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THESIS

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Option: Physico-Chemical Analysis and Reactivity of Molecular Species

Qualitative and semi-quantitative analysis of secondary metabolites produced by olive tree from various Algerian regions and evaluation of some spatio-temporal parameters.

Publicly presented and supported by :

Ahlem Tlili

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In front of the jury composed of :

Zehour Rahmani	Professor	Univ of Ouargla	President
Farah Ramdane	M.C.A	Univ of El Oued	Examiner
Abdelkrim Rebiai	M.C.A	Univ of El Oued	Examiner
Tarek Mekhalfi	M.C.A	Univ of Ouargla	Examiner
Mebarka Bouziane	Professor	Univ of Ouargla	Supervisor
Mahfoud Hadj Mahammed	Professor	Univ of Ouargla	Co-Supervisor

DEDICATION

A special dedication of my grateful feelings,

To my father: Mohammed Abdelkader

"May Allah have mercy upon him"

&

To my dear mother: Leila

"May Allah protect her"

Who encouraged me enormously.

To my brothers Adel, Abdelhak, Ayoub

&

My little sister Maram.

Who were always here for me.

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Thank you all

List of abbreviations

ACO	Acyl-CoA oxidase
AGE	Advanced Glycation End
ANOVA	Analysis of Variance
EC	Electrical Conductivity
F11	Volatiles of *O. europaea* flowers of cv. Chemlal from Mediterranean region
F12	Volatiles of *O. europaea* flowers of cv. Sigoise from Mediterranean region
F21	Volatiles of *O. europaea* flowers of cv. Chemlal from arid region
F22	Volatiles of *O. europaea* flowers of cv. Sigoise from arid region
GAE	Gallic Acid Equivalent
GC-EIMS	Gas Chromatography and Electron Ionization Mass Spectrometry
GLP-1	Glucagon-Like Peptide-1
HPLC-DAD	High-Performance Liquid Chromatography with Diode Array Detector
IC_{50}	The median Inhibitory Concentration
iNOS	inducible Nitric Oxide Synthase
L11	Volatiles of *O. europaea* leaves of cv. Chemlal from Mediterranean region
L111	Volatiles of *O. europaea* leaves of cv. Chemlal collected in July from Mediterranean region
L112	Volatiles of *O. europaea* leaves of cv. Chemlal collected in October from Mediterranean region
L113	Volatiles of *O. europaea* leaves of cv. Chemlal collected in January from Mediterranean region
L114	Volatiles of *O. europaea* leaves of cv. Chemlal collected in April from Mediterranean region
L12	Volatiles of *O. europaea* leaves of cv. Sigoise from Mediterranean region
L21	Volatiles of *O. europaea* leaves of cv. Chemlal from arid region
L22	Volatiles of *O. europaea* leaves of cv. Sigoise from arid region
MAE	Maceration with agitation Extraction
NO	Nitric Oxide
O111	*O. europaea* leaves of cv. Chemlal collected in July from Mediterranean region
O112	*O. europaea* leaves of cv. Chemlal collected in October from Mediterranean region
O113	*O. europaea* leaves of cv. Chemlal collected in January from Mediterranean region
O121	*O. europaea* leaves of cv. Sigoise collected in July from Mediterranean region

O122	*O. europaea* leaves of cv. Sigoise collected in October from Mediterranean region
O123	*O. europaea* leaves of cv. Sigoise collected in January from Mediterranean region
O211	*O. europaea* leaves of cv. Chemlal collected in July from arid region
O212	*O. europaea* leaves of cv. Chemlal collected in October from arid region
O213	*O. europaea* leaves of cv. Chemlal collected in January from arid region
O221	*O. europaea* leaves of cv. Sigoise collected in July from arid region
O222	*O. europaea* leaves of cv. Sigoise collected in October from arid region
O223	*O. europaea* leaves of cv. Sigoise collected in January from arid region
OLE	*O. europaea* Leaf Extracts
P1	Plot 1= 20 to 40 cm (depth)
P2	Plot 2= 40 to 60 cm (depth)
PCA	Principal Component Analysis
pH	Potential of Hydrogen
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
PRESS	Predicted Residual Sum of Squares
R	Coefficient of determination
RE	Rutin Equivalent
RSM	Response Surface Methodology
SMs	Secondary metabolites
ST	Soil Type
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
t_R	Retention Time
UARE	Ultrasonic Assisted-Reflux Synergistic Extraction
VOFs	Volatiles of *O. europaea* Flowers
VOLs	Volatiles of *O. europaea* Leaves
X_1	Concentration of Methanol, %
X_2	Solvent/Material Ratio, mL/g
X_3	Extraction Time, min
Y	Extraction Yield

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Abstract

The olive tree (*Olea europaea* L.) is mainly cultivated to produce olive oil for food purposes. However, the medicinal properties are attributed to its by-product especially the leaves which are currently the topic of a great deal of scientific research. The cultivation of *O. europaea* is very old and widespread in northern Algeria. Though, its appearance in the Saharan regions is quite recent.

This work aims to study the variation of leaves and flowers' chemical compositions through their production of certain secondary metabolites in two cultivars of *O. europaea* namely Chemlal and Sigoise, both cultivated in the Mediterranean and arid regions of Algeria and harvested, for this purpose, during different seasons.

To evaluate the extraction of the total phenolic content, both maceration with stirring and synergistic ultrasonic assisted-reflux extraction methods were used. A statistical study of the surface response methodology was carried out by the MINITAB software to optimize the extraction conditions of the two extraction methods. 12 fractions were obtained and analyzed by HPLC-DAD. The extraction of the volatile compounds by hydrodistillation was also carried out. 11 fractions were obtained and analyzed by GC-EIMS.

The statistical study by XLSTAT software showed that the climatic conditions, through different seasons, affected mainly the production of the major compounds in relation to the cultivation area or the cultivar type.

The present study revealed that cv. Sigoise cultivated in the arid region, has the highest contents of major compounds particularly oleuropein and luteolin-7-glucoside. In addition, the harvest season has a strong effect on the variation of polyphenols. The main substances in the volatile fractions from the Mediterranean region were (*Z*)-jasmone, nonanal, and aspiran II while those from the arid region were bornyl acetate, 3-ethenylpyridine, and (*E*,*E*)-2,4-heptadienal.

These results may provide valuable insights into the effect of arid climate conditions on the qualitative and quantitative production of bioactive natural products by *Olea europaea*. In addition, we have noticed an exceptional adaptation of the olive tree to the soil and climatic conditions of arid regions. On the agronomic level, new strategic crops should be encouraged in the vast Saharan regions of Algeria for their main products as well as for beneficial by-products such as olive leaves.

Keywords: *Olea europaea* L., Optimization, Extraction, Volatiles, Polyphenols, Oleuropein GC-MS, HPLC-DAD, Statistical, Arid climate.

Résumé

La principale utilisation de l'olivier (*Olea europaea* L.) est sans doute la production de l'huile d'olive à des fins alimentaires. Par ailleurs, les propriétés médicinales de *l'O. europaea* sont attribuées à ses feuilles qui font aujourd'hui l'objet de nombreuses recherches scientifiques. La culture d'*O. europaea* est très ancienne et très répandue dans le nord Algérien. Cependant, son apparition dans les régions sahariennes est assez récente.

Ce travail vise l'étude de la variation des compositions chimiques des feuilles et des fleurs de deux cultivars *d'O. europaea* (Chemlal et Sigoise), en particulier, leur production en métabolites secondaires dans deux zones différentes en Algérie (méditerranéenne et aride) durant différentes saisons.

Afin d'évaluer l'extraction du contenu en phénols totaux, les deux méthodes de macération avec agitation, et d'extraction synergique à reflux assisté par ultrasons ont été utilisées. Une étude statistique de la méthodologie de réponse de surface a été réalisée par le logiciel MINITAB pour optimiser les conditions d'extraction des deux méthodes d'extraction. 12 fractions ont été obtenues et analysées par HPLC-DAD. L'extraction des composés volatils par hydrodistillation a également été réalisée. 11 fractions ont été obtenues et analysées par GC-EIMS. L'étude statistique par le logiciel XLSTAT, a montré que les conditions climatiques, à travers les différentes saisons, affectaient principalement la production des composés majoritaires, en relation avec la zone de culture ou le type de cultivar.

Cette étude a révélé aussi, que le cultivar Sigoise cultivé dans la région aride a les teneurs les plus élevées en composés majoritaires notamment en oleuropéine et en lutéoline-7-glucoside. De plus, que la saison des récoltes a un fort effet sur la variation des polyphenols. Les principales substances dans les fractions volatiles de la région méditerranéenne sont la (*Z*)-jasmone, le nonanal et l'aspirane II alors que ceux de la région aride sont l'acétate de bornyle, la 3-éthénylpyridine et le (*E*,*E*)-2,4-heptadiénal.

Ces résultats peuvent fournir des informations précieuses sur l'effet des conditions climatiques arides sur la production qualitative et quantitative de produits naturels bioactifs par *Olea europaea*. Par ailleurs, nous avons constaté une adaptation exceptionnelle de l'olivier aux conditions pédologiques et climatiques des régions arides. Sur le plan agronomique, de nouvelles cultures stratégiques devraient être encouragées dans les vastes régions sahariennes de l'Algérie pour leurs principaux produits ainsi que pour les sous-produits bénéfiques tels que les feuilles d'olivier.

Mots clés : *Olea europaea* L., Optimisation, Extraction, Volatiles, Polyphénols, Oleuropéine GC-MS, HPLC-DAD, Statistique, Climat aride.

ملخص

تُزرع شجرة الزيتون (*Olea europaea* L.) بشكل أساسي لإنتاج زيت الزيتون للأغراض الغذائية. ومع ذلك، تُعزى الخصائص الطبية إلى منتجاتها الثانوية، وخاصة الأوراق، التي تعد حاليًا موضوع الكثير من الأبحاث العلمية. زراعة شجر الزيتون قديمة جدًا ومنتشرة في شمال الجزائر. و يعد انتشارها في المناطق الصحراوية من البلاد حديث لحد ما.

يهدف هذا العمل إلى دراسة تباين المركبات الكيميائية لأوراق وأزهار صنفين من شجرة الزيتون (شملال و سيقواز)، على وجه الخصوص، إنتاجهما لمركبات الايض الثانوي في منطقتين مختلفتين من الجزائر (البحر الأبيض المتوسط والجافة) خلال مواسم مختلفة.

من أجل تثمين استخلاص المركبات الفينولية، تم استخدام كل من النقع مع التحريك و الاستخلاص التآزري بالارتداد باستعمال الموجات فوق الصوتية (synergistic ultrasonic assisted-reflux). تم إجراء دراسة إحصائية لمنهجية الاستجابة السطحية بواسطة برنامج MINITAB لتحسين ظروف الاستخلاص لكلا الطريقيتين .ثم الحصول بعدها على 12 مستخلص وتحليلهم بواسطة الكروماتوغرافيا السائلة (HPLC-DAD). كما تم الحصول ايضا على 11 مستخلص عن طريق التقطير المائي و تحليلهم بواسطة الكروماتوغرافيا الغازية بالتزاوج مع مطيافية الكتلة (GC-EIMS).

أظهرت الدراسة الإحصائية التي أجريت ببرنامج MINITAB، ثم برنامج XLSTAT للمركبات المتطايرة التي تم تحليلها بواسطة GC- EIMS، و كذلك المركبات الفينولية التي تم تحليلها بواسطة HPLC-DAD، أن الظروف المناخية المختلفة، أثرت بشكل رئيسي على إنتاج المركبات مقارنة بمنطقة الزراعة أو نوع الصنف.

كشفت هذه الدراسة ايضا أن صنف سيقواز المزروع في المنطقة الجافة من الجزائر، يحتوي على نسب اكبر من المركبات الرئيسية، وخاصة ال oleuropein و luteolin-7-glucoside.

موسم الحصاد له كذلك تأثير قوي على تباين المركبات الفينولية. اما بالنسبة للمستخلصات المتطايرة، فتمثلت المركبات الرئيسية في منطقة البحر الأبيض المتوسط ب: (*Z*) -jasmone، nonanal، وaspiran II. بينما في المنطقة الجافة فكانت: bornyl acetate، 3-ethenylpyridine، و (*E*,*E*)-2,4-heptadienal .

قد توفر هذه النتائج معلومات جد مهمة حول تأثير الظروف المناخية الجافة على الإنتاج النوعي والكمي للمنتجات الطبيعية النشطة بيولوجيًا بواسطة *Olea europaea*. بالإضافة إلى ذلك، لاحظنا تكيفًا استثنائيًا لشجرة الزيتون مع التربة والظروف المناخية للمنطقة الجافة على المستوى الزراعي، و عليه ينبغي تشجيع الزراعات الاستراتيجية الجديدة في المناطق الصحراوية الشاسعة من الجزائر؛ لمنتجاتها الرئيسية وكذلك للمنتجات الثانوية المفيدة مثل أوراق الزيتون.

الكلمات المفتاحية: *Olea europaea* L. ، تحسين، استخلاص، المركبات المتطايرة، بوليفينول، أوليوروبين، GC-EIMS، HPLC-DAD، احصاء، مناخ جاف.

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General introduction

General introduction

Nowadays, there has been considerable interest in natural bioactive products derived from medicinal plants to replace synthetic products. Indeed, the olive tree (*Olea europaea* L.) is one of their main bioresources; it has been widely accepted as one of the species with the highest antioxidant activity via its oil, fruits, and leaves [1-3]. Literature on *Olea europaea* polyphenols has focused on olive oil consumption as a main dietary source [3]. However, *O. europaea* leaves (OEL) are distinguished by higher antioxidant value and are found to have higher phenolic compounds compared to the other parts of the plant [3-4]. OEL are considered both an important agricultural biomass and an easily available industrial (natural material of low cost).

OEL have been considered for centuries as an important herbal remedy in Mediterranean countries. They have been used in the human diet as an extract, herbal tea, and powder. Their benefits on human health are multiple: antioxidant, hypotensive, hypoglycemic, antimicrobial, antiatherosclerotic, antimycoplasmal, diuretic, antitumoral, anti-inflammatory, and antipyretic [3-5].

Generally, these properties are attributed to the presence of a great range of phenolic compounds, such as secoiridoids, triterpenes, lignans, flavonoids.. etc [6]. One of these potentially bioactive compounds is the secoiridoid Oleuropein, which can constitute up to 6-9% of dry matter in the leaves; for this reason, it was considered the most important compound concerning such properties [7].

The phenolic compounds in OEL can be affected by numerous intrinsic and extrinsic factors including; cultivar type, collection time, degree of ripeness, region of collection and method used for extraction [8].

In Algeria, the olive oil sector has the highest development potential, due to the increasing demand over the past two decades [9]. Although the *OE* tree is native to the Mediterranean region, the Algerian government was and still providing extra efforts encouraging extension services to extend *OE* cultivation acreage southward deep into the heart of the Sahara, which constitutes more than four-fifths of the country's area [10]. However, less exploration was held related to *OE* tree adaptation in this vast hard area, due to lack of quality control, lack certification, labelling, and analyses [11].

This thesis represents a contribution to the international scientific knowledge in the field of research on the chemical composition of medicinal plants, particularly their bioactive

natural products; it falls within the framework of the promotion of the various *OE* cultivars for sustainable development of the olive oil sector in Algeria.

Currently, the application of statistical methods is very helpful in providing insights about data in all fields of research. So many results concerning the chemical compositions, obtained during our experimental work, were effectively presented with a statistical significance; the latter has been very useful for interpretation and fruitful in highlighting the different groups of secondary metabolites/compositions relationships.

Overall, our study is divided into two parts:

- The first part is devoted to the bibliographic study; it contains two chapters:
  - The first one is dedicated to generalities on secondary metabolites.
  - The second is devoted to the chemical composition of *OE*.

- The second part represents the experimental part; which is divided into three chapters:
  - Chapter I: Optimization of extraction conditions of the phenolic compounds in *OE* leaves.
  - Chapter II: Phenolic compounds of *OE* leaves.
  - Chapter III: Volatile compounds of *OE* leaves and flowers.

The manuscript ends with a general conclusion and perspectives.

Part one:

Literature review

Chapter I:

Generalities on secondary metabolites

I.1. Introduction

The market for natural additives and ingredients is rapidly growing, with some natural products obtaining high prices. Moreover, the possible toxicity of certain synthetic compounds has led to an increased interest in natural product research from the cosmetic, pharmaceutical, and food additive industries [12-15].

Plant chemistry is the basis of the therapeutic uses of herbs [1, 16]. Good knowledge of the chemical composition of plants leads to a better understanding of its possible medicinal value.

***History:** The concept of SMs was first defined by Albrecht Kossel, Nobel Prize winner for physiology or medicine in 1910. Thirty years later, Czapek described them as end-products. According to him, these products are derived from nitrogen metabolism by what he called 'secondary modifications' such as deamination [17-18]. In the middle of the twentieth century, advances of analytical techniques such as chromatography allowed the recovery of more and more of these molecules, and this was the basis for the establishment of the discipline of phytochemistry [19-20].

I.2. Secondary metabolites

Secondary plant metabolites (SMs) are numerous chemical compounds produced by the plant cell through metabolic pathways derived from the primary metabolic pathways (**Figure I.1**) [1, 16]. They are molecules of low molecular weight with diverse chemical structures and biological activities.

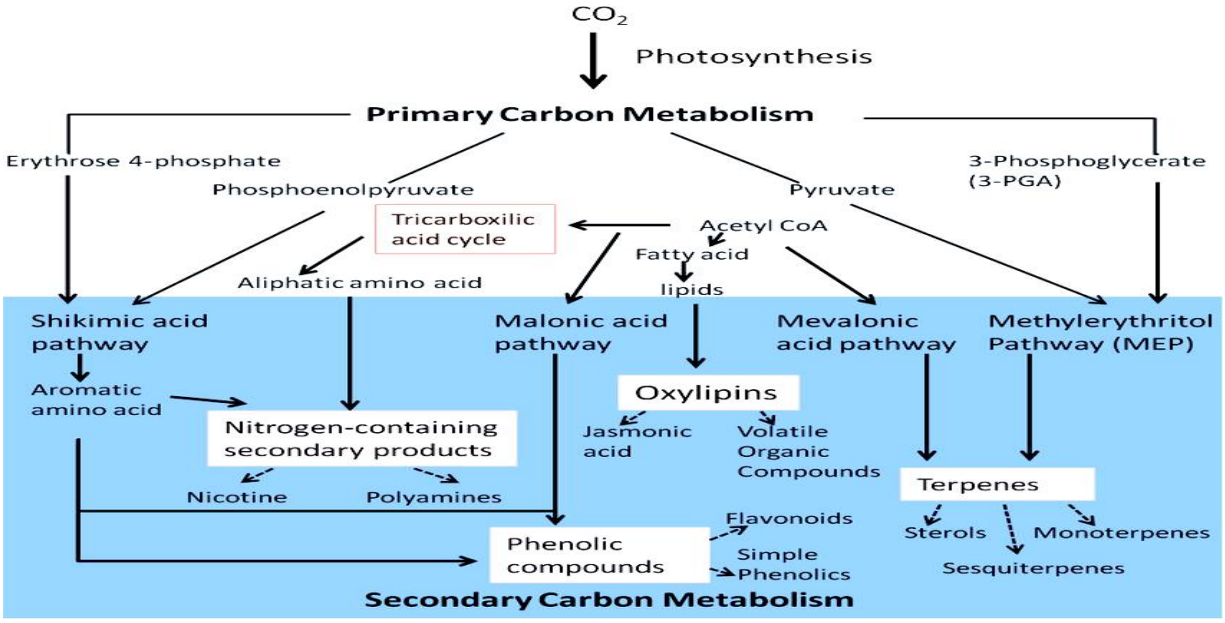


Figure I.1. Interrelationships between the primary and secondary metabolism [16].

Identification: The name secondary metabolite originates from the initial observation that their production is not necessary for the growth and reproduction of organisms, in contrast to primary metabolites which include lipids, amino acids, carbohydrates, and nucleic acids. However, SMs are far from being secondary and the term "specialized metabolites" is emerging to describe them. It is now accepted that SMs play key roles in the survival of the organisms that produce them because they determine interactions within their environment. Nowadays, SM production is a major research field for organic chemists, molecular biologists, and bioinformaticians alike [21].

I.3. Secondary metabolites biological effects

SMs have been shown to possess various biological effects, which provide the scientific base for the use of herbs in traditional medicine in many ancient communities. They have been described as an antibiotic, antifungal, and antiviral and therefore are able to protect plants from pathogens. Besides, they constitute important UV absorbing compounds, thus preventing serious leaf damage from the light. In fact, many researches proved the biological potential and medical use of SMs. They are medicines, used as a single compound or as a mixture that can be effective and safe even when synthetic drugs fail. Furthermore, they may even potentiate or synergize the effects of other compounds in the medicine [21-22].

I.4. Secondary metabolites classification

SMs are classified according to their chemical structures into four major categories as classified by British Nutrition Foundation. These four categories include phenolics (such as lignans, phenolic acid, tannins, coumarins, lignins, stilbenes, and flavonoids), terpenoids (such as carotenoids, sterols, cardiac glycosides, and plant volatiles), nitrogen-containing compounds (such as non-protein amino acids, cyanogenic glucosides, and alkaloids) and sulfur-containing compounds (such as glucosinolates, phytoalexins, thionins, and defensins) [22-23].

I.4.1. Phenolics

Phenolics constitute a large group of SMs. They share the presence of one or more phenol groups (**Figure I.2**) as a common characteristic and range from simple structures with one aromatic ring to highly complex polymeric substances [1, 24].

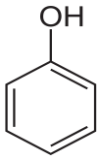


Figure I.2. Phenol structure [24].

Phenolics are widespread in plants where they contribute significantly to the color, taste and flavor of many herbs, foods and drinks. They protect plants from herbivory, pathogen attack and other animals due to their deterrent abilities. Their high concentration also imparts fungal resistance. Phenolic compounds often found attached to sugars which reduces their endogenous toxicity. They also shield plants from UV radiation and cold stress. Some phenolics are valued pharmacologically for their anti-inflammatory activities also effective antioxidants and free radical scavengers such as quercetin or antihepatotoxic properties such as silybin [23-24].

Phenolics classification

More than 8000 phenolic compounds have been discovered so far. They can be classified according to their biosynthetic origin or to their structure as presented in **Figure I.3** and **Table I.1** [22].

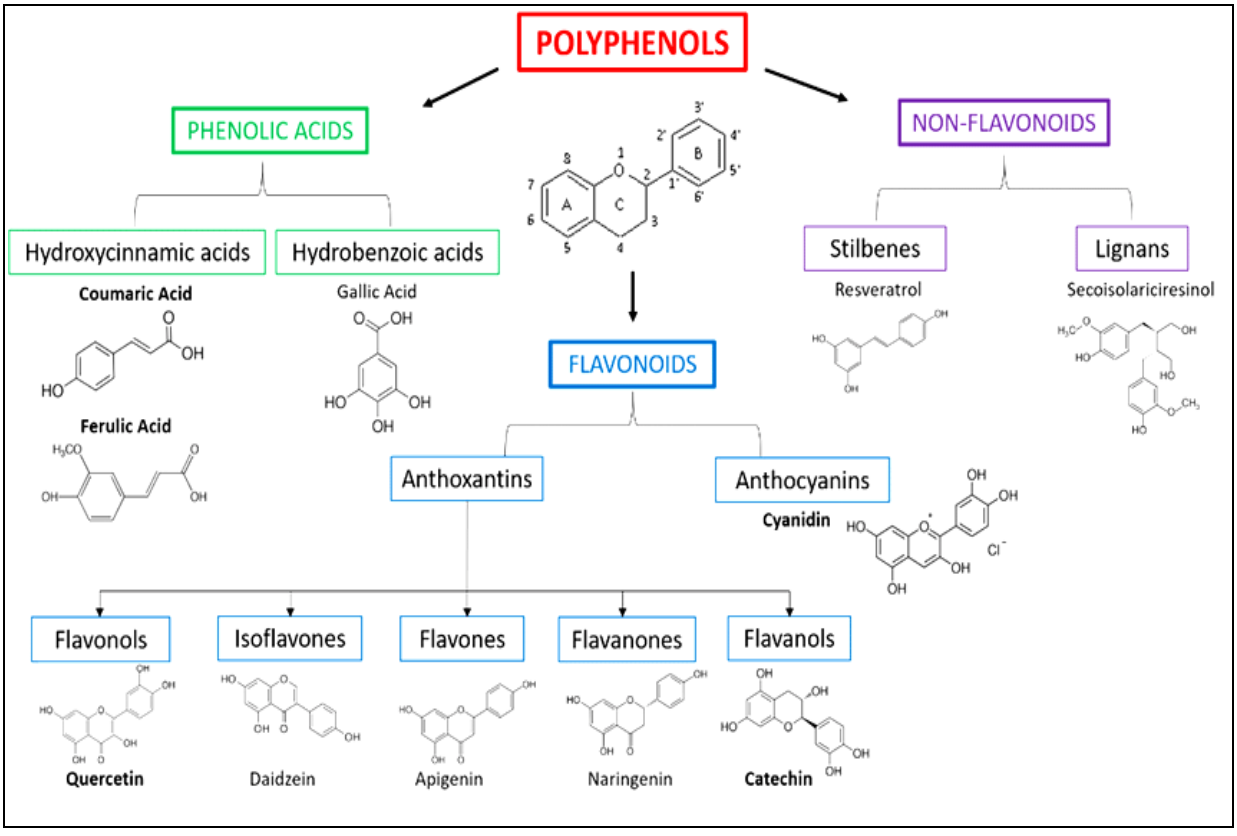
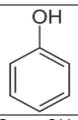
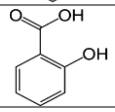
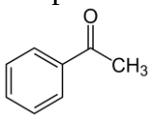
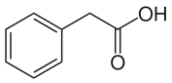
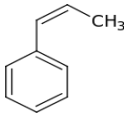
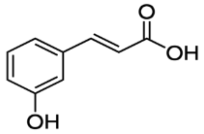
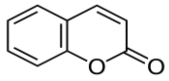
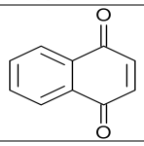
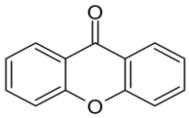
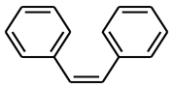
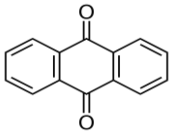
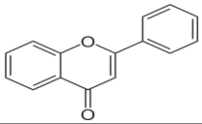
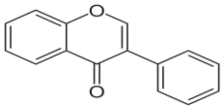
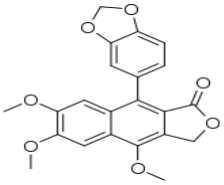
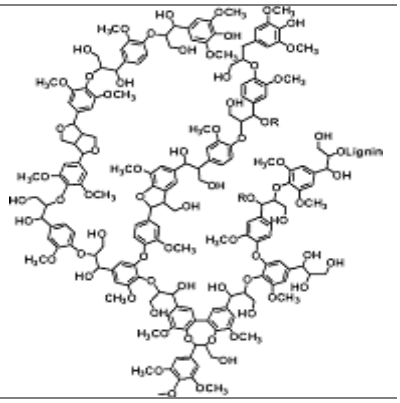
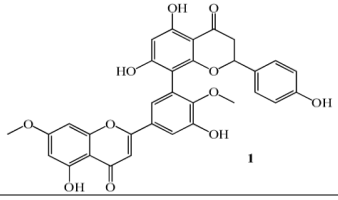


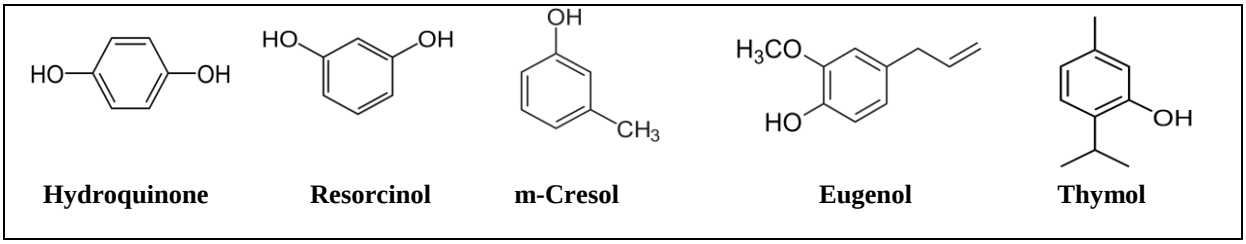
Figure I.3. Schematic classification of polyphenols [25].

Table I.1. The main classes of phenolic compounds [23-26].

No. of carbon atoms	**Basic skeleton**	**Class**
6	C_6	Simple phenols 
7	$C_6 - C_1$	Phenolic acids 
8	$C_6 - C_2$	Acetophenone,  Phenyle acetic acid 
9	$C_6 - C_3$	Phenylepropanoids, hydroxycinnamic acid, coumarins   
10	$C_6 - C_4$	Naphthoquinone 
13	$C_6 - C_1 - C_6$	Xanthone 
14	$C_6 - C_2 - C_6$	Stilbene,  anthraquinone 
15	$C_6 - C_3 - C_6$	Flavonoids,  isoflavanoids 
18	$(C_6 - C_3)_2$	lignans, neolignans  
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids 

- **Simple phenolics**

They are described as compounds having at least one hydroxyl group attached to an aromatic ring as a basic skeleton. The phenolic compounds in this group vary according to their functional group, which may be hydroxyl, aldehydic, or carboxylic group; these include eugenol (a phenolic phenylpropane), vanillin (a phenolic aldehyde), and salicylic, phenolic acids which can be divided into two classes: derivatives of benzoic acid such as gallic acid, and derivatives of cinnamic acid such as coumaric, caffeic and ferulic acid. Hydroquinone is also among the most widely distributed of the simple phenols, occurring in a number of plants as the glycoside arbutin. Glycoside formation is common, and the widely distributed glycoside coniferin and other derivatives of phenolic cinnamic alcohols are precursors of lignin [17, 23-26].



Hydroquinone **Resorcinol** **m-Cresol** **Eugenol** **Thymol**

Figure I.4. Examples of simple phenolics [17, 23].

- **Tannins**

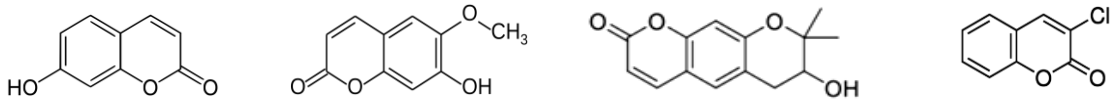
Tannins are polyphenols which have the ability to precipitate protein. There are two major types of tannins: hydrolyzable tannins and condensed tannins [24-26].

*Hydrolyzable tannins are formed from several molecules of phenolic acids such as Gallic and hexahydroxydiphenic acids, which are united by ester linkages to a central glucose molecule [17].

*Condensed tannins, or proanthocyanidins, are compounds whose structures are based on oligomeric flavonoid precursors and vary in the type of linkages between flavonoid units; hydroxylation patterns; stereochemistry of carbons 2, 3 and 4 of the pyran ring and the presence of additional substituents [17].

- **Coumarins**

Coumarins are derivatives of benzo-α-pyrone, the lactone of *O*-hydroxycinnamic acid, coumarin. More than 1300 natural coumarins have been identified. Coumarin itself has been found in about 150 species belonging to over 30 different families [26-27].



Umbelliferone **Scopoletin** **Decursinol** **Chlorocoumarin**

Figure I.5. Examples of coumarins [23-27].

- **Flavonoids**

Flavonoids are the largest group of naturally occurring phenolic compounds, which occurs in different plant parts both in free state and as glycosides [28].

The structural skeleton of flavonoids (**Figure I.6**) includes a chroman ring bearing an aromatic ring in position 2, 3 or 4. The structure consists of 15 carbon atoms (C_6–C_3–C_6). The aromatic ring A is derived from the acetate/malonate pathway, and ring B is derived from phenylalanine through the shikimate pathway [25]. Variations in substitution patterns to ring C (oxygenation, alkylation, glycosylation, acylation or sulfation) result in 13 flavonoid classes, being the most important flavonols, flavones, isoflavones, flavanones, flavanols (also called flavan-3-ols) and anthocyanidins or anthocyanins [23-26]. The chalcones are intermediate in the biosynthesis of flavonoids.

