	92	3.8	122	7.2
Tyrosol	101	2.0	92	4.0	84	6.1	107	13	113	3.5
Vanillic acid	103	13	111	4.0	96	6.8	105	4.5	108	7.1
Vanillin	97	13	97	0.9	91	13	113	20	111	6.7
naringenin	84	13	83	2.5	69	6.5	93	4.7	104	3.4
oleocanthalic acid	92	22	105	4.4	62	16	120	13	121	7.7
3,4-Dihydroxybenzoic acid	97	13	111	9.2	82	11	97	14	127	3.9

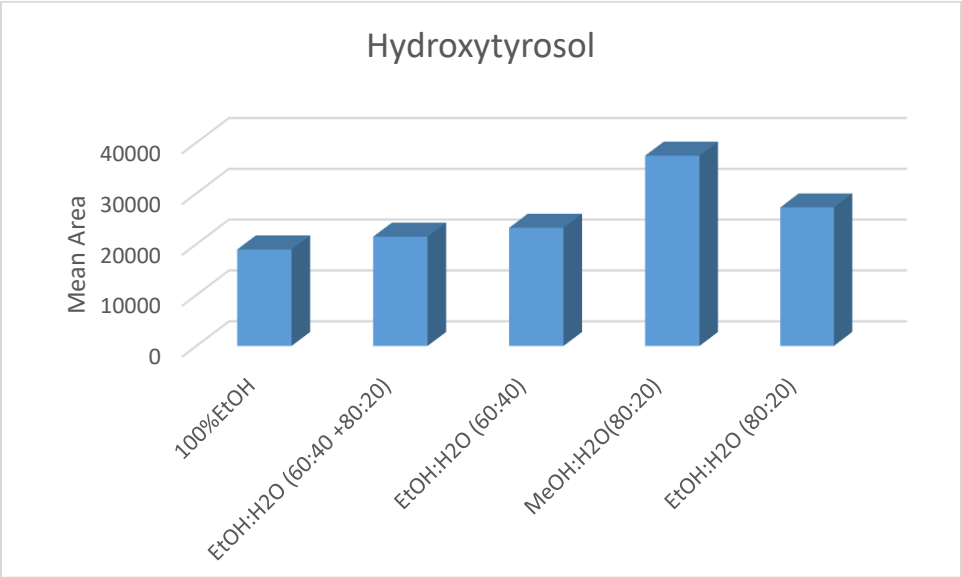


Figure 29: Bar chart of Hydroxytyrosol with different extractants

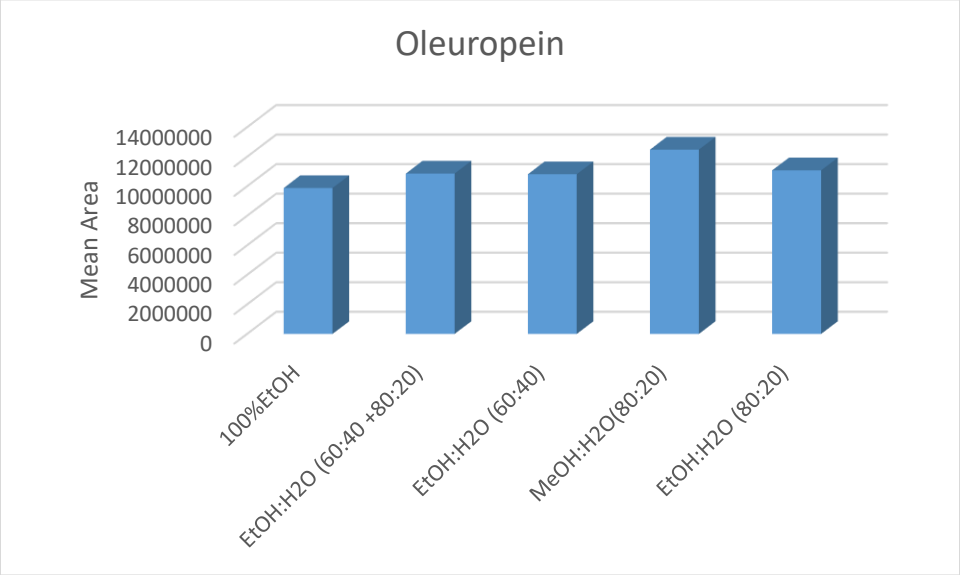