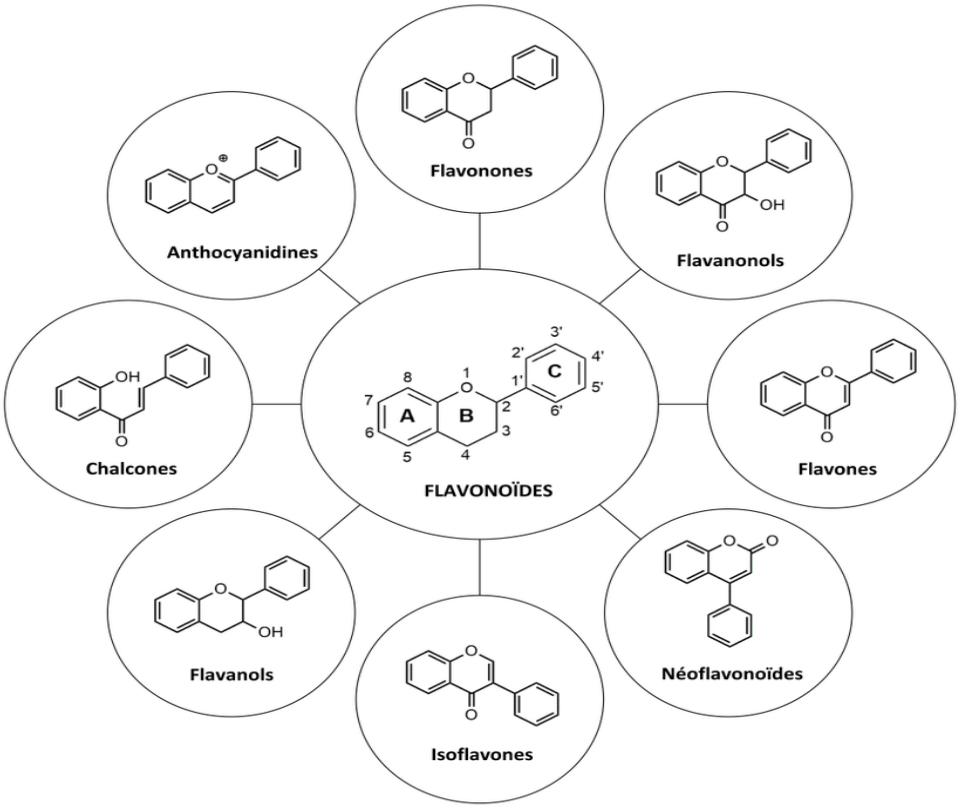


Figure I.6. Structures of the flavonoids classes [29].

Flavonoids have been found to prevent low density lipid peroxidation and thereby prevent atherosclerotic plaque formation, neurodegenerative diseases and ischemic injury. They also capture free radicals and chelate copper and iron ions, which can promote free radical generation. They also stimulate enzymes which are involved in detoxification of cancer causing substances and inhibit inflammation and aging processes [26, 29-30].

- **Stilbenes**

Stilbenes are a small group of phenylpropanoids characterized by a 1,2-diphenylethylene backbone. Most plant stilbenes are derivatives of the basic unit *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene). In plants that naturally produce stilbenes, these metabolites are generally accumulated in both free and glycosylated forms [31].

- **Lignans**

Lignans are formed of two phenylpropane units, which are commonly present in fruits, seeds, grains, trees and vegetables. Secoisolariciresinol and matairesinol (**Figure I.7**) were the first plant lignans identified, and later pinoresinol, lariciresinol and others [32].

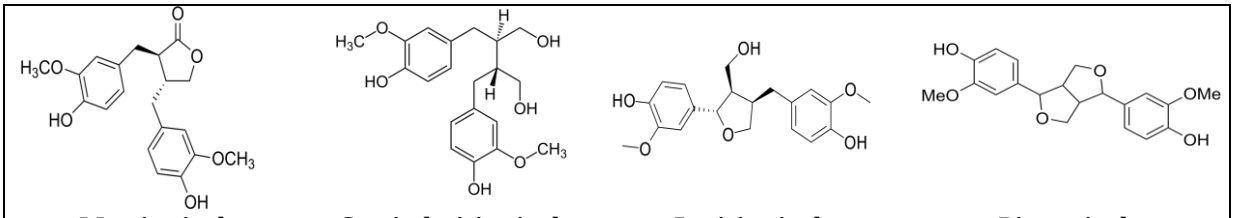
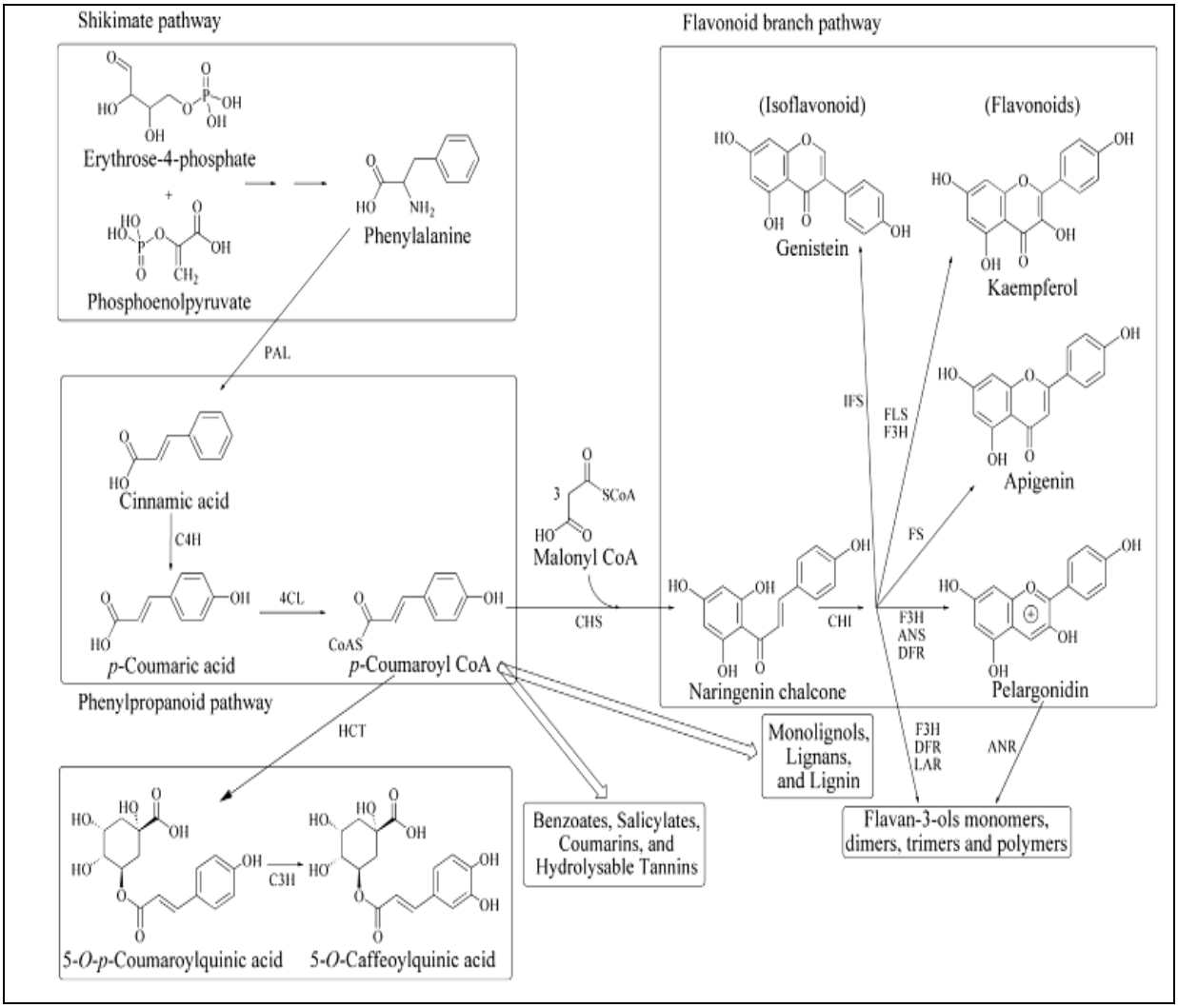


Figure I.7. Examples of lignans [32].

I.4.2. Phenolics biosynthesis pathway

Phenolic compounds are synthesized through two metabolic pathways: the shikimic acid pathway where phenylpropanoids are formed, and the acetic acid pathway, in which the main products are the simple phenols. The combination between both pathways leads to the formation of flavonoids, the most abundant group of phenolic compounds in nature, subsequently, producing PAs. **Figure I.7** is a summary for the whole pathway of phenolic compounds based on many references [26, 33-35].



PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; HCT, hydroxycinnamoyl transferase; C3H, *p*-coumarate-3-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; ANS, anthocyanidin synthase; DFR, dihydroflavonol reductase; FS, flavone synthase; FLS, flavonol synthase; F3H, flavanone 3-hydroxylase; IFS, isoflavone synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase.

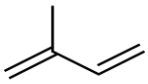
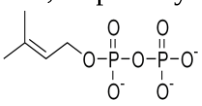
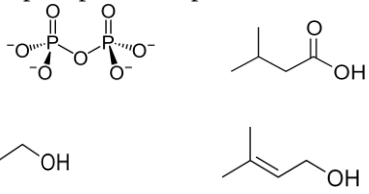
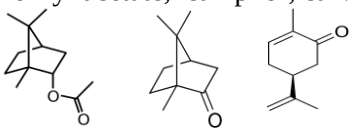
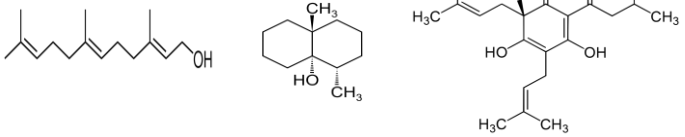
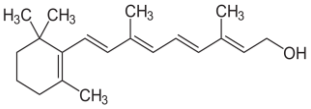
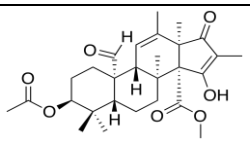
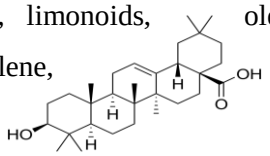
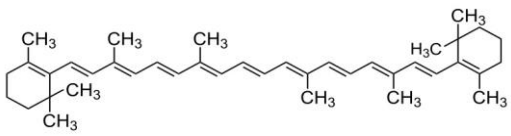
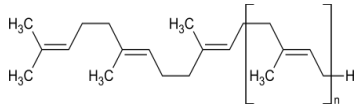
Figure I.8. Schematic of the major branch pathways of phenolics biosynthesis [34].

I.4.3. Terpenoids

Terpenoids are the largest class of plant secondary metabolites, representing about 60% of known natural products. Many terpenoids have substantial pharmacological bioactivity and are therefore of interest to medicinal chemists [36].

The terpenoids, also known as isoprenoids, derived from the 5-carbon compound isoprene, and the isoprene polymers called terpenes. While, sometimes used interchangeably with "terpenes", terpenoids contain additional functional groups, usually containing oxygen. The general formula of terpenes of $(C_5H_8)_n$. Classification is based on the number of isoprene units 'n' present in their structure (**Table I.2**).

Table I.2. Terpenoids classification with examples of chemical structures [36].

Terpenoids	**Analogue terpenes**	**General formula**	**Examples**
Hemiterpenoids	Isoprene  **01 isoprene Units**	C_5H_8	DMAPP, isopentenyl , Pyrophosphate, isoprenol,   isovaleramide, isovaleric acid, HMBPP, prenol
Monoterpenoids	Monoterpenes **02 isoprene units**	$C_{10}H_{16}$	Bornyl acetate, camphor, carvone, citral, citronellal,  citronellol, geraniol, eucalyptol, hinokitiol, iridoids, linalol, menthol, thymol
Sesquiterpenoids	Sesquiterpens **03 isoprene Units**	$C_{15}H_{24}$	Farnesol, geosmin, humulone 
Diterpenoids	Diterpenes **04 isoprene Units**	$C_{20}H_{32}$	Abieticacid, ginkgolides, paclitaxel, retinol, salvinorin A, sclareol, stevio 
Sesterterpenoids	Sesterterpenes **05 isoprene Units**	$C_{25}H_{40}$	Andrastin A, manoalide 
Triterpenoids	Triterpenes **06 isoprene Units**	$C_{30}H_{48}$	Amyrin, betulinic acid, limonoids, oleanolic acid, sterols, squalene,  ursolic acid
Tetraterpenoids	Tetraterpenes **08 isoprene units**	$C_{40}H_{64}$	Carotenoids 
Polyterpenoid	Polyterpenes >8 **isoprene units**	$(C_5H_8)_n$	Gutta-percha, natural rubber 

Terpenoids are normally produced in vegetative tissues, flowers, and occasionally roots. They are involved in the direct defense of plants against herbivores, and microbial pathogens.

I.4.4. Terpenoids biosynthesis pathway

Terpenoids are produced from the universal building blocks, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP and DMAPP are synthesized through two different biosynthetic pathways. One of these occurs in the plastids and supplies mostly the substrates for the production of monoterpenoids (often present in essential oils), diterpenoids, and tetraterpenoids (carotenoids). The other pathway, known as the mevalonate (MVA) pathway takes place in the cytosol. The IPP and DMAPP derived from this pathway are mostly used as substrates in the production of sesquiterpenoids and triterpenoids [34, 36-38].

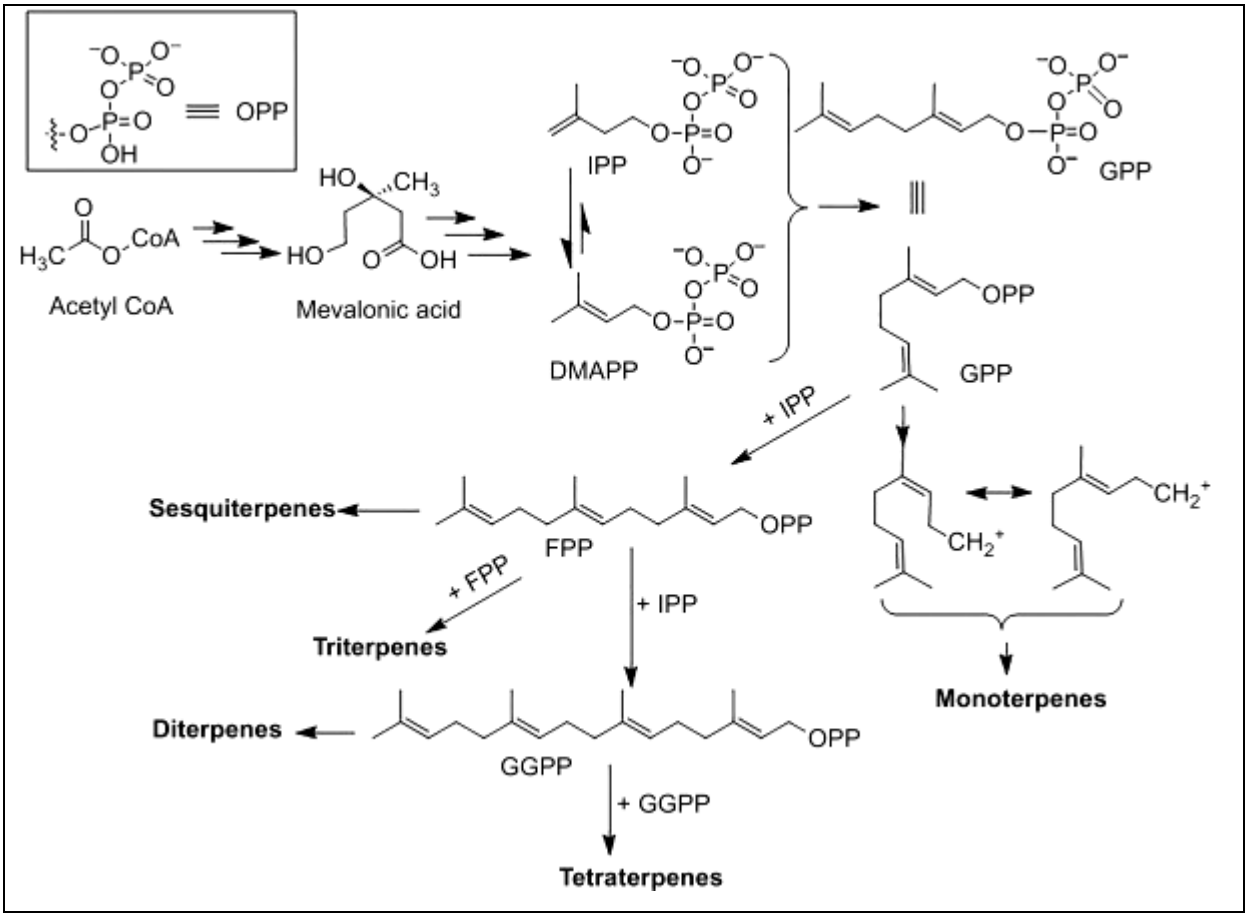


Figure I.9. Schematic of the terpenoids biosynthesis [37].

I.5. Methods for Analyzing Plant Metabolites

Plant metabolomics methods have been used for identifying functional secondary metabolites and metabolic pathways for both basic and applied research. Those methods help provide comprehensive perspectives on how plant metabolic networks are regulated. The most widely used methods include gas chromatography (GC) -mass spectrometry (-MS) (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis-MS (CE-MS), nuclear

magnetic resonance spectroscopy (NMR), Fourier transform-near-infrared (FT-NIR) spectroscopy, MS imaging (MSI), and live single-cell -MS (LSC-MS). These methods are often used in combination because they can provide largely complementary information with each other by analyzing different types of metabolites. A number of excellent technical reviews and detailed protocols regarding the utilization of these analytical tools in metabolomics experiments have been published [32-38].

I.6. Factors affecting secondary metabolites production

The plant growth and development are usually elicited or inhibited by different external factors or variables (light, temperature, soil water, soil fertility, and salinity). Therefore, the adaptation of plant morphology, anatomy, and physiological functions to the changes in biotic and abiotic may influence the accumulation of secondary metabolites (**Figure I.8**). Eventually leading to the change of overall phytochemical profiles which play a strategic role in the production of bioactive substances [39].

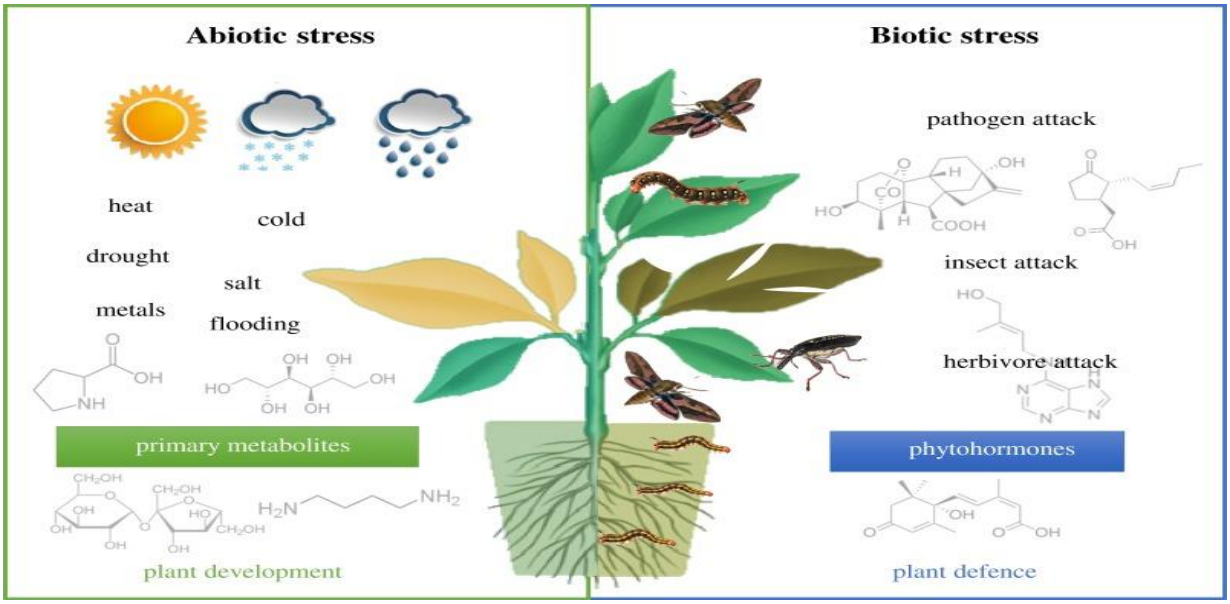


Figure I.10. Stress and defense responses in secondary metabolites production [39].

- **Root and stem:** Plant roots and stems are the main organs responsible for the accumulation of active components with important medicinal value. In root and stem herbs, the accumulation of active components is mainly affected by growth periods, growth seasons, and growth years. Some medicinal plants accumulate abundant of SMs mainly during their reproductive growth period [39].

- **Leaf:** Plants leaves are the main organs of plants for photosynthesis and play an important role in the life of plants. Leaves can also be used as a synthetic and storage organs for SMs. Leaf age, harvesting season, and growth stage, all affect the content of SMs in medicinal plant leaves [39].

- **Flower:** Most of the flowers of plants have an aromatic smell mainly composed of terpenes and aromatic compounds, whose synthesis and accumulation dynamics are mainly regulated by different development stages, circadian rhythm, biological and abiotic factors [39].

Currently, lots of literatures focused on the effect of environmental factors, the effect of the developmental growth, and the effect of genetic factors on the synthesis and accumulation of SMs of medicinal plants, which still have a lack of systematic classification and summary [39-41].

I.7. Conclusion

The use of herbal medicines should be based on comprehensive phytochemical studies for the determination of the chemical constituents of the herbs involved and the factors that affect their production. Hence the knowledge of the resultant pharmacological and toxicological effects can be deduced, as well as the possible synergistic or antagonistic effects due to the use of multiple component herbal formulae. For this reason, the isolation and structural elucidation of secondary plant metabolites, though ancient, is still a huge and fast-growing approach, and the techniques used for separation and analysis are advancing continuously.

Chapter II:

Generalities on Olea europaea L.

II.1. Introduction

Fossil evidence indicates the olive tree had its origins 20–40 million years ago in the Oligocene [42], in what is now corresponding to Italy and the eastern Mediterranean Basin [41]. Wild oleasters were present and collected in the East Mediterranean since ~19,000 BP. The genome of cultivated olives reflects their origin from oleaster populations in the East Mediterranean. The olive plant was first cultivated some 7,000 years ago in Mediterranean regions. The related literature aims to identify the olive tree compositions and their biological activities [41-42].

II.2. Generalities on *Olea europaea* L.

Olea europaea tree is found traditionally in the Mediterranean Basin. The species is cultivated in all the countries of the Mediterranean, as well as in Australia, New Zealand, North and South America and South Africa. *O. europaea* is the type species for the genus *Olea* [3-6, 41].

The olive's fruit, also called an "olive", is of major agricultural importance in the Mediterranean region as the source of olive oil; it is one of the core ingredients in Mediterranean cuisine. The tree and its fruit give their name to the plant family, which also includes species such as lilacs, jasmine, *Forsythia*, and the true ash trees (*Fraxinus*) [40].

II.3. *Olea europaea* classification

The olive, botanical name *O. europaea*, meaning "European olive", is a species of small tree in the family Oleaceae [43]:

Kingdom: Plantae
Subkingdom: Tracheophytes
Superdivision: Spermatophyta
Division: Magnoliophyta
Class: Magnoliopsida
SubClass: Asterids
Order: Lamiales
Family: Oleaceae
Genus: *Olea*
Species: *O. europaea*.

Figure II.1 *O. europaea* L. tree [44].

II.4. *Olea europaea* description

The *O. europaea* tree is an evergreen tree or shrub, short and squat, and rarely exceeds 8–15 m in height [45].

The silvery green leaves are oblong, measuring 4–10 cm long and 1–3 cm wide. The trunk is typically gnarled and twisted [45].

The small, white, feathery flowers, with tencleft calyx and corolla, two stamens, and bifid stigma, are borne generally on the previous year's wood, in racemes springing from the axils of the leaves [45].

Figure II.2 Botanical illustrations of *O. europaea* [42].

The fruit is a small drupe 1–2.5 cm long when ripe, thinner-fleshed and smaller in wild plants than in orchard cultivars. Olives are harvested in the green to purple stage [40].

II.5. Cultivars of *Olea europaea* tree

The large expansion area and long life of the *O. europaea* tree explain the vast number of existing cultivars (**Table II.1**), over 2600. The behavior of each variety in each region results from genetic determinism, which is expressed in the characteristics of each cultivar. These genetic traits are then expressed in phenology, fruit ripeness, resistance to stress, resistance to pests and diseases, final yield, and oil quality. Despite the different characteristics of each cultivar, it is known that most of these expressions are also strongly conditioned by the pedoclimatic conditions prevalent in each olive grove [46].

Table II.1. Top countries by olive production (2018–2020) and their most used cultivars [47].

Country	**Prod. (t)**	**Area (ha)**	**Main Cultivars**
Spain	6,559,884	2,573,473	Arbequina, Alorena, Cornicabra, Empeltre, Farga, Gordal Sevillana, Hojiblanca, Lechín de Sevilla, Manzanilla de Sevilla, Morisca, Negral, Nevadillo, Picual, Picudo.
Greece	2,224,096	887,177	Anphissis, Chalkidiki, Conservolia, Kalamon, Koroneiki, Kolybada, Lianolia, Mastoidis, Megaritiki

Italy	2,092,175	1,165,562	Ascolana, Bella di Cerignola, Biancolilla, Bosana, Canino, Carolea, Casaliva, Coratina, Frantoio, Leccino, Moraiolo, Nocellara del Belice, Nocellara etnea, Ogliarola, Pendolino, Peranzana, Taggiasca.
Turkey	1,730,000	845,542	Ayvalik, Domat, Erkence, Çakir, Memecik, Memeli, Uslu, Izmir Sofralik, Gemlik.
Morocco	1,416,107	1,008,365	Picholine Marocaine, Dahbia, Haouzia, Menara, Meslala.
Syria	899,435	765,603	Zaity, Sorani, Doebli, Khoderi, Kaissy, Abo satl Mossabi, Dan, Jlot.
Tunisia	700,000	1,646,060	Chétoui, Chemlali, Oueslati, Chemlali Tataouine, Zalmati, Gerboui, Baroni, Rkhami.
Algeria	696,962	424,028	Aaroun, Azeradj, Blanquette, Bouchouk, Chemlal, Ferkani, Khadraya, Hamra, Limli, Mekki, Sigoise, Roulette.
Egypt	694,309	67,293	Aggizi Shame, Kosiem, Maraki, Meloky, Hamed, Sebhawi, Sinawy, Toffahi, Wateken.
Portugal	617,610	355,075	Galega, Corbrançosa, Cordovil, Verdeal, Transmontana, Carrasquenha, Lentrisca, Madural.
World	20,337,435	10,185,151	

In Algeria, the olive trees and their names differ from one region to another. The best known cultivars are presented in the below table.

Table II.2. Top local Algerian olive cultivars and their oil yields (2007) [48].

Name	**Number of feet**	**Synonyms**	**Origin**	**Use**	**olive yields %**
Aghchren De Titest	3	No known synonym	Hamma Guergour (Setif)	Olive table	14 to 18
Aguenaou	4	Angaw	Bousselah (Setif)	Olive table	16 to 20
Blanquette De Guelma	4	No known synonym	Guelma	Olive oil	18 to 22

Chemlal	4	Achamlal, Achamli, Achemlal	Kabylie	Olive oil	18 to 22
Limli	4	Lmeli, Limeli	Sidi Aiche (Bejaia)	Olive oil	20 to 24
Rougette De Mitidja	4	No known synonym	Plaine De Mitidja	Olive oil	18 to 20
Sigoise	4	Olive de Telemcen, Olive du Tell	Plaine De Sig (Mascara)	Olive table	18 to 22

II.6. Pedoclimatic requirements of *Olea europaea* tree

In the Mediterranean Basin, traditional *O. europaea* orchards tend to have distinctive climatic conditions. *O. europaea* trees are considered one of the most suitable and best-adapted species to the Mediterranean-type climate. Long, warm and dry summers, with mild and wet winters, are general features of this climate [42, 46].

- **The tree geographical limits**

Globally, the *O. europaea* tree cultivation is approximately limited by the 30° to 45° parallels. This latitudinal belt suggests that climatic conditions are a key factor for the tree cultivation, and for its development cycle. The tree geographical limits, indicating that the *O. europaea* tree had to be cultivated at no more than 300 stages (53 km) from the Mediterranean coast [42, 46].

Pliny the Elder observed that the climatic limits were imposed by the sensitivity of the plant to low temperatures, winter frost and extremely high temperatures in summer [42, 46].

Fabiano stated that the olive tree will not grow either in very cold climates or in very hot ones, the most suitable climatic conditions for the cultivation of the olive tree are represented by what today is called a typical Mediterranean climate, which represents the transition between the arid climate of Northern Africa and the temperate rainy climate of Central Europe [42, 46].

- **Temperature**

As a matter of fact, an olive tree typically cannot withstand temperatures below −8 °C for more than one week. Very high summer temperatures may also limit its yield performances, maximum temperatures higher than ~30 °C, and its photosynthetic rate when exceeding 40 °C. A comprehensive climatological analysis over the Mediterranean Basin

indicated that olive cultivation areas are nowadays constrained by temperatures of the coldest (mean monthly temperature of January) and warmest months (mean monthly temperature of July), where the optimum monthly mean temperatures for its cultivation are centered on ~7 °C in January and ~25 °C in July [42, 46].

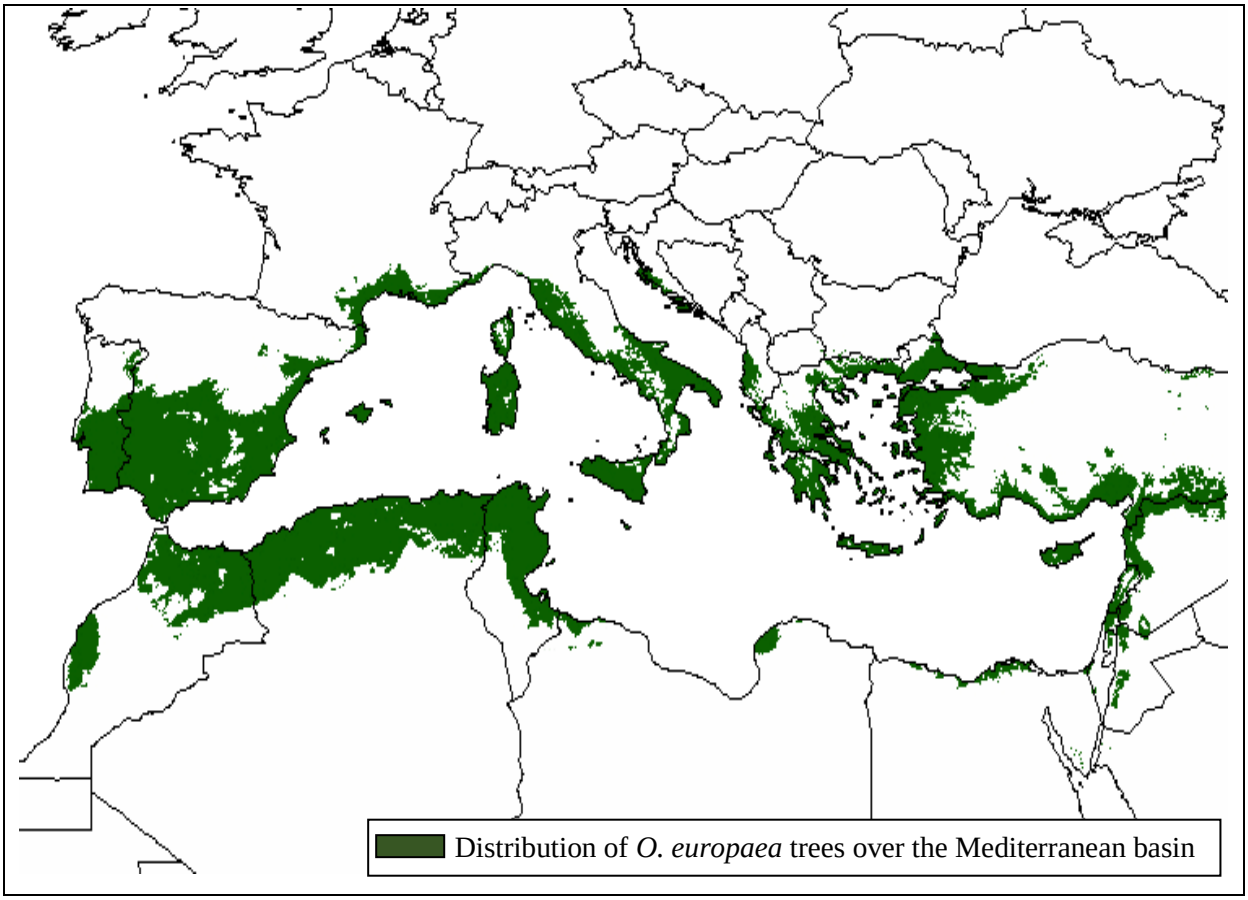


Figure II.3 Distribution of *O. europaea* orchards in the Mediterranean zone [49].

- **Precipitation**

About 90% of the olive trees grown in the Mediterranean Basin are primarily under rainfed conditions. Although olive trees are drought-tolerant species, their distribution in arid zones is limited by annual precipitation lower than 350 mm, and water availability is still considered an important resource to improve final yields. For this reason, olive growers employ management practices, such as sparse plantings and heavy pruning, to avoid severe water stress. This highlights the key role played by precipitation in the economic viability of this crop, which is exacerbated by the typically dry summers in their cultivation areas [42, 46].

Other atmospheric factors, such as solar radiation, relative humidity and wind, also influence the productivity of olive orchards.

II.7. *Olea europaea* tree medicinal uses

O. europaea tree leaves have an extensive use in traditional herbal medicine with the aim of preventing and treating several diseases particularly in Mediterranean region. They have been used for the treatment of many viral conditions, including (very topically) Swine Flu, herpes and shingles. Like olive oil, medicinal uses include reducing the risk of cardiovascular disease, thanks to the presence of an antioxidant, vasodilating and antiplatelet properties [50-62].

The main active component in *O.* europaea leaf and its extract is oleuropein, a natural product of the secoiridoid group. It is the reason of characteristic bitter taste of olive cultivars. Several studies have shown that oleuropein possesses a wide range of pharmacologic and health-promoting properties including antiarrhythmic, spasmolytic, immune-stimulant, cardioprotective, hypotensive, anti-inflammatory, antioxidant, and anti-thrombic effects. Many of these properties have been described as resulting from the antioxidant characters of oleuropein [58, 62].

Previously, oleuropein was reported also, to have an antihyperglycemic, lipid-regulating, and cardioprotective effects especially in cell culture and animal models [61].

- **Glycemia**

Antidiabetic effect of oleuropein, is shown in cell culture or animal models and limited number of studies conducted on humans [61]. Mechanisms correlated with the effects on glycemia and diabetes are shown in **Figure.II.4.**

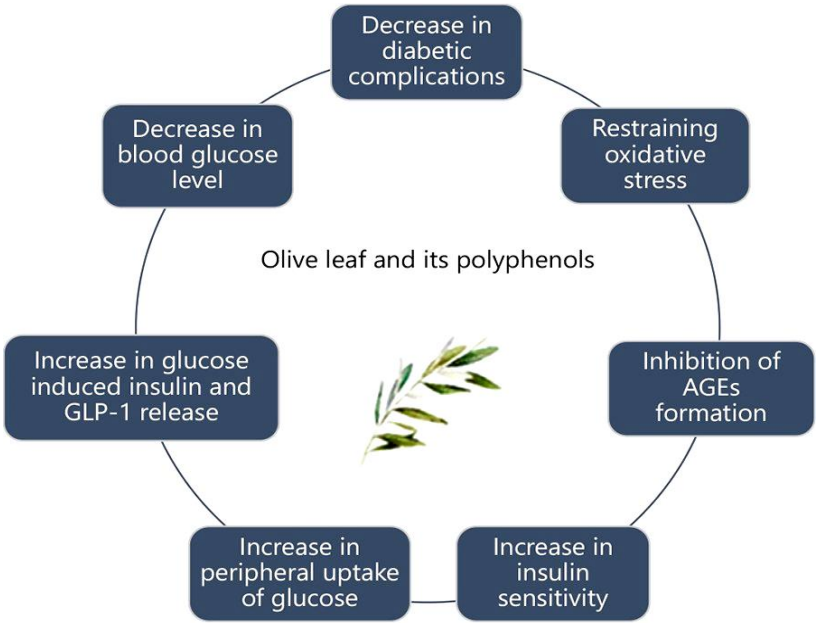


Figure II.4. Potential mechanisms and benefits of *O. europaea* leaves on glycemia [61].

- **Lipidemia and Cardiovascular Effects**

Lipid-regulating and cardioprotective effects of oleuropein and olive leaf extracts were examined in cell culture, animals, and a limited number of human studies/clinical trials [51-52, 60-61]. Potential effects and effect mechanisms of olive leaf extracts on lipid profile and cardiovascular parameters are shown in **Figure.II.5.**

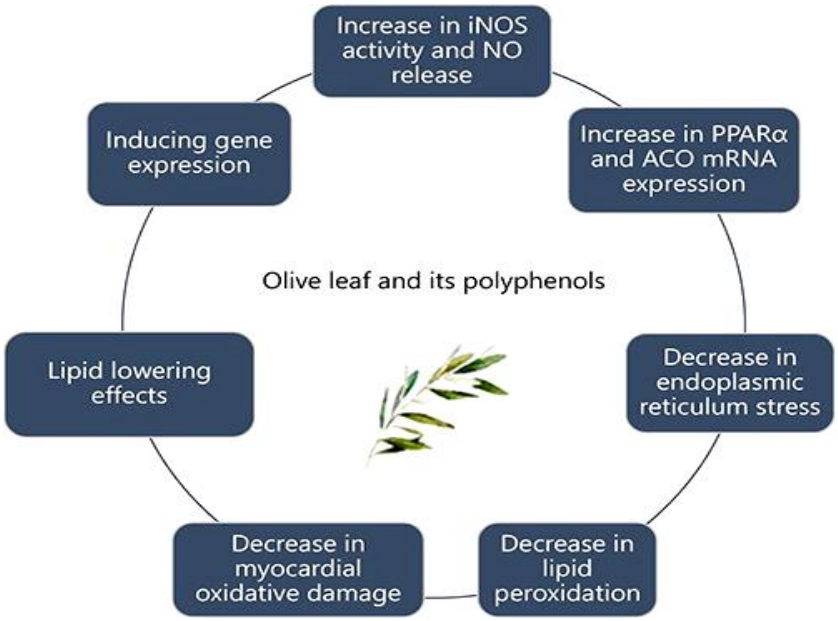


Figure II.5. Potential mechanisms and benefits of *O. europaea* leaves on lipidemia and cardiovascular disease [61].

II.8. *Olea europaea* leaves and their chemical compositions

O. europaea tree includes secoiridoids, carbohydrates, sugar alcohols, and terpenoids as biochemicals [51-60].

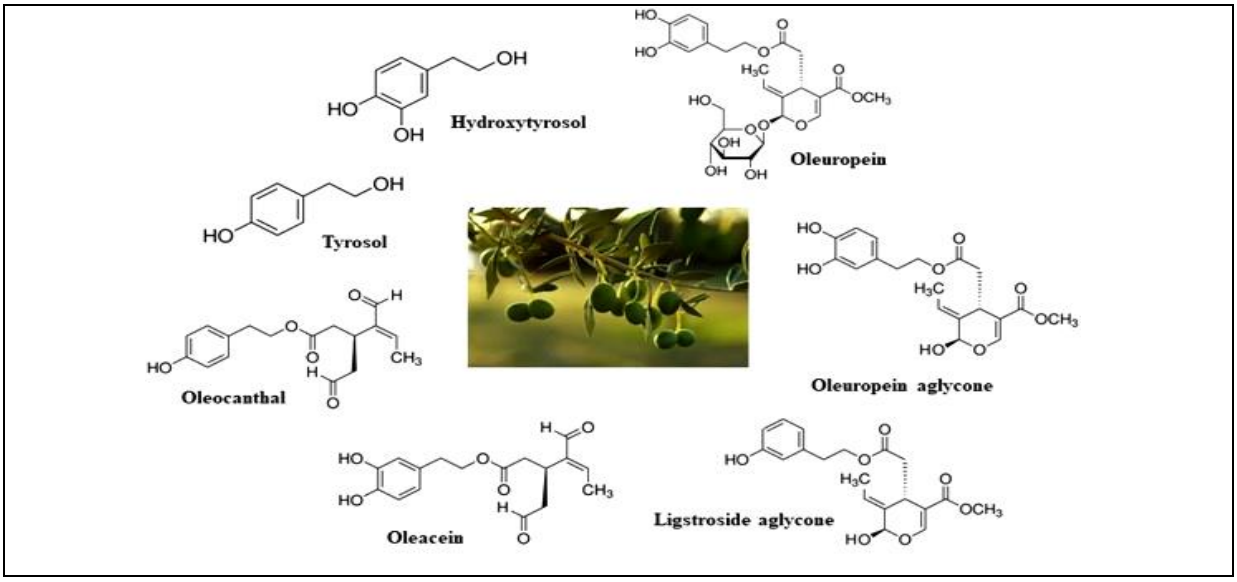
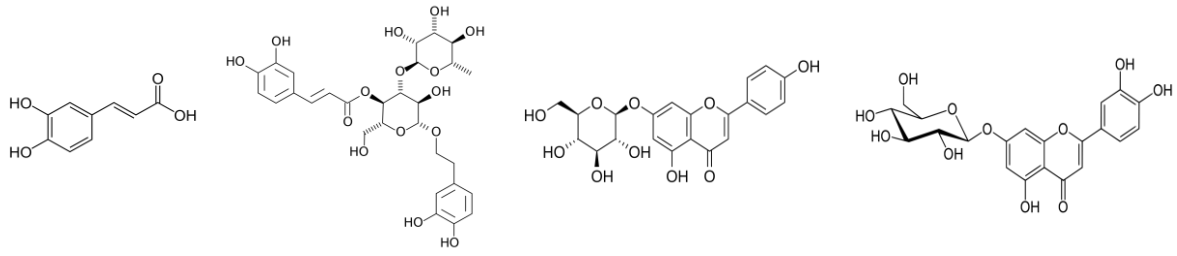


Figure II.6. The main compounds in *O. europaea* tree [50-60].

O. europaea phenolics are much more concentrated in the leaves compared with olive fruit or olive oil: 1450 mg total phenolics/100 g fresh leaf vs. 110 mg/100 g fruit and 23 mg/100 ml extra virgin olive oil [62-64].

As it was mentioned before the basic components in *O. europaea* leaf are secoiridoids such as oleuropein, ligstroside, I methyloleuropein, and oleoside; flavanoids such as apigenin, kaempferol, luteolin, and chrysoeriol; and phenolic compounds such as caffeic acid, tyrosol, and hydroxytyrosol [63-66].



Caffeic acid **Verbascoside** **Apigenin-7-glucoside** **Luteolin-7-glucoside**

Figure II.7. Chemical structure of the most abundant phenolic compounds in *O. europaea* leaf extract [62-66].

- **Secoiridoids**

Chemical components of *O. europaea* leaf, are glycosidically bound and produced by secondary metabolisms of terpenes as the pioneers of various indole alkaloids. Secoiridoids are generally derived from an oleoside type of glucoside oleosides that are characterized with the combination of elenolic acid and glucoside residues [67-68].

- **Oleuropein**

Oleuropein (**Figure II.9)** is an ester of 2-(3,4-dihydroxyphenyl) ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of Oleaceae, mainly in its aglycone form, which makes the sugar moiety insoluble in oil. Upon hydrolysis, oleuropein can produce elenolic acid, hydroxytyrosol, tyrosol, and glucose. The hydrolysis products found in olive leaf have also an important biological characteristics. It is considered that hydroxytyrosol is particularly correlated with health benefits of olive products [60-68].

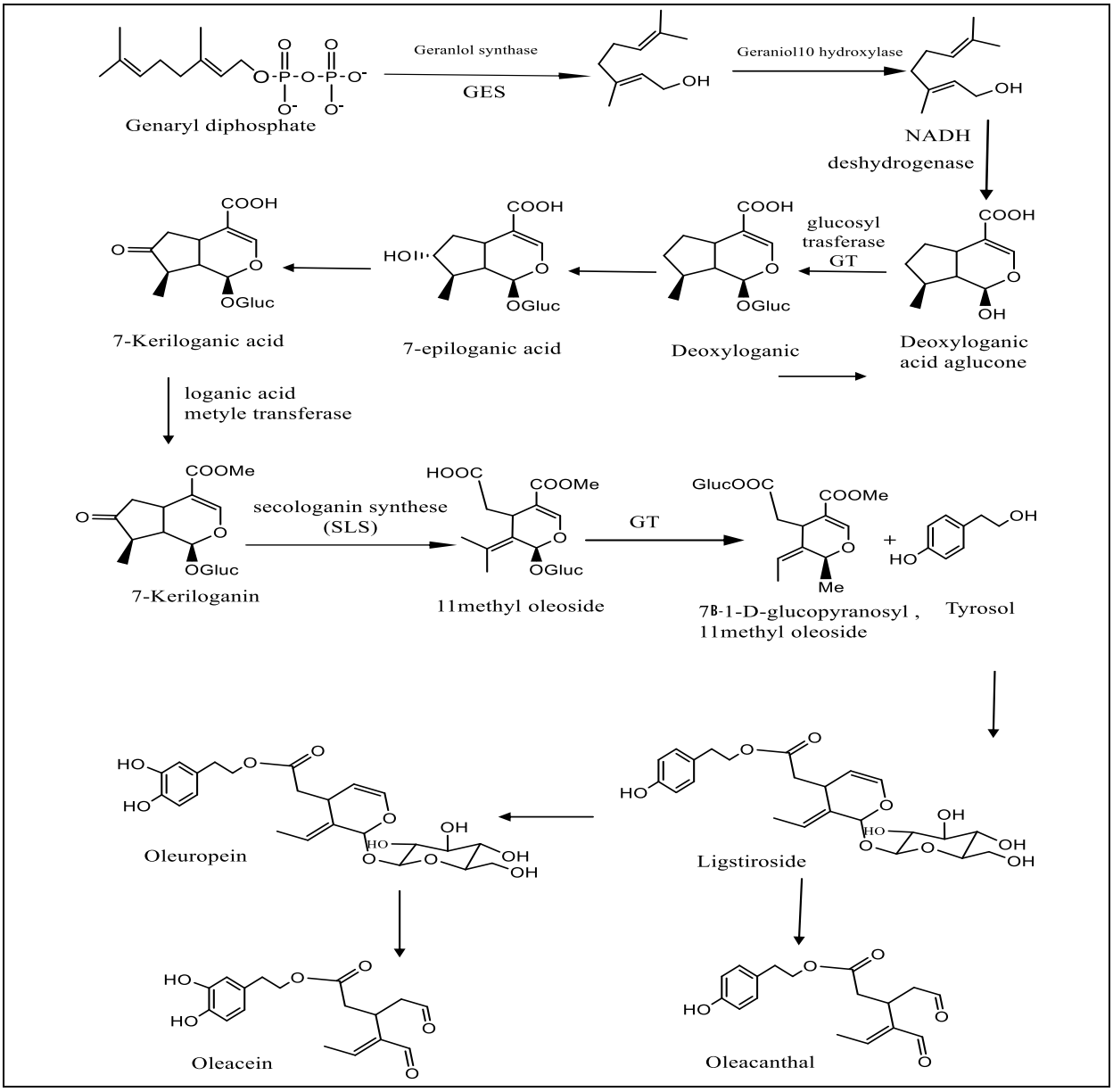


Figure II.8. Biosynthesis and biotransformation of secoiridoids in *O. europaea* [67].

Oleuropein content (6–14%) is very high in dry matter of *O. europaea* leaves. its amount in leaves may vary depending on harvest season and rise up to 17–23% [60-68].

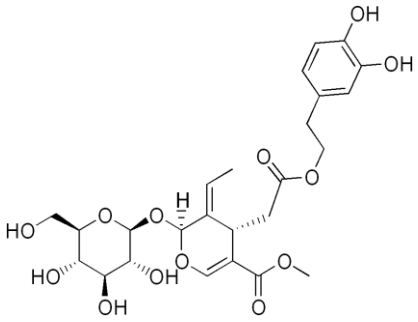


Figure II.9. Structure of oleuropein [68].

However, this complex phenol is found less in olive oil types except extra virgin olive oil. Table olive production processes increase the transformation of oleuropeins into hydrolysis products, and as a result, decrease non-hydrolyzed oleuropein forms. Its quantity is high in young olives. But during maturation, it metabolizes to hydroxytyrosol and its amount decreases [56-60].

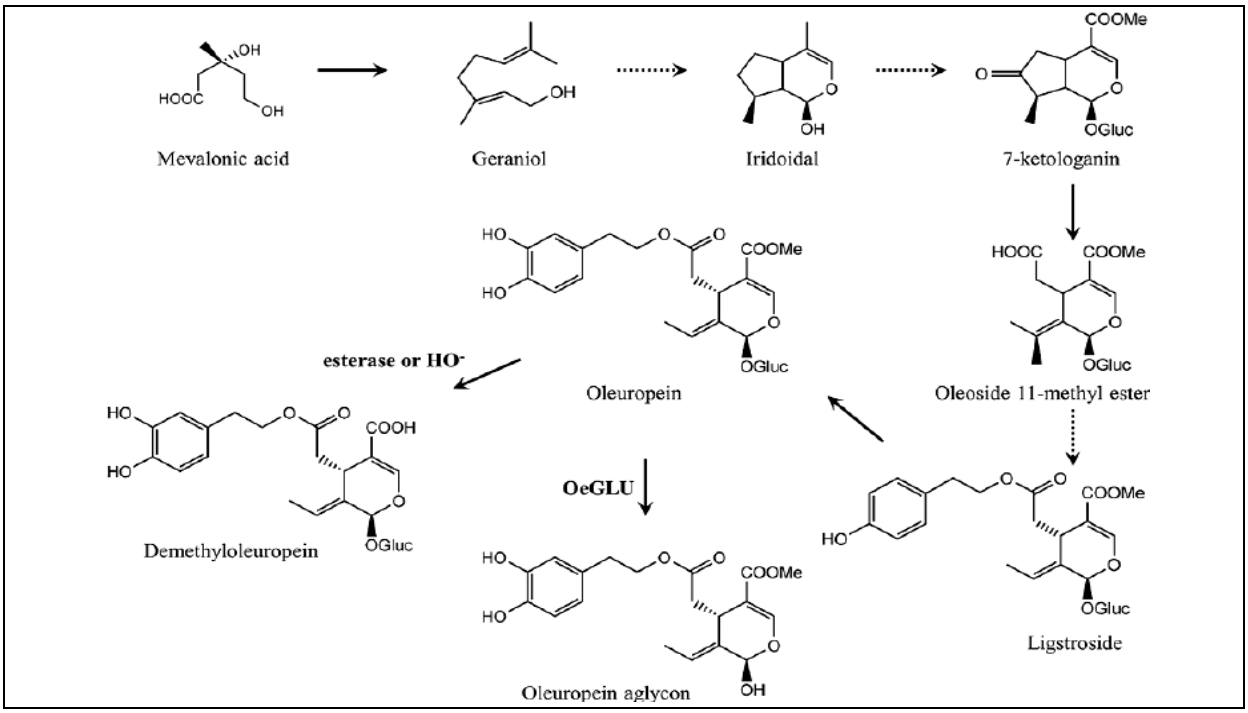


Figure II.10. Oleuropein biosynthesis and degradation pathway [68].

II.9. Safety of *Olea europaea* leaves and their extracts

Although there are strong evidence regarding biological activity of *O. europaea* leaf extract and its components (**Table II.2**), there is limited knowledge on their systemic toxicity and reliability [61]. Toxicity studies show that *O. europaea* leaf extracts are generally reliable and do not show toxic effect even at high doses [69].

Results of a study showed that supplementation of *O. europaea* leaf extract in male and female rats at single dose of 2,000 mg/kg (acute toxicity) and 100, 200, and 400 mg/kg doses (subacute toxicity) given for 28 days did not result in any toxicity [69].

Water soluble extract of *O. europaea* leaf was given at the doses of 360, 600, and 1,000 mg/kg/day for 90 days and did not cause any mortality and toxicity [69].

Only one study reported some histological changes such as fatty cytoplasmic vacuolation, necrosis of the hepatocytes, and a slight hemorrhage in both liver and kidneys of

rats after the supplementation of *O. europaea* leaf extract (0.9%) for 6 weeks. In addition, there is no adequate evidence about its genotoxic effects in the literature. Thus, more studies are needed to fully understand the safety of *O. europaea* leaf extracts for humans [69].

Table II.3. Amounts of phenolics in *O. europaea* leaf extract [70].

Phenolic compounds	**Amounts%**
Oleuropein	24.54
Hydroxytyrosol	1.46
Luteolin-7-glucoside	1.38
Apigenin-7-glucoside	1.37
Verbascoside	1.11
Tyrosol	0.71
Vanillic acid	0.63
Diosmetin-7-glucoside	0.54
Caffeic acid	0.34
Luteolin	0.21
Rutin	0.05
Diosmetin	0.05
Vanillin	0.05
Catechin	0.04

II.10. Conclusion

One of the natural antioxidant sources, *O. europaea* leaf has been used in order to prevent and treat some diseases in traditional medicine for ages. Furthermore, *O. europaea* leaf and its products such as olive oil are important components of the Mediterranean diet. A large number of studies in the literature report that *O. europaea* leaf has positive effects on the parameters related with diabetes and cardiovascular diseases through many mechanisms. It is possible to say that even the current results obtained until today seem promising. However, there are still numerous key points that need to be answered, and these will require further researches.

Part two:

Experimental

Chapter I:

Optimization of phenolics extraction

I.1. Introduction

Maximizing the extraction of phenolic compounds content has become a topic of interest in the industry since it could improve the profitability of crops and by-products.

As part of the development of natural resources in the arid zones of Algeria, we have chosen to explore the effect of three key parameters on the extraction of phenolic compounds of *O. europaea* leaves, cv. Chemlal, which are much appreciated for their biological activities.

The first chapter of the experimental part covers the used plant material, the characteristics of the study regions, and then the optimization of the extraction conditions of two extraction methods.

I.2. Presentation of the plant material

We chose two local Algerian cultivars (**Figure I.1**), which were of the same age (10 years old) and under the same cultivation practices. Among documented 36 cultivars throughout the country, these two were the most popular [71-74]:

- ***Olea europaea* L. cv. Chemlal:** the Kabylian olive oil cultivar, which occupies 40% of the national *O. europaea* orchards, presents mainly in Kabyle regions [72].

  ***Olea europaea* L. cv. Sigoise**: the dominated table olive in Algeria's western area, accounting for 25% of orchards [72].

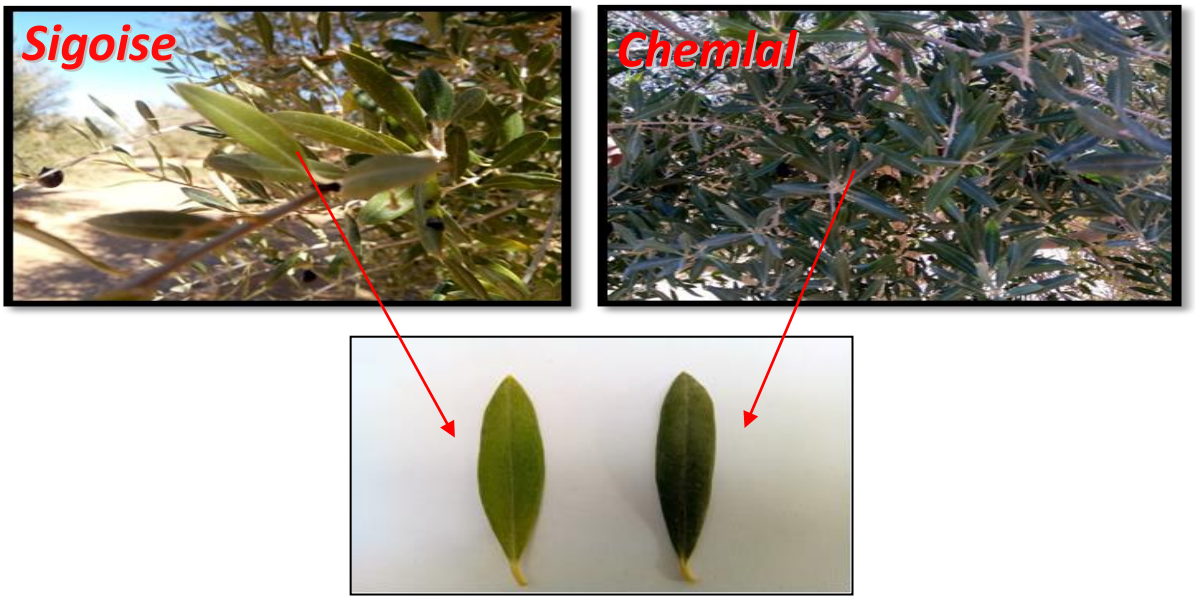


Figure I.1. *O. europaea* leaves of cv. Chemlal and cv. Sigoise (Ahlem Tlili, 2018).

The botanical identification of the collected material was performed at INRAA (National Institute of Agronomic Research of Algeria). These cultivars are very important in Algeria because of their agronomic characteristics as well as their socio-economic and

ecological interest [73-74]. The great lack of information on accelerated climate change and also at the level of central and local administration (encouraging the cultivation of certain Algerian cultivars like cv. Chemlal and cv. Sigoise) needs an examination of production, quality, resistance, and adaptation characteristics across the vast arid zone of Algeria.

I.3. Characteristics of the experimental sites

I.3.1. Geographical location

In order to estimate the chemical variability on the agro-systems regarding the climate conditions, the samples were collected from two different regions (**Figure I.2**).

a) The Mediterranean region:

This region (Cap Djinet) is located in the North of Algeria, at Boumerdes state (**Figure I.2**). Due to the typical Mediterranean climate (dry summers and wet winters), it has considerable agricultural potential especially in olive cultivation. The region is characterized by a mean annual temperature of 18°C, a high yearly rainfall of 739 mm, and a low evaporation rate (1214-1569 mm/year) [71].

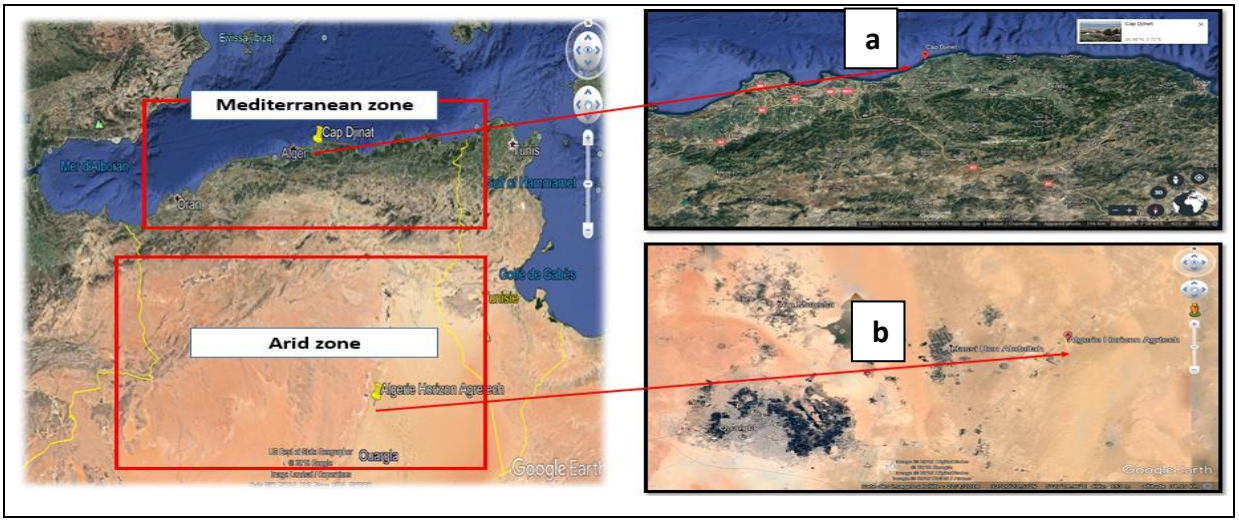


Figure I.2. The geographical location of the sampling sites [75].

b) The arid region :

The second region, named *Algeria Horizon Agritech*, is located in Southern Algeria, at Hassi Ben Abdallah, Ouargla state, which is considered a new olive crop with a project counts 100.000 olive trees in Algeria's desert (**Figure I.2**). This region is one of the hottest and driest regions in the world. Unlike the first region, Ouargla hyperarid climate has a very low precipitation rate (annual rainfall average, 38.7 mm), a very high evaporation (2138 mm/year), and a high thermal amplitude (annual temperature average 30 °C). It has very long, extremely hot, and dry summers, with short winters [71].

I.3.2. Pedoclimatic characteristics

a) Climatic conditions

Figure I.3 depicts the climatic conditions in Boumerdes and Ouargla regions during the study period (July 2018-April 2019).

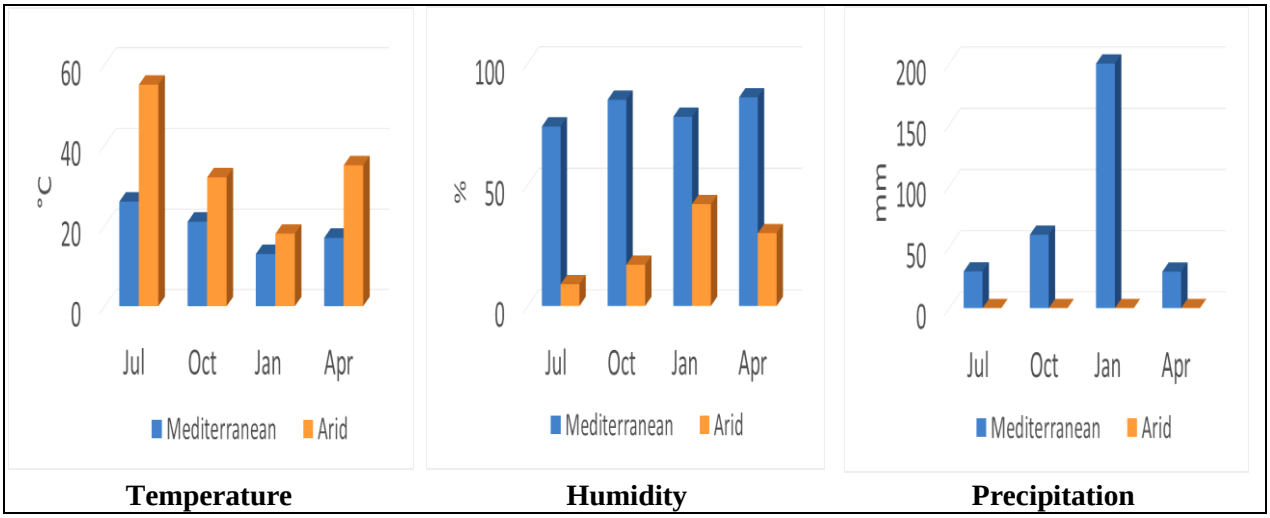


Figure I.3. The climatic conditions in Ouargla and Boumerdes regions (2018-2019) [71].

The leaves of five *O. europaea* trees were selected to cover one plot of the orchards. They were collected from each cultivar in accordance with the tree's annual cycle; olive ripening in July 2018, the hottest month of the year (summer, exceed 55°C in Ouargla), harvest in October 2018 (autumn), tree pruning in January 2019 (winter), and flowering in April 2019 (spring). As presented in **Figure I.3**, no precipitation was recorded in the arid region during the study period. Effectively, the leaves were dried in dark at room temperature for two weeks and grounded into a fine powder before being stored in paper bags until the laboratory work.

b) Soil characterization

Soil characterization was conducted by collecting samples from the two experimental sites from pits at different depths (20, 40, and 60 cm) near the olive trees of each cultivar. Each sample was characterized by the variables described in **Table I.1** by using a methodology that was based on previous methods of analysis. Soil samples were air-dried, crushed and sieved (2 mm). All analyses were carried out using a pH meter and a conductivity meter, on the aqueous extracts of the soil (soil/water ratio 1: 5) [71].

Table I.1. Soil characteristics of the experimental sites during the study period [71].

	pH	EC (dS/m)	ST	$CaCO_3$ (%)
Boumerdes region 3.46667 36° 46′ 0″ N, 3° 28′ 0″ E, 25 m	P1 7.92 to 8.19	0.44 to 0.6	Clayey silt	9.8
	P2 7.76 to 8.17	0.18 to 0.6	Clayey silt	8.2
Ouargla region 31° 56′57 ″ N, 5° 19′30 ″ E, 140 m	P1 7.51 to 7.67	0.6 to 0.8	Fine sand	12
	P2 7.95 to 8.21	0.2 to 0.6	Medium sand	14.9

P1: plot 1= 20 to 40 cm depth, **P2:** plot 2= 40 to 60 cm depth**, EC:** electrical conductivity at 25°C, **pH:** potential of hydrogen, **ST**: Soil type.