Figure 30: Bar chart of oleuropein with different extractants

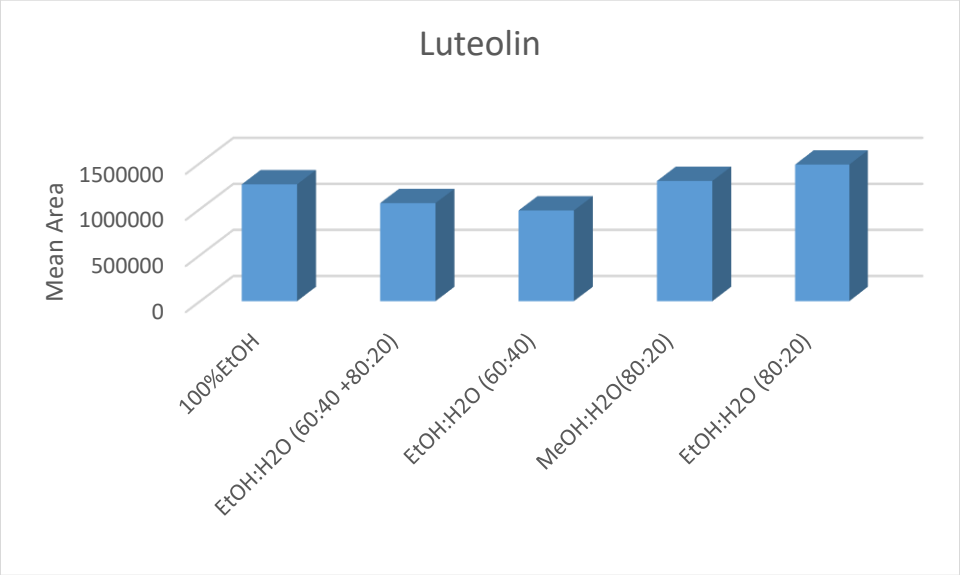


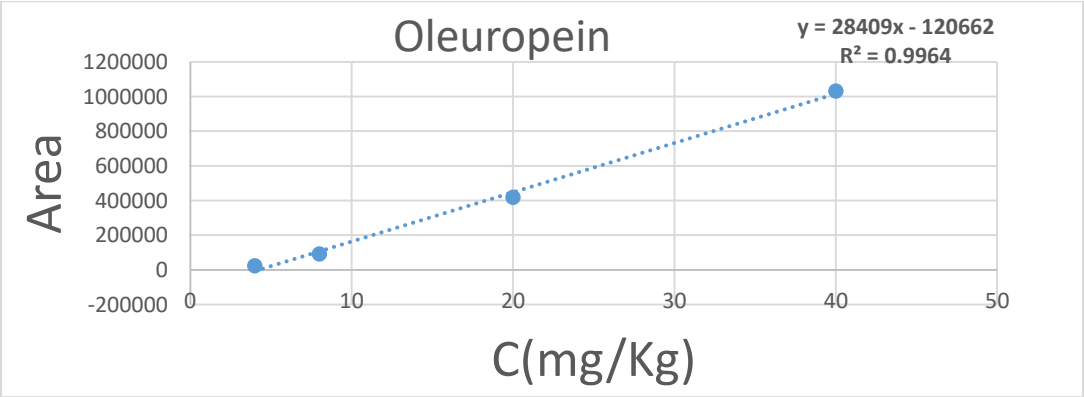
Figure 31: Bar chart of luteolin with different extractants

6.2 Validation

6.2.1 Linearity

The linearity was determined according to section 5.2.1, and the regression lines were determined by the least squares method. Below, the characteristic example of Oleuropein is presented, which is one of the most important analyte encountered in olive leaves **Figure 32.**