I.4. Experimental protocol

The experemintal part was carried out according to the following protocol:

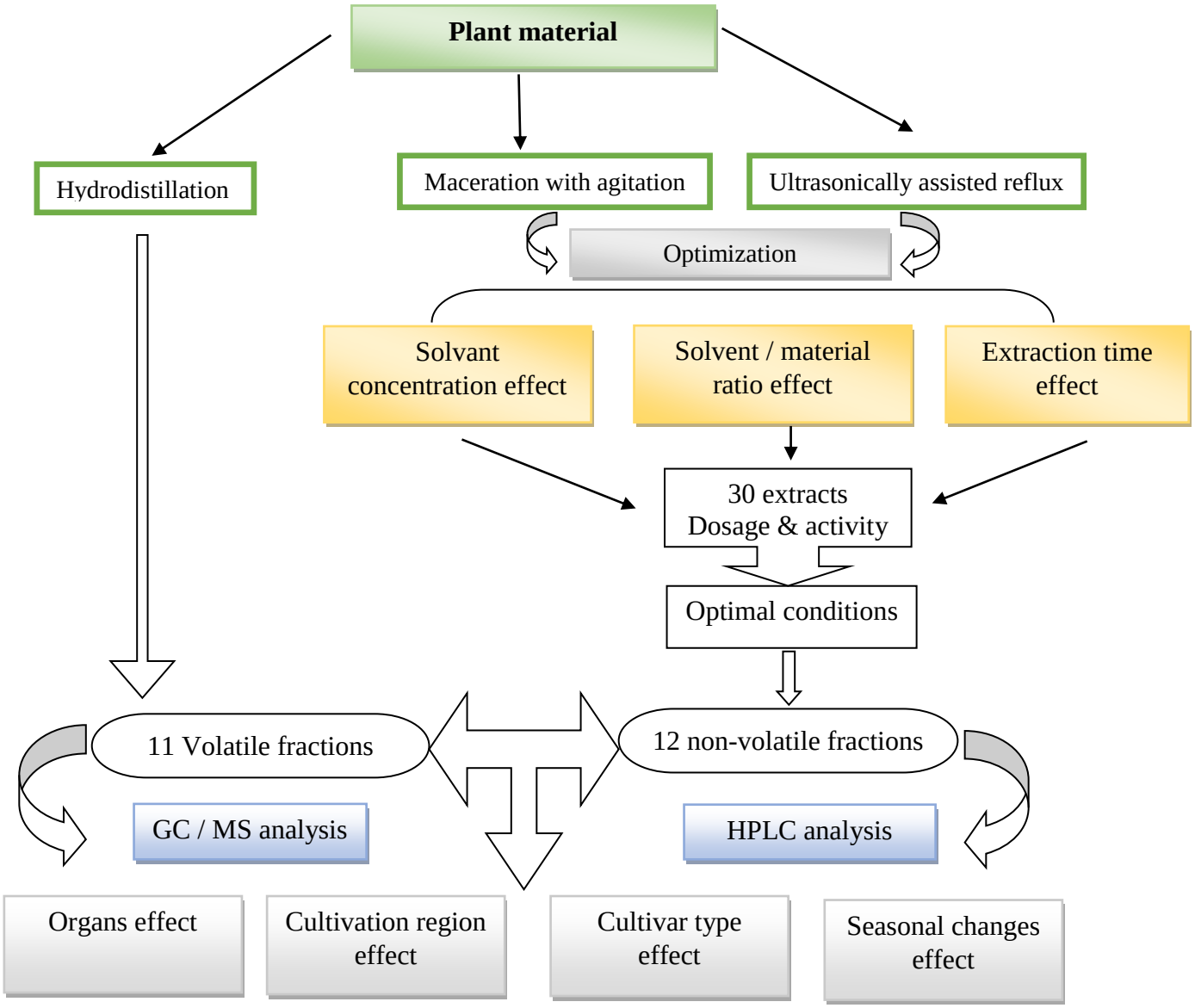


Figure I.4. Flowchart of the study methodology.

I.5. Optimization of extraction conditions

I.5.1. Response surface methodology

To evaluate the interaction of several experimental parameters of two extraction methods, response surface methodology (RSM) was used by MINITAB ® software (Version 19.1, USA). RSM is a relevant mathematical and statistical tool for process optimization [76].

Experiments were carried out according to a Box-Behnken design to investigate the effect of; methanol concentration, solvent/material ratio, and extraction time on the; extraction yields, total phenol contents, total flavonoid contents, and the antioxidant activities of the resultant extracts. The best possible combination of the previous parameters was examined.

a) Sample Preparation

The leaves of *O. europaea* (OEL) cv. Chemlal (March 2018) cultivated in the arid region were chosen for this experiment, depending on the phenolic profile of OEL from previous researches [52, 59]. OEL were dried at room temperature for 2 weeks, then ground to a fine particle (smaller than 0.5 mm) using a special grinder for processing food, after that stored until used.

b) Extraction methods

- **Maceration with agitation Extraction (MAE)**

The extraction method is a limiting process as the first stage impacts the isolation of phenolic compounds from OEL [77]. Since maceration extraction (ME), a traditional method resulting in a lower yield of phenolic compounds is still a main method to extract phenolic compounds from OEL [77]. We choose the method by adding agitation of 300 mot/min at ambient T=30° C to enhance the extraction.

- **Ultrasonic Assisted-Reflux Synergistic Extraction (UARE)**

In recent years, several novel techniques, such as supercritical fluid extraction, enzymatic extraction, microwave-assisted extraction, and ultrasonic-assisted extraction have been used for the extraction of phenolic compounds from plants instead of the conventional technique [77]. Among these methods, UAE has become more and more popular because it is a simple and eco-friendly method [77-78]. For this purpose, UAE with reflux synergistic was used also in this work with 35 kHz at 65° C.

The modified conditions for both MAE and UARE were based on previous studies.

c) Extraction conditions

The ranges and levels of variables are shown in **Table I.2**. The experimental design is presented in **Table I.3**. A total of 15 experimental runs with three replicates at the center points were carried out.

Table I.2. Values of the independent parameters employed in RSM.

Independent variable	Units	Symboles	Coded levels		
			-	0	+
Concentration of Methanol	% (v/v)	X_1	50	65	80
Solvent/Material Ratio	mL/g	X_2	5	15	25
Extraction Time	min	X_3	20	40	80

* Parameter coded forms -, 0 and + are the minimum point, centre point and maximum point (respectively) for the independent parameters.

Table I.3. Experimental design of response surface analysis recommended by MINITAB.

Run	X_1	X_2	X_3
1	65	15	40
2	80	25	40
3	80	15	20
4	50	5	40
5	65	25	60
6	65	5	60
7	65	5	20
8	50	25	40
9	50	15	20
10	80	15	60
11	80	5	40
12	65	25	20
13	65	15	40
14	65	15	40
15	50	15	60

I.5.2 Determination of extraction yields (Y)

Y were used as an indicator of the effects of the extraction conditions [79]. They were expressed by percentages as the following relation:

$$Y\% = (\text{mass of extract/mass of dry matter}) \times 100$$

I.5.3. Determination of Total Phenolic Contents (TPC)

TPC were carried out according to Folin–Ciocalteu methodology from previously published protocols, with minor modifications. Gallic acid (**Figure I.5**) was used as the reference standard (25 - 200 µg/ml), and the results were expressed as mg of gallic acid equivalents per g of sample (mg GAE/g) [80].

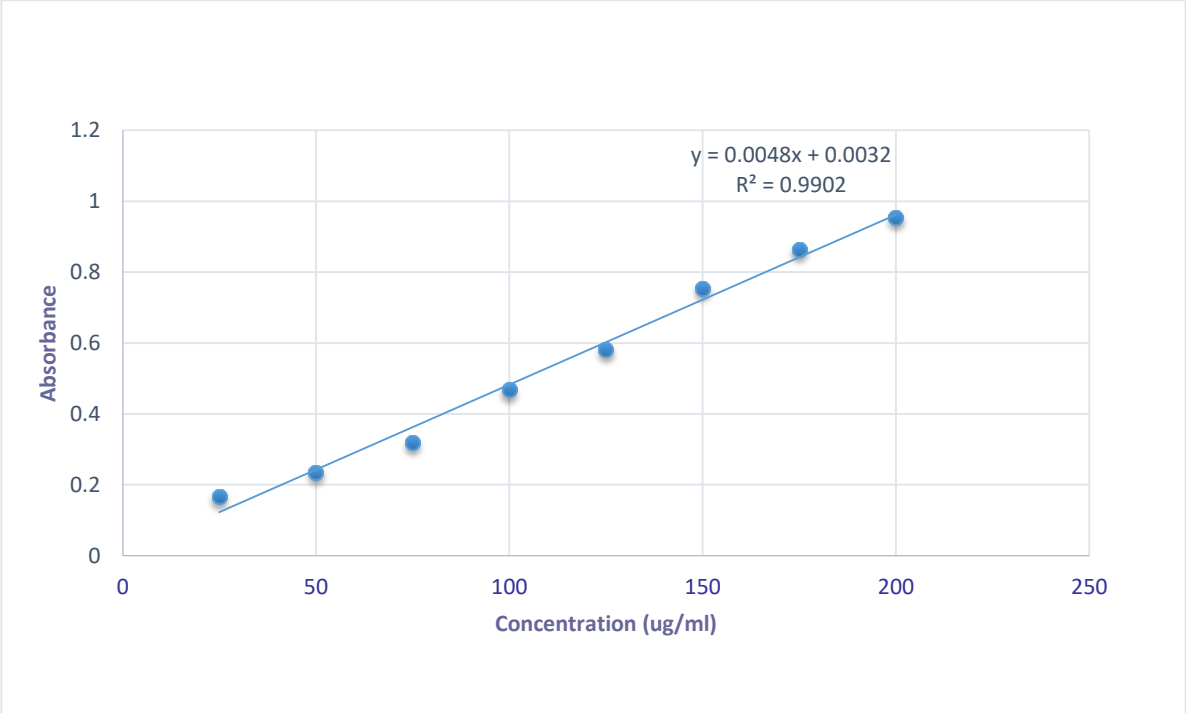


Figure I.5. Standard calibration curve of gallic acid.

Briefly, 150 µL of each sample (1 mg/ml) was added to 750 µL Folin–Ciocalteu's reagent (10%) and left to equilibrate for 10 min before adding 2 mL of 7.5% sodium carbonate (Na_2CO_3) solution and incubating in the dark for 30 min. Absorbance was then read at 765 nm using a UV spectrophotometer (UNICAM) [80].

I.5.4. Determination of Total Flavonoid Contents (TFC)

TFC in the extracts were determined using aluminum chloride colorimetric method described in previous protocols [80], with minor modifications.

In a test tube, 1.5 mL of extracts (1 mg/ml) was mixed with 1.5 ml of 20 % aluminum trichloride in distilled water. The absorption at 430 nm was read after 30 minutes. Rutin (**Figure I.6**) was used to draw the standard curve (1 -30 µg/ml) and the results were expressed as mg of Rutin Equivalent per gram of dried sample (mg RE/g) [80].

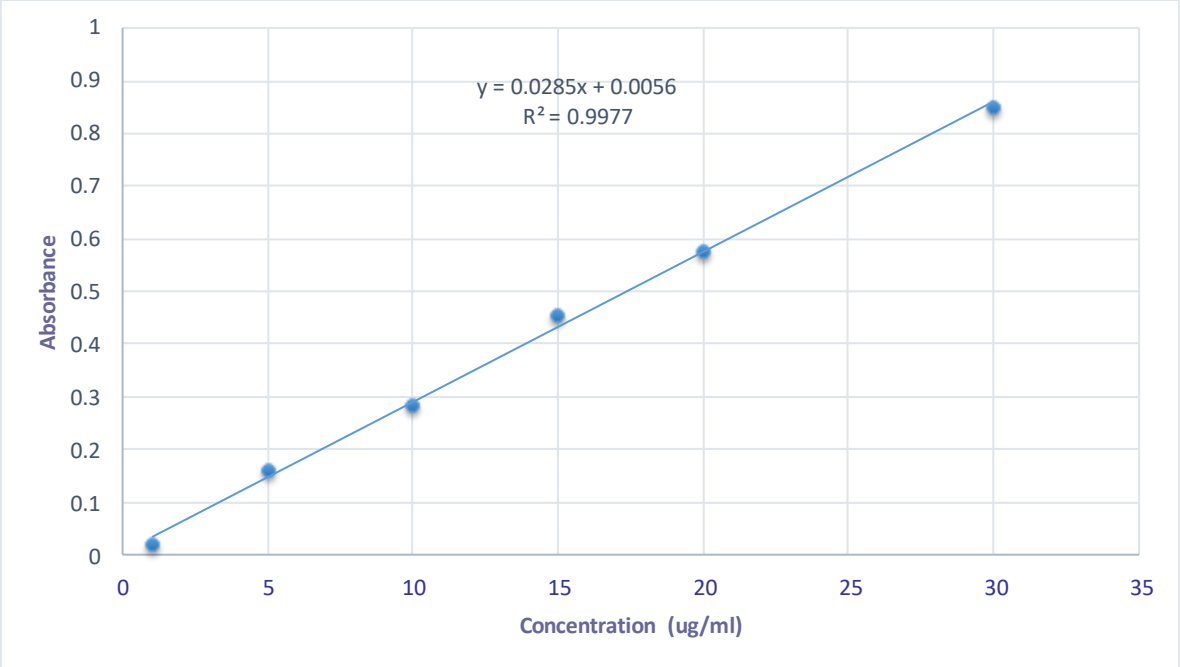


Figure I.6. Standard calibration curve of rutin.

I.5.5. Antioxidant Activity

- **DPPH free-radical scavenging activity**

The DPPH activity of the extracts was analyzed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay [81-82], with minor modifications. Briefly, 100 µL of the appropriately diluted samples (0.1 – 1 mg/ml), standard, and blank were added to 2.9 mL of DPPH solution (6 x 10^{-5} M). After 30 min of incubation at room temperature, the absorbance was measured at 517 nm.

IC_{50} values were calculated to determine the 50% inhibition of DPPH radicals.

All determinations of TPC, TFC, and antioxidant activities were carried out in triplicates.

I.5.6. Statistical Analysis

Besides RMS, MINITAB ® software was also used to establish the model equations and 3D plots of the responses. Also to predict the optimum values for obtainning the maximum TPC and TFC levels with the highest antioxidant activity.

I.6. Results and Discussion

To express Y, TPC, TFC or antioxidant capacities as a function of the independent variables, a second-order polynomial equation was used as follows [76]:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where various *Xi* values are the independent variables affecting the response. *Y*; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients.

I.6.1. Fitting the Model for the Prediction of Y, TPC, and TFC using UARE

The predicted and experimental Y, TPC, and TFC obtained by UARE with RMS experimental design are presented in **Table I.4**.

Table I.4. Experimental and predicated values of RSM analysis of UARE's Y, TPC and TFC.

RUN	Y%		TPC (mg GAE/g)		TFC (mg RE/g)	
	Predicated	**Experimental**	**Predicated**	**Experimental**	**Predicated**	**Experimental**
1	21.66	22.68	136.34	137.87	25.78	25.62
2	12.96	15.27	138.74	136.00	29.15	27.34
3	20.85	18.89	140.80	140.37	33.75	34.40
4	25.07	22.76	115.95	118.70	15.64	17.45
5	17.38	17.97	128.57	127.14	22.19	22.47
6	32.79	33.14	132.50	129.33	27.00	25.83
7	28.50	27.92	119.97	121.41	25.50	25.22
8	13.32	10.77	111.87	112.87	14.77	15.13
9	16.93	19.84	118.30	114.12	16.91	15.38
10	25.10	22.20	154.11	158.29	33.28	34.82
11	32.60	35.16	139.08	138.08	34.45	34.08
12	12.52	12.17	119.48	122.66	24.14	25.31
13	21.66	21.44	136.34	134.75	25.78	25.87
14	21.66	20.88	136.34	136.41	25.78	25.83
15	21.83	23.80	126.61	127.04	16.93	16.29

OEL have been found to contain a high concentration of phenolic compounds. Depending on the combination of extraction parameters, the software's predicted Y ranged from 12.52 to 32.79%. The TPC and TFC were ranging from 111.87 to 154.11 mg GAE/g and from 14.77 to 34.45 mg RE/g, respectively. These results are in good agreement with many studies have demonstrated that OEL have significant phenolic content [4-5]. For example, Lee et al. (2009) found that TFC and TPC of 80% ethanol extract of olive leaf were 58 mg/g NE and 148 mg/g TAE, respectively [83]. Other study by Skrget et al. (2005), in which total polyphenolic content of olive leaf extract was 144 mg/g [84].

- **Fitting the Model**

Table I.5 represents the analysis of the model depending on the responses. Where we can determine whether the association between the response and the term is statistically significant.

Table I.5. Analysis of variance for the determination of model fitting of UARE.

Sources of variation	**Y%**	**TPC (mg GAE/g)**	**TFC (mg RE/g)**
Lack of fit (*p*-value)	0.049*	0.089	0.003*
R^2	0.9199	0.9582	0.9747
Adjusted R^2	0.7757	0.8830	0.9292
PRESS	3.18910	4.03859	1.74644
F Ratio of Model	19.43	10.45	287.40
p of Model > F	0.843	2.44	0.018*

*Significant difference with $p < 0.05$

The coefficient of determination (R^2) value for the correlation between the predicted and actual values of Y was 0.9199, indicating that the model can predict 92% of the actual data for Y. R^2 is in reasonable agreement with the adjusted R^2 (0.7757) with a difference less than 0.2 as was suggested by Anderson and Whitcomb (2016) [85]. **Table I.5** also showed that the "lack of fit" for the model was significant ($p = 0.049$).

In addition, the PRESS (predicted residual sum of squares) was 3.1891 and the F-ratio was 19.43. PRESS is a measure of how well each point fits into the experimental design, further identifying the appropriateness of the model's fit. The lower the value of PRESS, the better the model describes the response [76, 85].

It was therefore concluded that the second-order polynomial equation for the three independent variables could be used. The predictive equation for the response Y is:

$$Y\ \% = -10.6 + 1.002\ X_1 - 0.100\ X_2 + 0.006\ X_3 - 0.00510\ (X_1)^2 + 0.0047\ (X_2)^2 + 0.00166\ (X_3)^2 - 0.0132\ X_1\ X_2 - 0.00054\ X_1\ X_3 + 0.00072\ X_2\ X_3$$

The *p*-values for the model fit were 0.089 and 0.003 for TPC and TFC, respectively. This shows that there was a significant difference between actual and predicted values for TFC only. This was supported with the lowest PRESS value (1.74644).

However, the coefficients of determination were 0.95 and 0.97 TPC and TFC respectively. This highlighted the close correlation between the actual and predicted values. This relationship is further supported with the values for PRESS and the F-ratios of the model: 4.03859 and 10.45 for TPC, 1.74644, and 287.40 for TFC.

This indicated that the mathematical models were reliable predictors of TPC and TFC of the OEL extracts. Therefore, the following second order polynomials could be used:

$$TPC = 58.8 + 0.61\ X_1 + 2.62\ X_2 + 0.331\ X_3 - 0.00025\ (X_1)^2 - 0.0988\ (X_2)^2 - 0.00333\ (X_3)^2 + 0.0062\ X_1\ X_2 + 0.00417\ X_1\ X_3 - 0.0043\ X_2\ X_3$$

$$TFC = -36.9 + 1.189\ X_1 + 0.916\ X_2 + 0{,}021\ X_3 - 0.00391\ (X_1)^2 - 0.01394\ (X_2)^2 + 0.00081\ (X_3)^2 - 0.00737\ X_1\ X_2 - 0.00041\ X_1\ X_3 - 0.00432\ X_2\ X_3$$

I.6.2. Fitting the Model for the Prediction of Y, TPC, and TFC using MAE

The predicted and experimental Y, TPC, and TFC obtained by MAE with RMS experimental design are presented in **Table I.6**.

Table I.6. Experimental and predicted values of RSM analysis of MAE's Y, TPC and TFC.

RUN	Y%		TPC (mg GAE/g)		TFC (mg RE/g)	
	Predicated	**Experimental**	**Predicated**	**Experimental**	**Predicated**	**Experimental**
1	15.38	14.72	130.31	130.58	16.63	16.92
2	08.59	08.63	136.64	134.95	18.12	16.92
3	13.12	13.74	125.81	129.12	20.66	21.76
4	19.06	19.02	106.18	107.87	14.46	15.66
5	09.59	09.53	139.53	141.83	16.39	16.50
6	22.41	23.08	138.76	140.37	18.88	18.78
7	17.63	17.70	128.08	125.79	15.95	15.83
8	08.64	09.32	110.81	111.83	14.95	15.94

9	12.23	12.22	96.23	96.83	17.38	16.29
10	14.34	14.36	139.51	138.91	22.85	23.94
11	18.80	18.12	142.64	141.62	19.98	18.99
12	09.84	09.18	125.94	124.33	17.07	17.17
13	15.38	17.90	130.31	130.37	16.63	16.69
14	15.38	13.52	130.31	130.00	16.63	16.29
15	15.54	14.92	106.80	103.50	17.43	16.32

MAE showed lesser levels of Y, TPC, and TFC compared to UARE. As the predicted values of Y were ranging from 8.59 to 22.41%. While the predicted ones of TPC and TFC ranged from 96.23 to 139.53 mg GAE/g and 14.46 to 22.85 mg RE/g, respectively. UARE higher yields may be attributed to acoustic cavitation phenomena, which could produce a strong impact on the solid surface resulting in the increased extraction rate [77].

- **Fitting the Model**

Table I.7 indicates that there was no significant difference between the actual and predicted values for Y (p >0.05).

Table I.7. Analysis of variance for the determination of model fitting of MAE.

Sources of variation	Y%	TPC (mg GAE/g)	TFC (mg RE/g)
Lack of fit (p-value)	0.909	0.006*	0.031*
R^2	0.9487	0.9848	0.8740
Adjusted R^2	0.8565	0.9575	0.6473
PRESS	1.60028	2.59542	1.40799
F Ratio of Model	5.108	176.18	31.63
p of Model > F	0.17	0.09	0.1023

*Significant difference with p < 0.05.

From **Table I.7**, the R^2 value was 0.9487, indicating that the model can predict 94% of the actual data for Y. In addition, the PRESS was 1.60028 and the F-ratio was 5.108. The p-values were 0.006 and 0.031 for TPC and TFC, respectively. This shows that there was a significant difference between actual and predicted values. While the pure errors had no significant values. The mathematical models are as below:

$$Y\ \% = -13.4 + 0.830\ X_1 - 0.204\ X_2 + 0.311\ X_3 - 0.00593\ (X_1)^2 - 0.00274\ (X_2)^2 - 0.00059\ (X_3)^2 + 0.00035\ X_1\ X_2 - 0.00173\ X_1\ X_3 - 0.00629\ X_2\ X_3$$

$$TPC = -163.4 + 7.624\ X_1 - 0.489\ X_2 + 0.501\ X_3 - 0.04942\ (X_1)^2 + 0.0487\ (X_2)^2 - 0.00527\ (X_3)^2 - 0.0177\ X_1\ X_2 + 0.00260\ X_1\ X_3 + 0.00364\ X_2\ X_3$$

$$TFC = 34.3 - 0.662\ X_1 + 0.741\ X_2 - 0.334\ X_3 + 0.00611\ (X_1)^2 - 0.01133\ (X_2)^2 + 0.00392\ (X_3)^2 - 0.00392\ X_1\ X_2 + 0.00178\ X_1\ X_3 - 0.00452\ X_2\ X_3$$

I.6.3. Fitting the Model for the Prediction of antioxidant activity

The model fit for the antioxidant activity of OEL extracts were also investigated. The predicted and experimental IC_{50} values of the experimental design RSM are presented in **Table I.8.**

Table I.8. Experimental and predicted values of UARE extracts' activity.

Run	IC_{50} (mg/ml)	
	Predicated	**Experimental**
1	0.47	0.44
2	0.37	0.37
3	0.37	0.37
4	0.44	0.43
5	0.49	0.49
6	0.49	0.49
7	0.46	0.45
8	0.48	0.47
9	0.45	0.46
10	0.47	0.46
11	0.46	0.47
12	0.41	0.40
13	0.47	0.47
14	0.47	0.50
15	0.46	0.46

IC_{50}: the median Inhibitory Concentration

High antioxidant activity was seen (**Table I.8**) in UARE. The predicted values of IC_{50} were ranging from 0.37 to 0.48 mg/ml.

Table I.9. Experimental and predicted values of MAE extracts's activity.

Run	IC_{50} (mg/ml)	
	Predicated	**Experimental**
1	0.40	0.40
2	0.33	0.31
3	0.29	0.30
4	0.31	0.33
5	0.33	0.37
6	0.25	0.25
7	0.32	0.28
8	0.40	0.37
9	0.43	0.45
10	0.34	0.32
11	0.30	0.32
12	0.36	0.36
13	0.40	0.40
14	0.40	0.41
15	0.28	0.27

IC_{50}: the median Inhibitory Concentration.

The predicted values of IC_{50} for MAE (**Table I.9**), were ranging from 0.28 to 0.40 mg/ml. Althought the UARE had larger levels of Y and TPC, the MAE had stronger antioxidant activity. For a comprehensive explanation of these results, the chemical profile of the obtained extracts is required.

- **Fitting the Model**

Table I.10 reveald that the *p*-values for MAE were significant but not for UARE. While those of MAE were 0.86 and 0.92. This emphasized the close correlation between the actual and predicted values.

In the two extraction methods we can see that the pure error for the IC_{50} was significant.

Table I.10. Analysis of variance for determination of model fitting for the activities results.

Sources of variation	Antioxydant Capacity	
	UARE	**MAE**
	IC_{50}	**IC_{50}**

Lack of fit	0.926	0.015*
R^2	0.9170	0.8608
Adjusted R^2	0.7677	0.6101
PRESS	0.0194196	0.0360539
F Ratio of Model	0.14	66.69
p of Model > F	0.0007*	0.00003*

The polynomial equation in terms of coded factors obtained from a regression analysis can be described as follows:

- **For UARE:**

$$IC_{50} = -0.010 + 0.01479\ X_1 + 0.01288\ X_2 - 0.00334\ X_3 - 0.000121\ (X_1)^2 - 0.000054\ (X_2)^2 - 0.000013\ (X_3)^2 - 0.000228\ X_1\ X_2 + 0.000076\ X_1\ X_3 + 0.000057\ X_2\ X_3$$

- **For MAE:**

$$IC_{50} = 0.115 + 0.0082\ X_1 + 0.0209\ X_2 - 0.00440\ X_3 - 0.000113\ (X_1)^2 - 0.000450\ (X_2)^2 - 0.000105\ (X_3)^2 - 0.000098\ X_1\ X_2 + 0.000167\ X_1\ X_3 + 0.000049\ X_2\ X_3$$

We use the surface plots to see how fitted response values relate to two continuous variables based on the model equations.

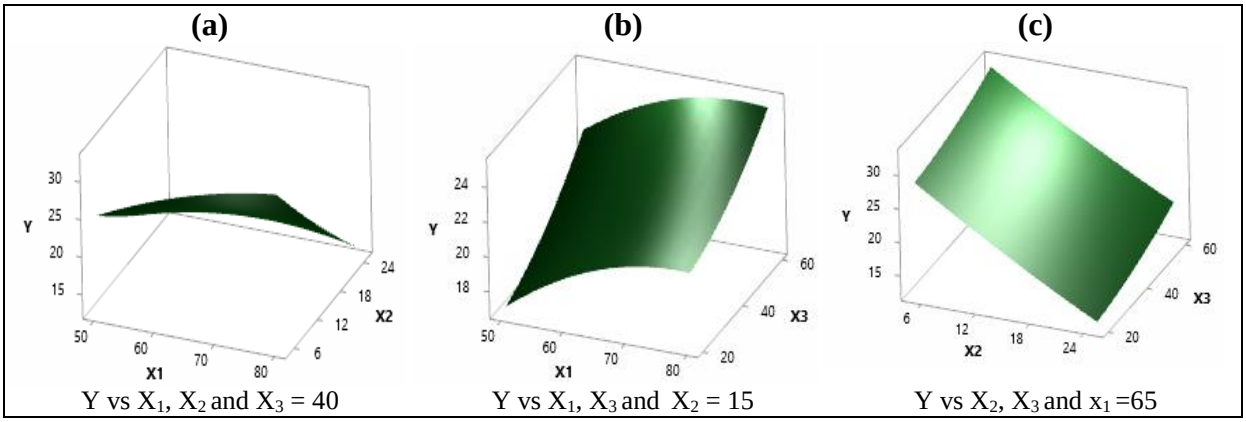
I.6.4. The Effect of the Different Variables on the yield

In the below surface plots the predictors are on the x and y axes. While the continuous surface represents the fitted response values on the z-axis. Because a surface plot shows only two continuous variables at a time, any extra variables are held at a constant level.

- **For UARE:**

The 3D graphic surface presented in **Figure I.7** shows the relationship between the Y and the variables studied. The response surface is curved because the model contains quadratic terms that are statistically significant.

Figure I.7 (a) shows that the highest Y values of OEL extracts are in the upper right corner of the plot, which corresponds to higher X_1 and lower X_2 values. The third predictor X_3, is not displayed in the plot. Minitab holds the values of X_3 at 40 min when calculating the fitted response values for Y of OEL extracts.



Y vs X_1, X_2 and X_3 = 40 Y vs X_1, X_3 and X_2 = 15 Y vs X_2, X_3 and x_1 =65

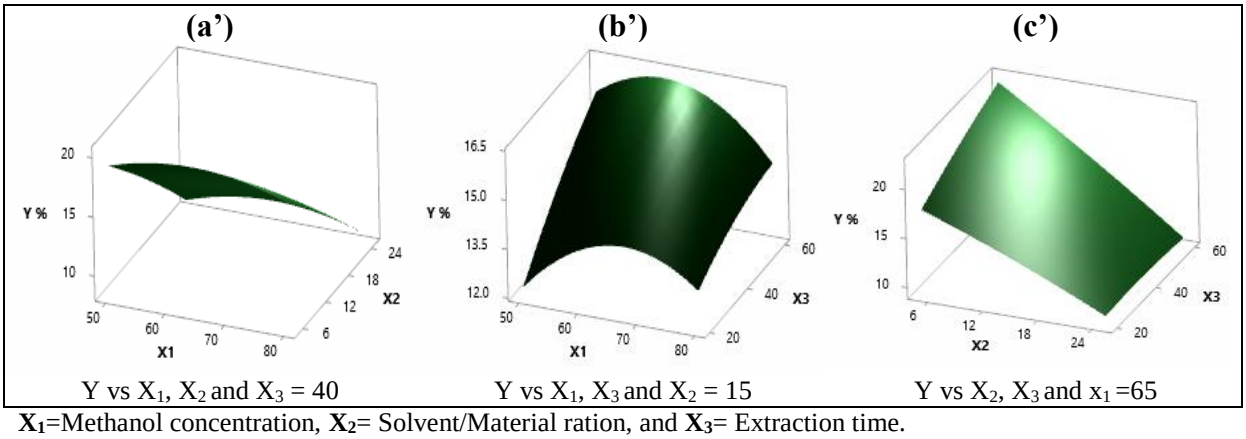
X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time.

Figure I.7. Surface plots of Y the extracts obtained by UARE.

In the plot **(b),** the highest values are in the upper right corner of the plot (**Figure I.7**), which correlates to higher X_1 values. The plot (**c**) confirms the prior plots while also illustrating that extraction duration has less effect on extraction yield.

- **For MAE:**

Identical UARE demonstration was shown in MAE's surface plots also (**Figure I.8**). This indicates that the results are accurate. It makes reasonable that factors, rather than the method used, determine the yield of polyphenols. According to the industry, UARE is the most likely to be used because it provides a higher extract yield with less extraction time.



Y vs X_1, X_2 and X_3 = 40 Y vs X_1, X_3 and X_2 = 15 Y vs X_2, X_3 and x_1 =65

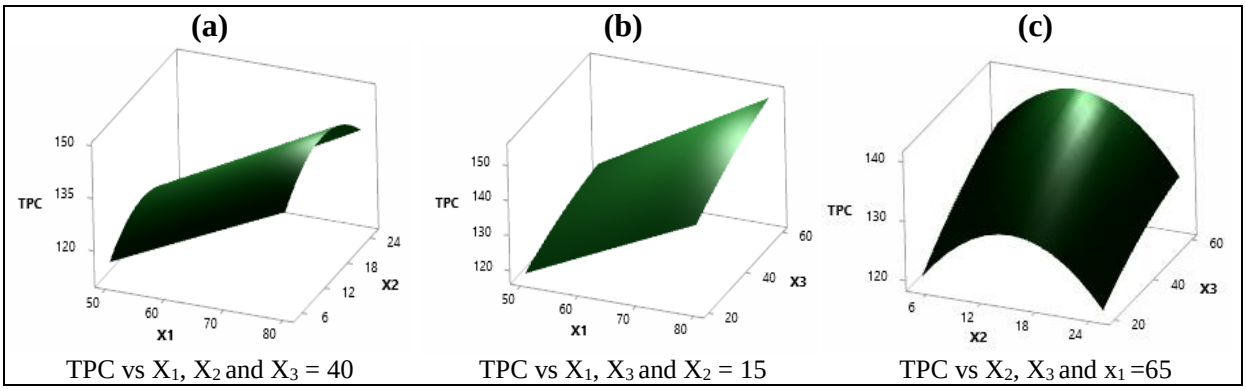
X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time.

Figure I.8. Surface plots of Y of the extracts obtained by MAE.

I.6.5. The Effect of the Different Variables on the Total Phenolic Content

In relation to Y plots, TPC values were higher also with the highest X_1 percentage. Also, the extraction time has less impact. However, we can observe clearly from **Figure I.9 (a)** and **(b)** plots that X_2=15% has the highest TPC values.

➢ **For UARE:**



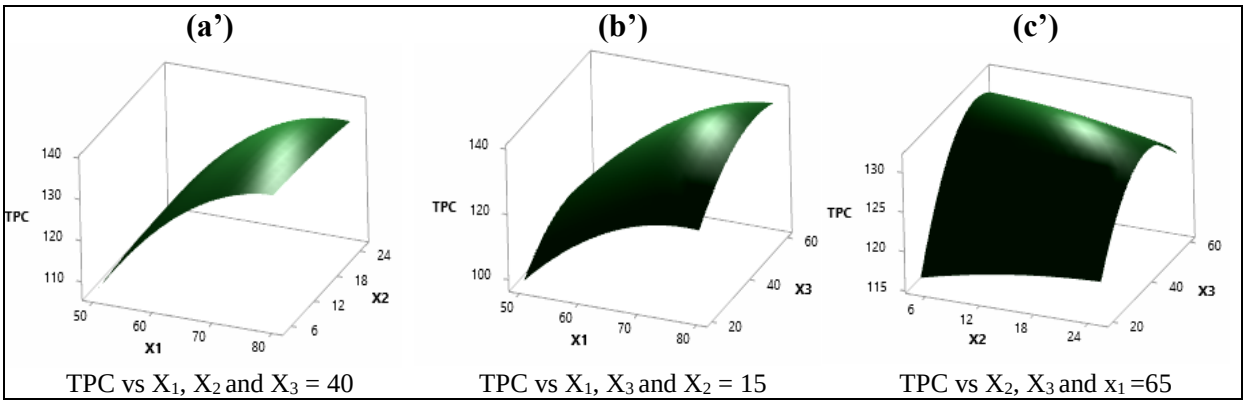
TPC vs X_1, X_2 and X_3 = 40 TPC vs X_1, X_3 and X_2 = 15 TPC vs X_2, X_3 and x_1 =65

X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time

Figure I.9. Surface plots of TPC of the extracts obtained by UARE.

➢ **For MAE:**

The MAE plots (**Figure I.10**) demonstrate that Y and TPC are significantly connected regardless of the used extraction method.



TPC vs X_1, X_2 and X_3 = 40 TPC vs X_1, X_3 and X_2 = 15 TPC vs X_2, X_3 and x_1 =65

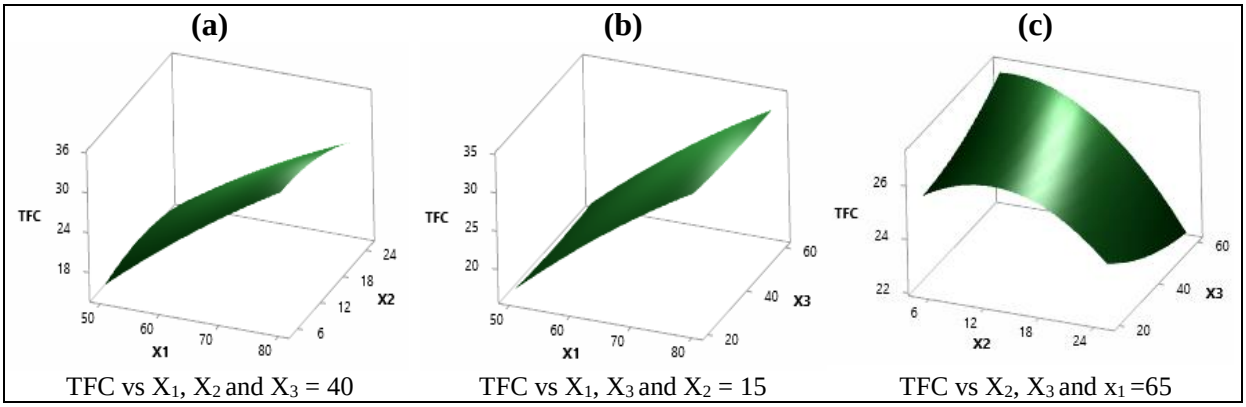
X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time.

Figure I.10. Surface plots of TPC of the extracts obtained by MAE.

I.6.6. The Effect of the Different Variables on the Total Flavanoid Content

According to TFC plots (**Figure I.11**), the response values increased as X_1 values increased, X_3 had no influence on TFC, and X_2 =15% enhanced TFC, just like TPC. For MAE, the X_2 with 15% enriches the extraction yield also.

➢ **For UARE:**



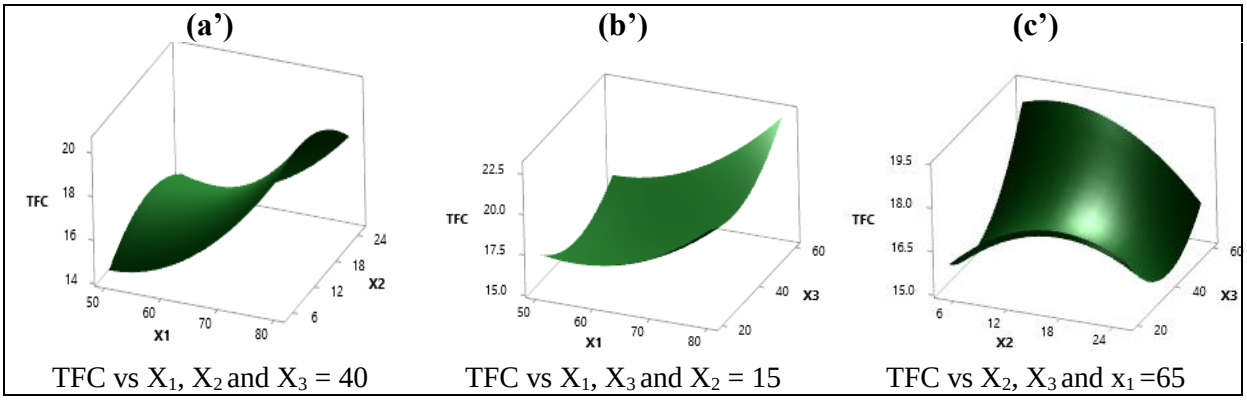
TFC vs X_1, X_2 and X_3 = 40 TFC vs X_1, X_3 and X_2 = 15 TFC vs X_2, X_3 and x_1 =65

X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time

Figure I.11. Surface plots of TFC of the extracts obtained by UARE.

- **For MAE:**

As the alcohol concentration increases and the content of water decreases (less polar), the content of phenolic compounds increases. This may suggest that compounds of different nature such as terpenoids, saponins and others may be extracted with the highest methanol concentrations. These molecules can interact with the phenols forming complex structures that interfere with the quantification of TPC. The results demonstrate that petroleum ether or hexane (non-polar solvents) washes should be applied before the phenolics extractions so these compounds will be eliminated from the extracts and will not quantified.



TFC vs X_1, X_2 and X_3 = 40 TFC vs X_1, X_3 and X_2 = 15 TFC vs X_2, X_3 and x_1 =65

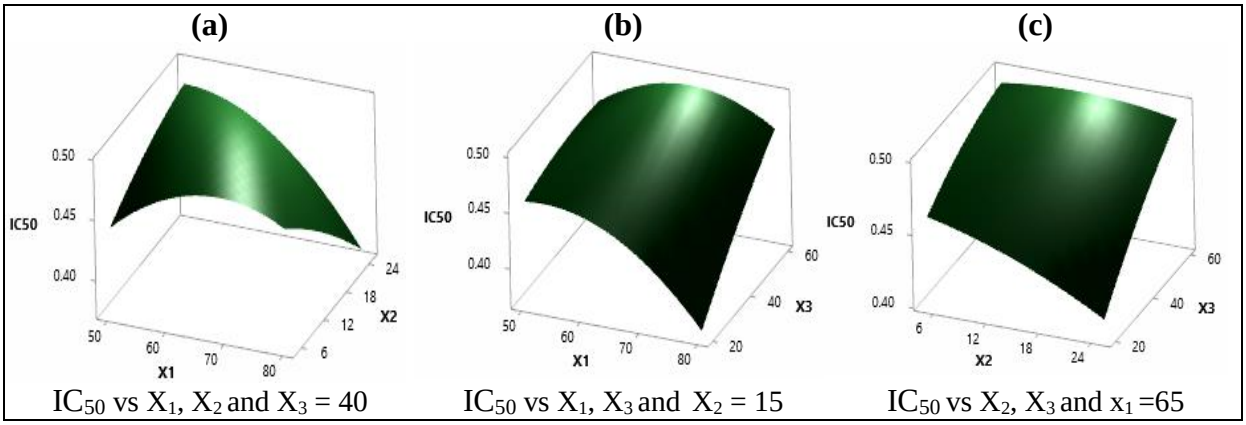
X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time.

Figure I.12. Surface plots of TFC of the extracts obtained by MAE.

I.6.7. The Effect of the Different Variables on the antioxidant activity

- **For UARE:**

Since the lowest IC_{50} represents the highest antioxidant activity, the lower responses in the plots (**Figure I.13**) indicate the optimal condition for extraction.



IC_{50} vs X_1, X_2 and X_3 = 40 IC_{50} vs X_1, X_3 and X_2 = 15 IC_{50} vs X_2, X_3 and x_1 =65

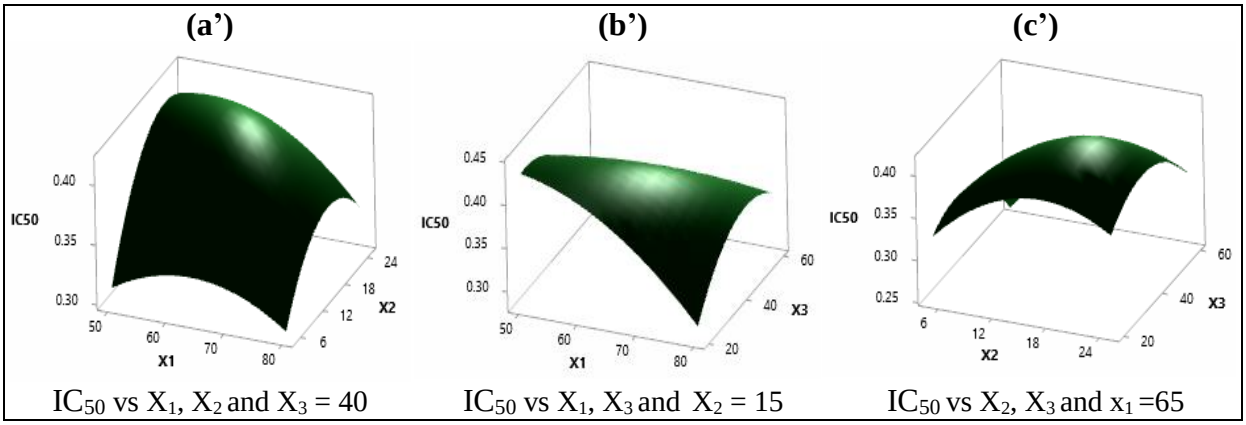
X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time

Figure I.13. Surface plots of IC_{50} of the extracts obtained by UARE.

Unlike the TPC and TFC, the IC_{50} was increasing with the increase of X_2. The response was higher with less extraction time also. This could be explained by the antagonistic effects due to the increase of multiple component by the extraction time.

- **For MAE**

The extracts obtained by MAE showed higher DPPH scavanging which is in consistent with the previous results (activity/compounds/extraction method). Also, the presence of various antioxidant compounds with different chemical characteristics and polarities.



IC_{50} vs X_1, X_2 and X_3 = 40 IC_{50} vs X_1, X_3 and X_2 = 15 IC_{50} vs X_2, X_3 and x_1 =65

X_1=Methanol concentration, **X_2**= Solvent/Material ration, and **X_3**= Extraction time.

Figure I.14. Surface plots of IC_{50} of the extracts obtained by MAE.

Hence the knowledge of the resultant of the possible synergistic or antagonistic effects due to the use of multiple component herbal formulae. In general, antioxidant molecules can react either by multiple mechanisms or by a predominant mechanism. The chemical structure of the antioxidant substance allows understanding the antioxidant reaction mechanism [81].

- **Extraction time effect on OEL compounds:**

The effects of the different extraction times on TPC of the extract was investigated in the range of 20 to 60 minutes. The impact was insignificant, we observed a slightly increase in the TPC as the extraction time increased. This could be explained by the short equilibrium time needed to establish the maximum TPC. During the initial stages of extraction (first 20 min), the combined effect of cavitation affect the material matrix swell, and the pores in the cell wall enlarge resulting in the enhanced solvent-solute contact, which in turn leads to the increase mass transfer rate resulting higher extraction efficiency. From the obtained results it is clear that the extraction efficiency increased for the first 30 min of extraction time. Our results were found to be in correlation with Al-Dhabi et al (2017) and Rostagno et al (2007) [86-87].

On other hand, several studies have reported insignificant increase in total phenolics content as the extraction time was prolonged [86-87]. Le and co-workers [88] also presented the impact of maceration extraction time on TPC from *Glycine max* L; TPC gradually increased from 5.4 to 12.8mg GAE/g of dried extract as the time increased from 15 to 60 minutes. However, from 60 to 150 minutes, the TPC remained unchanged at around 12.7 mg GAE/g of dried extract.

One of the essential independent variables in solid-liquid extraction is time due to its impact on the analyte's solubility and mass transfer which is associated with the compound's structure and molecular weight. Prolonged extraction time may lead to oxidation, epimerization, and degradation and structural destruction of extracted phenolic compounds of interest [89]. From the economical point of view shorter process time, results in more economic process.

- **Methanol concentration effect on OEL compounds:**

Increasing the methanol concentration enhances extraction yield. These results indicate that the solvent polarity should correspond with the targeted phytoconstituents. When solvents of different polarities are mixed, they tend to extract a wider spectrum of compounds. The combined use of water and organic solvent may facilitate the extraction of chemicals

which attributable to the higher solubility. Previous researches also found that the TPC of the extracts decreased with increasing water content in the aqueous solvent except for the methanol system [90-91].

Diem-Do *et al* 2014 [92], found that the TPC of the 80% aqueous methanol extract is higher than that of the 100% methanol extract and the 50% aqueous extract. This may be attributable to the content of more non-phenol compounds such as carbohydrates and terpenoids in water extracts than in other extracts. It may also be caused by the possible complex formation of some phenolic compounds in the extract that are soluble in methanol. These phenolic compounds may possess more phenol groups or have higher molecular weights than the phenolics in the water extract [81, 92].

- **Solvent/Material ratio effect on OEL compounds:**

In our study a strong positive correlation between the TPC and Solvent-Material ratio from 5% to 15% after that no significant increase was noticed. Theoretically, the Solvent-Material ratio significantly affects the extraction of the bioactive compounds due to its effect on the concentration gradient between the solute and the solvent at the surface of the raw material [93]. On another hand, the higher ratios (more than 15% in our case) extracted the lower TPC.

Researches concluded that the lowest Solvent-Material ratio was the most suitable for extraction of phenolics [94]. Moreover, it was found that the increase in the ratio only improved the yield for phenols (anthocyanins) in the case of acidified methanol. Higher solid-solvent ratio increases the concentration gradient, leading to an increased diffusion rate of the compounds from the extracted solid material into the solvent, but also determines the increasing of the necessary period of time to achieve equilibrium. Solvent-Material ratio could significantly affect the equilibrium constant and characterize the relationship between yield and solvent [93-94].

I.7. Predicted Values of the Model

In this research, the goal was to maximize TPC with the lowest IC_{50}. The optimum values of OEL extractions are exposed**.** The current variable settings are X_1 = 80%, X_2= 11%, X_3 = 20 min for UARE (**Table I.11**). While for MAE higher extraction time with less material mass is required. For higher yields in a short extraction time, it is better to use UARE instead of traditional maceration.

Table I.11. Predicted values of the responses at optimum conditions.

Extraction method	**Optimal conditions**			**Predicted responses**			
	X_1 %	**X_2 %**	**X_3 min**	**Y %**	**TPC (mg GAE/g)**	**TFC (mg RE/g)**	**IC_{50} mg/ml**
UARE	80	11	20	24	139	34	0.39
MAE	80	05	60	20	139	23	0.27

I.8. Conclusion

Response surface methodology is a useful tool to optimize the extraction process. It gives a better understanding and helps to lower the cost of the process. RMS has successfully applied for *O. europaea* leaves. The high correlation of the mathematical model indicated that a quadratic polynomia model could be used to optimize total phenolic and flavonoid content, as well as the antioxidant activity of OEL extracts. From an engineering perspective, it is very helpful to do mathematical modeling of extraction processes. It is used to model the experiment, relate the consumption of energy, time, solvent, and other parameters to the extraction. The closer the R^2 and error to unity and zero respectively, the higher the fit of predicted models to the experimental data.

Chapter II:

Phenolic compounds of O. europaea leaves:

analytical and statistical study

II.1. Introduction

The spread of olive trees cultivation in Algeria beyond the Mediterranean regions to arid regions will absolutely affect the plant's enzyme activity and physiology. Subsequently, the chemical composition and biological value of *O. europaea* trees would be affected.

In this context, the objectives of this chapter attempt to examine the leaf chemical variability of biological active compounds of two *O. europaea* cultivars (cv. Chemlal and Sigoise) as well as their production levels as they adapt to abiotic stress (hyperarid conditions) during three successive seasons; summer, autumn, and winter. To our knowledge, the scanning of the phenolic profile of cv. Chemlal and cv. Sigoise cultured in hyperarid region of Algeria will be investigated for the first time through this study.

II.2. Material and methods

II.2.1. Phenolic compounds extraction:

Based on the optimal conditions of phenolics extraction obtained from the previous chapter. 300 g of a fine OEL powder were extracted by UARE in each season for each cultivar. The filtered solution was concentrated in a rotary evaporator and then liquid-liquid extractions were applied firstly with dichloromethane after that with ethyl acetate [82]. The ethyl acetate residues were stored at −18 °C in an amber glass vials for HPLC analysis.

II.2.2. Chemicals and Standards

The solvents used in the extraction (methanol, dichloromethane, and ethyl acetate) were of analytical grade and obtained from Sigma–Aldrich. The ethyl acetate extracts were dissolved in methanol HPLC grade from Fisher Scientific (Houston, TX, USA). Oleuropein, tyrosol, luteolin, and luteolin glycoside standards were bought from Extrasynthese Co (Genay, France). The HPLC mobile phase mixture solvents were obtained from Wako pure Chemical Industries, Ltd. (Osaka, Japan).

II.2.3. High-performance liquid chromatography analysis

Chromatographic analysis was performed in Tunisia according to Souilem *et al.* (2014) [95]. The instrument consists of an Agilent series 1260 HPLC-DAD (Agilent, Waldbronn. Germany).

- Compounds separation was carried out on a ZORBAX Eclipse XDB-C18 column (4.6 mm I.D. x 250 mm x 3.5 µm particle size).

- The mobile phase was made of phase A (0.1% acetic acid in water) and phase B (100% acetonitrile).

- The elution conditions were: flow rate was set at 0.5 ml/min, injection volume of 5 µl, and operating temperature of 40 °C.

- The running gradient was as follows: 0-22 min, 10-50% B; 22-32 min, 50-100% B; 32-40 min, 100% B; 40-44 min, 100-10% B. Reequilibration duration lasted 6 min.

- The DAD detector scanned from 190 to 400 nm and detection was achieved at λ = 254 nm for oleuropein, at 280 nm for tyrosol, and at 330 nm for flavonoids (luteolin, apigenin-7-glucoside, and verbascoside)**.**

II.2.4. Identification of components

- Qualitative Characterization: compounds were identified according to their UV retention times.
- Quantitative Determination: quantification was performed for components, in particular oleuropein, by using external calibration with standards.

The concentrations of the phenolic compounds were expressed by µg/mg Extract.

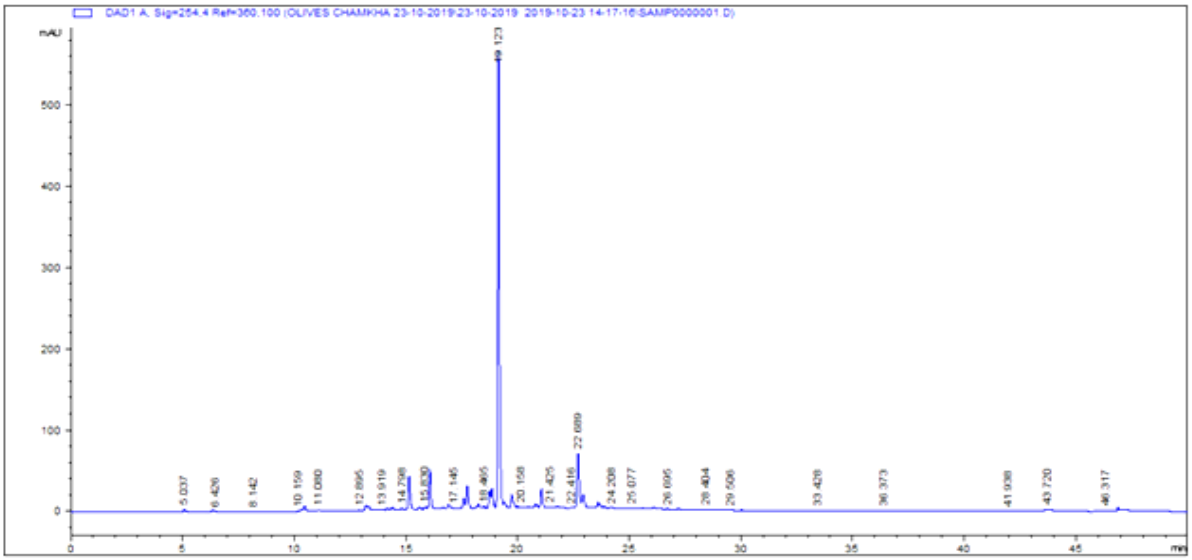
II.2.5. Statistical Analysis

In order to provide simple summaries about the measures, ANOVA test was applied on the obtained quantitative data for significant differences ($p \leq 0.05$). The data were compared using Tukey's test. All the statistics were performed by XLSTAT 2016 Addinsoft TM" software.

II.3. Results and Discussion

Out of 16 extracts, the phenolic profiles of 12 OEL extracts were identified by HPLC, one of the recorded chromatograms is presented in **Figure II.1**. While the phenolic concentrations are reported in **Table II.1** and **II.2** for cv. Chemlal, **Table II.3** and **II.4**, for cv. Sigoise. Thirteen identified compounds were tracked, namely; hydroxytyrosol, tyrosol, caffeic acid, vanillic acid, rutin, luteolin-7-glucoside, verbascoside, *p*-coumaric acid, apigenin-7-glucoside, oleuropein, luteolin, quercetin, and apigenin. We can clearly recognize the intense peak (t_R=19 min.) which represents the oleuropein with more than 50 % of the entire extract.

The difference in compositions and concentrations of the phenolic compounds among the studied OLE are obviously influenced by cultivars, season, and cultivation region.



t_R= 10.317: hydroxytyrosol, t_R= 12.948**:** tyrosol, t_R= 13,286**:** caffeic acid, t_R=13.919**:** vanillic acid, t_R=14.798: rutin, t_R=16.156**:** luteolin-7-glucoside, t_R= 16.455**:** verbascoside, t_R= 16.652: *p*-coumaric acid, t_R= 17.738**:** apigenin-7-glucoside, t_R= 19.123: oleuropein, t_R= 22.689: luteolin, t_R= 22.916**:** querciten, t_R= 25.071**:** apigenin. (t_R in min.)

Figure II.1. Chromatogram of the phenolic profile of *O. europaea* leaf.

II.3.1. Phenolic compositions in OEL of cv. Chemlal

a) Mediterranean region (O11)

Table II.1 represents the phenolic compositions detected in OEL during three successive seasons (1: July, 2: October, 3: January) from cv. Chemlal that has grown in the Mediterranean region.

Table II.1. Phenolics seasonal variation (µg/mg) of the fraction O11.

Compounds	O111	O112	O113
Hydroxytyrosol	0.5 ± 0.14^{e}	17 ± 1.63^{c}	8 ± 1.28^{f}
Tyrosol	0.7 ± 0.11^{e}	1.4 ± 0.40^{f}	0.4 ± 0.04^{gh}
Caffeic acid	0.6 ± 0.15^{e}	0.3 ± 0.17^{f}	-
Vanillic acid	-	0.2 ± 0.04^{f}	0.2 ± 0.08^{h}
Rutin	0.3 ± 0.22^{e}	-	3.4 ± 0.13^{g}
Luteolin-7-glucoside	28 ± 0.88^{c}	11.7 ± 0.17^{d}	32.8 ± 0.73^{c}
Verbascoside	0.2 ± 0.04^{e}	-	0.1 ± 0.04^{h}
***p*-Coumaric acid**	0.4 ± 0.04^{e}	0.06 ± 0.01^{f}	0.7 ± 0.13^{gh}
Apigenin-7-glucoside	39.2 ± 1.20^{b}	31.8 ± 1.80^{b}	42.6 ± 0.88^{b}
Oleuropein	583 ± 8.04^{a}	173.1 ± 4.77^{a}	577.6 ± 6.97^{a}
Luteolin	27.5 ± 0.35^{c}	7.1 ± 0.75^{e}	12.8 ± 1.64^{e}

Quercetin	12.4 ± 0.55^{de}	12.5 ± 1.11^{d}	18.6 ± 1.24^{d}
Apigenin	-	-	7.46 ± 0.53^{f}

Results are expressed as mean values ± standard deviation (SD). Letters in a column indicate the level of significant difference ($p < 0.05$) between compounds.

Discussion:

✓ **The main compounds**

Oleuropein is the most significant component among the phenolic compounds present in OEL [19-25, 44]. It has concentrations ranging from 173 to 583 µg/mg. Its highest concentration (583 µg/mg) was noted in July, while the olives were ripening. The lowest value (173 µg/mg), was recorded the next season, which corresponded to harvest time (October). When the olive trees were pruned the following season, the concentration climbed to 578 µg/mg (January), almost to the same level as July.

The second most abundant component in this cultivar was apigenin-7-glucoside, with concentrations ranging from 32 to 43 µg/mg. Unlike oleuropein, the lowest amount (32 µg/mg) of apigenin-7-glucoside was found in July and the highest (43 µg/mg) in January. Luteolin-7-glucoside, which ranged from 12 to 33 µg/mg, responded similarly to apigenin-7-glucoside.

Luteolin (from 7 to 28 µg/mg), also has the same behavior as the two previous glycosides. However, its highest concentration (28 µg/mg) was noted in July as oleuropein.

Quercetin **(**from 12 to 19 µg/mg), remained stable for the first two seasons, after that, it peaked in January.

✓ **The minor compounds**

Apigenin (7 µg/mg) was only detected in one season (January), while verbascoside and rutin were not present throughout harvest season.

During olive harvest, hydroxytyrosol and tyrosol reached their highest values, in particular, hydroxytyrosol with a high concentration (17 µg/mg) during the season when oleuropein was at its lowest. Probably due to the bioconversion of oleuropein to hydroxytyrosol. Other studies have confirmed that oleuropein is likely to decompose into hydroxytyrosol [56-58].

b) Arid region (O21)

Table II.2 represents the phenolic compositions detected in OEL during three successive seasons from cv. Chemlal that has grown in the arid region.

Table II.2. Phenolics seasonal variation (µg/mg) of the fraction O21.

Compounds	O211	O212	O213
Hydroxytyrosol	7.6 ± 0.82^{d}	2 ± 0.55^{bc}	$1.6 \pm 0.33f^{g}$
Tyrosol	0.8 ± 0.13^{d}	0.4 ± 0.06^{bc}	0.4 ± 0.11^{g}
Caffeic acid	-	-	-
Vanillic acid	0.1 ± 0.03^{d}	0.2 ± 0.04^{bc}	0.2 ± 0.06^{g}
Rutin	2 ± 0.28^{d}	1.4 ± 0.06^{bc}	2.8 ± 0.11^{f}
Luteoline-7-glucoside	-	0.4 ± 0.08^{c}	14 ± 1.06^{c}
Verbascoside	0.8 ± 0.08^{d}	0.6 ± 0.40^{bc}	0.8 ± 0.04^{g}
***p*-Coumaric acid**	0.6 ± 0.08^{d}	0.26 ± 0.05^{bc}	0.9 ± 0.08^{g}
Apigenin-7-glucoside	18.2 ± 0.53^{c}	$0.6 \pm 0.13b^{c}$	18 ± 0.48^{b}
Oleuropein	403 ± 22.53^{a}	173 ± 9.24^{a}	442.8 ± 2.84^{a}
Luteolin	25.5 ± 0.68^{bc}	0.8 ± 0.48^{b}	6.9 ± 0.74^{e}
Quercetin	30.8 ± 2.40^{b}	0.2 ± 0.11^{bc}	10.2 ± 0.88^{d}
Apigenin	23.7 ± 1.04^{bc}	0.13 ± 0.04^{bc}	-

Results are expressed as mean values ± standard deviation (SD). Letters in a column indicate the level of significant difference ($p < 0.05$) between compounds.

Discussion:

✓ **The main compounds**

Oleuropein, which ranged from 173 to 443 µg/mg in the arid zone, was responding similarly to the prior region. During harvest, oleuropein concentration was at its lowest, 173 µg/mg, and was identical to the first region. However, the Mediterranean region exposed higher concentrations of this chemotype. (**Figure II.2)**. According to the climatic conditions in the whole year of the study period, Boumerdes was stated to have more rainfall than Ouargla. Previous studies reported that the most significant factor that affects olive tree growing is temperature (15–20 °C), while water availability is the most significant factor that limits the tree yields [46, 49].

Despite being able to grow well even in poor, dry soils, in some cases, olive trees can grow with a rainfall of 200 mm year^{-1}, Proper olive cultivation areas should be above 400 mm year^{-1}, and values of 600 mm year^{-1}, 800 mm year^{-1} and 1000 mm year−1 are considered sufficient, moderate and good, respectively. Still, 500 mm year^{-1} is the lower limit for commercial olive yields under rainfed conditions [46, 96-98].

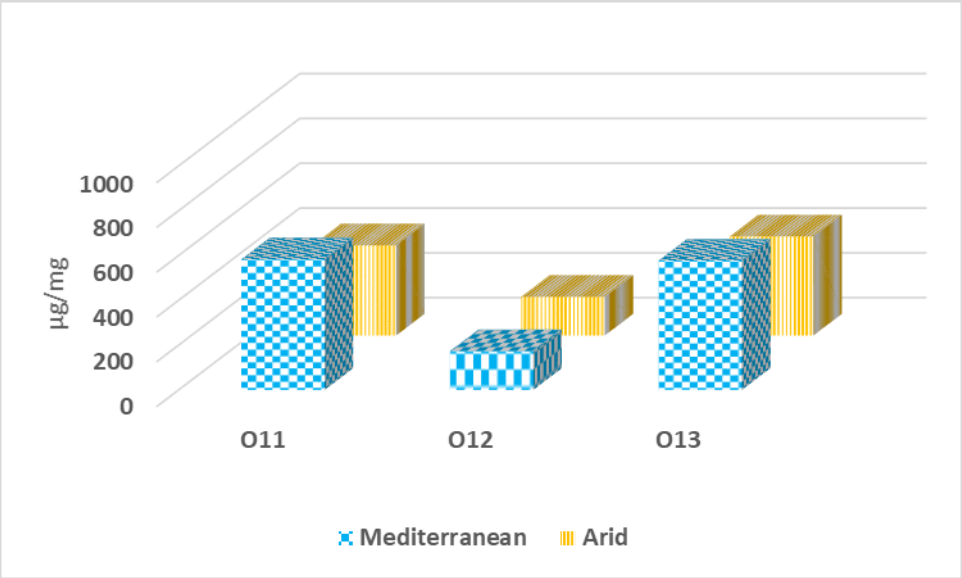


Figure II.2. Seasonal variation of oleuropein content (µg/mg) in cv. Chemlal.

In contrast to the first region, flavonoids rather than flavone glycosides were found to be the second main compounds. Quercetin concentrations ranged from 0.2 to 31 µg/mg, while luteolin concentrations ranged from 0.8 to 26 µg/mg. Their highest concentrations were observed in July, and the lowest in October.

Apigenin, which was detected in only one season in the first region, was found in two seasons in a row, with a significant concentration 24 µg/mg in July.

The flavone glycosides, apigenin-7-glucoside and luteolin-7-glucoside, were detected at lower concentrations compared to the Mediterranean region; the first compound ranged from 0.8 to 18 µg/mg, while the second one from 0.4 µg/mg in October to 14 µg/mg in January and it was totally absent in July.

✓ **The minor compounds**

Despite the absence of caffeic acid in this region, all of; verbascoside, *p*-coumaric acid, and rutin were found in higher concentrations than in the Mediterranean region.

On the other hand, hydroxytyrosol and tyrosol reacted similarly to each other but not to the region; their highest concentrations were noticed in July with 7.6 and 0.8 µg/mg, respectivly.

In general, all major compounds in cv. Chemlal were found to be higher in the Mediterranean region, except for quercetin and apigenin. While the arid region exhibited a higher concentration of the minor compounds.

II.3.2. Phenolic compositions in OEL of cv. Sigoise

a) Mediterranean region (O12)

Table II.3 represents the phenolic compositions detected in OEL during three successive seasons from cv. Sigoise that has grown in the Mediterranean region.

Table II.3. Phenolics seasonal variation (µg/mg) of the fraction O12.

Compounds	O121	O122	O123
Hydroxytyrosol	9.1 ± 0.51^{e}	7.2 ± 1.33^{ef}	8.4 ± 2.26^{c}
Tyrosol	0.86 ± 0.14^{f}	0.7 ± 0.19^{g}	0.6 ± 0.11^{d}
Caffeic acid	0.4 ± 0.04^{f}	0.3 ± 0.04^{g}	-
Vanillic acid	0.6 ± 0.08^{f}	0.5 ± 0.05^{g}	0.3 ± 0.08^{d}
Rutin	0.26 ± 0.08^{f}	2.6 ± 0.11^{g}	-
Luteoline-7-glucoside	42.8 ± 2.44^{c}	24.06 ± 1.37^{c}	0.3 ± 0.08^{d}
Verbascoside	-	0.26 ± 0.04^{g}	0.2 ± 0.13^{d}
***p*-Coumaric acid**	1.1 ± 0.31^{f}	0.3 ± 0.05^{g}	0.2 ± 0.017^{d}
Apigenin-7-glucoside	58.5 ± 10.31^{b}	35.1 ± 1.77^{b}	19.6 ± 1.86^{b}
Oleuropein	606.8 ± 6.44^{a}	757.8 ± 11.85^{a}	611.7 ± 11.56^{a}
Luteolin	29.7 ± 5.91^{d}	9.4 ± 2.71^{d}	5.33 ± 0.84^{c}
Quercetin	28.4 ± 2.71^{d}	10.8 ± 1.51^{de}	7.5 ± 2.66^{c}
Apigenin	-	-	-

Results are expressed as mean values ± standard deviation (SD). Letters in a column indicate the level of significant difference ($p < 0.05$) between compounds.