Figure 32: Calibration curve of Oleuropein



The results of the assessment of instrumental linearity and method linearity are summarized in the following table. In almost all cases, our data fitted very well in the linear model with correlation coefficients higher than 0.99. Nevertheless, the determination of method linearity in some cases like Eriodictyol, hydroxytyrosol, diosmetin, quinic acid and Luteolin etc. was impossible because the concentration of these analytes in the matrix was very high. Also, method linearity for some phenolic acids like gallic acid, p-coumaric acid and Syringic acid was not determined since early eluting peaks such as phenolic acids were subjected to strong ion suppression phenomena. Thus, we could not detect them in at least the low concentration levels or even more in any of them (gallic acid). For instance, p-coumaric acid and syringic acid were detected only at 20 and 40 mg/L.

Table 13: The results of instrumental and method linearity

	Olive leaves		Standards	
Analytes	Equation	R^2	Equation	R^2
2-cis,4-trans-Abscisic acid	y = 13486 x - 35870	0.996	y = 89455x - 21553	0.9996
2.5 dihydroxybenzoic acid	y=14951x - 53534	0.98	y = 87873x + 50149	0.98
Apigenin	-	-	y = 252531x + 453017	0.991
Caffeic acid	y = 8894.5x - 25037	0.98	y = 157915x + 73011	0.9993
Cinnamic acid	y = 8341.8x -28700	0.990	y = 34926x - 7865.9	0.9994
Eriodictyol	-	-	y=6808.8x-4030	0.995
Ferulic acid	y = 4720.3x + 6094.9	0.990	y = 43988x - 3950.5	0.9997
Gallic acid	-	-	y = 60297x + 102652	0.98
Hydroxytyrosol	-	-	y = 69549x - 25025	0.9996
Luteolin	-	-	y=211524x+54469	0.997
Oleuropein	y = 28409x - 120662	0.996	y = 191808x - 33740	0.998
Pinoresinol	y = 4581.5x +52640	0.98	y = 46648x - 17242	0.9994
Quercetin	y = 22638x +134019	0.997	Y=126969x-10066	0.990
Syringic acid	-	-	y = 16819x + 3059.5	0.997
Tyrosol	y = 6284.4x - 15411	0.98	y = 27192x – 9346.1	1.0
Vanillic acid	y = 2669.2x -4091.1	0.996	y = 18223x + 864.37	0.998
Vanillin	y=28262x+68882	0.97	y = 76802x - 20781	0.998
diosmetin	-	-	y = 41521x + 81317	0.92
eudesmic acid	y = 8305.2x - 18811	0.98	y = 53914x + 16734	0.991
naringenin	y = 25503x -45591	0.992	y = 257891x + 195969	0.998
p-coumaric acid	-	-	y = 42270x + 12679	0.994
quinic acid	-	-	y = 43026x + 35750	0.993

rutin	y = 1435.6x -2733.1	0.997	y = 12868x - 18116	0.98
salicylic acid	y = 51889x-68032	0.9991	y = 150599x + 120859	0.990
sinapic acid	y = 4394.1x - 9348.4	0.994	y = 44340x - 640.79	0.9991
taxifolin	y = 9764.1x +14302	0.998	y = 126056x - 73839	0.997
verbascoside	y = 3283x +55590	0.98	y=24150x-18948	0.997

6.2.2 Sensitivity

As has already been mentioned, the LOD and LOQ for each analyte were estimated both for the instrument (ILOD) and method (MLOD). So, linear regression analysis was used for external and matrix-matched calibration curves. The estimation of LOD and LOQ for each phenolic compound tested were calculated using the standard deviation of intercept from the linear regression analysis of both external and matrix-matched calibration curves. The MLOD and MLOQ have been calculated in the initial wet sample mass.