Discussion:

- ✓ **The main compounds**

cv. Sigoise, which grew in the same environment as the prior cultivar, had greater amounts of the major components.

Oleuropein was the only compound that showed different behaviors across the three seasons. Its concentration increased from 607 µg / mg to 758 µg/mg in the harvest and then decreased to 612 µg/mg.

From July to January, the concentrations of apigenin-7-glucoside and luteolin-7-glucoside decreased from 59 to 20 µg/mg and 43 to 0.3 µg/mg, respectively. Both luteolin and quercetin showed the same behavior, dropping from 30 to 5 g/mg and 28 to 7.5 g/mg, respectively.

✓ **The minor compounds**

Apigenin was completely absent in O21. In the other side, the production of hydroxytyrosol and tyrosol remained almost constant during the three seasons. Moreover, both caffiec and vanillic acids showed a smaller decrease from July to January.

b) Arid region (O22)

Table II.4 represents the phenolic compositions detected in OEL during three successive seasons from cv. Sigoise that has grown in the arid region.

Table II.4. Phenolic seasonal variation (µg/mg) of the fraction O22.

Compounds	O221	O222	O223
Hydroxytyrosol	6.3 ± 3.73^{ef}	3.2 ± 1.91^{d}	1.5 ± 0.93^{c}
Tyrosol	0.4 ± 0.08^{f}	0.1 ± 0.02^{d}	-
Caffeic acid	0.2 ± 0.01^{f}	0.26 ± 0.01^{d}	0.2 ± 0.02^{c}
Vanillic acid	-	0.4 ± 0.05^{d}	0.2 ± 0.04^{c}
Rutin	0.5 ± 0.01^{f}	2.53 ± 0.26^{d}	2.7 ± 0.48^{c}
Luteoline-7-glucoside	9.2 ± 0.10^{de}	0.3 ± 0.22^{d}	49.7 ± 4.60^{b}
Verbascoside	0.4 ± 0.26^{f}	31 ± 2.40^{b}	0.1 ± 0.66^{c}
***p*-Coumaric acid**	0.2 ± 0.08^{f}	0.06 ± 0.01^{d}	0.4 ± 0.13^{c}
Apigenin-7-glucoside	13.6 ± 1.95^{c}	0.13 ± 0.09^{d}	1.1 ± 0.31^{c}
Oleuropein	168.6 ± 11.06^{a}	827.86 ± 15.42^{a}	488.9 ± 16.04^{a}
Luteolin	29.7 ± 3.37^{b}	8.2 ± 0.26^{cd}	3.8 ± 1.06^{c}
Quercetin	11.9 ± 1.86^{cd}	14.06 ± 0.48^{bc}	6.2 ± 0.8^{c}
Apigenin	-	1.3 ± 0.44^{d}	-

Results are expressed as mean values ± standard deviation (SD). Letters in a column indicate the level of significant difference ($p<0.05$) between compounds.

✓ **The main compounds**

In October, this fraction showed the greatest increase in oleuropein concentration (828 µg/mg) (**Figure II.3)**. In parallel, the lowest value (169 µg/mg) was in the same fraction also in July. This droop was the only significant deviation compared to the other compounds. Unlike cv. Chemlal, the detection of this cultivar was higher in January (489 ug/ml) compared to July. In this month, the temperature in the arid region exceeded 55 °C with no rainfall. Other reports specified that the total precipitation content during the period until harvest time affected OEL phenolic substances [97-98].

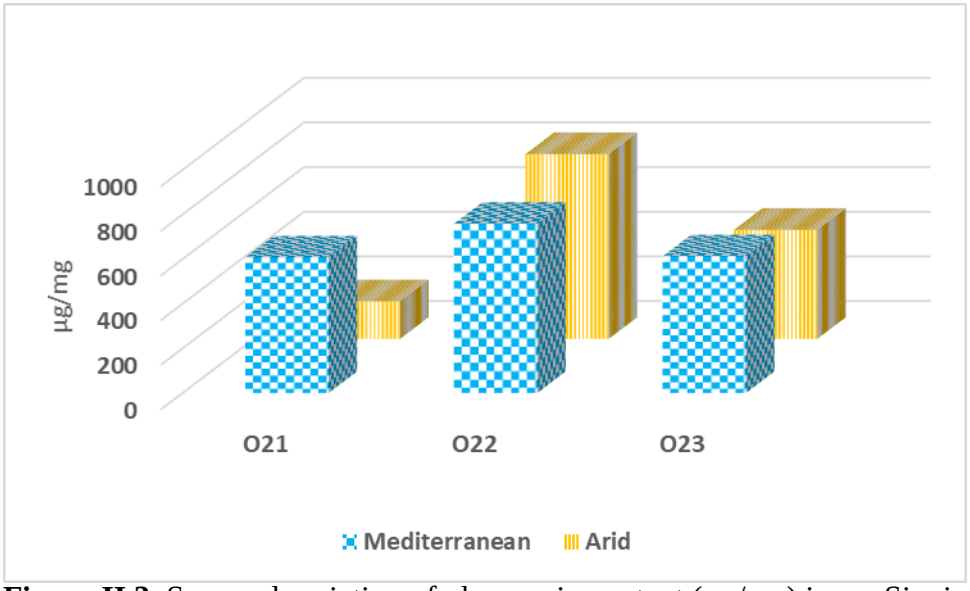


Figure II.3. Seasonal variation of oleuropein content (µg/mg) in cv. Sigoise.

Flavonoids identified in the second place also, similarly to the first cultivar in the same region, Luteolin concentrations were decreasing from 30 to 4 µg/mg. we can see that the production of this compound in both regions was at the same concentrations.

Among all the 12 OELs studied, the O22 fraction has the highest verbascoside concentration evaluated to 31 µg/mg during harvest, although its presence in the other seasons was almost insignificant.

In January, A significant presence for luteolin-7-glucoside (50 µg/mg) was noticed. While apigenin-7-glucoside highest value (14µg/mg) was found in July.

✓ **The minor compounds**

Unlike the first region, Apigenin (1.3 µg/mg) was recorded during harvest only. Both caffeic and vanillic acids were detected with almost the same concentrations (≈ 0.2 µg/mg).

In general, the production level of both cultivars in each region was almost identical. Flavone glycosides were the second main compounds in the Mediterranean region, while those of the arid region were flavonoids. In comparison, the concentration of phenolic compounds was slightly higher in cv. Sigoise samples collected from arid conditions. This finding reveals the cultivar's potential to produce secondary metabolites in an extremely hard desert climate.

For many socio-economic reasons cv. Chemlal is the widespread cultivar of *O. europaea* in Algeria (40% of the orchards). It is the main focus of most commercial entities

and researchers studies [73-74, 99]. However, another important aspect of cv. Sigoise as a high source of natural bioactive compounds was highlighted in the study.

Oleuropein was the only compound that showed different behaviors during the three successive seasons. The two cultivars were responding dissimilarly from each other concerning this constituent. The amounts decreased in cv. Chemlal from July until the olive harvest and increased again after that, contrariwise cv. Sigoise.

Şahin *et al*. (2012) [96], reported that the level of the oleuropein was generally lower in summer samples compared to spring ones in the leaves of twenty olive cultivars grown in Texas. This study was in agreement with cv. Sigoise results. In contrast, Fabbri *et al.* (2001) [97], found that the levels of the same compound in six Italian cultivars were low in the spring, which is in agreement with cv. Chemlal variation in the Mediterranean zone. These results suggest that the specific genetic makeup of Algerian cultivars was affecting the plant SM's production variation rather than the geographical location. Other studies reported the decrease of phenolic concentrations of olive oils from fruits of trees cultivated under high rainfall conditions when compared to the oils from low rainfall regions [98]. Contrary to cv. Chemlal and in agreement with cv. Sigoise in the season with the lowest rainfall recorded in both regions.

II.4. Conclusion

To the best of our knowledge, the phenolic compounds of OEL in unreported region of Algeria were evaluated for the first time in this study. Many other researches focused on cv. Chemlal, but cv. Sigoise was for the first time revealed with more importance. The two cultivars showed well production of phenolic compounds in a new growing area. The seasonal variation will determine the best period for local population, to gather OEL with the highest therapeutic potential.

The farm in the southern Algeria was started as a project since 10 years ago and now the analysis were for the first time established. This can evaluate the ecologic and economic perspectives for sustainable agriculture under arid climate conditions of the country.

Moreover, it can be concluded that cv. Sigoise cultivated in arid region is a rich source for the phenolic compounds of OEL extract. However, further researches are needed to determine action mechanism level of these chemical compounds.

Chapter III:

Volatile compounds of O. europaea flowers & leaves: analytical and statistical study

III.1. Introduction

In Algeria, less attention has been paid to *O. europaea* leaf volatiles (VOLs) [48, 72-73]. Therefore, the VOLs and those of flowers (VOFs) need detailed chemical clarification and estimates of possible changes related to the diversity of the Algerian climates [100-105].

The genetic factors for the major cultivars that can lead to the formation of different chemotypes with an important biological potential, also need to be taken into account [101-102, 105].

Consequently, this study is the first step to investigate the chemical variation of VOFs and VOLs in Algeria. Furthermore, it will provide information about the physiological characteristics of the major Algerian cultivars: Chemlal and Sigoise, in particular about the effect of geoclimatic conditions on the production of these compounds.

III.2. Material and methods

III.2.1. Preparation of the Volatile Samples

20 samples have been collected from the two; regions, cultivars, organs and the four seasons. Out of all the samples, only 11 of them (100 g each) were prepared from fresh *O. europaea* Flowers (F11, F12, F21 and F22) and dried Leaves (L11, L12, L21 and L22) at the full flowering stage (April 2019). In addition, to the study of volatiles seasonal variation of L11 (the main Algerian cultivar). In the codes, the first number represents the region (1: the Mediterranean, 2: the arid ones), while the second one represents the cultivar (1: cv. Chemlal, 2: cv. Sigoise).

The extraction of volatile compounds was carried out by hydrodistillation in a Clevenger apparatus for 3 h [106-111]. Volatile compounds were obtained by liquid-liquid extraction of the distillate with 2 ml of *n*-hexane. All the samples were stored in hermetically sealed glass vials at -18°C until analysis [110-115].

III.2.2. Gas Chromatography Analysis

The volatile fractions were analyzed at the Department of Pharmacy in the University of Pisa.

GC-EIMS analyses were performed with a Varian CP-3800 gas-chromatograph with the below conditions:

- A DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 μm).
- A Varian Saturn 2000 ion trap mass detector.
- The injector and transfer line temperatures were set at 220 and 240°C respectively.

- The oven temperature was programmed from 60 °C to 240 °C at 3 °C/min.
- Helium was used at 1 mL/min with a split ratio of 30:1.
- The MS acquisition parameters were: full scan mode, with a scan range of 35-350 amu, scan time 1.0 sec and fragmentation energy 70 eV.

III.2.3. Identification of components

Identification of the constituents was based on the comparison of their retention times with those of authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons, and on computer matching against commercial (NIST 14, Adams 2007) [116-117] and home-made library mass spectra built up from pure substances and components of known oils and MS literature data (Adams 2007) [117]. The levels of the identified volatiles were expressed relative abundance.

III.2.4. Statistical Analysis

A comparative study using the multivariate statistical analysis technique Principal Component Analysis (PCA) was applied to highlight the relationships between the chemical constituents cultivars and geographic site-cultivars based on the major compounds of VOFs (volatiles of *O. europaea* flowers) and VOLs (volatiles of *O. europaea* leaves) using the software XLSTAT 2016 from Addinsoft.

III.3. Results and Discussion

Overall, fifty-one compounds were identified accounting from 93.2 to 99.8% in VOFs and VOLs.

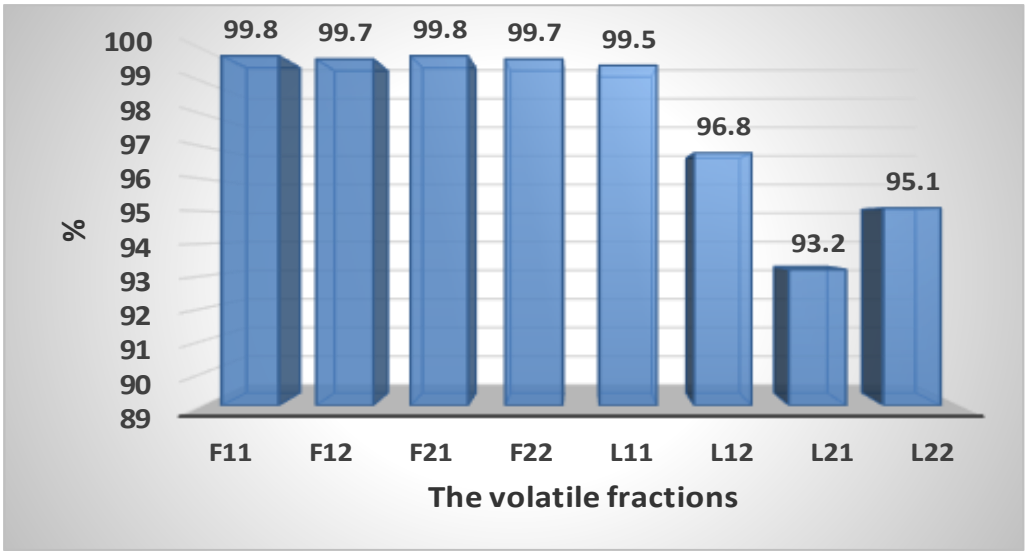


Figure III.1. The total identified compounds percentage of the studied volatile fractions**.**

III.3.1. Volatiles of *O. europaea* flowers (VOFs)

Table III.1 represents the twenty-four compounds that were identified in VOFs, accounting from 99.7 to 99.8% (**Figure III.1**). The number of each compound was based on the retention time of both leaves and flowers compounds compiled.

Table III.1. Volatile composition percentages of *O. europaea* flowers.

N°	Compound	*LRI*	*F11*	*F12*	*F21*	*F22*
1	hexanal	802	-	-	4.5	2.8
2	*(E)*-2-hexenal	856	-	-	4.4	-
3	*(Z)*-3-hexen-1-ol	857	-	-	2.7	-
5	*n*-nonane	900	-	-	0.8	0.8
6	heptanal	903	-	-	1.9	1.2
7	*(Z)*-2-heptenal	958	-	-	2.4	-
8	benzaldehyde	963	-	-	3.1	-
9	3-ethenyl pyridine	969	5.5	-	11.6	**17.2**
12	octanal	1002	-	-	1.9	-
13	*(E,E)*-2,4-heptadienal	1011	-	-	1.5	-
14	*p*-cymene	1028	3.7	8.1	2.7	2.8
15	limonene	1032	6.5	10.2	3.2	3.5
16	phenylacetaldehyde	1045	2.7	-	-	-
17	1-octanol	1071	-	-	1.6	-
18	*n*-undecane	1100	-	-	2.6	3.3
20	nonanal	1103	11.7	**32.4**	12.8	13.2
24	menthone	1154	-	-	4.5	4.5
27	methyl chavicol	1197	-	-	-	3
29	*n*-dodecane	1200	-	-	4.2	4.7
34	bornyl acetate	1287	-	-	**20.2**	**26.8**
37	*n*-tridecane	1300	-	-	-	3.7
41	phenylethyl propionate	1351	**17.9**	-	-	-
44	*(Z)*-jasmone	1395	**51.8**	**49**	13.2	9.4
45	*n*-tetradecane	1400	-	-	-	2.8

LRI: Linear retention indices (DB-5 capillary column), *F11: Flowers of Chemlal from the Mediterranean region; F12: Flowers of Sigoise from the Mediterranean region; F21: Flowers of Chemlal from the arid region; F22: Flowers of Sigoise from the arid region.

III.3.1.1. VOFs of cv. Chemlal cultivated in Boumerdes (F11)

In general, and without taking into account unidentified compounds, 7 compounds were identified representing 99.8% of F11, and classified into four chemical classes (**Figure III.2)**.

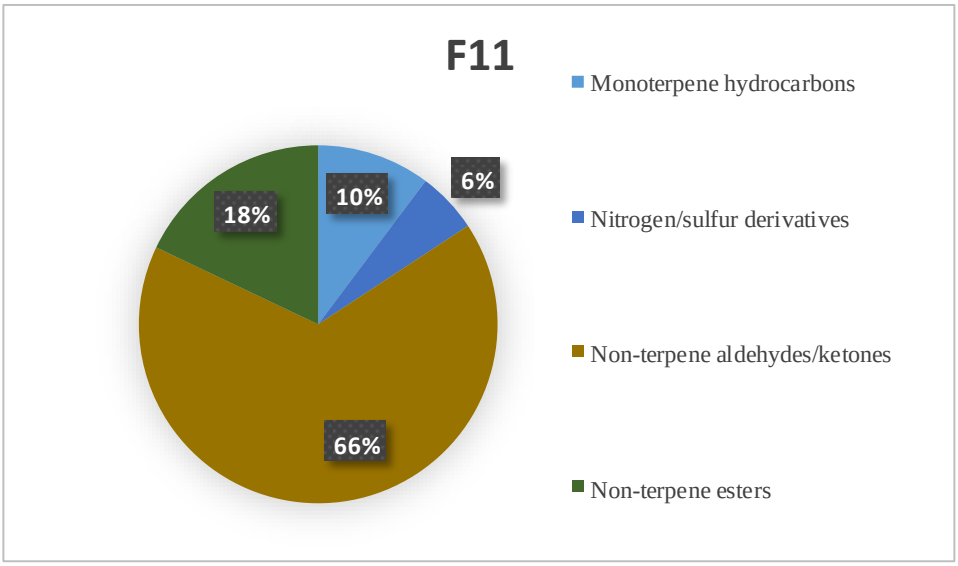


Figure III.2. Chemical classes percentage of **F11.**

This fraction showed a predominance of non-terpene aldehydes/ketones with 66% ; (*Z*)-jasmone was the major compound (51.8%) and nonanal with significant presence (11.7%). Followed by phenylethyl propionate (17.9%), which represents the class of non-terpene esters in the second place. The monoterpene hydrocarbons, which represented 10% of the sample, were *p*-cymene with 3.7% and limonene with 6.7% (**Table III.1)**.

III.3.1.2. VOFs of cv. Sigoise cultivated in Boumerdes (F12)

Unlike the previous cultivar, Sigoise cultivated in the Mediterranean region was distinct with two chemical classes representing 99.8%, and containing only 4 compounds. Non-terpene aldehydes/ketones predominantly constitute this fraction (82%) (**Figure III.3**).

As F11, (*Z*)-jasmone (49%) was also the major compound in F12, followed by nonanal (32.4%). The monoterpene hydrocarbons, which represent the second class with 18% of this volatile fraction, were as the previous cultivar, with *p*-cymene (8.1%) and limonene (10.2%). 3-ethenyl pyridine was absent only in this fraction (**Table III.1**).

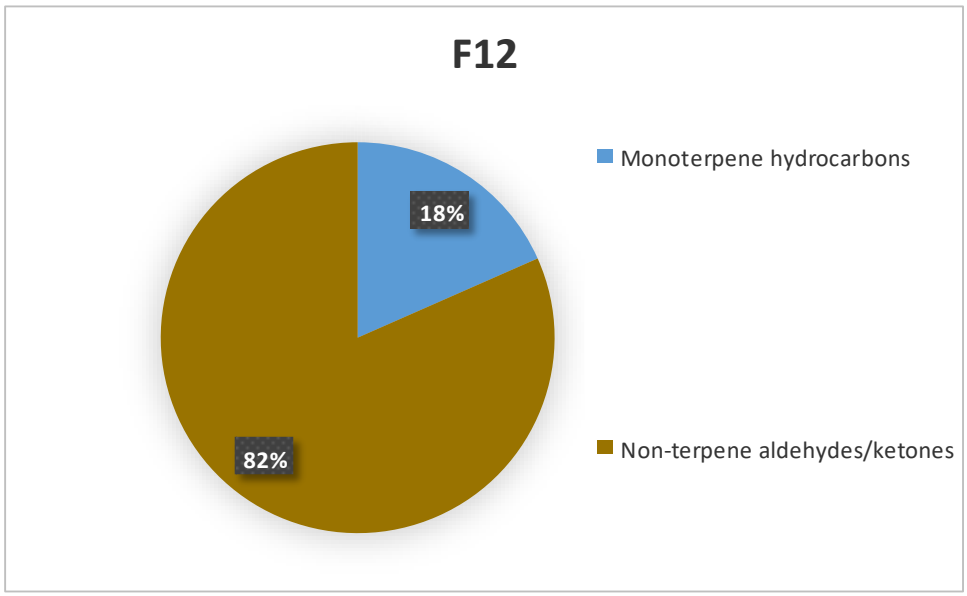


Figure III.3. Chemical classes percentage of **F12.**

III.3.1.3. VOFs of cv. Chemlal cultivated in Ouargla (F21)

19 compounds were identified representing 99.8% of F21, and classified into 6 chemical classes. The non-terpene aldehydes/ketones mainly constitute the fraction (46%). Oxygenated monoterpenes and Nitrogen/sulfur derivatives are respectively present with 25 and 12% as second and third classes. Besides non-terpene derivatives, corresponding 7% hydrocarbons and 4% alcohols/phenols/ethers. The hydrocarbons represent only 4% as the last class of total volatiles (**Figure III.4**).

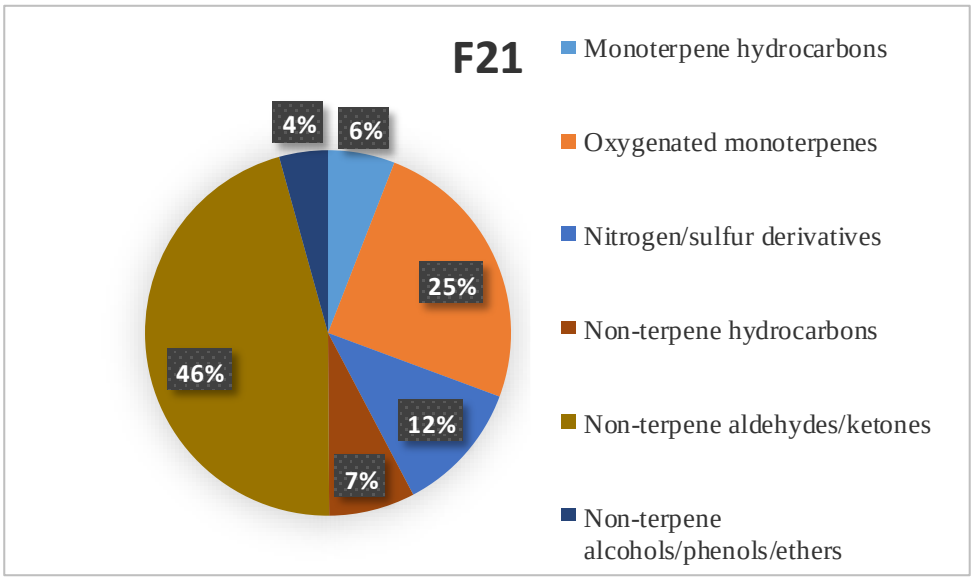


Figure III.4. Chemical classes percentage of **F21.**

Bornyl acetate was the main compound (20.2%) followed by (*Z*)-jasmone (13.2%) then nonanal (12.8) and 3-ethenyl pyridine (11.6%) (**Table III.1**).

III.3.1.4. VOFs of cv. Sigoise cultivated in Ouargla (F22)

15 compounds were identified representing 99.8% of F22. On the contrary the previous fractions, the oxygenated monoterpenes were dominant with the rate of 32% in this fraction, against only 6% hydrocarbons. The non-terpene derivatives were in the second class, the aldehydes/ketones with 27% and nitrogen/sulfur derivatives with 17% (**Figure III.5**).

As F21, bornyl acetate was the main compound (26.8%) of this fraction also, followed by 3-ethenyl pyridine (17.2%) then nonanal (13.2%). This fraction had the lowest proportion of (*Z*)-jasmone (9.4%) with an exclusive presence of phenylpropanoids class (3%) in VOFs (**Table III.1**).

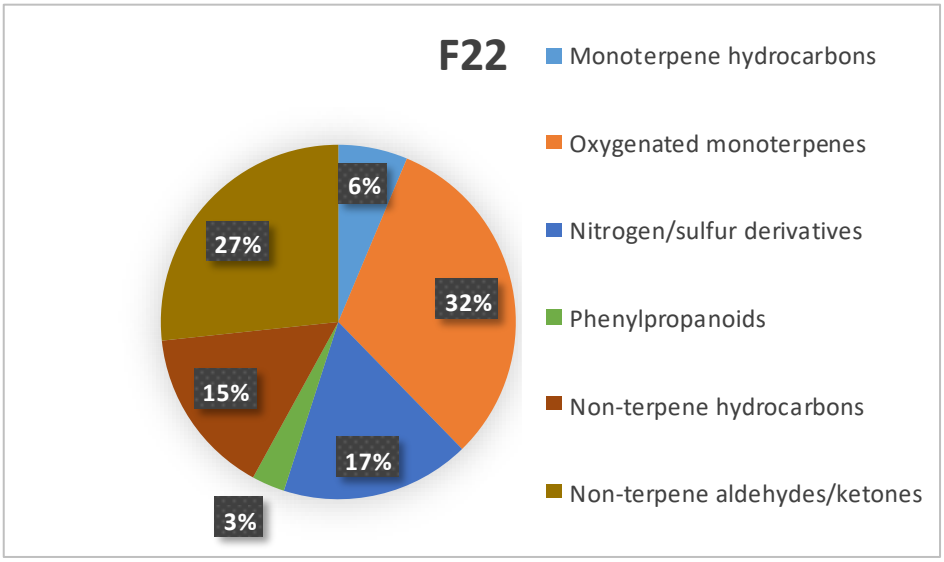


Figure III.5. Chemical classes percentage of **F22.**

III.3.1.5. Multidimensional statistical analysis (PCA of VOFs)

Principal Component Analysis (PCA) was applied to the VOFs data using the correlation matrix from **Table III.2** to obtain a more simplified view of the total compositions.

Table III.2 showed the results obtained from PCA for the 24 ones in VOFs. The cumulative percentage variance described by the first two principal components extracted from the VOFs (axes FC1 and FC2) explained 95.25% of the total variance.

The first principal component (FC1) accounted for 65.15% and the second one (FC2) accounted for an additional 30.10% of the total variance of flowers volatiles. The main

compounds were unrelated: each one fell in the same region quadrant. The minor compounds were placed in the upper and lower left quadrants, close to the centre, and none of them characterised neither the region nor cultivar.

Table III.2. Correlation matrix between the different compositions of VOFs.

Variables	F11	F12	F21	F22
F11	**1**	0,864	0,394	0,214
F12	0,864	**1**	0,505	0,301
F21	0,394	0,505	**1**	0,915
F22	0,214	0,301	0,915	**1**

F11: Flowers of Chemlal from the Mediterranean region; F12: Flowers of Sigoise from the Mediterranean region; F21: Flowers of Chemlal from the arid region; F22: Flowers of Sigoise from the arid region.

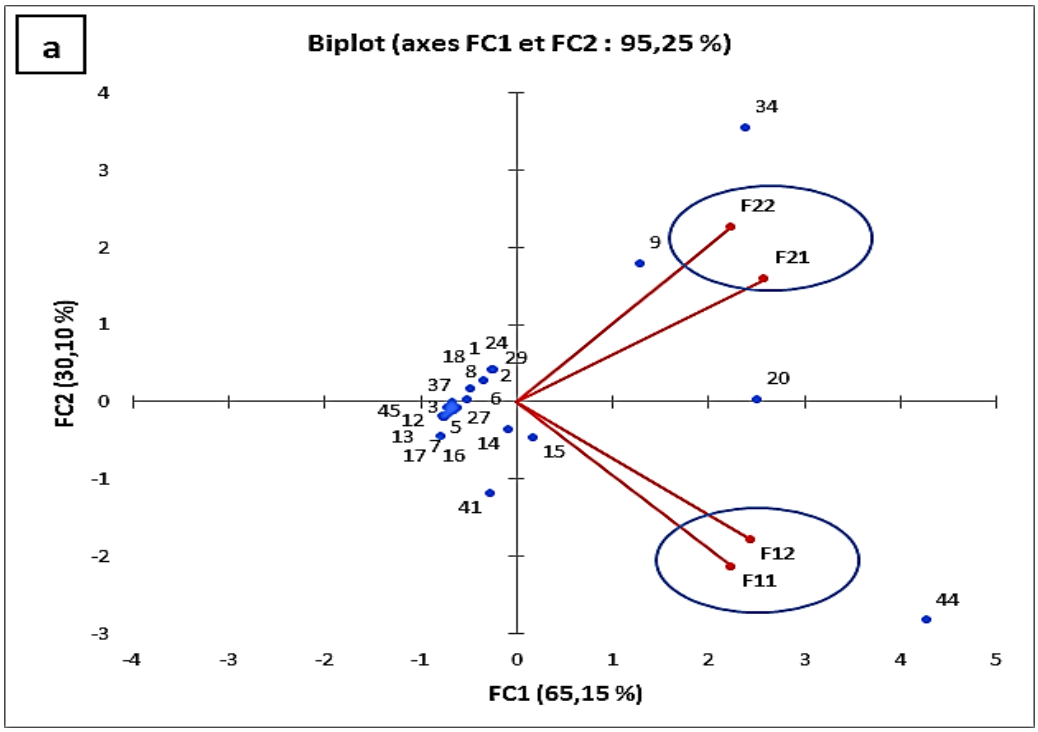


Figure III.6. Principal component analysis of VOFs.

The two regions were clearly separated from each other; the matrix of correlation from **Table III.2** shows that the correlation between F11 and F12 was 86.4% and between F21 and F22 was 91.5%. Also, the results indicate that the compositions of VOFs obtained from the same region are characterized by the same main compounds. In line with these results, several studies have demonstrated that the soil and the climate conditions have much more influence than the genetic factors among the cultivars [104, 108-110].

III.3.2. Volatiles of *O. europaea* leaves (VOLs)

The chemical compositions of VOLs are reported in **Table III.3.** Overall, forty-two compounds were identified, accounting from 93.2 to 99.5%.

Table III.3. Volatile compositions percentage of *O. europaea* leaves.

N°	Compounds	LRI	L11	L12	L21	L22
1	hexanal	802	2.4	-	-	-
2	*(E)*-2-hexenal	856	4.8	2.7	2.4	6.5
3	*(Z)*-3-hexen-1-ol	857	1.1	1.3	-	-
4	1-hexanol	869	0.8	-	-	-
5	*n*-nonane	900	0.5	-	0.3	-
6	heptanal	903	0.8	-	0.4	-
8	benzaldehyde	963	2.7	3	1.9	3.5
9	3-ethenyl pyridine	969	4.4	-	1.9	1.9
10	6-methyl-5-hepten-2-one	987	-	-	1.8	4.1
11	*(E,Z)*-2,4-heptadienal	997	-	-	1.5	7.1
13	*(E,E)*-2,4-heptadienal	1011	3.2	3	4.6	**16.9**
14	*p*-cymene	1028	0.7	2.7	-	-
15	limonene	1032	1.1	4.2	-	-
16	phenylacetaldehyde	1045	2.6	3.2	-	-
17	1-octanol	1071	1.4	1.7	2.3	3.6
19	linalool	1101	-	-	0.7	-
20	nonanal	1103	**26.9**	11.6	3.1	4.7
21	phenylethyl alcohol	1111	8.8	3.4	-	-
22	1,4-dimethyl-3-tetrahydroacetophenone	1150	-	-	0.9	-
23	*(E,E)*-2,6-nonadienal	1153	-	-	0,8	-
24	menthone	1154	-	1.7	0	-
25	*(E)*-2-nonenal	1163	-	-	1.2	-
26	2-phenylethyl formate	1177	3.2	-	-	-
28	safranal	1198	-	-	1	1.4
30	β-cyclocitral	1222	-	-	1	0.9
31	1,2-benzisothiazole	1223	2.4	1.6	-	-

32	carvone	1244	-	-	0.9	1.2
34	*(E)*-2-decenal	1262	1.3	-	1.2	0.6
35	*(E,Z)*-2,4-decadienal	1293	-	-	0.9	1.3
36	theaspirane I	1298	8.9	18.2	1.2	3.7
38	*p*-vinylguaiacol	1314	-	2.7	2.8	1.3
39	theaspirane II	1315	**17.7**	**23.7**	-	13.7
40	*(E,E)*-2,4-decadienal	1317	-	-	5.4	-
41	phenylethyl propionate	1351	-	3.4	1.2	-
42	eugenol	1358	1.6	-	-	
43	*(E)*-β-damascenone	1382	-	4	7.4	3.9
46	dihydrodehydro-β-ionone	1422	-	-	11.9	3.9
47	*(E)*-geranylacetone	1454	-	2	**14.4**	6.8
48	*(E)*-β-ionone	1488	2.2	2.7	9.7	4.8
49	dihydroactinidiolide	1536	-	-	5.1	3.3
50	caryophyllene oxide	1581	-	-	2.6	-
51	benzophenone	1627	-	-	2.7	-

LRI: Linear retention indices (DB-5 capillary column), *L11: Leaves of Chemlal from the Mediterranean region; L12: Leaves of Sigoise from the Mediterranean region; L21: Leaves of Chemlal from the arid region; L22: Leaves of Sigoise from the arid region.

III.3.2.1. VOLs of cv. Chemlal cultivated in Boumerdes (L11)

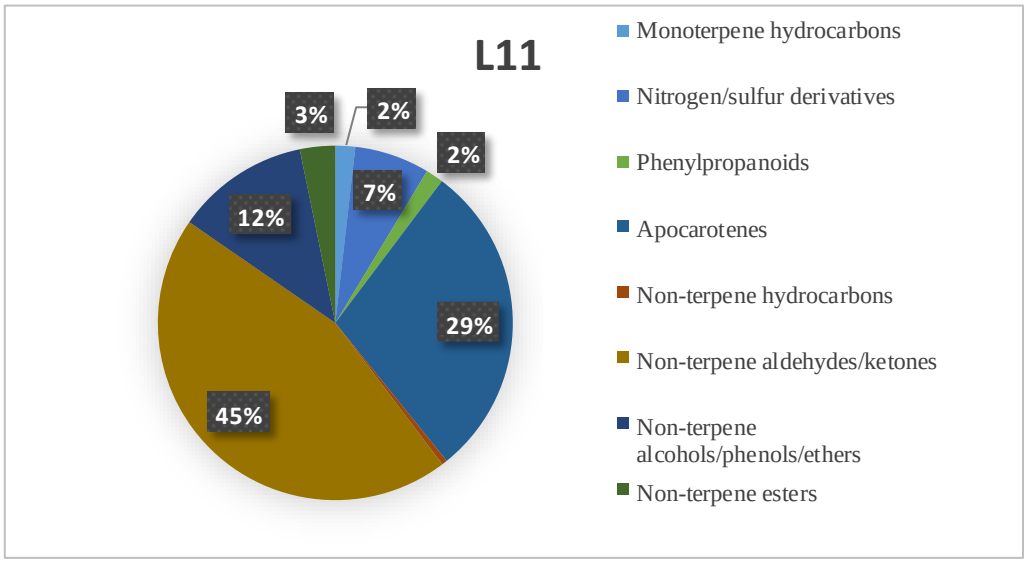


Figure III.7. Chemical classes percentage of **L11.**

22 compounds were identified representing 99.8% of L11. The non-terpene aldehydes/ketones mainly constitute the sample (45%) followed by Apocarotenes (29%) then non-terpene alcohol/phenols/ethers (12%) in second and third class, respectively. In all VOLs, this fraction has an axclusive presence of phenylpropanoids (2%) (**Figure III.7.**).

Nonanal was the major compound (26.9%) followed by theaspirane II (17.7%) then theaspirane I (8.9%) (**Table III.3**).

III.3.2.2. VOLs of cv. Sigoise cultivated in Boumerdes (L12)

19 compounds were identified representing 96.8% of the L12. The non-terpene alcohols/phenols/ethers mainly constitute the sample (51%) and the aldehydes/ketone (23%) then alcohols/phenols/ethers (9%). A significant presence of monoterpene hydrocarbons (7%) compared to the previous fraction (**Figure III.8**).

Theaspirane II was the major compound (23.7%) followed by theaspirane I (18.2%) then nonanal (11.7%) (**Table III.3**).

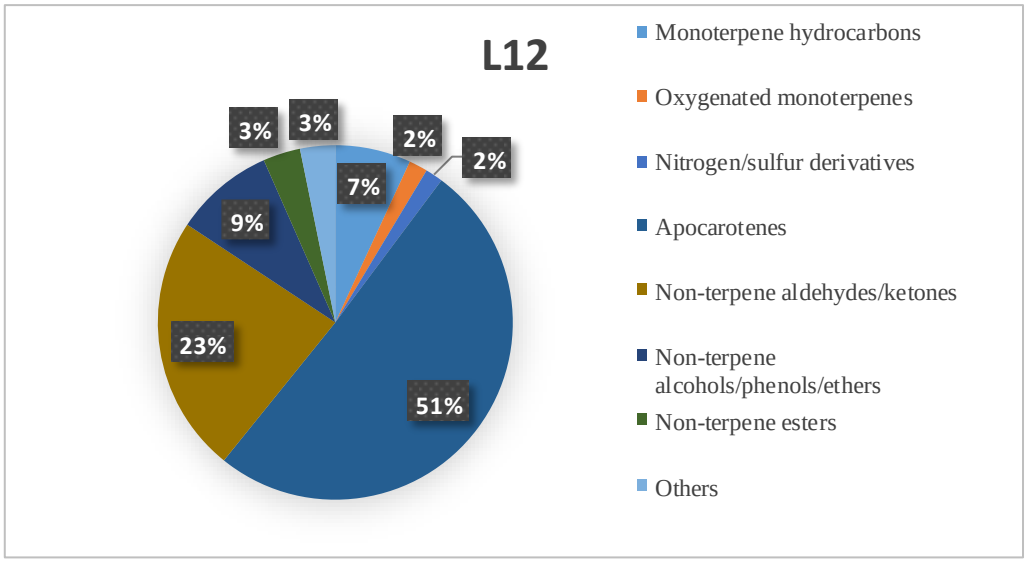


Figure III.8. Chemical classes percentage of **L12.**

III.3.2.3. VOLs of cv. Chemlal cultivated in Ouargla (L21)

31 compounds were identified representing 93.2% of L21. The Apocarotenes mainly constitute the sample (52%) and the non-terpene aldehydes/ketone (29%). Unlike the previous two fractions (L11 and L12), this fraction has 8 chemical classes, with the exclusive presence of oxygenated sesquiterpenes (3%). The total unidentified compounds were the lowest in this fraction (**Figure III.9**).

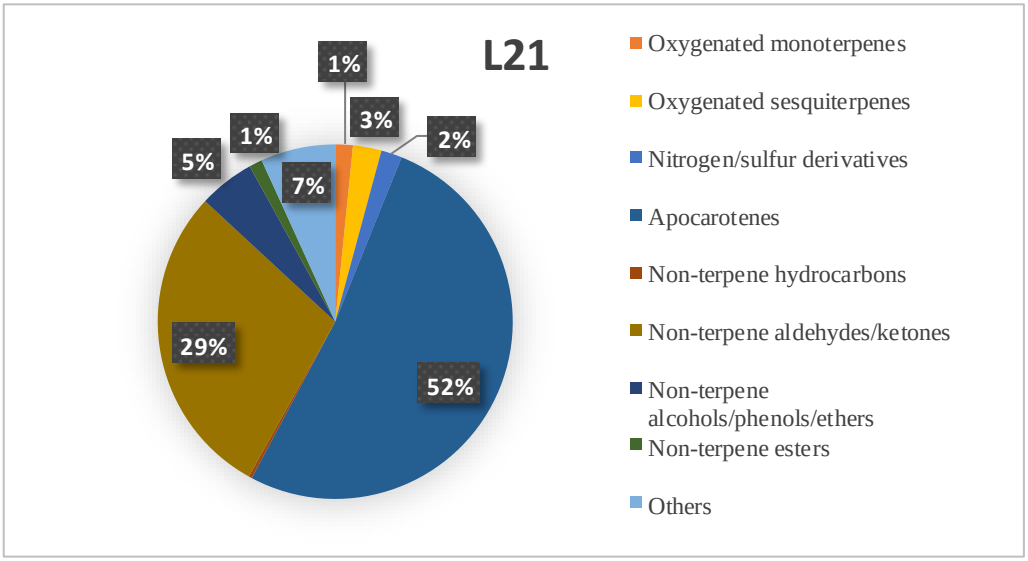


Figure III.9. Chemical classes percentage of **L21.**

(*E*)-geranylacetone was the major compound (14.4%) followed by dihydrodehydro-β-ionone (11.9%) as the second main constituent, then (*E*)-β-ionone (9.7%) (**Table III.3**).

III.3.2.4. VOLs of cv. Sigoise cultivated in Ouargla (L22)

21 compounds were identified representing 93.2% of the volatile fraction L21. The apocarotenes mainly constitute the sample (52%) and the non-terpene aldehydes/ketone (29%). Exactly as the previous fraction, the oxygenated monoterpenes had the last presence with 1% only (**Figure III.10**).

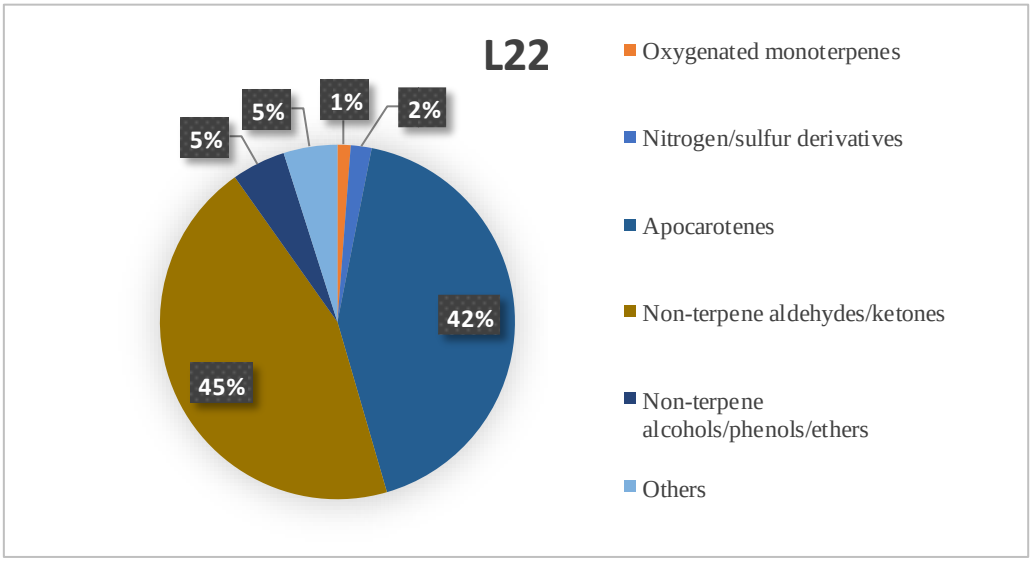


Figure III.10. Chemical classes percentage of **L22.**

(E,E)-2,4-heptadienal was the major compound (16.9%) followed by theaspirane II (13.7%) (**Table III.3**).

III.3.2.5. Multidimensional statistical analysis (PCA of VOLs)

Figure III.11 showed the results obtained from PCA for the 42 compounds in VOLs. The cumulative percentage variance described by the first two principal components extracted from the VOLs (axes LC1 and LC2), explained 83.85% of the total variance of VOLs.

Table III.4. Correlation matrix between the different compositions of VOLs.

Variables	L11	L12	L21	L22
L11	**1**	0.766	-0.030	0.417
L12	0.766	**1**	0.010	0.524
L21	-0.030	0.010	**1**	0.419
L22	0.417	0.524	0.419	**1**

L11: Leaves of Chemlal from the Mediterranean region; L12: Leaves of Sigoise from the Mediterranean region; L21: Leaves of Chemlal from the arid region; L22: Leaves of Sigoise from the arid region.

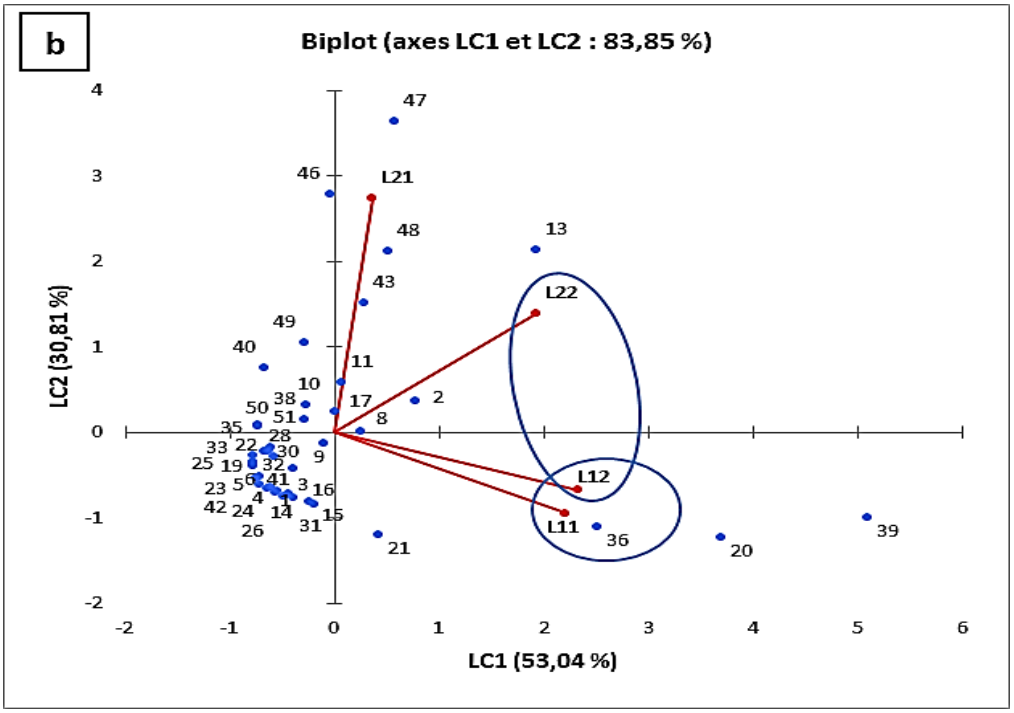


Figure III.11. Principal component analysis of VOLs.

The first and second principal components LCs explained 53.04% (LC1) and 30.81% (LC2) of the variance, across the samples. A correlation between L22 and L12 was noticed (52.4%) in **Table III.4**. Sigoise responded similarly in the two different geographic sites of

Algeria, probably because the environmental factors have less impact on the volatiles production of this cultivar, while for Chemlal this not applies (chemical similarity).

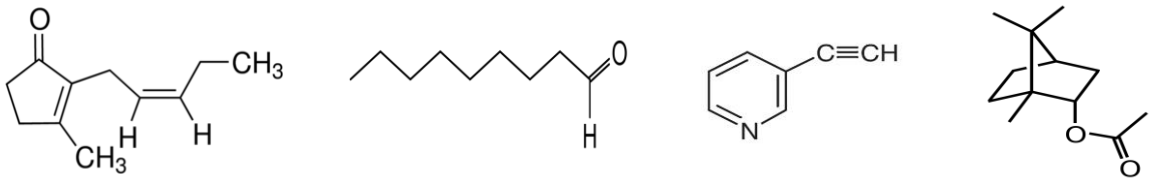
In general, the analysis allowed the identification of 51 compounds. As shown in the previous tables and figures, the identified constituents varied according to both type of cultivar (Chemlal or Sigoise), selected organ (leaves or flowers) and the geographic region (Mediterranean or arid).

However, most of the detected compounds exhibited low relative abundances (≤ 5%) and just a few of them were present as the major ones, such as *(Z)*-jasmone (51.8%), nonanal (32.4%), bornyl acetate (26.8%), theaspirane II (23.7%) and *(E,E)*-2,4-heptadienal (16.9%).

- **Comparaison between VOFs and VOLs**

By comparing, the VOLs (L11, L12, L21 and L22) and VOFs (F11, F12, F21 and F22), the former were richer in constituents, 42 vs. 24 volatiles. Among them, 15 compounds were common to both VOLs and VOFs, 27 compounds were found in VOLs only and 9 compounds were exclusive of VOFs.

From the previous graphs, VOLs and VOFs can be sorted into the 10 chemical classes. Aldehydes and ketones constituted the major class of VOFs, from 26.6% up to 81.4%, except for F22, which exhibited oxygenated monoterpenes as the most abundant class. Apocarotenoids (from 28.8 to 51.7%) followed by aldehydes/ketones (from 23.5 to 44.7%) were the most represented chemical classes of VOLs. On the other hand, apocarotenoids and oxygenated sesquiterpenes were not found in any of VOF samples.

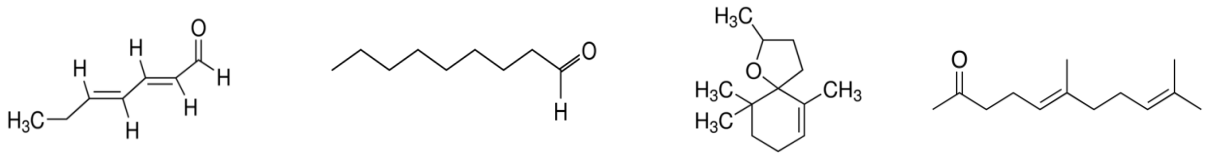


(*Z*)-jasmone **nonanal** **3-ethenyl pyridine** **bornyl acetate**

Figure III.12. Structures of the main compounds present in *O. europaea* flowers.

Several compounds exclusive of VOLs, with relatively high percentages, were theaspirane I (from 18.2 to 1.2 %) and theaspirane II (from 23.7 to 13.7%). These two compounds were among the main constituents characterizing cv. Sigoise.

Monoterpene hydrocarbons, limonene and *p*-cymene, which detected as the third chemical class in VOFs, were present at much lower levels in VOLs.



***(E,E)*-2,4-heptadienal** **nonanal** **theaspirane II** ***(E)*-geranylacetone**

Figure III.13. Structures of the main compounds present in VOLs.

Table III.5 showed that the PCA revealed a weaker inter-relationship between the compositions of VOFs and VOLs. Some compounds shared by leaves and flowers, such as nonanal, limonene and *p*-cymene, are an evidence that the olive tree release substantial quantities of the same volatiles from leaf and floral organs [118].

Table III.5. Correlation matrix between *O. europaea* leaf and flower volatiles.

Variable	F11	F12	F21	F22
L11	0.088	0.357	0.271	0.218
L12	0.031	0.138	0.022	-0.006
L21	-0.095	-0.064	-0.149	-0.144
L22	-0.090	-0.024	-0.048	-0.109

However, the study of the physiology and function of *O. europaea* floral organs is still in its infancy. More work is required for a detailed knowledge of the chemistry of floral fragrances of the olive tree [118].

- **Comparision the volatiles of the two cultivars: Chemlal and Sigoise**

Regardless of the harvesting area, Chemlal, the dominating cultivar in Algeria [99-101], showed a richer volatile composition compared to cv. Sigoise for both flowers (VOFs) and leaves (VOLs). It can be noted that the cv. Chemlal showed greater amounts of (*Z*)-jasmone than the cv. Sigoise in VOFs in both regions. In other hand, the other main compounds, nonanal, *p*-cymene and limonene showed more amounts in cv. Sigoise.

This can provide information about the plant physiology. Probably, the genetic characteristics of cv. Chemlal has different enzymes levels and enzyme activity than cv. Sigoise, which are in turn responsible for the qualitative and quantitative composition of volatiles compound.

Zhang *et al* [111] have found *(Z)*-jasmone in the flowers of *Olea europaea* L. of two Chinese and Mediterranean cultivars, but at much lower relative contents (from 7 to 0.15%). Therefore, the present study renowned that (*Z*)-jasmone, with its high levels in both cultivars, could represent a chemical marker for their flowers in the Mediterranean zone of Algeria. Due to this high relative amounts, and because it was demonstrated to be electrophysiologically active and repellent to herbivores, (*Z*)-jasmone may represent a defense compound for *O. europaea* [119-120].

- **Comparision the volatiles from the two regions**

Throughout the two regions, the main substances in the samples from the Mediterranean region were *(Z)*-jasmone (51.8%), nonanal (32.4%), theaspirane II (23.7%) and phenylethyl propionate (17.9%). While those of the arid region were bornyl acetate (26.8%), 3-ethenyl pyridine (17.2%), *(E,E)*-2,4-heptadienal (16.9%) and *(E)*-geranylacetone (14.4%), acetate (26.8%), 3-ethenyl pyridine (17.2%), *(E,E)*-2,4-heptadienal (16.9%) and *(E)*-geranylacetone (14.4%), with a significant correlation between the flower volatiles, more than 86% in each region.

By comparing all the volatile fractions, the ones collected from the arid region were more numerous and more diversified in terms of chemical classes in the plant material than the mediterannean region. In particular, monoterpenes, hydrocarbons, phenylpropanoids and alcohols were present only in VOFs collected from the arid region. Such as, *n*-dodecane, menthone, *n*-undecane, hexanal, heptanal and *n*-nonane. Similar relative amounts were noted for F21 and F22 but no significant differences for menthone and *n*-nonane. *n*-Dodecane reached here the highest amount (4.7%).

However, the flowers from the Mediterranean region were found to be rich in (*Z*)-jasmone, while the ones from the arid region were rich in bornyl acetate. Although, this compound was totally absent among the Mediterranean region volatiles. The oxygenated monoterpenes were absent in both F11 and F12. As published by Cano-Lamadrid *et al* and Veillet *et al*, bornyl acetate was detected in the volatile composition of olive oil (very low amounts) [120-121].

Many studies report the high defence level of these two compounds and that their production is related to environmental stress, which induces the expression of various genes to activate defense-related pathways that result in the release of defense chemicals [104-105, 121-123]. Our study confirmed that the environmental factor is affecting the chemical composition of *O. europaea* flowers, which led to different chemotypes.

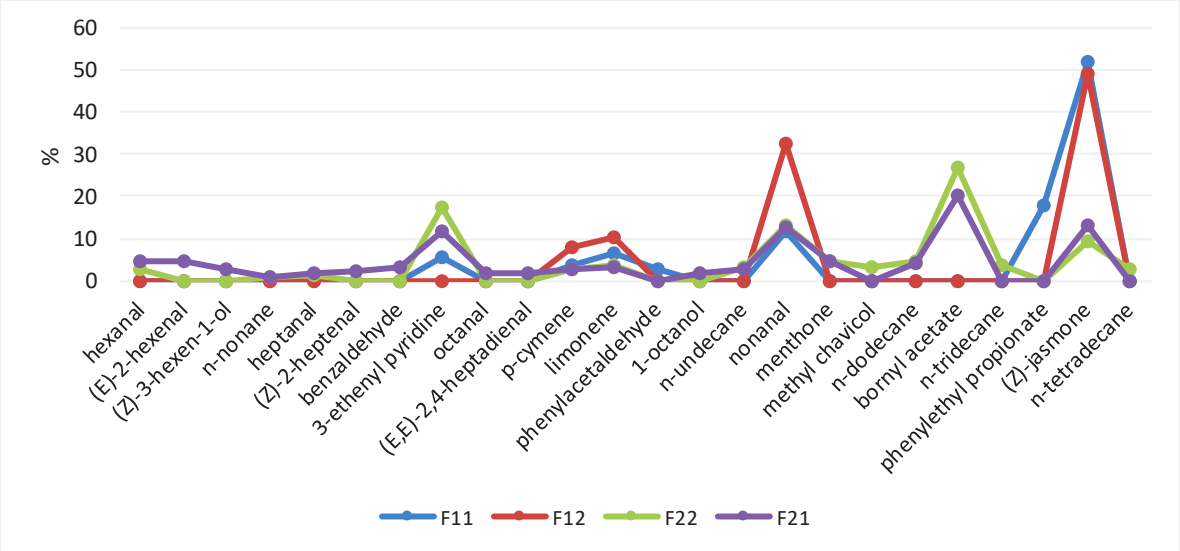


Figure III.14. Compositions variation of the flower volatiles.

Monoterpene hydrocarbons (limonene and *p*-cymene) which are detected as the third chemical class in VOFs were present in higher percentages in the Mediterranean region. Also, nonanal, the main compound in both VOFs and VOLs was more abundant in samples from the Mediterranean area. Previous studies have reported, nonanal as the main compound in leaves samples of different *O. europaea* cultivars from different regions (Italy, Tunisia and Portugal) [106-110].

Similar amounts of nonanal in Sigoise leaves from the Mediterranean region (11.6%) were noticed compared to the Tunisian cultivar Chemlali (11.23%) in the same season (April 2013) and the Italian cultivars Leccino (11.5%) and Cipressino (11.8%), but in November only [108, 110]. Other studies proved the antibacterial and antifungal potential of nonanal [124-126]. For example, Zhang *et al* reported that nonanal could significantly inhibit the mycelial growth of *Penicillium cyclopium*. Zavala-Sánchez *et al* demonstrated that nonanal isolated from *A. ludoviciana* was the compound responsible for the antidiarrhoeal activity [125-127].

Some other compounds were detected only in VOLs of the same region, particularly three of them, namely dihydroactinidiolide (5.1-3.3%), dihydrodehydro-β-ionone (11.9-3.9%) and phenylethyl alcohol (8.8-3.4%).

Comparing to the arid region, the Mediterranean climatic conditions stimulated much more the production of these natural products, particularly by cv. Sigoise for both VOFs and VOLs. Flamini *et al* detected both *p*-cymene and limonene among the volatiles of the Italian olive paste obtained by stone milling of the ripe fruits. Similarly, Malheiro *et al* reported the

detection of these two monoterpenes in the volatile composition of cv. Cobrançosa (Portuguese cultivar) olive leaf at different harvesting times. Limonene is a common constituent of various plant essential oils and it has been found to have numerous medicinal benefits (antioxidant properties, an excellent dietary source for cancer prevention and a slight cytotoxic activity toward normal cells) demonstrated both in human and animal studies [128-134].

On the contrary, Zhang *et al* did not report the existence of nonanal neither limonene nor *p*-cymene in VOFs of both the Chinese and the Mediterranean cultivars. So, this study is the first report for these compounds in *O. europaea* flowers [118].

III.3.3. Seasonal variation of the volatile fraction L11

Figure III.15 represents the composition variation of L11 during 4 successive seasons (1: July, 2: October, 3: January, 4: April). Overall, forty-seven compounds were identified, accounting from 98 to 99.1%.

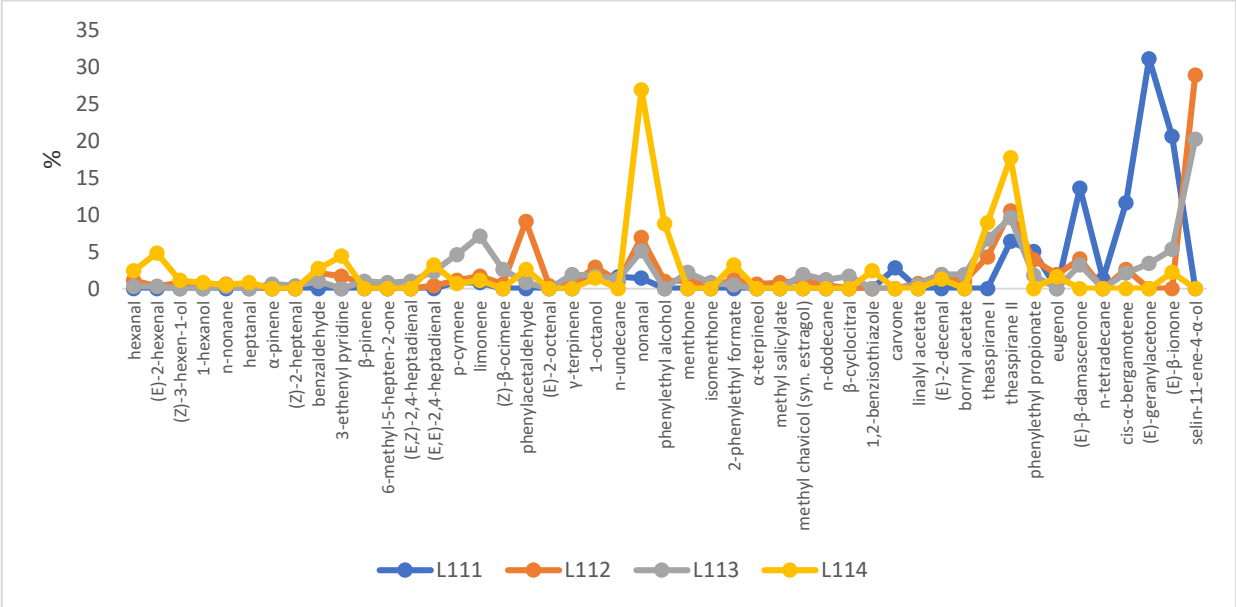


Figure III.15. Compositions seasonal variation of the volatile fraction **L11.**

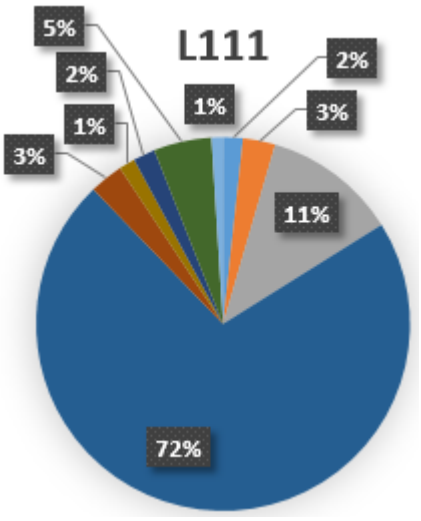
In general, the main compositions (nonanal and theaspirane II) were present in all the seasons with considerable amounts. They were increasing from July 2018, which corresponds to olive growing season until April 2019 which corresponds to flowering season. Also, their production remained almost stable in the third season corresponding the olive trees pruning.

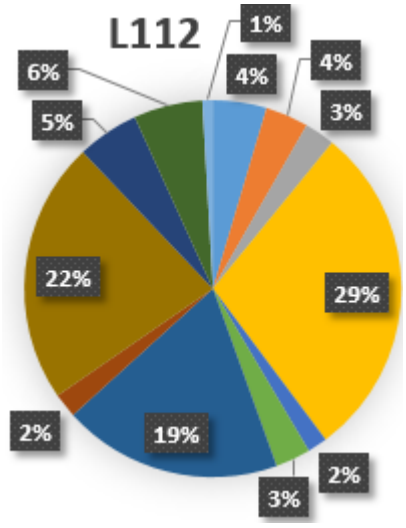
The main compound of the first season L111 was (*E*)-geranylacetone with more than 30%, recording the highest amount among all fractions. During the following season L112, the previous compound completely disappears and the main compound became selin-11-ene-4- α-ol with more than 28%. This compound remains the highest compound even in the following season L113, although it was completely absent in the first season L111. Nonanal was the major compound with over 26% in the last season L114.

Both limonene and *p*-cymene were present throughout the seasons, they increased from the first season to the third one, where they reached their highest values and decreased by the fourth season.

The second season L112, which coincided with the olives harvest, was more numerous and varied in its compositions, since 37 compounds were detected and classified into 11 chemical classes. This diversity in volatiles remains almost the same during the following season L113.

From **Figure III.16** which represents the chemical classes of L11 seasonal fractions, the Apocarotenes predominantly constitute the L111 and L113 fractions with 72 and 30%, respectively. The oxygenated monoterpenes with 29% were dominant in the second fraction L112 and the non-terpene aldehydes/ketone with 45% in the last fraction L114. The oxygenated sesquiterpenes were completely absent in the first and last seasons. Moreover, the phenylpropanoids which were absent in the first season were present in all other seasons with almost the same ratio ~ 2 to 3%.