Table 14: Total results of LODs and LOQs

	Method		Instrumental	
Analytes	LOD	LOQ	LOD	LOQ
2-cis,4-trans-Abscisic acid	3.1	9.4	0.20	0.60
2.5 dihydroxybenzoic acid	5.1	15	1.2	3.5
Apigenin	-	-	1.8	5.4
Caffeic acid	6.3	19	0.52	1.6
Cinnamic acid	5.0	15	0.25	2.5
Eriodictyol	-	-	0.65	1.9
Ferulic acid	3.6	11	0.17	0.51
Gallic acid	-	-	1.7	5.0
Hydroxytyrosol	-	-	0.20	0.60
Luteolin	-	-	0.6	1.9
Oleuropein	3.1	9.2	0.33	1.0
Pinoresinol	6.7	20	0.23	0.70
Quercetin	1.9	5.7	0.97	2.9
Syringic acid	-	-	0.64	1.9
Tyrosol	8.7	26	0.08	0.23
Vanillic acid	2.2	6.7	0.32	1.0
Vanillin	13	39	0.39	1.2
diosmetin	-	-	2.8	8.5

eudesmic acid	5.2	16	0.9	2.8
naringenin	3.2	9.6	0.68	2.0
p-coumaric acid	-	-	0.71	2.1
quinic acid	-	-	1.6	4.7
rutin	2.0	5.9	2.4	7.2
salicylic acid	1.1	3.3	1.0	2.9
sinapic acid	3.9	12	0.30	0.89
taxifolin	1.3	3.8	0.51	1.5
verbascoside	4.8	14	0.6	1.7

According to the table, method LODs and LOQs were very good for most analytes. So, the method is proved to be suitable for the detection of phenolic compounds in low concentrations. The values for method LODs ranged between 1.1 to 8.7 mg/kg, while for LOQs ranged between 3.3 mg/Kg to 39 mg/Kg, respectively. Regarding the values for instrumental LODs and LOQs, they ranged between 0.08 to 2.8 mg/Kg and 0.23 to 8.5 mg/kg, respectively.

6.2.3 Trueness

The trueness was assessed by measuring each analyte's recovery rate after adding known amounts of the analytes to an olive leaf sample (blank matrix), as already mentioned and compared to a matrix-based standard at the same concentration level.

According to the following table, most of the studied analytes' recoveries ranged between 80-110%, indicating the developed method's accurate determination. Compounds that exist in high concentration in olive leaves, such as hydroxytyrosol and Luteolin, could only be assessed in the high concentration level while others such us diosmetin and quinic in none of the concentration levels. For almost all phenolic acids, including caffeic acid, sinapic acid, 2.5 dihydroxybenzoic acid, 2-cis,4-trans-abscisic acid, eudesmic acid and p-coumaric acid, the mean recovery at the lowest concentration (2 mg/Kg) level was not calculated. The main reason is that LODs of these compounds were proved to be higher than 2 mg/Kg. Moreover, gallic acid was not detected in any concentration level due to

high ion suppression phenomena in the matrix. So, this method is not selective for the determination of this analyte.

Table 15: % Recoveries at three concentration levels

	% Recovery		
Analytes	C=2mg/Kg	C=8mg/Kg	C=40mg/Kg
2-cis,4-trans-Abscisic acid	-	101	93
2.5dihydroxybenzoic acid	-	108	84
Apigenin	-	-	80
Caffeic acid	-	85	63
Cinnamic acid	-	75	82
Eriodictyol	-	-	105
Ferulic acid	98	108	90
Gallic acid	-	-	-
Hydroxytyrosol	-	-	88
Luteolin	-	-	95
Oleuropein	-	131	94
Pinoresinol	-	96	92
Quercetin	-	126	81
Syringic acid	-	-	89
Tyrosol	-	118	86
Vanillic acid	104	107	93
Vanillin	-	114	88
diosmetin	-	-	-
eudesmic acid	-	104	86
naringenin	-	100	78
p-coumaric acid	-	-	99
quinic acid	-	-	-
rutin	-	119	101
salicylic acid	-	113	95
sinapic acid	-	103	81
taxifolin	-	104	103
verbascoside	-	65	80

6.2.4 Matrix effect

In order to measure the ME, the matrix factor (MF) is necessary. The matrix factor was determined by comparing the analytes' response between matrix-matched standards and standard solutions at three different concentration levels (2, 8 and 40 mg/Kg). The results of ME% were shown in the following table.

The matrix effects were considered to be high enough for most of the analytes. As previously mentioned, the early eluting phenolic acids were shown the higher ME% values. Furthermore, the matrix seems to significantly affect the ion intensity, and thus a cleanup step may be considered a suitable means to reduce the matrix effect and increase the quantification's confidence.

Table 16: Matrix effect at three concentration levels

	ME%		
Analytes	C=2mg/Kg	C=8mg/Kg	C=40mg/Kg
2-cis,4-trans-Abscisic acid	-	58	41
2.5dihydroxybenzoic acid	-	76	37
Apigenin	-	-	65
Caffeic acid	-	93	98
Cinnamic acid	-	44	8.7
Eriodictyol	-	-	54
Ferulic acid	-21	58	54
Gallic acid	-	-	-
Hydroxytyrosol	-	-	23
Luteolin	-	-	-56
Oleuropein	-	76	45
Pinoresinol	-	2.0	46
Quercetin	-	-46	14
Syringic acid	-	-	48
Tyrosol	-	6.3	8.5
Vanillic acid	65	61	43
Vanillin	-	-147	-63
diosmetin	-	-	-
eudesmic acid	-	69	41
naringenin	-	87	64
p-coumaric acid	-	-	81
quinic acid	-	-	-

rutin	-	34	51
salycilic acid	-	30	-26
sinapic acid	-	74	61
taxifolin	-	48	66
verbascoside	-	-129	-77

6.2.5 Precision

Precision was assessed by the evaluation of repeatability (intra-day precision) and reproducibility (inter-day precision). The results for the estimation of precision presented in the following table (Table 17). Specifically, the vast majority of analytes showed RSDr < 10% and RSD_R < 20% indicating the satisfactory precision achieved with the developed method. It has to be mentioned that for some analytes like 2-cis,4-trans-abscisic acid, syringic acid, vanillin, eudesmic acid, p-coumaric acid and sinapic acid, the precision of the method were not determined at low or/and middle concentration levels (2, 8 and 40 mg/Kg) due to the low detection limits. Moreover, analytes' precision with high concentrations in the matrix has been evaluated using a relative standard deviation of the spiked samples' absolute area.

Table 17: The repeatability and the intermediate precision of the method

	Precision (%RSD)					
	Repeatability			Intermediate precision		
Analytes	C=2mg/L	C=8mg/L	C=40mg/L	C=2mg/L	C=8mg/L	C=40mg/L
2-cis,4-trans-Abscisic acid	-	8.9	4.4	-	13	10
2.5dihydroxybenzoic acid	-	5.5	4.9	-	9.0	8.0
Apigenin	-	-	9.7	-	-	10
Caffeic acid	-	5.0	13	-	41	39
Cinnamic acid	-	3.1	9.9	-	8.8	11
Eriodictyol	-	-	13	-	-	17
Ferulic acid	7.8	9.9	0.9	8.6	8.3	8.4
Gallic acid	-	-	-	-	-	-
Hydroxytyrosol	9.1	9.5	7.2	6.0	10	8
Luteolin	6.6	8.5	6.7	8.2	9.4	8.9
Oleuropein	-	7.4	4.4	-	22	5.4
Pinoresinol	-	0.7	17	-	21	26

Quercetin	-	8.8	6.9	-	15	15
Syringic acid	-	-	3.6	-	-	8.1
Tyrosol	-	26	5	-	20	7.7
Vanillic acid	6.0	8.8	3.4	9.1	8.9	7.7
Vanillin	-	-	4.4	-	-	5.0
diosmetin	17	17	13	15	11	16
eudesmic acid	-	6.0	7.0	-	7.7	9.3
naringenin	-	28	6.5	-	30	6
p-coumaric acid	-	-	6.2	-	-	12
quinic acid	3.7	18	10	9.2	16	28
rutin	-	6.6	2.8	-	14	6
salycilic acid	-	10	4.1	-	16	8.7
sinapic acid	-	3.6	5.6	-	7.6	9.8
taxifolin	-	23	8.5	-	29	8
Verbascoside	-	16	17	-	15	17