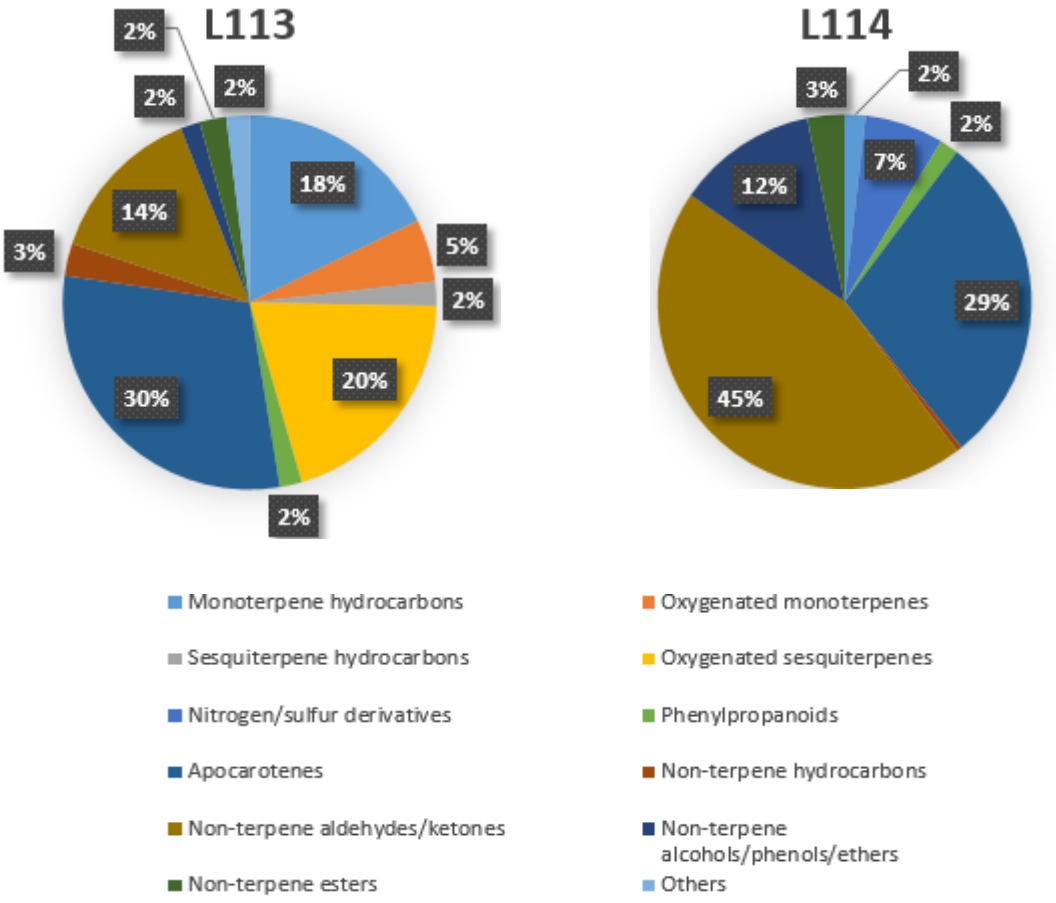


Figure III.16. Seasonal variation of chemical classes of the volatile fraction **L11.**

III.4. Conclusion

This chapter has provided for the first time important information about the volatile profiles of *Olea europaea* L. flowers and leaves of the most abundant cultivars in Algeria, cultivated in Mediterranean and Saharan regions. This can lead to an important source of variable natural products, potentially bioactive. Besides, it revealed the relationship between the volatiles and their geographic origin that caused the different volatile bouquets. This work could be a key to the large cultivation project of 100.000 olive trees in southern Algeria by providing information on how the plant physiology changes in the adaption to extremely hard conditions, such as those present in the arid state Ouargla. Certainly, the present research will be very helpful for sustainable agriculture in the Algerian Sahara. Cosmetics, food and pharmaceutical industries will benefit from the presence of the main volatile compounds in *O. europaea*, in particular of jasmone in the production of perfumes and as a food flavoring, or bornyl acetate as an analgesic.

General conclusion and perspectives

The beneficial properties of *Olea europaea* leaves have been attributed to their composition, especially to their content in phenolic compounds.

The aim of this study was to demonstrate the influence of an arid environment on the production of secondary metabolites by *O. europaea* widespread Chemlal and Sigoise cultivars in Algeria. Our results provided a contribution to the study of the relationships: composition/extraction method, composition/region, composition/cultivar, composition/organ, and composition/season.

The first experimental chapter pointed to maximize the phenolic content with higher biological capacity depending on response surface methodology. For industrial insight, the ultrasonic- assisted reflux method has many advantages compared with conventional methods like maceration due to its reduced extraction time, higher extraction efficiency, less labor and high extraction selectivity with less raw material ratio. These advantages make it the best method to extract phenolic compounds from *O. europaea* leaves.

The results of the second and third experimental chapters showed a great variability in the composition of the leaves of *O. europaea* extracted from local Algerian cultivars compared to other studies hold other cultivars in other countries (Spain, Italy and Tunisia).

The entire comparative study of the composition of volatile compounds and polyphenols of cv. Sigoise and cv. Chemlal, by the principal component statistical analysis allowed us to draw the following conclusions:

- ✓ The volatile and non-volatile fractions obtained from *OE* trees cultivated in the arid region; Ouargla, are chemically richer compared to those in the Mediterranean region; Boumerdes.
- ✓ Sigoise cultivar has higher oleuropein content than the leading cultivar Chemlal in Algeria.
- ✓ Highlighted potential relationships of the cultivation environment and volatile chemotypes. Effectively, (*Z*)-jasmone for VOFs in the Mediterannean region and bornyl acetate and 3-ethenyl pyridine in the arid region.

- ✓ The olive harvest period affects, strongly, the level of the phenolic compounds. Especially, the decrease of the major compound oleuropein. On other hand, this probably conducted to valorize oleuropein throughout bioconversion into hydroxytyrosol which is a high added value product.
- ✓ The statistical analysis allowed significant values ($p < 0.05$) to highly significant ($p < 0.001$), which confirms the success of our study.

Finally, the results of this study are expected to be of importance for the development of a wide range of products based on *O. europaea* leaves extract. Particularly that, any Changes in the volatiles and phenolic profiles can cause changes in the biological activities of olive leaf extracts.

In terms of prospects and following the obtained results, it would be interesting to conduct a more detailed investigation of the survey in the other cities of the southern region of Algeria, in order to properly assess the state of cultivated medicinal plant in the vast arid area of Algeria.

The results of this work can also be supplemented by:

- ➢ Evaluation of the biological activity of our extracts.
- ➢ The study of the insecticidal effect of the volatile fractions.
- ➢ The analysis on a wide range of cultivars.

The environmental impact of olive oil production, such as energy and water use, gas emissions, and waste generation, has increased as the olive oil industry has grown. Olive leaves are the most significant source of waste, as they are a plentiful and unavoidable by-product of olive oil production due to the need for tree-pruning. In Spain alone, an estimated 1.25 million tons of olive leaf waste are produced each year, accounting for over half of global production. Our findings can be used by industry to help them make environmentally sustainable decisions on how olive leaf waste can be utilized and optimized.

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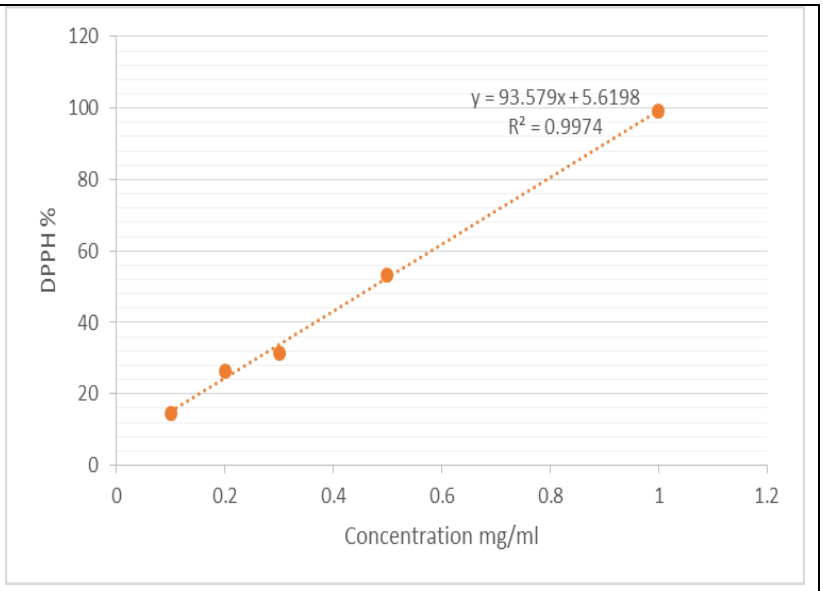
Annexes

Annex 01. Some IC_{50} curves obtained from UARE extracts (1st and 7th concentrations).

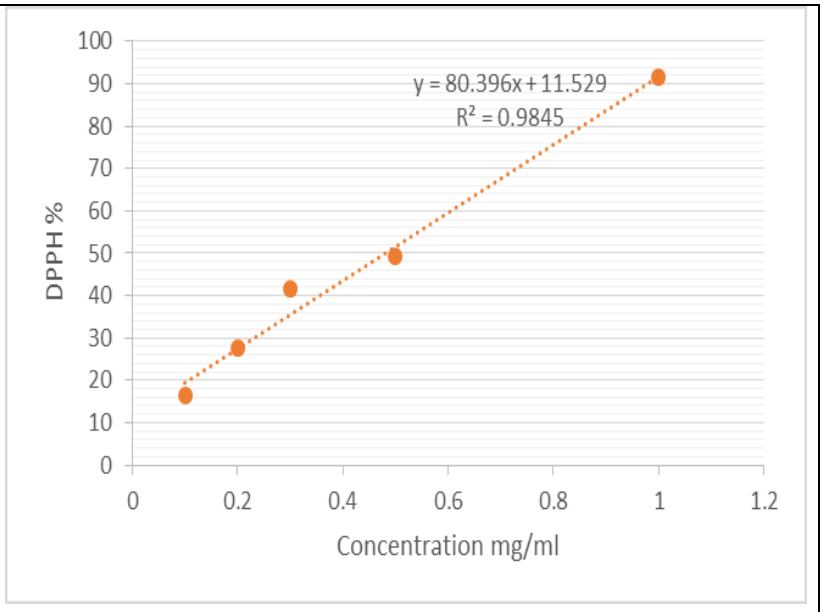
C_1 mg/ml	Abs	DPPH %
0.1	0.566	14.37216
0.2	0.486	26.47504
0.3	0.454	31.31619
0.5	0.308	53.40393
1	0.0063	99.0469

IC_{50} = 0.474253839

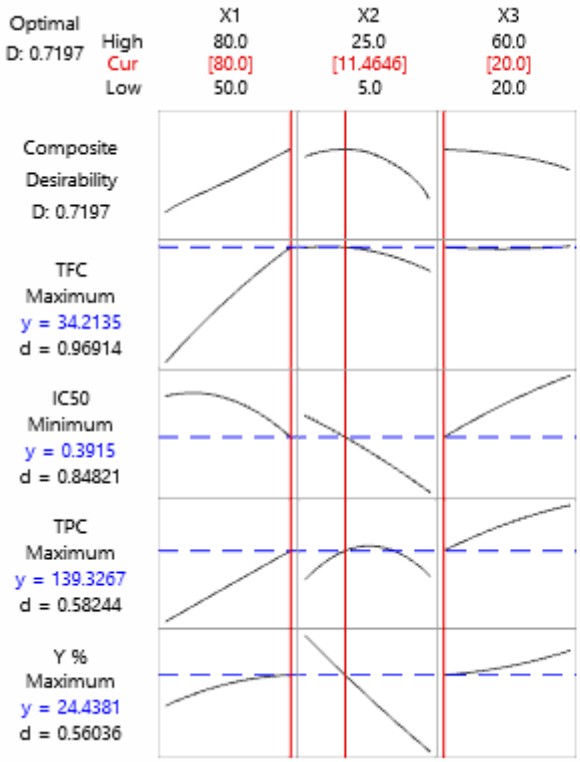


C_7 mg/ml	Abs	DPPH %
0.1	0.553	16.33888
0.2	0.479	27.53404
0.3	0.386	41.60363
0.5	0.335	49.31921
1	0.055	91.67927

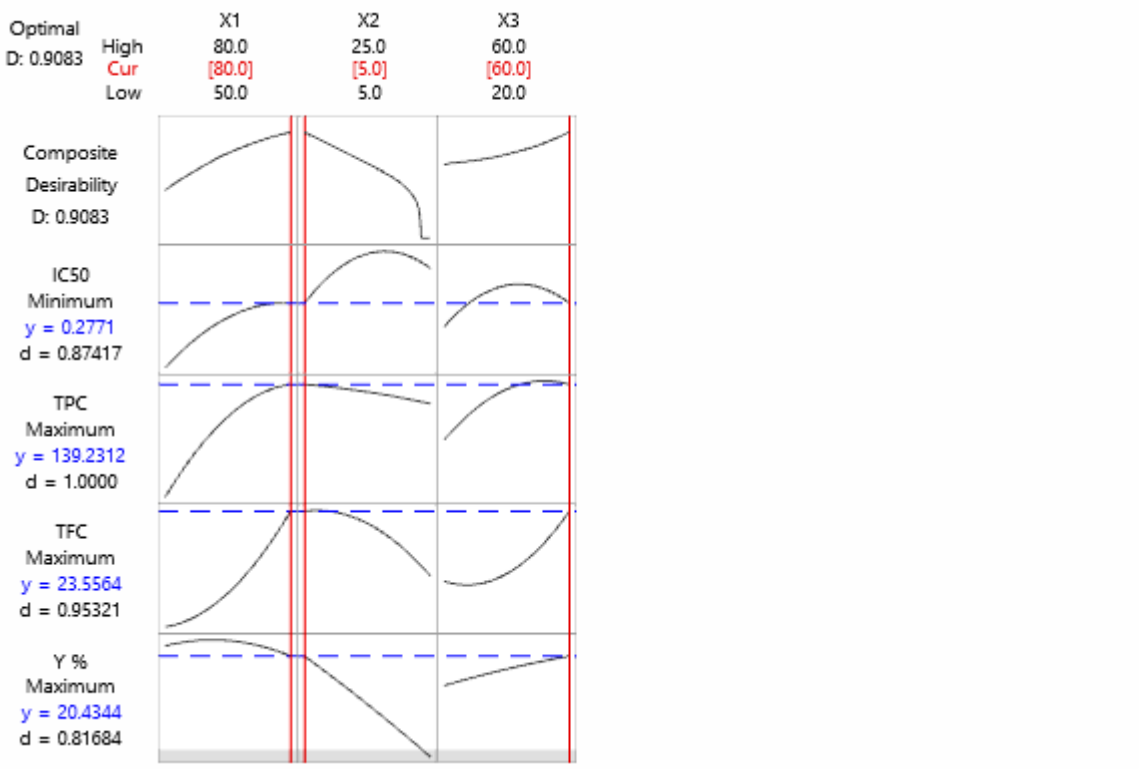
IC_{50}= 0.478518832



Annex 02. The optimization plot displays the fitted values for the predictor settings by RSM.



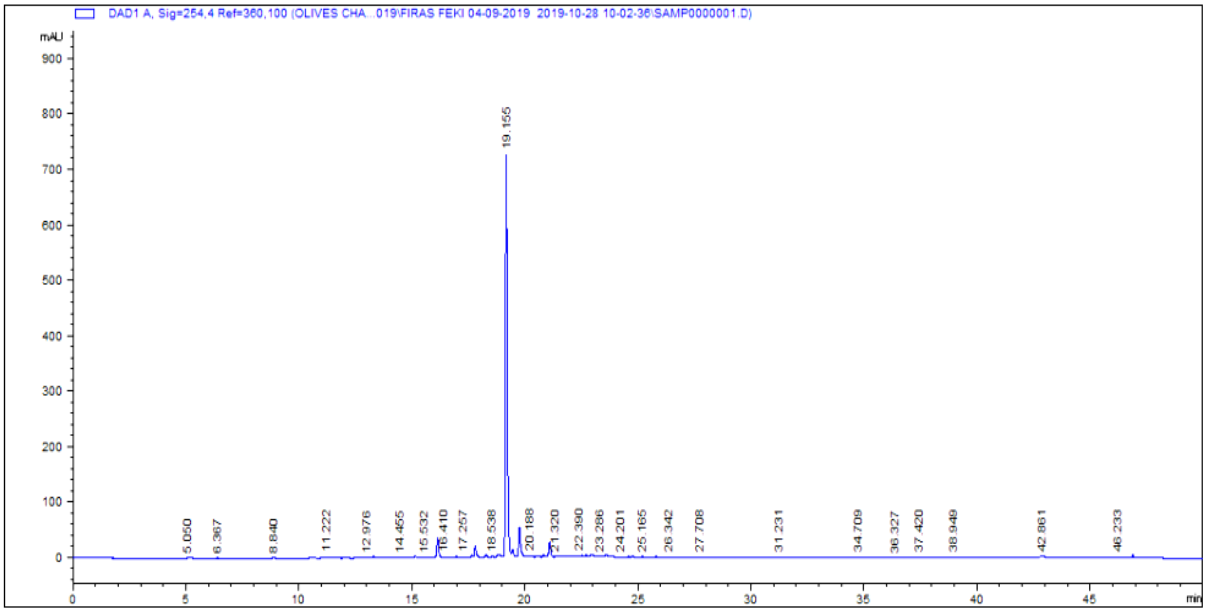
UARE optimal conditions



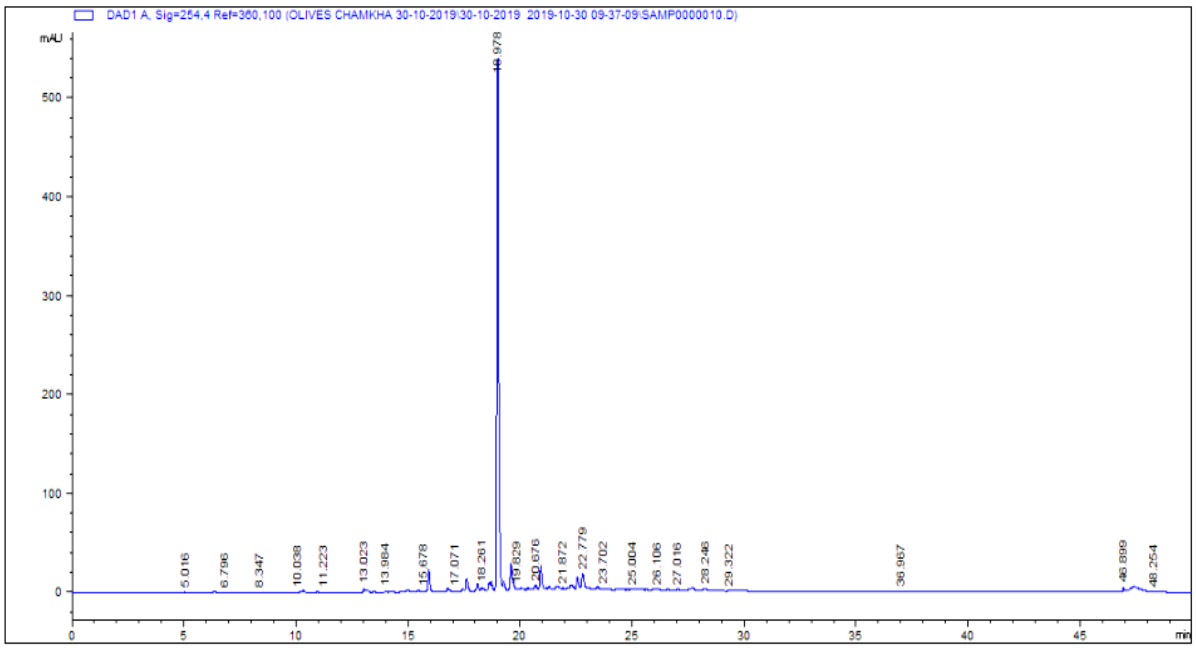
MAE optimal conditions

Annex 03. Chromatograms of cv.Chemlal in two different cultivation regions.

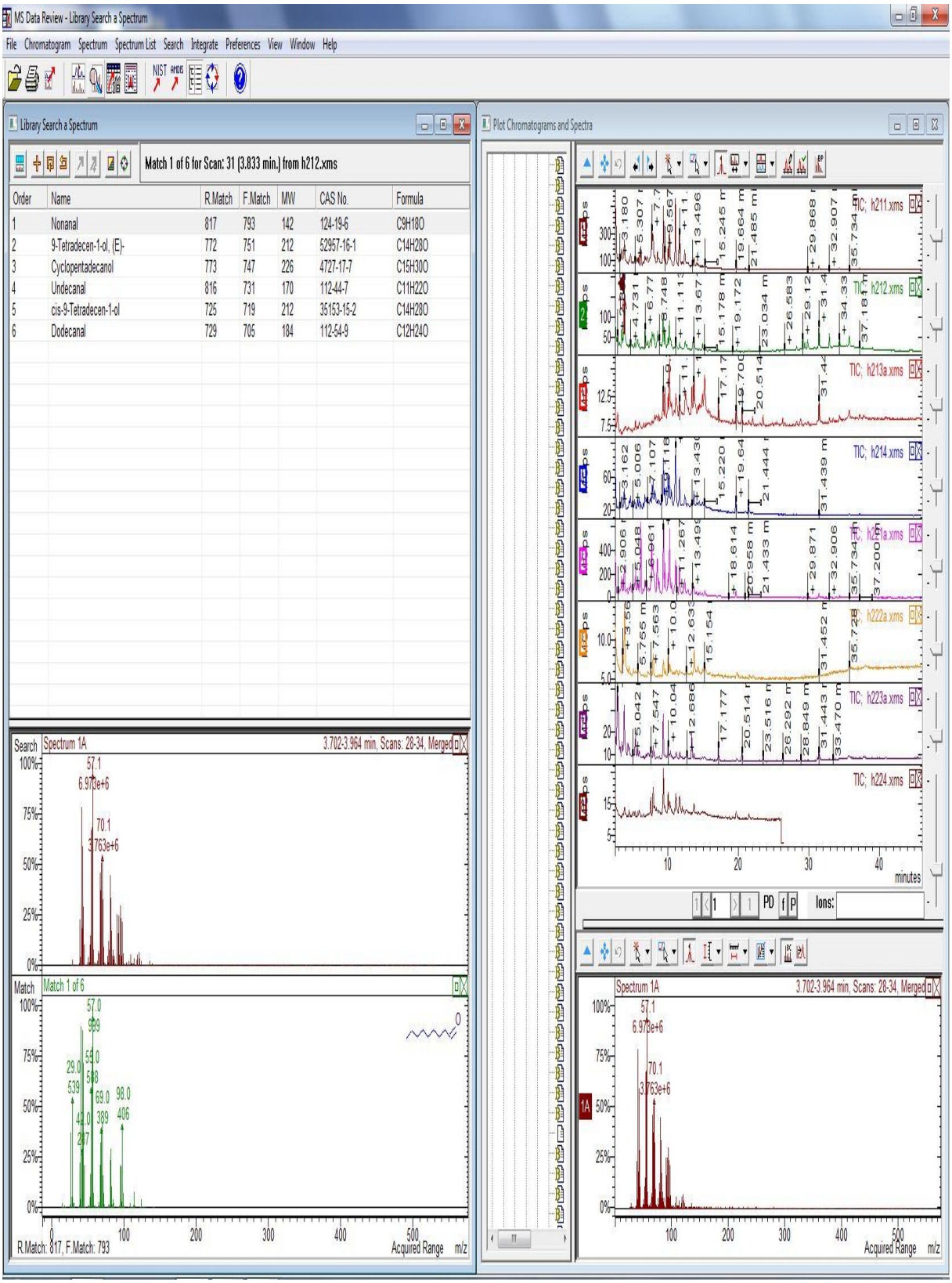
Chromatogram of cv.Chemlal cultivated in Mediterannean region.



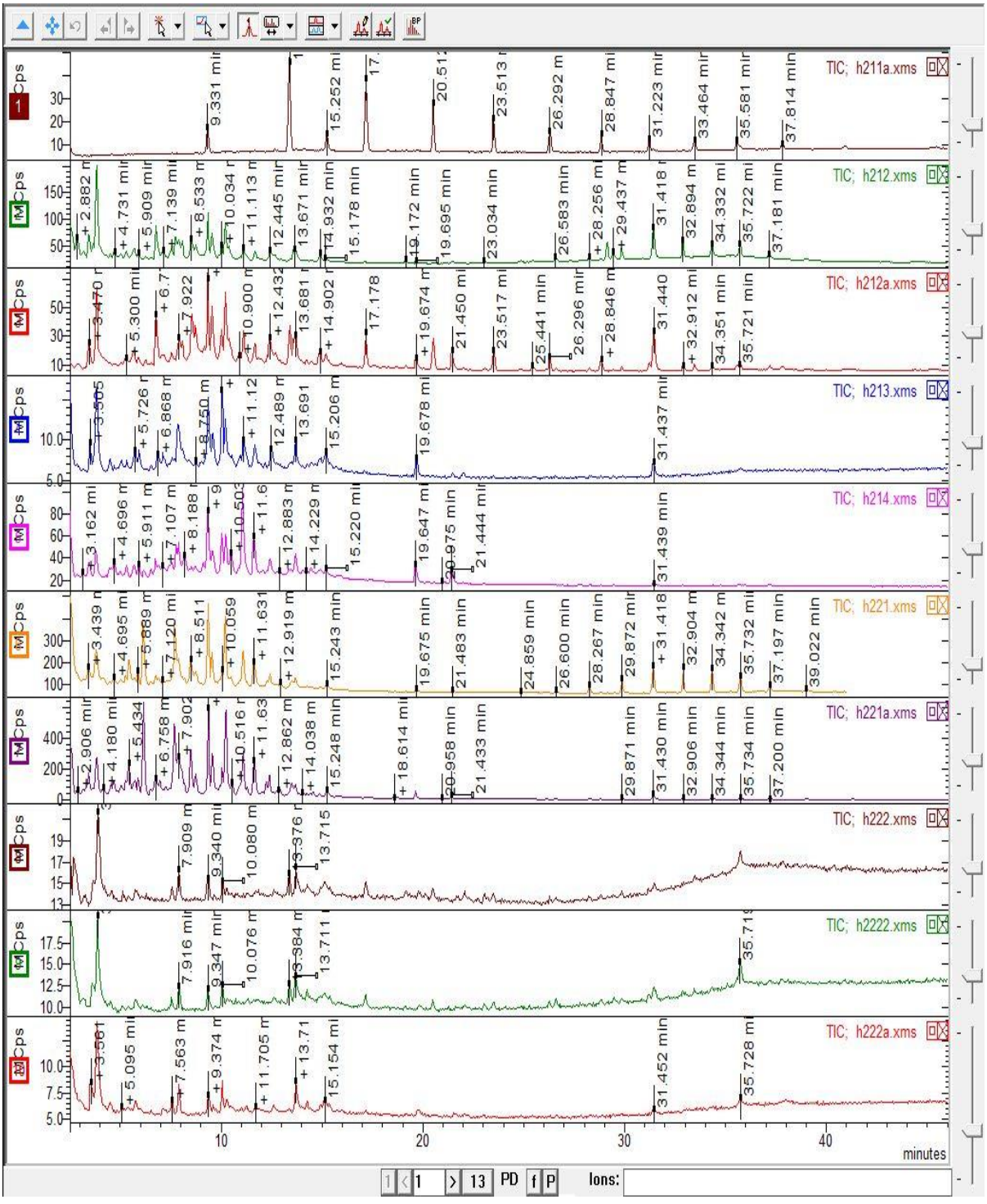
Chromatogram of cv.Chemlal cultivated in arid region.



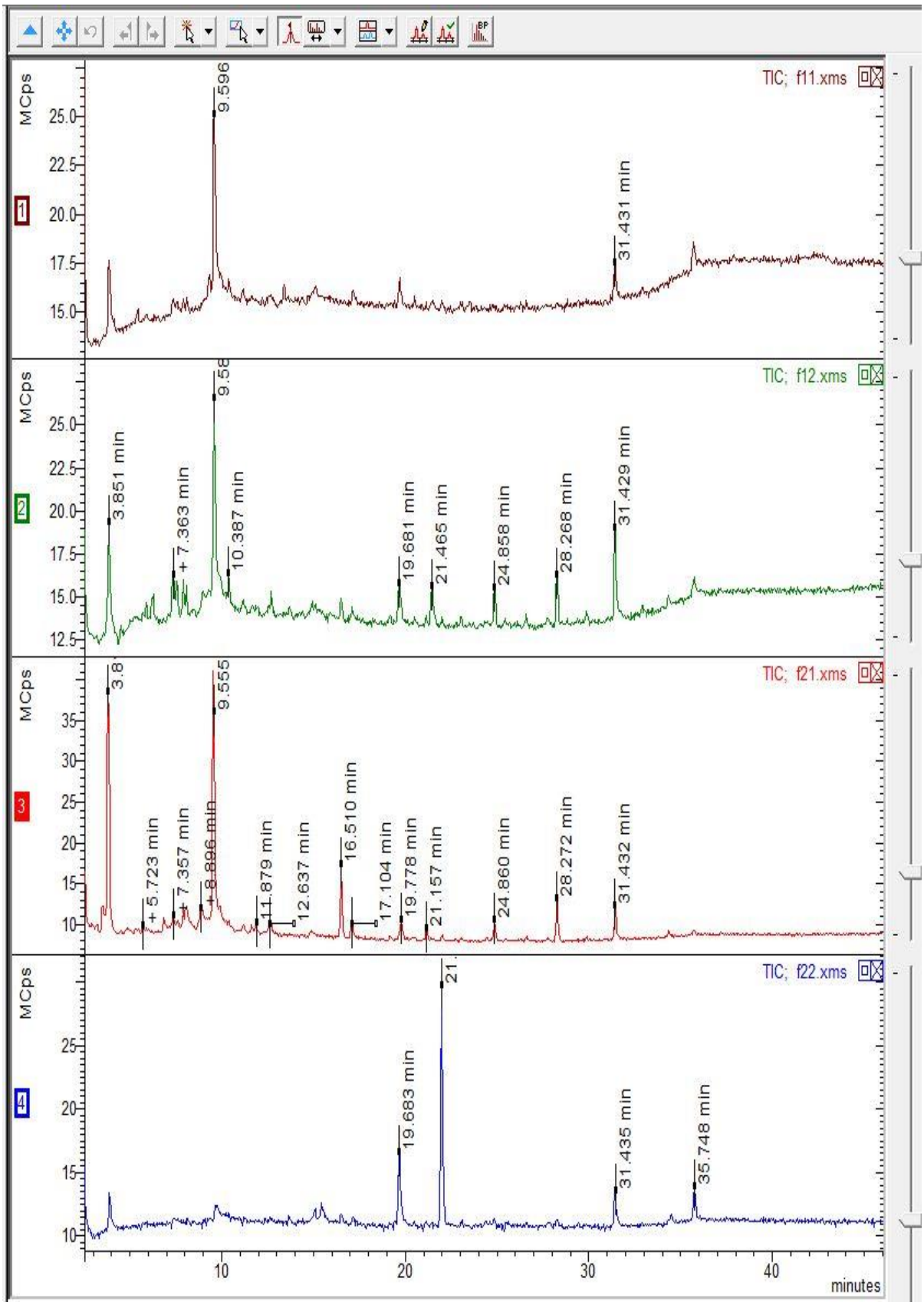
Annex 04. MS Data review- Library Search a Spectrum (The identification of the major compound, nonanal in VOLs.)



Annex 05. The chromatograms GC/MS of VOLs.



Annex 06. The chromatograms GC/MS of VOFs.



Annex 07. Statistical study of VOFs using ACP.

Summary statistics

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
F11	24	0	24	0.000	51.800	4.158	11.071
F12	24	0	24	0.000	49.000	4.154	11.807
F21	24	0	24	0.000	20.200	4.158	5.119
F22	24	0	24	0.000	26.800	4.154	6.515

Bartlett's sphericity test

Chi-square (Observed value)	77.903
Chi-square (Critical value)	12.592
DF	6
p-value	< 0.0001
alpha	0.05

Test interpretation:

H0: There is no correlation significantly different from 0 between the variables.

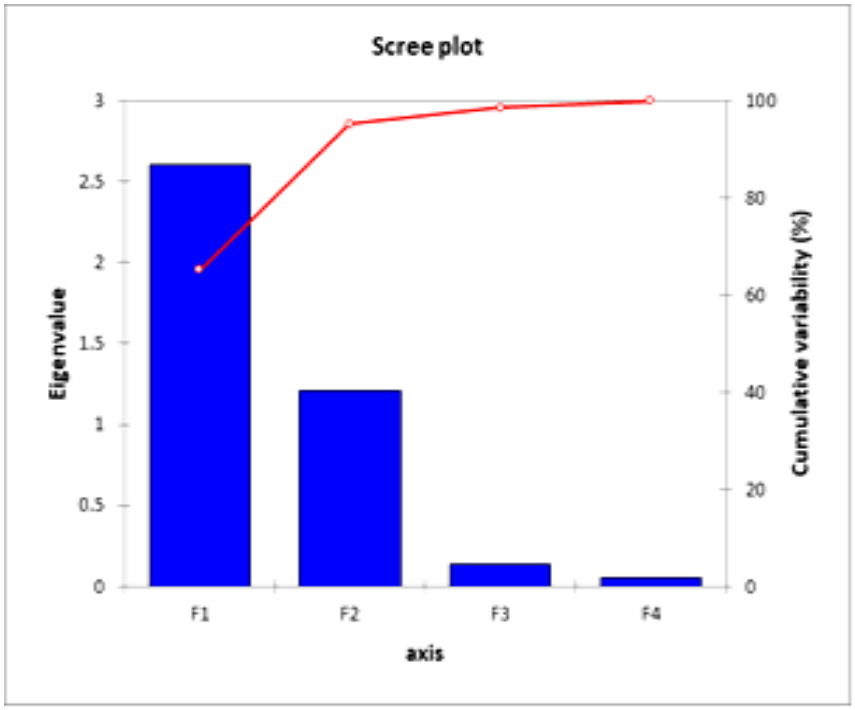
Ha: At least one of the correlations between the variables is significantly different from 0.

As the computed p-value is lower than the significance level alpha=0.05, one should reject the null hypothesis H0, and accept the alternative hypothesis Ha.

The risk to reject the null hypothesis H0 while it is true is lower than 0.01%.

Kaiser-Meyer-Olkin measure of sampling adequacy

F11	0.593
F12	0.561
F21	0.556
F22	0.512
KMO	0.554



Annex 08. Dendrogram of VOLs by XLSTAT.