6.3 Target screening in olive leaf samples

A number of 15 analytes were determined in all samples, namely apigenin, elenolic acid, eriodictyol, hydroxytyrosol, luteolin, oleuropein, oleuropein aglycone, quercetin, tyrosol, diosmetin, oleacin, oleomissional, rutin, salicylic acid, sinapic acid and taxifolin. The following table summarizes the average concentration of each compound along with its standard deviation in the varieties of Adramitiani and Kolovi. According to the following table (**Table 13),** it can be concluded that eriodictyol was the most abundant phenolic compound, followed by oleuropein and diosmetin in both Adramitiani and Kolovi variety. The average concentration of eriodictyol in Adramitiani variety was about 1148 ± 668 mg/kg and 494 ± 519 mg/kg in Kolovi variety. The average concentration of the second most abundant analyte, oleuropein, was about 414 ± 662 mg/kg for Adramitiani and 307 ± 245 mg/kg for Kolovi. Lastly, the average concentration for diosmetin was 184 ± 246 mg/kg for Adramitiani and 141 ± 71 mg/kg for Kolovi. Other analytes found in high concentrations were luteolin, elenolic acid, hydroxytyrosol and quercetin for the Adramitiani variety and oleacein, elenolic acid and rutin for the Kolovi variety.

In much research concerning phenolic profile determination in olive leaves, oleuropein is the most abundant compound, followed by hydroxytyrosol. Other important analytes that have been reported in the literature were oleuropein aglycone, luteolin, apigenin, rutin, elenolic acid, tyrosol and quercetin. However, the concentration level and the specific phenolic compounds found in olive leaves varies depending on several conditions, including the variety. For example, Mehmet Musa Ozcan et al. (2016) concluded that the concentration of oleuropein varies between 4,7–143.200 mg/Kg, of hydroxytyrosol between 2,1–1.120 mg/Kg, of luteolin between 10,1–5600 mg/Kg, of rutin between 13,8–3.500 mg/Kg, of apigenin between 4,6–339,5mg/kg, of tyrosol between 90-660 mg/Kg and oleuropein aglycone between 170–280 mg/kg. These ranges are based on the analysis of different varieties originated in Spain and Italy, including serana, sikitita, verdial, frantoio, arbequina and picual [78]. In another study conducted by Mustafa Cittaan et al. (2018), olive leaves from Manisa in Turkey were analyzed in order to find their phenolic profile. The results showed lower levels of eriodictyol (6,19 -7,35 mg/kg) and sinapic acid (3,65-6,13 mg/Kg) while analytes like quercetin (37,07- 43,73 mg/kg), luteolin (215,67- 223,53 mg/kg) and apigenin (66,53-118,49 mg/Kg) were detected in higher levels than that to our study. Finally, the taxifolin concentration was found at the same levels (7,03-9,39 mg/Kg) [76]. Furthermore, the analysis of 18 Portuguese olive leaf cultivars by Julieta Merinhos et al. (2014) showed that the concentration of rutin ranged between 21- 298,6 mg/Kg, luteolin between 44,7-778,8 mg/kg, apigenin between 4,6-339,5 mg/Kg and diosmetin between 18,9-350,8 mg/Kg [79]. From all the above, it can be concluded that the phenolic profile of olive leaves shows a wide variation between different varieties and depends on many other factors that include not only the origin of each olive leaf but also storage conditions, climatic conditions, moisture content and maturity stage.

Table 18: The average concentration (mg/kg) of phenolic compounds in various olive leaf samples

Olive leaf matrix	Adramitiani		Kolovi	
	n=12		n=12	
Analytes	avg	SD	avg	SD
Apigenin	15	14	23	10
Elenolic acid	40	30	64	46

Eriodictyol	1148	668	494	519
Hydroxytyrosol	37	34	21	13
Luteolin	68	51	125	67
Oleuropein	414	662	307	245
Oleuropein aglycone	1.7	1.3	2.5	1.6
Quercetin	31	20	24	15
Tyrosol	14	8.0	15	5.7
diosmetin	184	246	141	71
oleacein	8.1	7.2	79	139
rutin	22	8.3	32	19
salycilic acid	8.7	7.5	10	7.4
sinapic acid	18	6.8	15	9.4
taxifolin	10	3.7	6.3	3.5

CHAPTER 7

CONCLUSIONS

The relationship between diet and health has increased interest in natural antioxidants such as bioactive components of natural raw materials. Olive leaves are a good and cheap source of phenolic compounds. Phenolic compounds in olive leaves are numerous and of diverse nature. As a result, it is important to be able to determine the phenolic compounds in olive leaves precisely.

In this master, a generic method for determining the main phenolic compounds with HPLC-ESI-QTOF-MS in olive leaves was developed and validated. After several experiments, the method selected included microwave drying of 0,25g initial olive leaf sample and extraction using MeOH:H20 (80:20). The developed method was then validated for 27 analytes concerning linearity, sensitivity, trueness, matrix effect, and precision. In more detail, linearity was excellent in most cases with correlation coefficients higher than 0.99, whereas LODs and LOQs raged between 0.08-8.7 mg/kg and 0.23-39 mg/kg, respectively. The vast majority of analytes have shown RSDr%< 10 and RSDR% <20, whereas the recoveries ranged between 80-110%, proving the method's acceptable trueness. The matrix effects were ranged from -77 % to 98%.

Additionally, target screening of 24 olive leaf samples from Lesvos Island was performed. Half of the samples belonged to the Adramitiani variety and the other half to Kolovi. The quantification was performed using matrix match calibration curves, and the phenolic content of these olive leaf matrices was measured as average concentration for each variety. The average concentration of determined polyphenols ranged from 3.5 to 1148 mg/Kg. Overall results showed that eriodictyol, oleuropein and diosmetin were the analytes found in higher concentrations. Finally, the mean concentration for oleuropein and eriodictyol were higher in the Adramitiani variety.

The results obtained in the present work show that olive leaves are a natural product with rich phenolic content that could be furtherly used in the cosmetic, food and pharmaceutical industry.

ABBREVIATIONS AND ACRONYMS

Table 1: Abbreviations and acronyms.

OMWW	Olive Oil Waste Water
TOPW	Table Olive Processing Wastewater
HIV	Human Immunodeficiency Viruses
UV	Ultra-Violet
DNA	Deoxyribonucleic acid
ROS	Reactive Oxygen Species
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium
LDH	Organochlorine pesticides
LDH	lactate dehydrogenase
PA	Proanthocyanidins
OD	Oven Drying
FD	Freeze Drying
VD	Vacuum Drying
MD	Microwave Drying
PLE	Pressurized liquid extraction
SHLE	Superheated liquid extraction
SFE	Supercritical Fluid Extraction
LLE	Liquid-liquid extraction
SPE	Solid-phase extraction
MAE	Microwave-assisted extraction
USAE	Ultrasound-assisted extraction
HRMS	High-Resolution Mass Spectrometry
LRMS	Low-Resolution Mass Spectrometry
UV-Vis	Ultraviolet-Visible
NMR	Nuclear Magnetic Resonance
ESI	Electrospray Ionization
HPLC	High-Performance Liquid Chromatography
MS	Mass Spectrometry
QqQ	Triple Quadrupole
IT	Ion trap
TOF	Time of Flight
DDA	Data Dependent Acquisition
DIA	Data Independent Acquisition
SRM	Single Reaction Monitoring
bbCID	Broadband collision-induced dissociation

DAD	Diode Array Detector
AC	Alternating Current
DC	Direct Current
CID	Collision-induces Dissociation
RC	Regenerated cellulose
CRM	Certified Reference Material
LOQ	Limit of Detection
LOQ	Limit of Quantification
ME	Matrix Effect
MF	Matrix factor
R	Recovery
RSDr	Repeatability
RSD_R	Reproductivity
GC	Gas Chromatography
LC	Liquid Chromatography
QTOF	Quadrupole time of flight mass spectrometer
LE	Low Energy
RP	Reversed-Phase
ACN	Acetonitrile
MeOH	Methanol
EtOH	Ethanol

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